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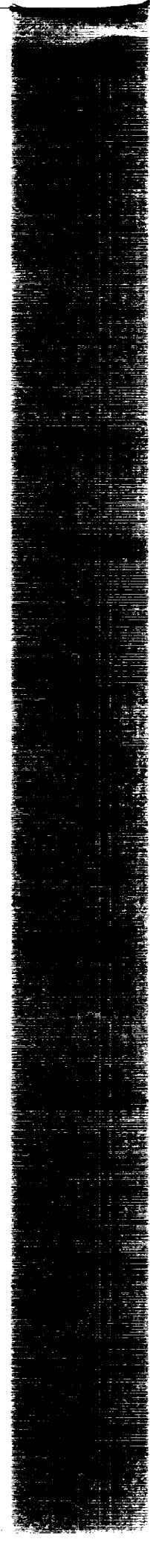

NASA Technical Memorandum

1992-1993 NASA Space Biology Accomplishments

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PREFACE

This publication has two objectives: first, to provide the scientific community and NASA with a summary of the accomplishments of the research pursued under the auspices of the Space Biology Program, and second, to stimulate an exchange of information and ideas among scientists working in the fields of gravitational and space biology.

Individual technical summaries of the research conducted under the tasks within the NASA Space Biology Program for the calendar years of 1992 and 1993 are presented in this publication. Each summary, prepared by the principal investigator, consists of a description of the research and the project's accomplishments, as well an explanation of the significance of the accomplishments and a list of the publications over the last 2 years resulting from the research. Since Small Payload Shuttle middeck space flight experiments have become an integral part of Space Biology research, reports on these experiments are incorporated in the document. Accomplishments of the scientists in the NASA Space Biology Research Associates Program, which provides opportunities for postdoctoral scientists to conduct research in the fields of gravitational and space biology at host university laboratories, are also included. The participants in this program have been outstanding and merit independent recognition.

Thanks are due to the Program participants and postdoctoral scientists whose research and cooperative response to our requests for information made this report possible. Julianna Klejnot played a principal role in organizing and editing the publication with the support of Mary Lou Burnell, Bruce M. Hather, Carla J. Howard, Richard M. Poley, and Joshua Singer. Their significant and essential contributions which made this publication possible are gratefully acknowledged.

Thora W. Halstead, Ph.D.

INTRODUCTION

THE NASA SPACE BIOLOGY PROGRAM

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One of the major features of the physical environment of the surface of Earth is the constant presence of the force of gravity. We can study the importance of gravity to life on Earth by using the unique biological research tool provided by the phenomenon of near-weightlessness encountered on spacecraft during their free-fall flight. Access to space provides an opportunity to manipulate the force of gravity from its normal value of one down to almost zero for prolonged periods of time. This capability has laid the foundation for the Space Biology Program to support research using hypergravity, Earth-normal gravity, and microgravity as environmental tools to advance fundamental knowledge about biological systems; the role of gravity and the effects of microgravity on biological processes; and the interaction of gravity and other environmental factors on biological systems.

The Program emphasizes research in cell, developmental, integrative and plant biology, structural biosystems, and gravity sensing that seeks an understanding of basic mechanisms affected by gravity. Studies include higher and lower plants and animals as subjects, as well as cell and tissue cultures. The scope of the research and scientific objectives in each of these areas is described below. While the research supported by the Program is totally ground-based, much of it must ultimately lead to flight experiments that can confirm or refute the fidelity of ground-based models and hypotheses. The Space Shuttle middeck Small Payload Program has been the main source of opportunities for such flight experiments.

Plant Biology. Gravity has profound influences on the development, growth, and physiological processes of plants; yet, the basic mechanisms underlying gravity's effects are still not well understood. The goals of plant gravitational biology are to achieve both a fundamental scientific understanding of the effects of gravity on plants, and to provide basic knowledge of plant development and physiology that can contribute to the utilization of plants in space and on Earth. Research is focused in three primary areas: understanding the basic mechanisms whereby plants perceive, transduce, and respond to a gravitational force; elucidating the role of gravity and microgravity on developmental and reproductive processes in plants; and determining the role of gravity and microgravity in metabolism, photosynthesis, and transport processes in plants. Integral to each of these areas is how gravitational force and other environmental stimuli are integrated biologically.

Cell Biology. Cells, whether as single unicellular organisms or as the basic structural and functional unit of multicellular biological systems, have been shown to be sensitive to gravity. The scientific goal of gravitational cell biology is to understand how the weak force of gravity can act as a biological regulator that functions at the cellular level, and thereby understand how a physical force is transduced to produce a biological effect. To attain this goal, research is focused on identifying how cells "sense" gravity both directly and indirectly, how this information is transduced into a biological response, and how cells respond to both acute and long-term variations in gravity.

Developmental Biology. This research area examines the influence of gravity and microgravity on reproduction, genetic integrity, differentiation, growth, development, life span, senescence, and subsequent generations of animals. The goals are to determine whether specific animal species have evolved developmental mechanisms that depend on a gravity force or vector for normal function and, if so, to identify the mechanisms; to determine if altered gravity induces phenotypic or genotypic changes; and to determine if gravity affects the capacity of animal species to reproduce and develop normally over serial generations.

Gravity Sensing. Animals have developed gravity-sensing systems that facilitate orientation and locomotion within Earth's environment. The goals of this discipline are to understand how the brain processes information by understanding the organization and functioning of gravity-sensing organs; to test mechanisms of adaptation by exposure to variable gravity; to elucidate the stages by which the gravity-sensing system evolved, from invertebrates through vertebrates; and to begin to understand where in the developmental process gravity influences genetic transcription/translation to direct production of a specific functional architecture in gravity-sensing organs.

Structural Biosystems. In response to the force of gravity under which all biological species on Earth evolved, organisms have developed structures to withstand gravity loads. The goal of this discipline is to understand the role of gravity in modulating biological processes that regulate musculoskeletal systems. Research focuses on identifying how gravity level and direction influence the type, pattern, and amount of biomineralization and muscle in structural biosystems; understanding interactions between gravity and internal (e.g., metabolic regulatory substances) and external (e.g., environmental) factors in these systems; and specifying the time course of musculoskeletal adaptation to altered gravity and readaptation to normal Earth gravity.

Integrative Biology. Living systems have evolved in a gravitational field and are regulated to function within tightly controlled limits. Asymmetries in the environment (including presumably gravity) result in disturbances in these regulatory mechanisms to which living systems adapt and evolve. The goal of integrative biology is to understand how gravity affects mechanisms regulating homeostasis, adaptation, and the ability of living systems to respond to internal and external signals. Research focuses on understanding the role of gravity on animal regulatory mechanisms: the generation and/or entrainment of circadian rhythms, the internal synchronization of several circadian functions, and one or more selected homeostatically controlled systems; e.g., the regulation of body temperature and the associated neuroendocrine regulation of energy and water.

The use of the unique microgravity environment of spaceflight is an integral part of these research efforts. Flight experiments in gravitational biology permit identification, confirmation, and understanding of effects due to microgravity. At the same time, ground-based research activities lay the groundwork for validation by flight experiments, identify gravity-sensitive biological systems, and utilize modern research techniques and instrumentation to target analyses at the most fundamental levels.

ACCOMPLISHMENT HIGHLIGHTS

SPACE BIOLOGY
ACCOMPLISHMENTS HIGHLIGHTS

PLANT

Gravitropism: Sensing

Nature of the Receptor

- The Potential Gating Theory, a working hypothesis, attempts to link the internal bioelectric fields of the plant to the transport of the growth hormone, indole-3-acetic acid (IAA). Briefly, the theory states that the transport of IAA from the vascular stele of the plant to the cortical cells is through plasmodesmatal connections and is regulated by the bioelectric potential difference between the stele and cortex. (Desrosiers/Bandurski)

- In order to study the role of amyloplasts in plant gravisensing, amyloplast manipulation by forces other than gravity is desirable and achieved using a high gradient magnetic field. The magnetic field generates a ponderomotive force that is superimposed to the force of gravity and allows the application of various forces to all or a subset of amyloplast in, for example, the root cap. The extent of amyloplast displacement correlates with the subsequent growth direction (curvature) of roots. (Hasenstein)

- The statolith compartment in *Chara* rhizoids is more complex than prior reports indicate. In addition to containing the previously reported barium sulfate crystals, this compartment has an organic matrix consisting of protein and carbohydrate moieties. (Kiss)

- The response to gravity is directly correlated with the number of statoliths in *Chara* rhizoids. (Kiss)

- Gravitropic roots of the aquatic angiosperm, *Limnobium*, have sedimented amyloplasts in their elongation zone but not in their rootcap. If amyloplast sedimentation is responsible for gravitropic sensing, then the site of sensing in *Limnobium* roots is the elongation zone and not the root cap. Comparison of several different moss genera establishes that amyloplast sedimentation is present whenever the protonema is gravitropic. (Sack)

- Hydrostatic pressure mimics gravitational pressure. Finding that a unidirectional-applied hydrostatic pressure mimics gravitational pressure in inducing the physiological response. (Wayne)

- Gravireceptor may be an integrin-like protein. Demonstrates that the receptors are localized at the plasma membrane-extracellular matrix junction at the ends of the cell and that the mechanoreceptor for gravitational and hydrostatic pressure is an integrin-like protein. (Wayne)

- Gravireceptor is distinct from touch-receptor in the same cell. The touch receptor is distinct from the receptor involved in sensing gravitational and hydrostatic pressure. (Wayne)

Role of Membranes

- Cell-to-cell communication through plasmodesmata (PDM) is under metabolic control. When adenosine triphosphate (ATP) levels are reduced in wheat roots, the maximum size molecule that passes through PDM increases from about 800 to over 5,000 MW. Under normal circumstances ATP is able to pass from cell to cell through the PDM. (Cleland)

- Cell-to-cell communication through PDM is also modulated by gravity. In oat coleoptiles, the movement of carboxyfluorescein from subepidermal cells to cortical cells is greatly increased when a vertical coleoptile is placed in a horizontal position. (Cleland)

- Cytoskeletal protein extracts from *Phycomyces* sporangiophores contain protein bands reacting with monoclonal antibodies to g-actin, myosin, and integrin in western blots. These proteins are being considered in a cytoskeletal framework and the anchoring of that framework to the membrane and cell wall which could, in conjunction with the vacuole, serve in the detection of gravity stress in this organism. (Edwards)

- It has been proposed that mechanical tension is passed from cell wall to the plasma membrane and its mechanosensory calcium channels by way of adhesion sites that are functionally similar to those of animal cells. To explore this concept, cell membranes were exposed by digesting away wall polymers so that antibodies to the animal adhesion molecules integrin, vitronectin, and fibronectin could be applied. These antibodies were then stained with fluorescent secondary antibodies and covisualized using total computation, optical sectioning microscopy. The antigens occur in punctate loci, with extensive overlap. This and other evidence supports the idea that adhesion sites in plants have many similarities to those in animals. (Pickard)

- A change in membrane potential is not involved in the signal transduction chain involved in sensing gravitational or hydrostatic pressure. (Wayne)

Role of Phytochrome

- Towards understanding the role of phytochrome in regulating root gravitropism, there is evidence for the hypothesis that a phytochrome-associated protein kinase modulates light-regulated root gravitropism *in vivo* and that the protein kinase may function to regulate receptor activity. (Feldman)

Gravitropism: Transduction

Role of Calcium

- It was determined that the cells that mediate curvature induced by application of calcium gradients near the root tip are cells of the postmitotic isodiametric growth (PIG) zone. The PIG zone is a group of cells between the meristem and the main elongation zone. This is the same group of cells that plays the primary role in the response of roots to gravity and to touch. (Evans)

- The calmodulin gene from *Phycomyces* has common homologies to both higher plant calmodulins and calmodulins from other fungi. A 339 base pair (bp) fragment of the gene has been obtained by polymerase chain reation (PCR) and will be used in an effort to sequence the entire gene (Braam and Davis, 1990). (Edwards)

- To measure signal-induced changes in the level of cytosolic Ca^{2+} concentration, transgenic plants carrying the aequorin gene from jellyfish were produced. Using confocal image analysis, signal-induced changes in free Ca^{2+} concentration in these transgenic plants were detected. (Poovaiah)

- Two distinct classes of Ca^{2+} channels are involved in gravisensing, a third is not. Ca^{2+} channels located at the ends of the cells are sensitive to organic channel blockers and are involved in gravisensing (Classes I and II). A third class of Ca^{2+} is found throughout the plasma membrane, is insensitive to organic channel blockers, and is involved in sensing electrical stimulation and touch (Class III). (Wayne)

- Use of lanthanides to probe the structures of the three classes of Ca^{2+} channels indicates that Class I is an elastic peristaltic channel with three binding sites within the pores for Ca^{2+}. Likewise, Class III is an elastic peristaltic channel with three binding sites for Ca^{2+}; however, the binding sites are definitely multidentate. By contrast, Class II channels have an external vestibule that can only be blocked by large cations. (Wayne)

Role of Calmodulin

- To study the consequences of altered levels of calmodulin on plant growth and development and signal transduction, transgenic potato plants were generated carrying the calmodulin cDNA (PCM-1) in sense and antisense orientations driven by constitutive and inducible promoters. These transgenic plants exhibit striking differences in growth and development. (Poovaiah)

Role of Calcium/Calmodulin Regulated Kinases

- Towards understanding the hypothesized role of calcium/calmodulin-regulated kinases in root gravitropism, a cDNA encoding a homologue of mammalian CaMK II was isolated from root caps of maize. This suggests a physiological function for a CaMK in light-regulated root gravitropism. (Feldman)

- A Ca^{2+}/calmodulin-dependent protein kinase from corn root tip has been purified. This protein (56 kDa) autophosphorylates in the presence of Ca^{2+a} and calmodulin. In addition, a Ca^{2+}/calmodulin-dependent protein kinase has been cloned from lily. (Poovaiah)

Role of Electrical Signaling

- The response of maize roots to applied electrical fields initially shows curvature toward a negative electrode followed by curvature toward a positive electrode. The electrical response of maize roots to thigmostimulation was tested, and dramatic touch-induced depolarizations of surface potential were found. (Evans)

Role of Hormones

- Roots inhibited by auxin show rapid, strong gravitropic curvature. This curvature is mediated exclusively by gravistimulated enhancement of elongation on the upper side of the PIG zone and tells us that gravi-induced rapid elongation on the upper side of the PIG zone is not mediated by a gravi-induced shift in auxin distribution. (Evans)

- In red-pretreated pea epicotyls, a decrease in ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), paralleled increased ACC-malonyl (MACC) conjugation within 30-minute gravistimulation. This red light regulation of ethylene biosynthesis may be responsible for altering cell growth in the stem after gravistimulation. (Harrison)

- The oxidation of ethylene precursor, ACC, is altered in pea seedlings grown in one-liter closed canisters within a 5-day incubation period. This result was accompanied by ethylene accumulation within the canisters and indicates a reduction in oxygen during the incubation period. (Harrison)

- Phytochrome is the pigment involved in regulating the light-induced reversed gravitropic response of stems of the *lazy-2* gravitropic mutant of tomato. The reversed gravitropic response of *lazy-2* shoots results from stimulation of growth on the upper side of gravistimulated stems. (Lomax)

- Wild type and *diageotropica* tomato seedlings do not respond to gravity when submerged in high concentrations of indole-3-acetic acid (IAA) or IAA transport inhibitors N-1-naphthylphthalamic acid (NPA) and 2, 3, 5 - triiodobenzoic acid (TIBA). This finding suggests that IAA transport plays an important role in the gravitropic response of tomato seedlings. (Rice)

Role of Proteins

- Towards understanding the hypothesized role of calcium-dependent protein kinases (CDPKs) in root gravitropism, it has been shown that root caps contain four CDPK peptides; two of which co-migrated with those found in other tissues, and two of which appear to be unique to the root cap. (Feldman)

- An investigation of developmentally regulated calmodulin-binding proteins in *Vicia faba* (fava bean) seedlings led to the identification of glutamate decarboxylase as a calcium-dependent calmodulin-stimulated enzyme in roots. The product of glutamate decarboxylase activity, gamma-aminobutyrate (GABA), is known as a stress-induced metabolite in plants and as an inhibitory neurotransmitter in animals and insects. The biochemical linking of calcium-mediated signal transduction to stress physiology offers new directions of research in plant gravitropism. (Ling)

- Phytochrome, the same pigment that mediates the effects of light on gravitropism, induces increased guanosine triphosphate (GTP)-binding protein activity in the envelope of pea nuclei. There results implicate nuclear GTP-binding proteins in the signaling pathway by which phytochrome regulates changes in plant growth and development. (Roux)

- Confocal laser microscopy of immunostained rhizoid cells of *Dryopteris* has revealed that Ca^{2+}-binding annexin proteins are concentrated at the extreme tips of these cells during their initiation and elongation. Ca^{2+} is also concentrated in this region. These results suggest that annexins may play a major role in Ca^{2+}-mediated exocytosis events critical for the polar growth of rhizoids, and may participate in the asymmetric growth response of gravitropism. (Roux)

Use of Mutants

- In general, there is a close correlation between auxin sensitivity and gravitropic sensitivity; i.e., the greater the reduction in sensitivity to growth inhibition by auxin, the greater the reduction in graviresponsiveness. (Evans)

- Roots of the tomato (*Lycopersicon escalentum*, Mill.) mutant *diageotropica* (*dgt*) exhibit an altered phenotype. We speculate that the insensitivity of *dgt* root growth to auxin transport inhibitors and ethylene is an indirect result of the loss of sensitivity to auxin in this single gene-recessive mutant. (Rayle)

- A tomato mutant, *diageotropica*, displays a developmentally regulated response to gravity. Young (5–20 day old) seedlings which are turned horizontally will curve upward to vertical, although they are slower than wild type. After development of the first internode, the maximum attainable gravicurvature declines substantially. (Rice)

Gravitropism: Growth Response

- Specific wall proteins, *expansins*, that mediate the acid-extension responses of isolated walls have been identified. These novel proteins are important for the extension growth of most plant cells and may be ubiquitous components of plant cell walls. (Cosgrove)

- A new method has been developed for studying the old problem of how roots bend down in the Earth's gravitational field. Method allows continuously following of the amount of force the root generates as it tries to bend down. This will allow building a biomechanical model of root performance in terms of work done and power output. (Galanes/Lintilhac)

- Auxin binding protein (ABP) in graviresponding oat (*Avena*) shoots has been isolated and has had its action related to the signal transduction process and to increases in auxin sensitivity in lower halves of leaf-sheath pulvini that are responding to gravistimulation. Key components of the signal transduction process are: (a) Ca^{2+} requirement, (b) a plasma membrane ATPase-driven proton pump, and (c) an increase in the number of high affinity binding sites on the ABP for auxin. (Kaufman)

- Differential gene expression occurs early during the gravitropic response in cereal grass pulvini. (Kaufman)

- Antibodies against complex polysaccharides of higher plants cross-react with *Chara* rhizoids in a specific manner. These antibodies will allow investigation of the vesicle blockage hypothesis (for the response mechanism) since apical vesicles can be divided into several categories depending on size, staining patterns, and antibody labeling. (Kiss)

- Stem segments and intact seedlings of the *diageotropica* tomato mutant do not elongate in response to exogenous IAA at the time of development. However, *diageotropica* seedlings can respond to gravity, which suggests that IAA-induced elongation and the asymmetric growth produced during gravistimulation are different. (Rice)

- A second gene encoding a putative Ca^{2+}-ATPase has recently been identified and cloned. (Wimmers)

Cytoskeleton

- Cortical cells and columella cells of graviresponding roots show significant reorientation of cortical microtubules. Reorientation is most explicit in the outer cortex along the lower side of the elongation zone. The upper, convex side of roots is not affected. The specificity of the reorientation indicates that the outer cortex, not the stele or epidermis, is the tissue most influenced by the growth inhibition that leads to gravicurvature. (Hasenstein)

Gravity and the Plant Cell

- Nuclei, as well as amyloplasts, sediment consistently and completely in the cells of the elongation zone in roots of *Limnobium*. The location of cells containing nuclear sedimentation is under tight developmental control. (Sack)

- Apical cells of protonemata of the moss *Ceratodon* are unusual among plant cells with sedimentation in that only some amyloplasts sediment and these do not fall completely to the bottom of vertical cells. Application of cytoskeletal inhibitors to protonemal cells of the moss *Ceratodon* suggests that microtubules restrict the sedimentation of plastids along the length of the cell and that microtubules are load-bearing for all plastids in the apical cell. (Sack)

Plant Metabolism

- Changes in soybean cotyledon starch concentration due to altered gravity treatments (lower in clinorotated and higher in centrifuged plants) appears to be directly related to ADP glucose pyrophosphorylase activity. This enzyme is known to be rate limiting for starch synthesis in many plants under other conditions. This is the first time ADP Glucose Pyrophosphorylase has been suggested to play a role in gravity-mediated changes in starch concentration. (Brown, C.)

- By using cell cultures of *Pinus taeda* (Eberhardt, *et al., Journal of Biological Chemistry*, 1993), it has been possible to attain a cell line capable of undergoing a developmental-like transition from an unlignified primary wall to a lignified secondary wall (~S_1 deposition). (Lewis, N.)

Plant Cell and Developmental Biology

- Alterations of the orientation of isolated segments of *Chara* plants affect the differentiation pattern of rhizoids which develop from these segments. While rhizoids normally have oblique division planes which occur at some distance from the tip, occasionally they develop a pattern of periclinal and anticlinal divisions at the tip which are more similar to the pattern seen in shoot tissue. (Bisson/Hollingsworth)

- Orchard grass (*Dactylis glomerata* L.) leaf segments cultured *in vitro* can be preincubated at 4°C for at least 7 days without affecting somatic embryogenesis and plant regeneration. (Conger)

- A system was developed to maintain orchard grass leaf segments in contact with agar medium in Petri dishes. Leaf segments are overlaid with 1800 µm Teflon™ mesh. The mesh is secured with a polypropylene ring which is slightly smaller in diameter that the Petri dish and which fits between the lid and the mesh. (Conger)

- Strategies have been devised for handling embryogenic plant cells and tissues prior to the initiation of an experiment in space from the perspective of having no or absolutely minimum developmental progression on Earth. Low temperature strategy have also been tested to enable investigations with totipotent plant cells to be set up and kept quiescent and thus enable an investigator to initiate an experiment with cultures by changing or modifying a single parameter. This provides a simple and direct means of initiating an experiment once it is in space. (Krikorian)

- The CHROMEX-4 payload had several scientific components and represented a coordinated effort to optimize science return. One part dealt with reproduction and seed formation; the second dealt with cell division, chromosomes, and genetic stability; the third part dealt with cell wall formation and gene expression; the fourth with non-structural carbohydrates and biochemical partitioning of carbon. Only those cells that were absolutely clearly scorable as aberrations were recorded. The number of aberrations found were far fewer than has been found in roots of other species grown in earlier space experiments. Karyotype analysis of chromosomes which were made from root cells in metaphase in both ground controls and in space flight was also carried out. No distinctive changes in karyotype were detectable. (Krikorian)

- A unifying hypothesis based on stress in space-grown plant cells and systems has been developed taking into account data gained from cytological and karyological examinations of space grown plant materials. Stated in its barest essentials, *the more developmentally advanced a system is, the less likely it is to suffer catastrophic stress effects in the space environment, the less advanced or morphologically complex it is, the greater the vulnerability.* (Krikorian)

- Data gained from nuclear examinations suggest that cells of species with large nuclei, large chromosomes and DNA content, with variously located centromeres, show signs of considerable perturbation in space. Although interpretation has been drawn from only a few species, cells of polyploids with large chromosomes that are essentially metacentric show very few disturbances. The younger the somatic embryo stage in terms of its developmental progression, the more sensitive it is; the more advanced, the less sensitive. The more polyploid the system, the more resistant to perturbation it seems to be but the higher the DNA level in the nucleus the more sensitive it seems to be. Cells of species with small chromosomes and low DNA content show far fewer mitotic anomalies. (Krikorian)

- The smaller the responding embryogenic initial, the greater the vulnerability to perturbation and hence more "damage" is shown in space (daylily). Embryogenic cells dispersed in a semi-solid medium show less perturbation than those in liquid or on semi-solid media (carrot). (Krikorian)

- A growing procedure has been developed that permits the utilization of pre-flowering plants in the Plant Growth Unit (PGU). The flexibility of the PGU has been extended for use in all stages of the plant life cycle. (Musgrave)

Gravitaxis of Micro-Organisms

Methods

- A direct computer-based numerical method for analyzing videotape images of swimming cells, acquired through a microscope, was adapted (mechanically and through the generation of dedicated software) for the task of analyzing the statistical information required for a complete description of the distribution of swimming

velocities (speed, angle, spread, sensory input correlations). The method can be used live or with video records acquired and stored previously. It is therefore suitable for analyzing microgravity and hypergravity data. (Kessler)

- Invention of the rotating cuvette method for analyzing (a) gravi-phototaxis without statistical bias, (b) gyro-phototaxis, (c) shear + gravity addition as function of centrifugal force, and (d) for analyzing sedimentation and sedimentation interactions among small particles (applications in materials science, gas/grain studies). A prototype apparatus was constructed and shown to operate as expected. A final version was shown to require much improved (but available, costly) bearings. (Kessler)

Theory

- Theoretical predictions were developed for the variation of angular width of the micro-organisms' velocity distribution with magnitude of the effective acceleration of gravity. This theory can be applied in the entire range of micro –> hypergravity. The theory is ready for experimental test, using the computer/microscope instrumentation and the NASA ARC centrifuges for initial runs at hypergravity. (Kessler)

Environmental Factors

- The mechanostimulus-inducible *TCH3* gene of *Arabidopsis* encodes a unique calmodulin-related protein that harbors 6 potential Ca^{2+}-binding EF hands. The protein is abundant in the meristematic dome and vascular tissue of 8-day-old *Arabidopsis* shoots. *TCH3* also accumulates at branch points, suggesting that *TCH3* may be regulated not only by externally applied mechanical stress, but also by physical strains that occur during morphogenesis. (Braam)

- The mechanostimulus-inducible *TCH3* gene of *Arabidopsis* encodes a protein with significant sequence to xyloglucan endotransglycosylase (XET). This is intriguing because XET has been proposed to function in cell wall loosening and cell expansion, processes necessary for thigmomorphogenesis and gravitropism. (Braam)

- The rapid (<1 minute) initial collapse of growth rate (60%–90%) by dark-brown soybean seedlings in response to a single episode of thigmic stress applied to the tip of the hook was accompanied by a 20%–50% reduction in turgor in the cell elongation zone of the hypocotyl during the first hour following treatment. Increasing calcium from 1–5 mmol in the growth medium enhanced seedling growth rate and shortened the time of recovery to the pre-stress elongation rate. (Myers/Mitchell)

- Few studies have investigated plant responses to the low irradiances available in current growth chambers for space experiments. Results emphasize the importance of increasing the light in such chambers. Although plants may appear relatively normal when grown close to the light compensation point, development is greatly delayed and yields are reduced— although yields are somewhat higher than expected because of the longer exposure to the low light. These adverse affects make it very difficult to evaluate the actual effects of microgravity. High CO_2 and substrate-moisture problems compound these difficulties. (Salisbury)

- Excellent equipment has been developed to monitor such environmental factors as CO_2, O_2, light, air and leaf temperatures, pressure, and substrate moisture in future space experiments with plants. This will make it possible to diagnose and better understand the observed plant responses. (Salisbury)

ANIMAL

Gravity Receptors and Neurophysiology

- Hair cells with differing hair bundle morphologies differ in their voltage-dependent conductances and the rate and extent of their adaptation to their bundle displacement. Vestibular nerve afferents innervating the otolith organs may derive their low-frequency response dynamics from the adaptation kinetics of their innervated hair cells. (Baird)

- Mechanisms responsible for mediating gravitationo-ocular reflexes in the bullfrog tadpole were investigated by utilizing the isolated head preparation. A method has been developed to quantify spontaneously occurring events such as action potentials recorded from extraocular motor nerves. Nose-up tilt of the isolated head results in an increase in activity of trochlear motoneurons and a reciprocal decrease in the activity of medial rectus motoneurons. Iontophoresis of antagonists to the excitatory transmitter glutamate into the extraocular motor nuclei silences motor nerve activity. Similarly, excitatory post-synaptic potentials (EPSPs) evoked by electrical stimulation of afferent pathways are reversibly abolished by these same glutamate antagonists. (Cochran)

- Galvanic polarization of the labyrinth in pigeons can selectively silence the most irregular firing afferents while leaving the regular firing afferents either unaffected or only slightly reduced. These effects have been effectively used to determine how convergence of regular and irregular firing afferents upon vestibular nuclei neurons produces responses to rotational stimulation. (Dickman)

- The anatomical orientation of the semicircular canals in pigeons were determined in order to specify the alignment between canal activation directions and afferent response directional selectivity. (Dickman)

- A correspondence between expression of S-100ß and the appearance of mature afferent synapses and myelin implies an association between S-100ß and these processes. (Fermin)

- Afferent vestibular neurons that connect the inner ear to the brain express the calcium binding protein/neurotrophic factor S-100ß in their nuclei, whereas afferent auditory neurons in the same nerve of the same animal during the same embryonic stage, express this protein diffusely in the cytoplasm and nuclei. (Fermin)

- Gravity-related otoconia in the chick inner ear are perfect calcite crystals with an organic matrix that in fixed material is distorted, but nevertheless has a close resemblance to a computer-generated model derived from assembling various two-dimensional plates of organic matrix chemically fixed and stained histochemically. (Fermin)

- Objective quantification of afferent neuron-related molecules is facilitated by color thresholding, which, contrary to gray levels of intensity densitometry, does not require a prior knowledge of specified filter combinations. (Fermin)

- Modulation of hippocampal cell activity is maintained in animals that have been exposed to hypergravic fields. Thus, even though anatomical evidence shows that the number of receptors for serotonin is decreased, there appears to be a surplus of receptors so that neural information processing is maintained and electrical activity is not altered. (Horowitz)

- Thresholds fall markedly following 19 days of incubation in the chick embryo, suggesting that normally developing gravity receptors become more sensitive to stimuli as development proceeds before and after hatching. (Jones)

- The use of purely morphological features to distinguish subclasses of type I hair cells belonging to different afferent classes provides a significant contribution to studies of the vestibular periphery. The general trends in regional synaptic innervation observed in the crista of the chinchilla, as well as in the monkey, a primate model, seem to be valid. This has implications for studies of human synaptic innervation of the vestibular periphery. (Lysakowski)

- Type I-like and type II vestibular hair cells have now been identified in two taxonomically very distinct teleost fishes, providing strong evidence that these are ubiquitous among at least telosts. This suggests that hair cell heterogeneity is very important for the function of the fish ear, and supports the suggestion that multiple hair cell types arose far earlier than with the origin of amniotes. (Popper)

- Two types of vestibular hair cells, resembling the mammalian type I and type II hair cells, are found in both the saccule and utricle of the goldfish, *Carassius auratus*. The two hair cell types are in discrete epithelial regions, lending the preparation to physiological studies of hair cell function. The morphologically different hair cell types can be closely correlated with physiologically distinct hair cells that have been reported in the literature for goldfish hair cells. (Popper/Lanford)

- Biochemical studies strongly suggest that hair cell heterogeneity may extend to the fish lateral line. Only one type of hair cell in the lateral line is damaged by the ototoxic drug genamicin sulphate, and these cells also regenerate, even in the presence of the drug. (Popper/Song)

- A study of rat macular hair cell synaptic plasticity conducted on the Space Life Sciences 1 (SLS-1) mission demonstrated that plasticity is retained into adult stage. Synapses were increased by ~55 percent in type II cells and by ~41 percent in type I cells in animals that had flown compared to ground animals. Tissues were collected beginning 4.5 hours after landing of the Shuttle (R + 0). (Ross)

- Much more transmission electron microscopical data were collected, illustrating the origin of efferent terminals from afferent nerve fibers and calyces. These terminals end on type II hair cells, other nerve fibers, and on calyces. This means that the number of efferent terminals of extrinsic origin are fewer in number than is commonly believed. (Ross)

- There is a shift toward the sphere-like central body in ribbon synapses of both kinds of hair cells in maculas of R + 0 flown rats ($p = <0.0001$). This may indicate a generation of new synapses since the sphere-like ribbon is considered to be the primitive form by developmental biologists. There is also an increase in pairs and in clusters of synapses in type II hair cells of R + 0 flight rats compared to controls ($p = <0.0001$). Stress may influence synaptic number in the macular hair cells. (Ross)

- The development of the statocyst of *Aplysia* was studied in embryos and larvae reared at 1, 2, 3, and 5.7 g. The statolith in embryos becomes progressively smaller as the g force is increased. At increasing g-levels, the percentage of statocysts with no statolith increases. Similarly, post-metamorphic specimens produce fewer and smaller statoconia at high g-levels. (Wiederhold)

- The time course of appearance and growth of the otolith organs and semicircular canals in larvae of the Japanese red-bellied newt, *Cynops pyrrhogaster*, has been established. Three morphologies of otoconia have been identified: in the adult utricle and early larval utricle and saccule, barrel-shaped otoconia are made of calcite; at a later stage, first fusiform and then prismatic otoconia appear in the saccule, and both of these forms are made of aragonite. (Wiederhold)

Development

- Hair cells in the bullfrog vestibular otolith organs regenerate following aminoglycoside ototoxicity. Regenerating hair cells, recognizable by their short, well-formed hair bundles, can be classified into distinct hair cell types by the same morphological criteria used to classify their mature counterparts. The 5-bromo-2'-deoxyuridine (BrdU) labeling studies suggest that hair cell recovery in the vestibular otolith organs takes place via both mitotic and non-mitotic mechanisms. (Baird)

- The frog egg develops a functional bilateral symmetry before fertilization. The source of this axial polarity does not arise during oogenesis as previously thought but is acquired during meiotic maturation, when reorganization of the cytoskeleton permits gravity-driven cytoplasmic rearrangements to occur. This finding provides an explanation for the observed wide variability in the spatial relationship between the point of sperm entry and the process of dorsal-ventral axis specification. (Danilchik)

- Parallel experiments were performed on slow clinostat and in space flight to examine the effects of altered gravity on the aggregation of the nicotinic acetylcholine receptors and the structure of the cytoskeleton in cultured *Xenopus* embryonic muscle cells. Concordance of results between space flight and the clinostat show the slow clinostat is a relevant simulation paradigm. (Gruener)

- Space-flown cells showed marked changes in the distribution and organization of actin filaments and had a reduced incidence of acetylcholine receptor aggregates at the site of contact with polystyrene beads. The sensitivity of the synaptic receptor aggregation and cytoskeletal morphology suggests that in the microgravity of space cell behavior may be importantly altered. (Gruener)

- Gravity normally plays a role in the establishment of the dorsal ventral axis during early development of the amphibian *Xenopus laevis*. A clone was isolated for an RNA found to be dorsally enriched during the first cleavage cycle in *Xenopus*. Sequence analysis indicated that this clone represented the small subunit of mitochondria. *In situ* hybridization analysis showed that mitochondria become localized to the presumptive dorsal side of the embryo shortly after fertilization. The relocation of mitochondria to the presumptive dorsal side of the embryo was found to be gravitationally sensitive. (Phillips)

- Results show that excitable cells are sensitive to alterations in the perceived gravity field. The function of acetylcholine receptor (AChR) channels, however, is not drastically affected, possibly due to a process of cellular adaptation. Taken together, these results indicate that the development of the neuromuscular junction may be affected during exposure to microgravity. (Reitstetter)

- The identification of neuroregulatory peptides, neurotransmitters, and pituitary hormones at key stages of development is critical to the fundamental understanding of reproductive system maturation and function, the control systems that orchestrate these processes, and how they may be affected by conditions in space. The data we are gathering are essential to recognize and correctly interpret the influence of hypogravity

and other environmental conditions of space flight on the neuroendocrine regulation of the reproductive system. (Schreibman)

- Tobacco hornworm (*Manduca sexta*) pupae, which normally undergo adult development in a horizontal position, are sensitive to a head-up vertical position as evidenced by (1) more rapid development within 7 days of inversion, (2) elevated hemolymph concentrations of alanine and phenylalanine, and (3) increased protein content of the fully developed dorsolongitudinal flight muscle. (Tischler)

- The *hsp70.2* gene is testis-abundant, not testis-specific. It is expressed at lower levels in other adult murine tissues and in predominantly extra-embryonic tissues in the mid-gestation conceptus. At the level of *in situ* hybridization, we confirmed the developmentally regulated pattern of expression of *hsp70.2* during mouse spermatogenesis. The expression of Hsp70 and Hsp90 in the adult testis was confirmed, at the protein level, to be most abundant in mouse germ cells from pachytene spermatocyte to round spermatid stages. Hsp90 protein was found to co-immunoprecipitate with a protein of 70 kDa, which appears to be a member of the Hsp70 family. Studies on the expression of *hsp70.2* have revealed the surprising observation of transcripts in both the sense and antisense orientations from this area of the genome. (Wolgemuth)

Bone and Muscle

- Alendronate, a bisphosphonate, can prevent the bone loss subsequent to skeletal unloading. The data suggest that alendronate or like drugs may be useful for preserving bone during long-term space flight. However, this drug inhibited normal bone modeling and may be detrimental to the growing skeleton. (Bikle)

- Determining the mRNA and protein levels of insulin-like growth factor-1 (IGF-1), IGF-2, and their receptors from fetal life into old age permits us to gauge changes in these important skeletal growth factors at different ages and provides correlative information regarding the role of these factors in skeletal development. (Bikle)

- Space flight and hindlimb elevation increases IGF-1 mRNA and protein. The MRNA levels for IGF-1 do not decrease with skeletal unloading as was expected from the fall in bone formation; rather, they increase, suggesting a compensatory mechanism for resistance to IGF-1 observed during skeletal unloading. (Bikle)

- Hindlimb elevation results in a decreased ability of IGF-1 and growth hormone to stimulate bone growth. Understanding the nature of this resistance could provide the key to understanding the decline in bone formation with skeletal unloading. (Bikle)

- Differences were demonstrated in stromal cell proliferation in cells obtained from the bones of young and old rats. If such cells remember their history of loading *in vivo* (in addition to age) when evaluated *in vitro*, this model will permit us to perform a number of experiments *in vitro* to attempt to unravel the decline in osteoblast function during skeletal unloading. (Bikle)

- Parathyroid hormone is a potent stimulator of bone resorption and could be involved in the process of microgravity-induced bone loss. Two immediate early gene responses which may be fundamental to PTH-altered gene expression in the osteoblast have been identified and characterized. (Clohisy)

- Observations derived from non-beating, passively loaded, rabbit heart cells indicate that catecholamines can maintain and even promote the fractional rate of heart cell growth

and modulate the turnover of contractile proteins, but adrenergic activation is incapable of inducing the assembly of these nascent proteins into myofibrils in non-beating rabbit cardiac myocytes. (Decker et al.)

- Endosteal osteoblasts of compact bone of the tibia from 9-day SLS-1 flight show less alkaline phosphatase activity than the adjacent precursor cells. Pro-collagen-containing secretory granules in osteoblasts showed an increase in numbers during 9-day recovery period following SLS-1 flight. (Doty)

- Preliminary results from the 14-day SLS-2 flight indicate that the alkaline phosphatase activity of endosteal osteoblasts was reduced by space flight. (Doty)

- The micromass cultures of embryonic mouse limb mesenchyme flown aboard International Microgravity Laboratory 1 (IML-1) showed little, if any, matrix produced during space flight. Until late in the mission (4–5 days) the flight cells were abnormally smooth, having no matrix associated with the cells. (Duke)

- Chronic, intermittent mechanical strain (CMS) applied to osteoblasts increases whole-cell conductance, mediated through the mechanosensitive channel. These data suggest that mechanical strain may prime the mechanosensitive channel to tonically respond to continued strain. Furthermore, CMS also up-regulates the production of bone matrix proteins, type I collagen, osteopontin, and osteocalcin. The up-regulation of osteopontin and osteocalcin is independent of Vitamin D stimulation. These observations suggest that CMS produces a more differentiated osteoblast and that the signaling mechanism for CMS may be transduced through the mechanosensitive channel. (Duncan)

- Modeling the endosteum and periosteum of bone as a monolayer of osteoblasts grown on filters shows osteoblasts represent a significant barrier to flux of fluid, and the hydraulic conductivity can be regulated by calcitropic hormones, calcitonin, and parathyroid hormone. (Frangos)

- Studies have provided strong evidence that the ATP-dependent proteolytic system involving ubiquitin and the proteasomes is the major degradative pathway and is activated by a variety of physiological stimuli that lead to muscle wasting, including stress (e.g., cortisol) and disuse. (Goldberg)

- Results indicate that unweighting of the soleus muscle by tail-cast suspension causes a marked increase of insulin-stimulated glucose transport activity, with no enhancement of the insulin-independent pathway for glucose transport. (Henriksen)

- Prostaglandin E_2 (PGE_2) acts as bone cytokine. When prostaglandin is added to serum deprived osteoblasts, cell growth is increased from 2–3 times the control. Prostaglandins induce oncogene message synthesis. Prostaglandin causes a 20-fold increase in expression of c-fos; in addition, the c-fos response of the osteoblast is proportional to the amount of PGE_2. The rise in c-fos expression is followed by an increase in c-jun. Also, messages for ß-actin and γ-actin are induced with the addition of prostaglandin. (Hughes-Fulford)

- The decrease in cytoplasm to nuclear ratio in atrophied muscle fibers could suggest that when lower cytoplasmic levels are maintained the availability of myonuclei are no longer a modulatory factor in controlling fiber size. (Kasper)

- Eccentric exercise training (lengthening muscle contractions) during non-weight bearing attenuated, but did not prevent, the loss of soleus muscle wet weight and non-collagenous protein by 77 and 44 percent, respectively. (Kirby)

- The absence of increases in skeletal muscle cAMP during the first 4 hours after a single 10- or 60-minute bout of running by untrained rats suggests that either cAMP is not part of the mechanochemical link between endurance exercise and increased mitochondrial density or that the response of the other elements in the adenylate cascade may correlate better with run duration. (Kirby)

- An investigation of the temporal relationship between extracellular matrix formation, collagen assembly, and mineral deposition in a bone cell (osteoblast) culture of embryonic chicken calvaria showed that mineral formation is dependent on the state of maturation or degree of assembly of collagen fibrils. (Landis)

- Collagen deposition in a bone cell (osteoblast) culture of embryonic chicken calvaria has been studied and was shown to be controlled at several post-translational stages during fibrillogenesis, including cross-link formation, lateral fibril formation, and increasing fibril stability. (Landis)

- Studies of a bone cell (osteoblast) culture of embryonic chicken calvaria grown in the presence of vitamin D_3 [$1,25(OH)_2D_3$] have demonstrated that different initial osteoblast cell populations yield more mature cells of a greater heterogeneous nature. The effects appear to depend upon the embryological or developmental stage. (Landis)

- The mineral crystals from bone cell (osteoblast) cultures of embryonic chicken calvaria and normal calcified tissues have been described for the first time by high-voltage electron microscopy and three-dimensional image reconstruction methods. (Landis)

- Alendronate dramatically increased cancellous bone mass in growing animals by decreasing both bone and cartilage resorption; however, the drug also inhibited bone formation. (Morey-Holton)

- During a 9-day space flight, individually housed growing rats showed more dramatic bone changes than group-housed animals. During recovery, the group-housed animals appeared to recover more rapidly that the individually housed rats. (Morey-Holton)

- Within 24 hours of return from space flight, young, growing rats showed ~300 percent increase in steady-state levels of mRNA expression of proteins associated with bone matrix formation. (Morey-Holton)

- The morphology of the rat osteosarcoma cells flown on Spacelab-Japanese was slightly different compared to our ground controls, suggesting that microgravity causes slight retraction of osteoblastic cells from the substratum. Rat tissue inhibitor of metalloproteinases-2 was cloned, and regulation in osteoblastic cells was examined. Parathyroid hormone increases transcription of this gene as a primary response through cAMP-dependent pathway. Collagenase is expressed as a late-differentiation gene in normal, differentiating, mineralizing rat osteoblasts. (Partridge)

- A specific domain within the type I collagen promoter is utilized in bone cells. This region is distinct from sequences required for promoter activity in other type I collagen producing tissues. This is called the basal bone element, and it is believed that it will be crucial in understanding the molecular control of collagen formation by activated bone cells. (Rowe)

- The type I collagen promoter transgene containing 3.5 kb of 5 ft. flanking sequence responds to the signal for the osteoblast to increase bone formation in response to mechanical loading. (Rowe)

- Satellite cell mitotic activity of the rat soleus and extensor digitorum longus muscle decreases below weight-bearing control levels within 24 hours of the initiation of hindlimb unweighting. Hindlimb unweighting reduces the quality and quantity of regeneration in rat soleus muscles. The reduction appears to be related to a slowed development of fibers during regeneration rather than a slowed formation of fibers, the result of reduced satellite cell proliferative activity during the early phase of regeneration. (Schultz)

- Focal contractions and hyperstretch lesions demonstrated weaker, gradually diminishing, staining pattern; while wide-A, opaque, and missing-A regions demonstrated abrupt absence of staining. (Thompson, J.)

- Lesions produced as the result of the application of eccentric loads to atrophic muscle show compromised immunostaining with antimyosin antibody, indicating alterations in the myosin protein conformation between lesioned and non-lesioned areas. (Thompson, J.)

- The effects of hindlimb unloading on single-fiber function (fiber atrophy, decreased peak tension, and increased maximum shortening velocity) of the slow type I fibers of the soleus were confirmed. As pH was lowered from 7.0 to 6.2, both single-fiber force and velocity decreased significantly, suggesting that the fibers from hindlimb unloading animals are less resistant to H^+ ion. Hindlimb unloading induces a shift from type I fibers with slower velocities to fibers with faster velocities. The fibers exhibiting the fastest velocities showed the greatest reduction in force as pH was lowered. This suggests that the increased fatigability following hindlimb unloading is in part caused by an increased susceptibility of the contractile apparatus to H^+ ions. (Thompson, L.)

- Juvenile rats flown on STS-48 showed a similar pattern of hindlimb muscle protein changes (i.e., soleus atrophy, reduced growth of plantaris and gastrocnemius), a comparable intensified effect of insulin on soleus glucose uptake, and elevated soleus interstitial fluid volume as has been observed using the tail-cast hindlimb suspension model. (Tischler)

- Atrophy of the juvenile soleus muscle due to hindlimb unweighting is characterized by a preferential loss of myofibrillar proteins, which show accelerated protein degradation not evident for non-myofibrillar proteins in the same muscle. (Tischler)

- Heightened effects of insulin, insulin-like growth factor, and catecholamines were demonstrated to be physiologically significant in the soleus of unweighted hindlimbs by injecting the muscle and conducting in situ measurements of hormone responsiveness. (Tischler)

- Previous voluntary running does not lessen the effect of unweighting on atrophy of the soleus muscle. (Tischler)

- Studies with glucocorticoid antagonist, RU 38486, showed that unweighting atrophy of the soleus is definitely not a consequence of elevated circulating glucocorticoids or increased muscle binding capacity for these hormones. (Tischler)

- Older rats exposed to short-duration microgravity showed fewer changes in bone structure and material properties than younger rats. Therefore, animal age and endocrine status are important factors in predicting the duration of microgravity exposure that would induce significant changes in bone structure and material properties. The load history of the muscle plays an important role in the compartmentalization of collagen and its maturation during growth and development. (Vailas)

- There is a residual amount of lysylpridinoline in hypertrophic cartilage of newly hatched chicks. These findings suggest that the unusual presence of this crosslink of non-mineralized fibrocartilage may be associated with a change in calcification. (Vailas)

- Significant interactions occur between mechanical forces and growth factors in regulating skeletal muscle size when tissue-cultured skeletal myofiber cultures are used. Mechanical stimulation increases both the sensitivity to, and efflux of, anabolic autocrine/paracrine cytokines and growth factors such as prostaglandins and insulin-like growth factor-1. Stress-related glucocorticoids cause muscle atrophy, and mechanical stimulation protects myofibers from this atrophy by generating anabolic cytokines of the prostaglandin family. Two molecular mechanisms have been identified by which muscle stretch increases cytokine production: (1) increased synthesis and activity of cyclooxygenase, the enzyme which synthesizes prostaglandins; and (2) activation of several phospholipases, which increases the availability of arachidonic acid from phospholipids. Both processes are dependent on stretch-activated G proteins. (Vandenburgh)

- The protein matrix of sea urchin embryo calcitic spicules is a collection of over 30 different proteins. Specific antibodies have been raised against two of them; anti-SM50 and anti-SM30. Antibody localization studies show that these proteins are located in different portions of the spicule matrix. These same antibodies have been used to show that adult mineralized tissues contain these same matrix proteins. Epitope tagged versions of the mRNA for SM50 are expressed in the embryo after the mRNA is microinjected into the zygote. (Wilt)

Regulatory Biology

- Hypophysectomy blocks the xylazine-induced increase in angiotensinogen, as does adrenalectomy, but replacement treatment with glucocorticoids in adrenalectomized rats restores the response. Therefore, it appears that xylazine acts by an extrapituitary route but requires the presence of glucocorticoids for the response to occur. (Ganong/Reid)

- Recent studies have (a) confirmed the localization of nitric oxide synthase in the macula densa of rabbits using the nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemical technique, (b) demonstrated that inhibition of nitric oxide synthesis with L-N^{g}-nitro-L-arginine methyl ester (L-NAME) in conscious rabbits markedly suppresses the renin secretory response to activation of the macula densa control of renin secretion by furosemide, and (c) demonstrated that inhibition of nitric oxide synthase increases vasopressin secretion in conscious rabbits. These observations provide new evidence that nitric oxide participates in the regulation of renin and vasopressin secretion. (Ganong/Reid)

Cell Biology

- Dermal fibroblasts detect mechanical force vectors and rearrange their cell bodies to align parallel to the applied force. Corneal fibroblasts fail to re-align. Force perception/response requires integrity of the actin cytoskeleton and may also depend on intercellular connections and cell/matrix adhesion. (Grymes)

- Mechanical force interacts with growth factor signal transduction pathways in dermal fibroblasts of a genetic disease condition (Werner's syndrome). A normal platelet-derived growth factor (PDGF) response profile is recovered during mechanical stretch and through adaptation to a pliable culture substrate. (Grymes)

- Clinostat g-force averaging inhibits contraction of collagen gels by corneal stromal fibroblasts, while application of oscillating rotational forces enhances contraction. Environmental forces interact with cellularly generated forces in matrix reorganization behaviors. (Grymes/Johnson-Wint)

- A magnetic twisting device was developed which allows one to apply controlled mechanical stresses directly to specific transmembrane receptors and simultaneously measure the cellular response in living cells. Using this device, it was demonstrated that integrin receptors act as cell surface mechanoreceptors and transmit mechanical signals to the cytoskeleton via a specific molecular pathway. (Ingber)

- Mechanical measurements obtained with the magnetic twisting device provide experimental data in support of the tensegrity (tensional integrity) model of cell architecture described in previous research. New studies with three-dimensional tensegrity models demonstrate that use of this form of architecture by cells could explain how remodeling of the cytoskeleton occurs at the molecular level in response to mechanical stress application. (Ingber)

- The concept of "solid state" signaling is proposed, in which mechanochemical transduction would be mediated simultaneously at multiple locations by force-induced cytoskeletal rearrangements that result in redistribution of associated elements of the cell's metabolic machinery. (Ingber)

- In both 3T3 mouse osteoblasts (Hughes-Fulford) and *Xenopus* muscle cells (Gruener) grown on small coverslips in the Materials Dispersion Apparatus flown on STS-56, the actin cytoskeletal filaments were coalesced, disorganized, and showed significantly altered morphology compared to ground controls. This strongly suggests that the cytoskeleton may be involved in altered cell response to space flight and that microgravity sensing directly involves the cytoskeleton. (Lewis/Hughes-Fulford/Gruener)

- Mono- and polyclonal antibodies were generated for the characterization of microtubules, microtubule-organizing centers, and the centrosomes, as well as to investigate the dynamic microtubule reorganizations necessary for the union of sperm and egg nuclei, the formation of the mitotic apparatus and further development. The role of calcium for these processes was investigated by interfering with calcium-requiring events using inhibitors such as dithiothreitol. These inhibitors interfered to varying degrees with the organization of cytoskeletal proteins, with membrane fusions, and with the formation of spicules during later development. (Schatten)

- Developed the optical and electronic hardware, as well as the software of a powerful and inexpensive system to detect changes of intracellular Ca^{2+} concentration with high temporal and spatial resolution. (Verdugo)

- Discovered that the free ionized fraction of Ca^{2+} inside the endoplasmic reticulum of ciliated cells does not remain constant at millimolar levels as it had been thought. Instead it stays at submicromolar concentration, but it can reach submillimolar levels following stimulation. (Verdugo)

Immunology

- The *in vitro* activation of B-lymphocytes was tested on STS-56 (April 1993) with human spleenic cells exposed during flight to lipopolysaccharide (LPS), *S. aureus cowan* (SAC), anti CD3, and CD40 ligand. Post-flight analyses showed no significant difference between flown and ground control cells. This is in agreement with other reports that B-cells do not appear to be affected by microgravity. (Lewis/Neil)

- Mouse T-lymphocytes stimulated with *Staphylococcal Enterotoxin* (SEB) of anti-CD3 monoclonal antibody in the presence of MHC-presenting cells on small coverslips were flown in the Materials Dispersion Apparatus on STS-56. The T-cells initiated DNA synthesis indicating that T-cell receptor-mediated activation is not impaired during space flight. This is in contrast to the consistently blunted response of T-lymphocytes flown in the presence of Con-A. This differential activation of T-cells in microgravity can provide a tool for investigating mechanisms of abnormal or altered cellular responses to mediators. (Lewis/Principato)

- Antiorthostatic suspension by the tail enhanced the capacity of mice to resist a primary infection with virulent *Listeria monocytogenes*. It also enhanced the capacity of mice to express protective immunological memory during a secondary infection with virulent *Listeria monocytogenes*. (Miller)

- Thymus cells harvested from rats flown on STS-54 produced enhanced titers of interleukin-3 (IL-3) and interleukin-6 (IL-6) compared to ground controls. Spleen cells harvested from the same rats produced enhanced levels of IL-6 but normal levels of IL-3. (Miller)

- Whole body antiorthostatic suspension did not influence the capacity of neutrophils harvested from rats to generate the oxidative burst. (Miller)

- Gentle dispersion and clinorotation had similar inhibitory effects on *in vitro* lymphocyte activation by mitogenic lectins. Data suggest that dispersion of cells and other indirect environmental effects significantly contribute to the inhibition of lymphocyte activation observed in clinostats and that inhibition occurs at very early steps regulating entry into the activation sequence. (Sams)

- Phorbol esters and ionophores, which bypass the surface events and directly activate intracellular signal transduction systems, allow the initiation of early activation events in suspended or clinorotated lymphocytes. The data suggest that alteration in surface signaling is responsible for the inhibition of lectin-induced lymphocyte activation in clinostats. (Sams)

- Current study suggests that space flight can inhibit the differentiation of precursor cells to mature macrophages. This result is in accordance with previous human and animal

studies which suggest that the macrophage is a key cell in space-flight-induced immune dysfunction. (Sonnenfeld/Miller)

PLANT PROJECTS

GRAVITY-INDUCED ALTERATION IN DIFFERENTIATION IN *CHARA*

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Description of Research

Conversion Structures:

Chara corallina is a freshwater alga with a complex morphology with rhizoids and shoots comprised of multicellular nodal complexes separated by long intermodal cells. The peripheral cells and basal branchlet cells of the node can give rise to axillary shoots and rhizoids. Shoots are thick, with a diameter around 250 μm, and green. They show a characteristic pattern of anticlinal and periclinal tip divisions, and they are negatively gravitropic. Rhizoids are thin, having a diameter around 30 μm, and colorless. They display oblique division planes often far from the apex, and are positively gravitropic. Isolated plant segments consisting of internodes with the nodal complex on each end can be experimentally cultured so that rhizoids and axillary shoots initiate from both nodes.

Occasionally, anomalous differentiation occurs, in which some rhizoids later develop shoot-like properties. We call these shoot-like rhizoids "conversion structures." Two types of conversion structures can take place: tip division and greening. Only tip division was affected by orientation with respect to gravity (Table 1). We call this phenomenon "gravi-morphogenesis."

Table 1. Summary of normal and anomalous rhizoid development in isolated *Chara* nodal complexes
[Mean ± SD, summary of 4 experiments]

Condition	Total Rhizoids	Dividing, %	Greening, %
Normal	549±302.16	3.62±3.35	5.40±5.07
Inverted	641±358.79	8.34±5.43	5.40±3.68

In order to determine the time at which cellular differentiation is fixed, one-half of the nodes were reversed in orientation after a certain number of days. Nodes that were originally normal were switched to inverted, and those originally inverted became normal. In two experiments, one-half of the segments were reversed in orientation on day 7, when no conversion structures were apparent.

Accomplishments and Significance of the Accomplishments

Segments which were reversed in orientation developed as expected for the final orientation. That is, segments whose orientation was changed from normal to inverted had a greater percentage of rhizoids with dividing tips than segments in the normal orientation. Segments whose orientation was changed from inverted to normal had a lower percentage than segments maintained in an inverted orientation. This indicates that the signal resulting in enhanced tip division was not fixed at 7 days. Inversion on day 11 or day 16 resulted in an enhanced conversion rate in segments whose orientation was altered from normal to inverted. Segments whose orientation was altered from inverted to normal at these later days, however, did not show a decreased number of conversion structures. This suggests that a decision made to convert, between 7 and 11 days, is

not readily reversed; but a normal rhizoid can still convert on a later day if its orientation occurs later.

Molecular Biology:

Since changes in differentiation entail changes in gene expression, we began experiments to characterize gene expression in *Chara*. We have generated cell-specific cDNA libraries from shoot and rhizoid tissue by the Haselkorn method (Bauer et al., 1993, PNAS (USA)90: 8812), using N_6-oligomers to generate small (200 bp) cDNA from rhizoids and shoots, as well as larger cDNA libraries by conventional methods. Whole alga DNA has also been isolated for generation of a genomic library.

We are at present screening the cDNAs to determine whether there is differential expression of ATPase isoforms, which are known to be differentially expressed in higher plants both in a tissue-specific manner and in response to environmental stimuli.

Publications

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CALMODULIN-RELATED PROTEINS IN MECHANOSTIMULATED PLANTS

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Description of Research

The long-term goal of this research is to understand the role(s) of the TCH proteins of *Arabidopsis thaliana* in the plant's responses to mechanical stimuli, including gravity, touch, wind, and rain. The TCH genes were discovered due to their strong and rapid induction of expression following stimulation of plants with mechanical stimuli. Three of the TCH genes encode calmodulin-related proteins, strongly implicating involvement of a calcium ion signaling system in the mechanosensory pathway of *Arabidopsis*. Very recently we have found that a fourth TCH gene product is closely related to a xyloglucan-specific endotransglycosylase. This is exciting because these enzymes may be involved in cell wall loosening and cell expansion. To gain insight into the functions of the TCH proteins, we are overproducing them in *E. coli* for direct functional assays and also for generating antibodies as specific tools for monitoring the accumulation, stability, localization, and post-translational modifications of the proteins. As we dissect the functions of the TCH proteins in response to varied mechanical stimulation, we hope to shed light on the complex and undoubtedly important roles of the calcium ion as a second messenger in plant responses to its environment. Through an understanding of the cellular events that occur in plants following mechanical stimulation, the mechanism(s) and signal transduction pathways that lead to mechanostimulus-induced developmental changes will be elucidated.

Accomplishments

(1) We have isolated, mapped, and sequenced the genomic locus and nearly full-length cDNA of the TCH3 gene. The sequence information obtained indicates that TCH3 is novel in several respects: (a) It encodes a calmodulin-related protein (60 percent identity) that has six potential calcium ion binding sites. (b) The conceptual protein product contains linker regions joining pairs of calcium ion binding sites that are distinct from the linker region of calmodulin in that they are significantly longer (17 amino acids). (c) The TCH3 gene may have evolved relatively recently, as the 5' end of the gene (including sequence encoding of the first two calcium ion binding sites and the first intron) is a nearly identical duplication of the region encoding the second pair of calcium ion binding sites.

(2) We have isolated, mapped, and sequenced the genomic locus and nearly full-length cDNA of the TCH4 gene. Although TCH4 expression is regulated similarly to the TCH1-3 genes, it is unique in that the conceptual protein sequence is unrelated to calmodulin. However, our analysis indicates that the conceptual TCH4 protein is significantly similar to regions of *Arabidopsis Meri-5* (80 percent identical) and to a xyloglucan-specific endo 1,4 b-D glucanase enzyme (50 percent identical) postulated to function in cell wall expansion. We have already succeeded in generating antibodies against a portion of the TCH4 protein that is unrelated to *Meri-5*. Our preliminary evidence suggests that this antibody recognizes a single protein from *Arabidopsis* cells and is therefore likely specific for the TCH4 protein. This antibody will be a valuable tool in assessing the accumulation, localization, and function of the TCH4 protein. We are currently generating transgenic antisense TCH4 *Arabidopsis* plants and TCH4-gusA fusion plants for tools to examine the *in vivo* functions of the TCH4 protein and the regulation of expression of the TCH4 gene.

(3) We have generated polyclonal antibodies directed against the region of the TCH3 protein containing the first through fourth calcium-binding sites. This antiserum is able to recognize the TrpE-TCH3 fusion protein used as antigen and the b-galactosidase-TCH3 fusion protein (demonstrating recognition of the TCH3 portion of the protein). This antibody is specific in that it is unable to detect the TCH1 and TCH2 fusion proteins, indicating that the antibody does not recognize a nearly full-length calmodulin protein (TCH1) or a calmodulin-related protein (TCH2). This is important because of the presence of multiple expressed calmodulin genes and calmodulin-related proteins in plants. These data cannot rule out the possibility that other calmodulin-related proteins in *Arabidopsis* will be recognized by the TCH3 antibody; however, as shown by western analysis, a single band of the expected size (33-kD) is detected in total protein from cultured *Arabidopsis* cells. Our immunolocalization experiments demonstrate that the TCH3 protein is abundant in the meristematic dome and vascular tissue in 8-day-old *Arabidopsis* shoots. TCH3 also accumulates at branch points. These studies of TCH3 are actively continuing to determine the developmental and tissue-specific localization and accumulation after stimulation in *Arabidopsis* plants.

Significance of the Accomplishments

Finding 1: Complete characterization of the TCH3 genomic locus provides us with information concerning the primary structure of the conceptual protein product. As described above, TCH3 has several features indicating that, although it is a calmodulin-related protein in that it possesses EF-hand structures predicted to be capable of binding calcium ions, it is significantly distinct from calmodulin itself and therefore very likely has distinct functions. Indeed, the region of TCH3 corresponding to the domain believed to be important for interaction with target enzymes diverges from calmodulin. This feature suggests that, if TCH3 protein functions to regulate target enzymes, the targets are likely distinct from those of calmodulin.

Finding 2: The molecular characterization of the TCH4 gene is also a valuable step forward in determining the identity, functions, and control of this mechanostimulus-regulated gene. Sequence analysis indicates that the conceptual protein product of the TCH4 gene is significantly similar to enzymes involved in cell wall modifications and possibly cell expansion. This is intriguing because it would be expected that thigmomorphogenesis and gravitropism (mechanostimulus responses) would require either an alteration in the orientation of cell expansion or asymmetric cell wall expansion, respectively.

Finding 3: Immunodetection of the TCH3 protein using the newly generated polyclonal antiserum has demonstrated that, in young *Arabidopsis* shoots, the protein is abundant in the meristematic dome, vascular tissue, and branch points. The significance of the localization to the meristem is not yet clear, and it should be noted that accumulation of protein or RNA in this region may reflect the relative abundance of small cells rich in cytoplasm (this localization is often seen with "housekeeping" molecules). The accumulation in branch points is especially intriguing, as these are the regions that would be expected to be under mechanical strain during development, thus suggesting that TCH3 may be regulated not only by externally applied mechanical stress, such as wind or touch, but also by physical strains that occur during morphogenesis. These studies of TCH3 are actively continuing to determine the developmental and tissue-specific localization and accumulation after stimulation in *Arabidopsis* plants.

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KENNEDY SPACE CENTER PLANT SPACE BIOLOGY LABORATORY FLIGHT EXPERIMENT SUPPORT

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Description of Research

Growing plants in the microgravity of space comes with a unique set of problems and opportunities. For example, the lack of biologically significant gravitational force has been shown to impact the growth, metabolism, and reproductive capability of plants. Additionally, there are unusual horticultural concerns in microgravity which must be addressed, including appropriate nutrient delivery and lighting subsystems.

The overall goal of the research effort at the Kennedy Space Center Plant Space Biology laboratory is to provide scientific advice and testing of plant flight hardware. To this end, studies are conducted (1) to determine if gravity influences photosynthesis and carbohydrate metabolism in higher plants by using the ground-based techniques clinorotation, centrifugation, and/or tropistic stimulation; (2) to determine the impact of microgravity on carbohydrate metabolism and photosynthesis in higher plants; and (3) to investigate nutrient delivery and lighting systems which could have application for plant culture in microgravity.

Accomplishments

(1) In 6-day-old soybean cotyledons, the activity of the starch biosynthetic enzyme ADP glucose pyrophosphorylase was reduced in clinorotated plants and was increased in centrifuged plants relative to the control plants (Figure 1). None of the five other starch metabolic enzyme activities measured were affected.

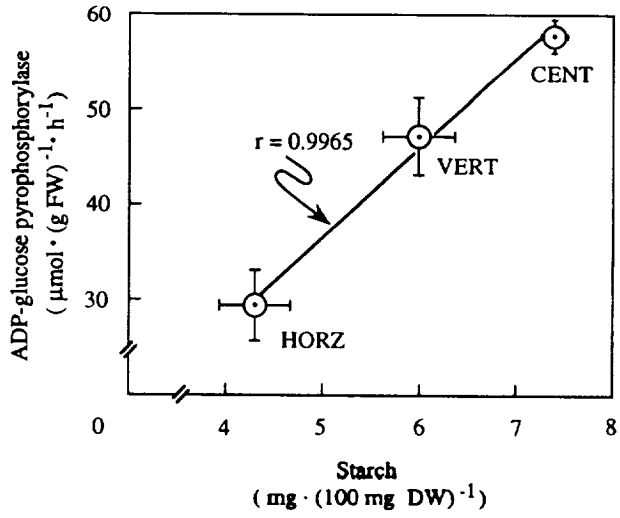

Figure 1. Relationship between ADP glucose pyrophosphorylase activity and starch concentration in soybean cotyledons exposed to altered gravity conditions. Horizontal clinorotation (HORZ), vertical rotation (VERT) and centrifugation at 5g (CENT).

(2) Using *in vivo* protein labeling with 1-D and 2-D PAGE analysis, no changes in soluble protein expression were found in chronically rotated *Arabidopsis* (21-day treatment) and maize (6-day treatment) plants compared to the non-rotated control plants. There were slight differences in growth.

(3) The porous tube nutrient delivery system was configured for testing during parabolic flight aboard the KC-135. Control of tube surface moisture levels was maintained on all of the tubes, regardless of pore size (0.3, 0.7, and 2.0 μm) at all of the gravity levels (0.01 - 1.8g).

(4) Investigations into the use of light emitting diodes (LEDs) for supporting plant growth commenced. Reductions in growth, photosynthetic rates, and disease resistance were noted in pepper plants grown under red (660 nm) or red+far red (660 +735 nm) compared to the metal halide-grown control plants. The addition of just 4.2 μmol m^{-2} s^{-1} of blue light (1.4 percent of the total photosynthetically active radiation [PAR]) significantly reduced these differences.

(5) Wheat seedlings grown under high-intensity red light from LEDs did not synthesize chlorophyll in their shoots. The root-perceived inhibition of chlorophyll biosynthesis in the leaves was caused by the impairment of the Mg-chelatase enzyme working at the origin of the Mg-tetrapyrole pathway.

Significance of the Accomplishments

Finding 1: Changes in soybean cotyledon starch concentration due to altered gravity treatments (lower in clinorotated and higher in centrifuged plants) appears to be directly related to ADP glucose pyrophosphorylase activity. This enzyme is known to be rate limiting for starch synthesis in many plants under other conditions. This is the first time ADP glucose phosphorylase has been suggested to play a role in gravity-mediated changes in starch concentration.

Finding 2: Although clinorotation can be a severe stressor to plant growth and metabolism, we found that chronically clinorotated *Arabidopsis* and maize plants do not show physiological and biochemical symptoms of severe stress (reduction or retardation of growth, marked changes in protein banding patterns). Although clinorotation is not a substitute for microgravity, it may be suitable for certain ground-based studies.

Finding 3: The porous tube nutrient delivery system, which utilizes the capillary movement of water through ceramic tubes to supply plant roots with moisture and soluble nutrients, is under consideration as a possible microgravity nutrient delivery technique. The KC-135 parabolic flight results provide strong evidence that the system will contain and supply water under conditions other than Earth normal gravity. Plans for testing the system under microgravity conditions on the Space Shuttle are underway.

Finding 4: Because of their minimal mass, volume, and wavelength specificity, LEDs are promising as a radiation source for intensive plant culture systems, such as space-based research chambers or bioregenerative life support systems. For pepper plants, it appears that while red light (660 nm) alone will support plant growth, low levels of supplemental light in other regions of the spectrum (blue, 300-500 nm) are necessary for optimal growth.

Finding 5: The use of high-intensity LEDs has been suggested for the culture of plants in space. However, there may be developmental problems to be overcome, such as the inhibition of chlorophyll biosynthesis in leaves caused by high-intensity red light. Blue light appears to mitigate this response and may be an important addition to the spectrum.

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PLASMODESMATA AND THE CONTROL OF GRAVITROPISM

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Description of Research

Gravitropic curvature occurs when there is an unequal rate of cell elongation on the two sides of a non-vertical plant organ; e.g., in stems the lower side grows faster than the upper side with the result that the stem curves upward. This difference in rate of cell elongation is due to an asymmetric distribution of the growth hormone, auxin. The normal downward polar auxin transport stream becomes diverted laterally in non-vertical stems. I have proposed a new theory to explain how this occurs. My research is directed toward a direct test of this theory.

Polar auxin transport occurs when auxin, taken up symmetrically into cells, is exported by auxin efflux carriers restricted to the base of these cells. This results in a directional flux of auxin from cell to cell. However, plant cells are interconnected by protoplasmic tubes, the plasmodesmata (PDM), which are believed to pass any molecule smaller than the size exclusion limit (SEL) of about 800 Da. If so, auxin should backflow into the exporting cell through the PDM and equalize the auxin, preventing polar transport. I propose that auxin moves through cells which perceive gravity and that in these cells the PDM are closed in the vertical direction but open in the horizontal direction. This prevents backflow of auxin and permits polar auxin transport in a basipetal direction. When the stem is reoriented horizontally, the lateral PDM close, because they are now the "vertical" PDM, and polar auxin is now lateral rather than longitudinal. This idea can be tested by microinjecting fluorescent dyes into such cells and determining the direction of movement by epifluorescence microscopy. It is predicted that if a small amount of dye is injected into cells of a vertical stem, it will move laterally because the PDM are open in this direction. It will not move vertically because PDM are closed in this direction. When the tissue is placed in a horizontal orientation, the injected dye will now move longitudinally rather than laterally.

In order to learn this technique, I participated in a project in the laboratory of Dr. W.J. Lucas, at the University of California, Davis. I examined the question as to whether the size exclusion limit of PDM of wheat roots is affected by intracellular adenosine triphosphate (ATP), as there had been a suggestion that reduction in ATP levels would cause PDM to close. I therefore determined the effect of anaerobic conditions and azide on the SEL of these roots. It was shown that both treatments resulted in a reduction of intracellular ATP to less than 10 percent of control levels. In addition, I examined the possibility that ATP, itself, might be able to move from cell to cell via the PDM.

Accomplishments

(1) I have shown that when the intracellular ATP levels are reduced by azide or by anaerobiosis, the size exclusion limit of wheat root epidermal and subepidermal cells increases from <1 kDa to 5-10 kDa within 30 minutes.

(2) I have shown that when a fluorescent analogue of ATP, 2'0-(trinitrophenyl) adenosine-5'-diphosphate (TNP-ADP) (mol wt 681), is injected into wheat root cells, it is able to move freely through the PDM to neighboring cells.

Significance of the Accomplishments

Finding 1: The fact that a reduction in cellular ATP causes the SEL of PDM to increase nearly 10-fold indicates that cell-to-cell communication in plants is under direct metabolic control. This raises the possibility that constriction of PDM to prevent movement of molecules >1 kDa is due to phosphorylation of some specific PDM protein by a protein kinase. The idea that PDM might be able to regulate cross-talk between cells in this way is a new one.

A second point is that these data indicate that when root cells are subjected to localized anaerobiosis, a frequent stress for plant roots, they are able to compensate for the stress by increasing PDM conductance. This should facilitate movement of sugars into the anaerobic region, increasing anaerobic respiration. In addition, it should permit reduced beta-nicotinamide-adenine dinucleotide (NADH) to escape the anaerobic cells and move to an aerobic environment where it can be converted to NAD^+, further increasing anaerobic ATP production.

Finding 2: The fact that TNP-ADP can move freely from cell to cell through the PDM means that cells are interconnected in terms of their energy charge, since ATP and ADP should be able to equilibrate from cell to cell through the PDM. The idea that plant cells have a common pool of ATP has never been considered before. It means that cells can cooperate in production of ATP, with cells with a high ATP demand not having to have a high rate of ATP production themselves.

These results are going to have a major impact on the way that PDM are considered in plants and the mechanisms of cell-to-cell communication in plants.

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EMBRYOGENESIS OF GRAMINEAE IN MICROGRAVITY

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Description of Research

The overall objective of this research is to provide information on the influence of microgravity on embryo initiation, differentiation, development, and the ultimate reproductive capacity of resultant plants utilizing an *in vitro* culture system in orchard grass (*Dactylis glomerata* L.) in which the target cells remain *in situ*. Somatic embryos form directly from mesophyll cells in cultured leaf segments and develop fully to a germinable stage. The basal 3 cm of the youngest two leaves are split down the midvein. These are cut transversely into segments approximately 3 mm square. Segments from one-half leaf are used for various treatments, and the corresponding sister segments serve as controls. This provides a precise control for each treated leaf segment and the opportunity to use paired statistics for the analyses of data. Observations and data will be collected on quantity and quality of embryo formation, axis determination, and polarity. This will involve estimation of embryo number using an already developed formula and extensive histology. Plants will be established from somatic embryos and used for mitotic chromosome analyses. The same plants will be transferred to the field and used for meiotic chromosome analyses and estimation of pollen fertility.

The findings will aid in corroborating or refuting those results previously obtained with other plant tissue culture systems, as well as provide new information. In addition to the advantages and uniqueness of the system already mentioned, the experiments provide the opportunity to obtain data on a species representing the *Gramineae*.

Accomplishments

We have been conducting work on this project for approximately 9 months. During this time, the research has been concentrated on developing our system into a flight experiment. Among the most important factors is adherence of the leaf segments to the medium during vibration and vertical orientation as might be encountered during different phases of a space flight. We have devised a system in which the leaf segments are overlaid with either polypropylene or Teflon™ mesh after they have been placed on agar solidified medium. To hold the mesh in contact with the medium and leaf segments, a polypropylene ring, approximately 8 mm wide and slightly smaller in diameter than the Petri dish containing it, is placed on the mesh inside the dish before covering the dish with the lid. The lid holds the polypropylene ring firmly to the mesh. This scheme is illustrated in Figure 1. Segments from one-half of each of the innermost and next leaf outward are plated serially from the basal to the distal portion. The corresponding segments from the sister leaf halves are plated identically except that they are not overlaid with mesh; these, as mentioned above, serve as controls.

So far, we have conducted experiments with mesh having openings of 710 µm, 1800 µm, or 2200 µm. The mesh was kept on top of the leaf segments for 2 weeks and then removed. This time period was chosen because the duration of Shuttle missions is usually about 10–14 days.

Preliminary data indicate either no influence from overlaying with mesh or a slightly improved response. Response in terms of relative growth (qualitative observations) and plant regeneration appears to be better with mesh having larger rather than smaller openings. Segments overlaid

with mesh respond equal to, or better than, those not overlaid with mesh (controls). We are still in the process of conducting experiments and collecting data.

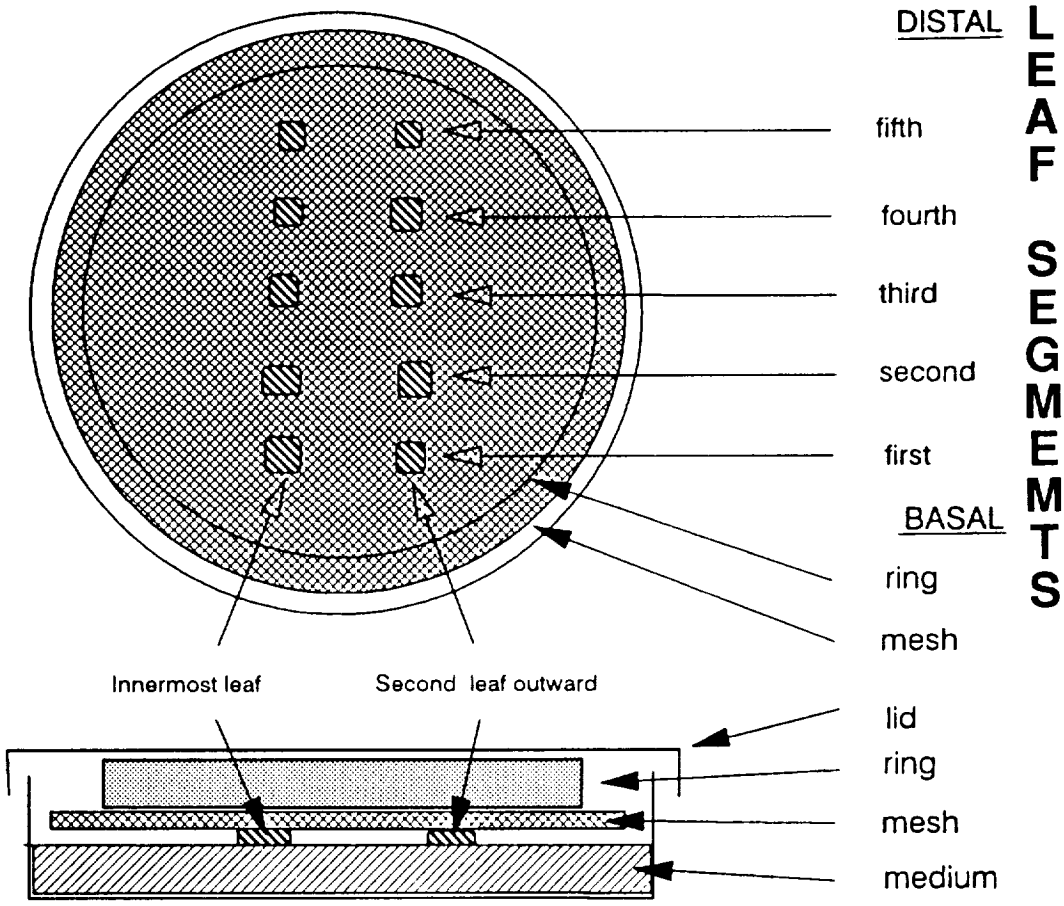

Figure 1. Diagram of mesh scheme to hold orchard grass leaf segments in contact with the medium. After the segments are plated onto the medium, a piece of mesh of exact diameter as the inside of the Petri plate is placed on top of them. A polypropylene ring of slightly smaller diameter is then placed on top of the mesh. The width of the ring is such that it fits exactly between the mesh and the lid of the Petri plates.

Significance of the Accomplishments

Adherence of the experimental material, in this case, orchard grass leaf segments, to agar medium during various stages of a space flight is extremely important to success of the experiment. The frequently observed improved response by the overlay of our leaf segments with mesh may be due to the mesh holding more of the leaf surface in contact with the medium. Our system may improve agar stability and, moreover, have application, in either its present or modified form, to other biological systems.

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MECHANISM OF DIFFERENTIAL GROWTH DURING STEM GRAVITROPISM

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Description of Research

Plant growth is responsive to a number of internal and external stimuli, including gravity. Gravity modulates the biophysical and biochemical processes that govern plant cell expansion, such that plants tend to grow in a fixed orientation with respect to gravity and they revert to this orientation following displacement. This gravity response, known as gravitropism, is mediated by a bilateral asymmetry in growth of roots and stems. We are investigating the cellular and biochemical mechanisms underlying gravitropic growth asymmetries to gain insight into the fundamental mechanisms controlling plant growth and to learn how gravity modifies these processes.

We study young cucumber seedlings (*Cucumis sativus* L.) because their stems display a vigorous gravitropic bending upward after they are turned on their side. This reorientation is accomplished by a transient cessation of cell elongation on the upper stem surface and a simultaneous increase (twofold) of the growth rate on the lower stem surface. The growth asymmetry begins to reverse itself well before the stem attains a vertical position. These fine modulations of cell growth occur in a stem that is only 1.5 mm in diameter; that is, cells can be quite close to one another and still react in opposite fashion to a gravitropic stimulus. By studying this gravitropic response, we hope to elucidate how plants integrate sensory stimuli and use this information to control their growth and form.

Our previous studies showed that cell turgor pressure and membrane hydraulic conductivity play a secondary, passive role, not a controlling role, for this gravitropic response. Our studies showed that water transport poses an insignificant limitation to cell expansion in cucumber seedlings and that the major control point for plant growth rests in the biophysical relaxation of the wall.

We are now studying the biochemical basis for wall relaxation, with the working hypothesis that wall enzymes catalyze the wall modifications that give rise to wall stress relaxation. A major puzzle in this field is the nature of these wall enzymes and the mechanisms by which their activity may be regulated. We have developed a cell-free system for studying the biochemistry of cell wall expansion (see Figure 1), and we recently reported the first isolation of wall proteins responsible for the "acid-growth" response of wall.

Accomplishments

(1) A novel class of proteins was identified with the ability to induce extension of isolated walls. We call the proteins "expansins." They are found in an active form in cucumber walls obtained from the growing region of the cucumber hypocotyl. Cucumber walls contain two active proteins of 29 kD and 30 kD nominal molecular mass (determined by sodium dodecyl sulfate-polyacrilamide gel electrophoresis [SDS-PAGE]). These proteins induce extension of walls that are held in tension in an acidic buffer. They exhibit many of the peculiar biochemical sensitivities that characterize "acid-growth" of native walls; e.g., acidic optimum, activity enhancement with thiol reducing reagents, activity inhibition with 1 mmol aluminum, copper and mercury ions, and survival of brief treatment in boiling methanol.

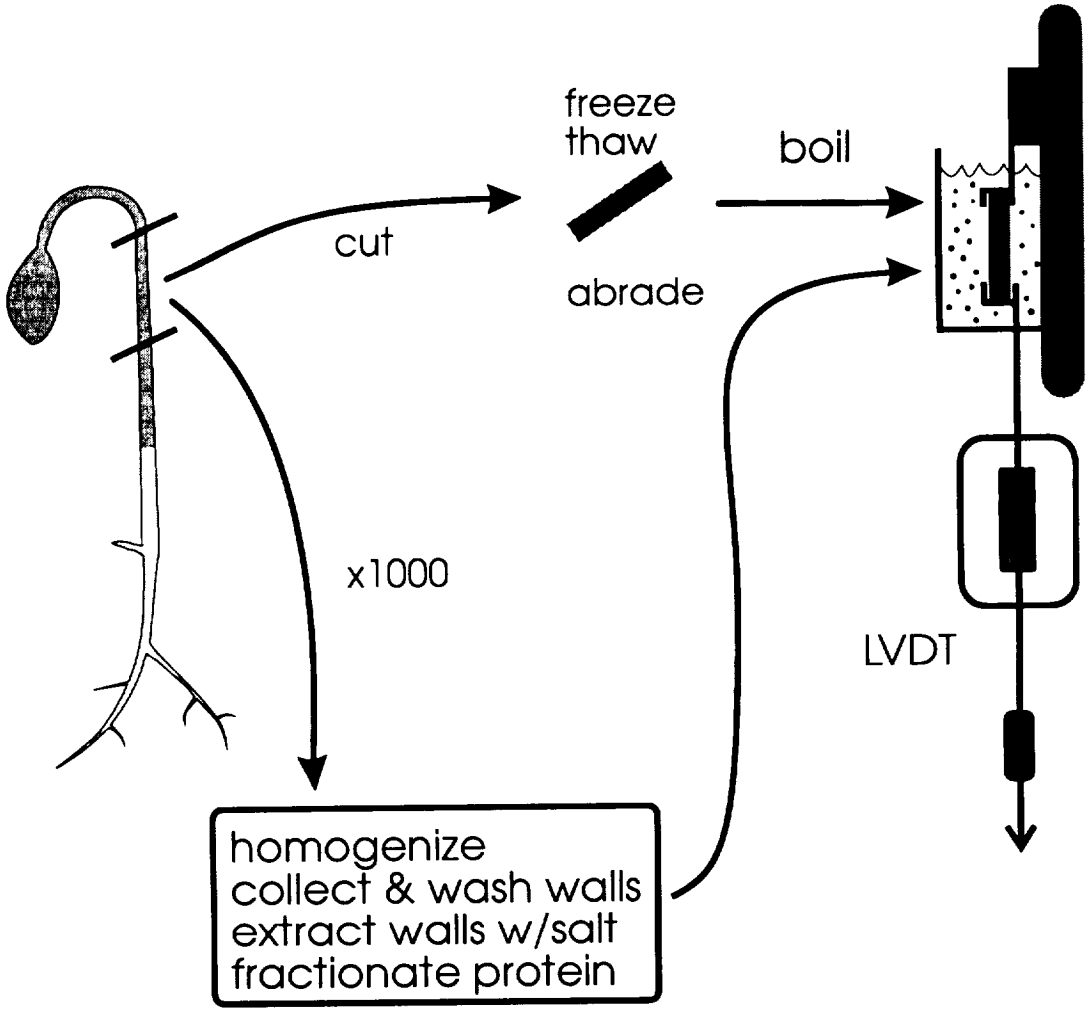

Figure 1. Diagram of our reconstitution system for identifying proteins that catalyze wall extension. The growing region of etiolated cucumber seedlings are used as the biological material because their walls display stable and sensitive wall-extension characteristics. As substrate for the reconstitution assay, the growing hypocotyl is frozen and thawed to kill the cells, abraded with carborundum powder to permeabilize the cuticle, boiled in water (15 seconds) to inactivate the endogenous proteins, and clamped in a constant-force extensometer. Protein fractions with suspected extension activity are added to the cuvette surrounding the wall sample, and extension responses are measured with a position transducer (linear variable displacement transducer [LVDT]) attached to the bottom clamp.

(2) A wall protein antigenically related to cucumber expansin-29 can be extracted from oat coleoptile walls. We purified wall extension proteins from oat coleoptile walls by high-pressure liquid chromatography (HPLC) and related methods in combination with our wall extension reconstitution assay (Figure 1). The single active fraction contained a single major protein band of 29 kD. In Western blots, this protein was recognized by an antibody raised against cucumber expansin (the 29 kD form).

(3) Treatment of cucumber hypocotyls with 1 μmol auxin leads to a change in the redox state of the cell wall, such that wall protein sulfhydryls become more reduced. This conclusion was established by labeling tissues (with or without auxin pretreatment) using an impermeant fluorescent thiol reagent. Wall-bound proteins were then extracted with 1 mol NaCl. The protein-associated fluorescence doubled in the auxin-treated samples, yet the total molar amount of protein remained little affected.

(4) Light treatment of oat coleoptiles reduces their growth rate and also reduces their ability to grow in response to auxin, fusicoccin, and acidic buffers. Moreover, their walls lose responsiveness to acidic buffers and to exogenous expansins. They also reduce their rate of gravitropic bending. In other words, it appears that the ability of the walls to respond to various growth-stimulating agents is reduced after light treatment.

Significance of the Accomplishments

Finding 1: ***Identifies specific wall proteins that mediate the acid-extension responses of isolated walls.*** This is the first identification of proteins with this ability. Because acid-growth responses are nearly ubiquitous in plants with cellulosic walls, we infer that these novel proteins are important for the extension growth of most plant cells. Acid growth has been implicated in the action of auxin, light, and gravity on plant cell enlargement. Therefore, it is plausible that these stimuli affect growth via effects on expansin action.

Finding 2: ***Indicates that expansins are found in grass walls, and thus may be ubiquitous components of plant cell walls.*** It is notable that the polymeric composition of matrix polysaccharides in dicot walls and grass walls is very different; yet expansins are effective on both types of walls. This implies that the biochemical mechanism for acid-induced wall expansion is common to these distantly related plants. Both dicot and grass shoots are thought to respond gravitropically via an auxin-induced differential acidification of the wall space; we postulate that expansins are the wall protein factors that translate a wall pH asymmetry into a wall expansion asymmetry. (We recognize that other mechanisms of wall expansion may additionally be involved).

Finding 3: ***Is important because it shows that auxin can cause changes in the thiol redox state of wall proteins.*** This fact, in combination with the observation that expansin activity is stimulated by thiol reducing reagents, suggests that a part of auxin action and gravitropism may involve control of wall redox state. We are now testing for asymmetries in wall redox potential during gravitropism.

Finding 4: ***Indicates that changes in wall susceptibility to expansin action may prove a mechanism for controlling plant growth.*** The results also provide correlative evidence in favor of the idea that expansins play an important role in plant responses to light, auxin, and gravity.

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SEARCH FOR STRUCTURAL PROTEINS HAVING POTENTIAL FUNCTION IN GRAVITY SENSING AND FOR DIFFERENTIAL EXPRESSION OF GENES IN GRAVITROPISM

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Description of Research

Gravitropism displayed in plants and fungi involves detection, transduction, growth response, and resolution. The molecular foundations of each of these phases is arguably more complex in higher plants, whose responsive organs are multicellular and, often, where the tissue of gravity detection is separated from the tissue undergoing the growth response. These gravitropic mechanisms, however, are just as likely to have some common evolutionary cores with those organisms that have single-celled gravitropic structures, such as the sporangiophore of the terrestrial fungus, *Phycomyces*, which has been the focus of our research.

Several approaches are being undertaken to understand the mechanisms for the detection of gravity, the secondary messenger mechanisms with a particular emphasis on calcium-related functions, and the genetic interplay in the response. Until recently we have been attempting to understand various membrane ion channels in *Phycomyces* sporangiophores whose characteristics might inherently be useful in one or more aspects of gravitropism. Our inability to satisfactorily identify the membrane or stabilize certain channels that appeared to be stretch-activated has lead us to reassess this approach.

Last year we (a) investigated the *in situ* immunolocalization of cytoskeletal proteins, the properties of which might lend themselves to a fretwork used to detect alterations of the cell's orientation to the gravitational field; (b) began work to sequence *Phycomyces* calmodulin; and (c) continued fine-tuning our study of the effect of gadolinium on gravitropism of *Phycomyces* and some of its gravitropic mutants. Calmodulin continues to hold our interest because it has been found to be differentially expressed in higher plants with respect to mechanical stimulation such as touch (and, likely, gravity), and a protein partially purified from *Phycomyces* has shown calmodulin-like activity (Martinez, et al., 1982).

This year we (a) are confirming the presence of cytoskeleton and related proteins of protein extracts using immunoidentification procedures; (b) preparing to perform an mRNA subtraction or possibly differential display in order to determine what, if any, genes are activated/expressed upon exposing the fungal sporangiophore to gravity stress; and (c) probing for second messenger proteins likely involved in coordinating orientation to gravity and growth response.

Accomplishments

(1)We have *in situ* localized polyclonal antibodies to globular (g)-actin and chicken spectrin to a thin-lined area at the plasma membrane of the *Phycomyces* sporangiophore using immunofluorescence. Though control tissues were negative, the use of polyclonal antibodies could result in an anomalous reaction. These results are being confirmed using the more specific monoclonal antibodies.

(2) Total protein extracts from *Phycomyces* sporangiophores and cytoskeletal protein extracts using variations of an extraction procedure developed for yeasts have revealed the presence of two 40–50 kDa bands in the range expected for g-actin in Western blots using monoclonal actin

antibody. The cytoskeletal extraction has produced Westerns with two 180–200 kDa proteins in the expected weight range for myosin with monoclonal myosin antibody and two faint bands in the range of 100–120 kDa with monoclonal integrin immunoassay. Assays for spectrin have been inconclusive.

(3) Wheat calmodulin cDNA (gift of R. Zielinski, University of Illinois at Urbana-Champaign) hybridized with two closely associated bands from *Phycomyces* genomic DNA in a Southern blot indicates that a calmodulin gene resides in *Phycomyces* and bears homology to wheat calmodulin. Although a protein with calmodulin-like activity has been described from *Phycomyces*, neither the protein nor the gene has been sequenced. In order to sequence, DNA primers were constructed from two regions homologous in the calmodulin cDNA sequences from wheat and soybean, and three fungal species, *Neurospora, Candida,* and *Achlya*. Using these primers, we have obtained, using PCR, a 339-base pair (bp) fragment that hybridizes to both wheat calmodulin cDNA and genomic *Phycomyces* DNA in Southern blots. Thus, we believe this fragment to be a portion of the *Phycomyces* calmodulin gene, and we are presently sequencing the fragment. We will be able to extend this fragment and/or will be able to use the fragment as a probe for the differential expression of calmodulin in gravity-stressed and unstressed cells.

Significance of the Accomplishments

Findings 1 and 2: The presence of actin and myosin is to be expected in *Phycomyces*, since cytoplasmic streaming is evident in the sporangiophore. Our results support this assumption. Integrins are a group of proteins involved in linking or anchoring the cytoskeleton to an extracellular matrix in many animal cells. The presence of an integrin-like protein in *Phycomyces* sporangiophores leads us to speculate on its possible role in rigidly anchoring the cytoskeleton to the cell wall. We further speculate on the possibility of its serving to locate/activate proteins in the cell membrane that would assist in the growth response to altered stress in the cytoskeleton due to the orientation of the sporangiophore with respect to gravity.

Finding 3: The calmodulin gene from *Phycomyces* has common homologies to both higher plant calmodulins and calmodulins from other fungi. The 339-bp fragment of the gene obtained by PCR, after being sequenced, will be used in an effort to sequence the entire gene. The fragment alone will be a useful probe in determining whether calmodulin is differentially expressed in gravity-stressed sporangiophores as it is in stressed *Arabidopsis* stem tissue (Braam and Davis, 1990).

ROOT GRAVITROPISM IN MAIZE AND *ARABIDOPSIS*

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Description of Research

This research is directed toward understanding the influence of gravity on plant growth, in particular how roots become oriented with respect to gravity (gravitropism). The detection of gravity occurs at the tip of the root while adjustments in growth occur about 2-5 mm behind the tip. We are interested in the nature of the signal moving from the cap to the responding zone, and we want to determine whether gravity sensing is restricted to cells in the cap. We seek to identify the cells showing the earliest growth changes following stimulation. This will allow us to concentrate our efforts on these cells in regard to the influence of gravity on factors likely to control growth (e.g., hormone distribution, hormone sensitivity, electrical properties).

Our recent research has centered on the following: (1) Analysis of the differing effects of the growth regulating hormone, auxin, on cells in different regions of the root; (2) Analysis of the nature of the gravitropic response in roots pretreated with high levels of auxin, the hormone thought to control the differential growth leading to curvature; (3) Modification of the video digitizer system to allow analysis of the small roots of *Arabidopsis* seedlings, so we may utilize auxin response mutants of this species to analyze gravitropism in plants with reduced auxin sensitivity; and, (4) Characterizing the surface electrical potential pattern in maize roots with emphasis on gravity-induced changes in this pattern and its spatial and temporal correlation with growth pattern changes during gravitropism.

Accomplishments

(1) Auxin inhibits the elongation of cells in the main elongation zone but promotes the elongation of a group of cells close to the meristem after a lag of about 1 hour. This region of the root has been referred to as the Postmitotic Isodiametric Growth (PIG) zone or, alternatively, as the Distal Elongation Zone (DEZ). The promotion of elongation in the DEZ accounts for the ability of roots to adapt to inhibiting levels of auxin. The root resumes growth because of the delayed promotion of elongation in the DEZ. Cells in the elongation zone remain inhibited.

(2) Roots treated with a concentration of auxin sufficient to inhibit elongation 100 percent show strong curvature upon gravistimulation. The curvature results from strong enhancement of growth along the top of the root in the DEZ, just as occurs in untreated controls. Growth along the bottom is nil because of the high auxin concentration.

(3) We successfully modified the video digitizer system to allow analysis of growth in roots of *Arabidopsis*. The timing of the auxin response in these roots is similar to that reported in other species. We have analyzed the auxin dose/response relationship in wild type seedlings as well as the auxin resistant mutants axr1-3, axr1-12, aux1, and axr2. We find that there is no shift in the dose-response curve along the concentration axis but that the mutants show enhanced promotion by low doses of auxin, as well as reduced inhibition by high doses. The mutants show impaired gravitropism, but the degree of gravitropic responsiveness appears to correlate more with absolute growth rate than with auxin sensitivity per se.

(4) There is a distinct surface potential distribution along the apical region of maize roots. The potential is rapidly but transiently reduced in a biphasic pattern upon stimulation of the root

surface (e.g., touching at a localized point). The region of the root most sensitive to electrical perturbation upon stimulation corresponds with the DEZ; i.e., it is the same region showing the earliest growth response following gravistimulation.

Significance of the Accomplishments

Finding 1: The finding that auxin promotes elongation in the DEZ but inhibits in the elongation zone is significant because it alters our perception of this hormone as one which can only retard the elongation of intact roots. It is also significant because we know that there are periods during the gravitropic response when growth is strongly enhanced in localized regions of the root. The observation that the elongation of certain cells can be enhanced by auxin raises localized auxin elevation as a possible regulator of growth acceleration during gravitropism.

Finding 2: The finding that roots with elongation rates reduced to zero by treatment with auxin can exhibit gravitropic curvature with kinetics similar to that of control roots is an especially significant observation. We have shown that the pattern of growth acceleration along the top of the root is essentially the same in gravistimulated auxin-inhibited roots as in controls. This tells us that the acceleration of elongation in the early stages of gravitropism is not auxin mediated and that gravistimulation can cause strong growth enhancement in cells on the upper side of the DEZ even in the presence of high concentrations of auxin. The conclusion is that gravity-induced growth acceleration is auxin-independent. The major theory of root gravitropism until now holds that differential growth is accounted for by auxin redistribution. This finding shows that this idea is wrong, at least as applied to the growth acceleration component of gravitropism.

Finding 3: Our successful modification of the digitizer system to allow analysis of *Arabidopsis* mutants opens the door for a more detailed analysis of the role of auxin in root growth and gravitropism. This will allow us to thoroughly analyze gravitropism in a number of recently generated mutants.

Finding 4: The finding that DEZ cells show the largest electrical perturbation in response to localized stimulation suggests that these cells may be particularly important in root sensory responses. Cell-to-cell electrical transmission has been suggested as a possible mode of signal transmission during root gravitropism. Further characterization of electrical changes in the cells which show the earliest growth response to gravistimulation should add insight regarding the role of membrane potential changes in signal transmission or alteration of growth patterns.

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PHYSIOLOGICAL, BIOCHEMICAL, AND MOLECULAR PROCESSES ASSOCIATED WITH THE TRANSDUCTION OF GRAVITY IN ROOTS OF MAIZE

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Description of Research

Our research focuses on understanding the mechanisms used by plants to convert the physical stimulus of gravity into a developmental response. This work is performed with roots of corn. Normally, when roots are reoriented to the horizontal, they perceive this changed orientation and grow downward. We know that this change in orientation is perceived and then biochemically processed in a special region at the tip of the root known as the root cap. Within the root cap it is hypothesized that certain biochemical steps, collectively called signal transduction steps, occur. We are investigating the hypothesized involvement of specific proteins, known as kinases, in signal transduction. Based upon models developed from animal and microbial systems, it is suggested that kinases in the root cap function to convert the physical stimulus of gravity into a developmental response. Although the mechanism of operation of kinase has been well defined from other non-plant organisms, there is no reason to believe that the basic elements of kinase operation differ in plants. These basic elements include a compound called a second messenger, which regulates the activity of the kinase (i.e., does it function?), and a target (usually another protein) which the "turned-on" kinase then affects. For this effort we are, (1) investigating the presence of second messenger kinases in root caps, (2) investigating what proteins are regulated by kinases, and (3) determining whether root cap kinases mediate the biochemical processing of gravity.

Accomplishments

We undertook a molecular approach in order to determine whether second messenger kinases operate in root caps. We constructed a cDNA library from root caps and then screened this library with oligonucleotides fashioned after conserved regions of other second messenger kinases. Using this approach, we have obtained several putative second messenger kinases. One, which we have called 90.7 (Biermann and Feldman, 1992), has all the features characteristic of kinases which fall into calcium/calmodulin, cAMP-regulated kinases. In addition, 90.7 autophosphorylates and is expressed to a high level in the root cap. In order to test effects of possible regulators on 90.7 protein kinase activity, we developed a sensitive method for detecting regulation of autophosphoryl labeling of protein kinases in unfractionated corn root protein extracts. We tested a wide variety of potential second messengers, including calcium and plant hormones. Although we were not able to show that any of these compounds increased 90.7 activity, we could demonstrate that intermediates of carbohydrate metabolism (e.g., sugar phosphate intermediates) down-regulated the autophosophorlyating activity of the 90.7 kinase. This indicates that the regulation of kinases in plants, specifically the 90.7 kinase, is different from that known in animals and fungi, further suggesting operation of unique protein kinase regulatory mechanisms in plants. Details of this work are in Biermann and Feldman (1992). In addition to finding the 90.7 kinase, we have also recently sequenced a kinase which has all the characteristics of a calcium/calmodulin II kinase (CaMK II). This kinase was isolated from a root cap library, and we are now in the process of defining how the activity of this kinase is regulated. Based upon the finding of a CaMK II in root caps, we undertook a study of the effects of drugs which are supposedly specific inhibitors of CaMK II. One such drug, KN-93, was applied to roots which

were then reoriented with respect to gravity. We found that this drug prevented the root from completing the gravity response, curving downward, but did not inhibit root elongation. These results are detailed in the publication by Lu, Feldman, and Hidaka (1993) and provide strong evidence that a CaMK II pathway, or one involving CaMK II homologues, operates in root caps and is involved in processing the gravity signal.

Significance of the Accomplishments

During the previous year, we have investigated the hypothesized role of protein kinases in mediating the conversion of the physical stimulus of gravity into a developmental response. A significant accomplishment of this work has been the demonstration of several different kinases in root caps as well as our having shown that, when we inhibit the activity of a kinase apparently belonging to the calcium/calmodulin subfamily of kinases, we simultaneously affect the gravitropic response. These data thus suggest that not only are second messenger regulated kinases present in roots but also, when the activity of one of these kinases is inhibited, we can prevent roots from responding to gravity.

Publications

Lu, Y-T, Feldman, L.J., and Hidaka, H. 1993. Inhibitory effects of KN-93, an inhibitor of $Ca2^{+}$/calmodulin-dependent protein kinase II, on light-regulated root gravitropism in maize. *Plant Physiology and Biochemistry* 31: 857-862.

A NEW METHOD FOR FOLLOWING THE GRAVITROPIC RESPONSE IN MAIZE ROOTS

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Description of Research

The primary objective of this work has been to develop a continuous measure of the bending force output of gravitropically responding roots. Our long-term goal is to develop a means of following the mechanical parameters associated with the gravitropic bending response in maize roots in order to build a true biomechanical picture of the process. Additionally, we hope to provide a more representative study of gravitropic bending by providing mechanical constraints against which the bending root must act.

Traditional methods for following the gravitropic response involve goniometric measurements of unrestrained roots bending in humid air. Besides being a highly unnatural environment for roots, the traditional method affords no opportunity to analyze mechanical factors in the bending response.

Accomplishments

We have developed a method which allows us to follow the bending force output of the responding root.

We place the root between two narrowly separated horizontal Teflon™ surfaces which act as an artificial crevice to restrain downward bending. The forces generated in the vertical direction are measured continuously with a Vitrodyne V-200 mechanical testing frame. The load generated by the root is measured as a restoring force necessary to maintain constant displacement between the two Teflon™ surfaces as the root tries to force them apart by bending downward.

Initial characterization of the system shows pH, calcium, and light dependence of the response. Decapping also eliminates all bending force production by the root. Simultaneous axial growth measurements reveal a link between gravitropic bending and root elongation.

Characteristic curves representing bending force production over time are shown in Figures 1-3. Figure 1 shows root bending force output in distilled water buffered to pH 7.0. Figure 2 shows a dramatic increase in force output in 10 mmol $CaCl_2$. Figure 3 shows an equally dramatic depression of force output when available calcium in the medium is bound by EGTA* . These curves illustrate the dynamic nature of force production by the root and show a typically multi-modal character. Superimposed on these plots are curves illustrating overall root extension determined by simultaneous time-lapse photography.

* Ethylene Glycol-bis (β-Aminoethyl Ester) N,N,N',N',-Tetraacetic Acid.

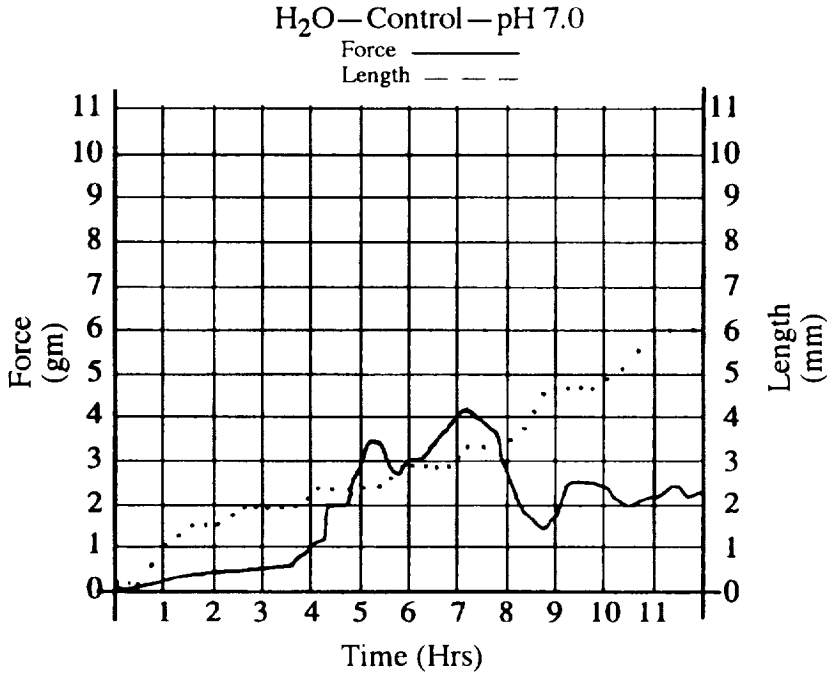

Figure 1. Root bending force output in distilled H_2O.

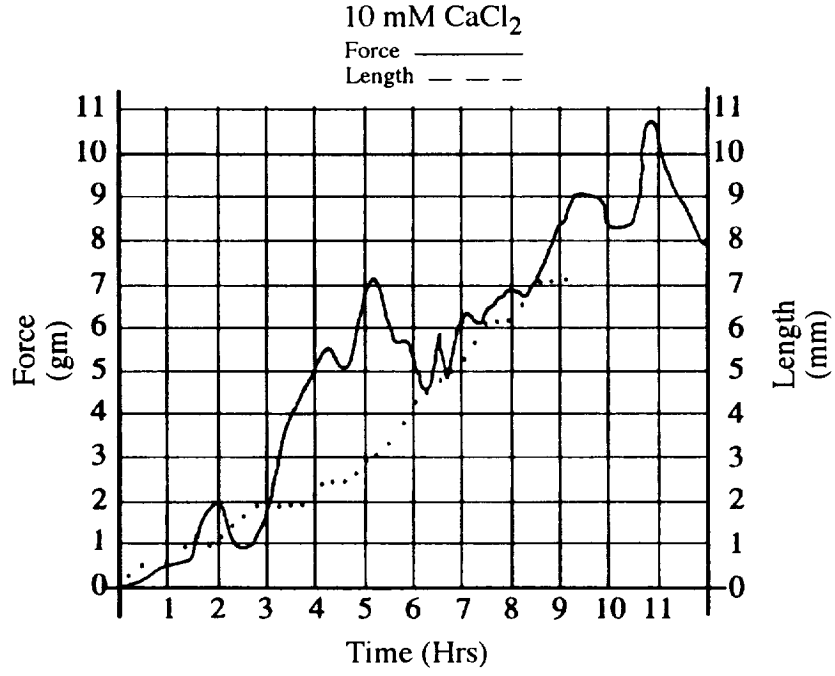

Figure 2. Increase in root bending force output in $CaCl_2$.

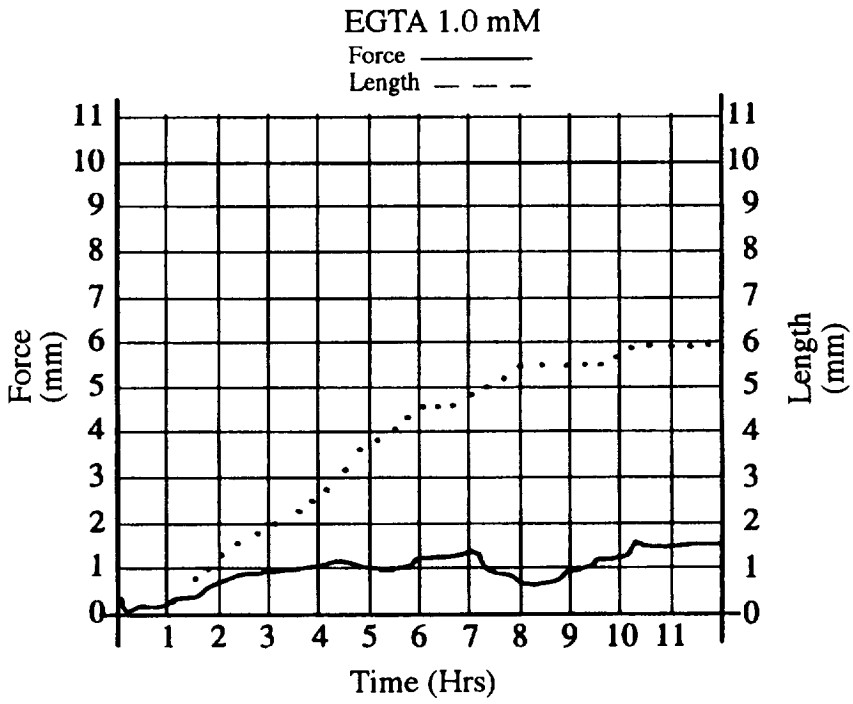

Figure 3. Depression of root bending force output with calcium in the medium bound by EGTA.

Significance of Accomplishments

The forces produced during gravitropic bending by the root axis are a reflection of differential growth in the zone of elongation, and they serve two purposes: First, they move the root tip in space, thereby guiding its growth; and, second, they allow the root to move small obstacles which lie in its path and aid the root in soil penetration.

With our measurement system, we can follow small changes in the gravitropic response which reflect the dynamic nature of the controls involved. This may allow us to break down the time course of the response into changes in root stiffness versus overall root growth. In the future, we hope to be able to relate the time-variable character of bending force output to local variations in root extensibility on the upper and lower surfaces of the root.

Other measurements made possible by this system include axial growth rate measurements under continuous controlled tip load. We hope to extend these measurements to generate work and power output measurements for gravitropically responding roots.

INTERACTIONS OF LIGHT AND ETHYLENE IN STEM GRAVITROPISM

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Description of Research

Increased ethylene production occurs after gravistimulation in many plant stems. However, it is generally thought that ethylene is primarily involved in modifying the later phase of curvature rather than regulating early, upward bending.

Conflicting reports exist concerning the effect of light on ethylene biosynthesis. Studies focusing on etiolated pea shoots have reported light-inhibited as well as light-stimulated ethylene production. In our previous studies using etiolated pea stems, red-light-inhibited ethylene production has altered the kinetics and locus of curvature during gravistimulation. Thus, red-light treatment of etiolated tissue may alter gravitropism by affecting ethylene biosynthesis.

In this study, we examined the regulation of ethylene production during stem gravitropism in etiolated (given dim green light daily) and red-pretreated, etiolated pea seedlings. We evaluated the levels of ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), the malonyl-conjugate of ACC (MACC), and *in vivo* ACC oxidation.

Accomplishments

(1) In red-pretreated pea epicotyls, decreased ACC paralleled increased MACC levels within 30 minutes of gravistimulation. Tissue levels of ACC and MACC did not significantly change during the time course of gravistimulation in etiolated seedlings.

(2) ACC oxidation did not change in either etiolated or red-treated pea epicotyls during gravitropic curvature.

Significance of Accomplishments

In this research, red-pretreated etiolated pea stems exhibited changes in ACC and MACC levels during gravitropism. Ethylene biosynthesis was not significantly changed in gravistimulated etiolated epicotyls. These results suggest light regulation of ACC malonylation and/or ACC synthesis.

It is proposed that gravistimulation causes a decrease in ethylene biosynthesis via conjugation of ACC. An ethylene decrease within a restricted zone of the stem's subapical region could result in the localized gravitropic bending observed in red-pretreated plants. These findings support the previous observation that inhibition of ACC synthesis (by aminoethoxyvinylglycine) also alters the kinetics and locus of curvature in etiolated stems in a manner similar to red pretreatment.

REGULATION OF ETHYLENE BIOSYNTHESIS IN PLANTS GROWN IN CLOSED CANISTERS

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Description of Research

Growth space is a limiting factor in NASA's Space Shuttle experiments and will be critical for the cultivation of plants in space. Small closed environmental canisters are currently used for plant studies aboard the Space Shuttle middeck locker facility. Ethylene accumulation in small closed chambers may limit plant growth and affect the interpretation of results from Shuttle experiments.

Altered ethylene production can result from a change in ACC synthesis, conversion of ACC to ethylene, or from the conjugation of ACC into MACC. The conversion from ACC to ethylene is oxygen dependent and requires the enzyme ACC oxidase. Malonylation of ACC serves as a mechanism to dissipate excess ACC and participates in the regulation of ethylene biosynthesis.

This investigation (supported by the WV Space Grant Consortium) was conducted to determine changes in ethylene biosynthesis in pea seedlings grown in closed, 1-liter canisters, using measurements of ethylene, ACC oxidase, ACC, and MACC.

Accomplishments

(1) ACC oxidase activity decreased in closed canisters after 5 days of incubation. This result is most likely due to decreased atmospheric O_2 and/or increased ethylene levels within the chamber. Overall slowing of ethylene biosynthesis may occur in longer term experiments within closed chambers.

(2) Tissue ethylene production and ACC levels did not change in seedlings grown in closed canisters over the 5-day incubation period. Overall, ACC levels in roots were found to be greater than those in stems. The accumulation of ACC in roots is consistent with previous reports that ACC is produced in the roots and transported to the stem where it is aerobically converted to ethylene.

(3) MACC in roots of closed canisters was greater than MACC in roots of open canisters after 5 days of incubation. This was accompanied by a slight decrease in MACC in stems of closed chambers. High conjugation activity in the roots may result in lowered ethylene production by reducing the amount of substrate (ACC) for ACC oxidase.

Significance of the Accomplishments

The findings suggest that slight changes in ethylene biosynthesis (ACC oxidation and MACC formation) begin to occur in etiolated pea seedlings grown in small enclosed canisters over the relatively short incubation period of 5 days. Within this time, ethylene accumulation and oxygen depletion may begin to affect the ethylene biosynthesis system as well as ethylene-sensitive enzymes and ethylene-regulated genes involved in plant growth.

Publications

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PROBING THE GRAVISENSING AND GRAVIRESPONSE SYSTEMS IN ROOTS

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Description of Research

Gravisensing is typically associated with amyloplast displacement. However, an analysis of the mode of action of amyloplasts is lacking. It is unknown which percentage of amyloplasts needs to be displaced or whether the act of displacing, or a particular position inside the receptor cells, constitutes gravisensing. Since reorientation always involves the entire plant and not just amyloplasts, we attempt to isolate amyloplast movement from other factors.

Instead of displacing the amyloplasts by reorientation, we induce displacement by a high gradient magnetic field (HGMF). The HGMF is generated between permanent magnets by inserting a magnetically soft particle in a wedge-shaped form. The transition from high-field intensity in the vicinity of the wedge to the uniform field between the permanent magnets generates a gradient that exerts a ponderomotive force on magnetic substances. If a substance, such as amyloplast starch, which is more diamagnetic than the cytoplasm, is positioned in sufficient proximity to the gradient, it will be repelled from the gradient (or wedge). This technique is being used to manipulate amyloplasts in roots and correlate curvature and growth rate with amyloplasts distribution and forces.

Among other parameters, plant growth depends on the amount and direction of deposition of new cell wall material. There is substantial evidence suggesting that the orientation of cellulose fibrils is correlated with the orientation of microtubules (MTs). We study the behavior and possible role of cortical MTs in growth control in curving roots. Our studies attempt to characterize the time frame and extent of changes in the cytoskeleton of graviresponding roots. The investigations are based on indirect immunofluorescence and confocal microscopy.

Accomplishments

(1) The measurement of the parameter that determines the magnitude of the ponderomotive force, the magnetic susceptibility, is not complete; but we were able to visualize the phenomenon of a magneto-ponderomotive force acting on starch grains (see Figure 1).

Figure 1. Sedimentation of starch grains in the presence a high gradient magnetic field (left) and in the absence of a high gradient field (right). A ferromagnetic cone (C) was positioned next to a water-filled capillary (0.5 mm diameter) between two 10 mm thick SmCo magnets (not shown). This distorted the otherwise uniform magnetic field such that the starch grains moved away from the cone leaving a space devoid of starch grains (arrows). In the absence of a cone, the starch grains sediment uniformly (right).

We tested various combinations of generation of HGMF and the effect on root curvature and found a high correlation between the fraction of amyloplasts that are displaced and curvature in vertically oriented or clinostatted roots. We compared roots that were either positioned horizontally (i.e., control, showing natural graviresponse, no magnetic gradient applied) or clinostatted (1 rpm) in two configurations. Vertical clinostatting is when the (horizontal) axis of rotation is perpendicular to the root/shoot axis (i.e., gravity vector changed longitudinally) of vertically oriented seedlings. Horizontal clinostatting indicates rotation parallel to the root/shoot axis of horizontally placed seedlings. Table 1 summarizes the data on growth and curvature obtained in controls and magneto-stimulated roots.

Table 1. Curvature (degrees, ±SE) and growth (mm) of flax (*Linum utitatissimum*) roots in the absence or presence of an HGMF.

Time	Horizontal Orientation (no magnetic field)		Vertical Clinostatting (HGMF)		Horizontal Clinostatting (HGMF)	
	Angle	Growth	Angle	Growth	Angle	Growth
1 h	34.9±11.2	0.76±0.5	16.5±7.4	0.59±0.16	23.8±11.6	0.49±0.24
2 h	47.1±10.2	1.46±0.59	32.7±10.4	1.25±0.32	32.7±11.6	1.23±0.34

The presence of the high-gradient magnetic field induces curvature, and the distribution of amyloplasts (as measured by microscopical analysis of root caps) is highly correlated with the extent of curvature. The results suggest that the mode of action of amyloplasts in gravisensing can be investigated by substituting the force of gravity by a magneto-ponderomotive force. It is also conceivable that a magnetic field can physiologically substitute the gravity signal under microgravity conditions.

(2) The studies of cortical MTs of graviresponding corn roots (cv. Merit) revealed a wealth of information. The most important result is that there seems to be a graviresponse-specific reorientation of MTs in the lower cortex but not lower epidermis of roots, while MTs of the upper cortex are not affected. MTs in cap cells also reorient after gravistimulation. Dark-grown roots of this variety typically do not curve and also do not exhibit MT reorientation along the lower side of the cortex. IAA application to vertically growing roots resulted in MT reorientation in regions ranging from 1.8 mm (10^{-6} mol) to the maturation zone (10^{-8} mol). However, IAA causes MT reorientation in light and dark grown roots.

Significance of the Accomplishments

We were able to induce amyloplast displacement using HGMF in root caps of flax. The curvature obtained under conditions of dynamic weightlessness (clinostatting) or vertically oriented roots suggests that the amyloplast position, rather than directional movement, induces changes in differential growth.

We have established that cortical microtubules behave in a highly specific manner during gravicurvature of roots.

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GRAVITROPIC RESPONSE MECHANISM IN CEREAL GRASS SHOOTS

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Description of Research

The primary goal of our NASA Space Biology Program research project is to unravel the gravitropic response mechanism in cereal grass shoots. Our objectives are threefold: (1) to show how gravity perception occurs, (2) to understand the signal transduction process, and (3) to delineate the key physiological and molecular steps represented in the response mechanism cascade. The working model we are currently using, which depicts the key steps for gravity perception, signal transduction, and response in cereal grass shoot pulvini, is shown in Figure 1. Portions outlined in solid-line rectangular boxes have been demonstrated by us, and others, to occur in graviresponding cereal grass shoot pulvini. Portions outlined by dotted rectangular boxes are assumed to occur but have not been directly characterized.

In our cereal gravitropism research, we are focusing on two major components of this model: (1) how auxin-mediated signal transduction occurs in graviresponding pulvini, and (2) early molecular events involving gravity/hormone-enhanced invertase gene expression that starts the response mechanism cascade in cereal grass pulvini.

Accomplishments

(1) ***Gravitropic tissue elongation of oat shoot leaf-sheath pulvini at different pHs***: The effects of the buffered pH treatment on the gravitropic response and pulvinus elongation in oat shoots has been measured. After 24 hours of the gravistimulation under buffered pH conditions, the gravitropic curvature response and gravitropic tissue elongation of the bottom flank of the pulvini were determined. Figure 2 shows the gravitropic bending response and the gravitropic elongation growth of the bottom half of oat pulvinus segments. The following points can be made: (a) The most acidic conditions (pH 4.5) promote the elongation of "bottom" halves of pulvini by 52.4 percent of their original length. (b) "Bottom" halves at pH 5.5 and 6.5 elongate by 45.2 and 35.7 percent of their original lengths, respectively. (c) The highest tissue elongation of the unbuffered control "bottom" halves (by 64 percent of the original length) is not significantly different from pulvinus elongation at pH 4.5. (d) Under these buffered pH conditions, gravitropic curvature responses of pulvinus segments containing "bottom" halves are reduced to 62.9 to 55.7 percent of that of the unbuffered control pulvinus segments. However, such variation in the reduction of gravitropic curvature is not statistically significant.

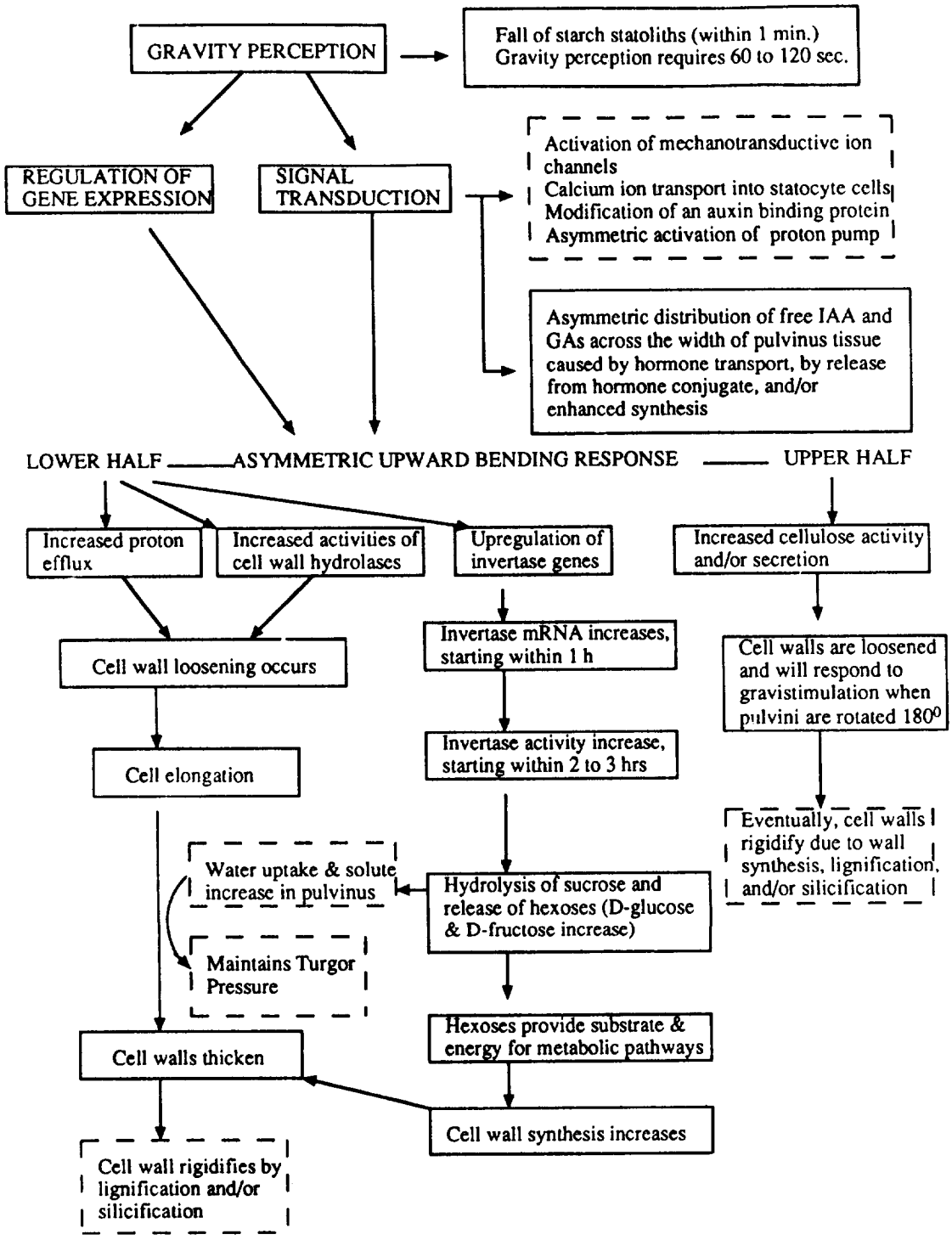

Figure 1. Working model for gravitropic response mechanism.

(2) ***Changes in sensitivity to exogenous indole-3-acetic acid (IAA) of gravistimulated oat shoot pulvini:*** The elongation of oat pulvini has been measured after 24 hours of gravistimulation treatment in the presence of various concentrations of IAA. Figure 3 shows the relative growth of the top and bottom sides of the gravistimulated pulvini, as well as vertically-held control pulvini, as compared to their original lengths. The following points can be made: (a) The maximum growth of vertically-held pulvini occurs at 10^{-5} M IAA. (b) The top part of the graviresponding pulvini shows the same pattern of dose-response tissue growth induction by IAA as vertically-held pulvini. (c) The bottom parts of the graviresponding pulvini show maximum growth induction by IAA as vertically-held pulvini. (d) The bottom parts of the graviresponding pulvini show maximum growth at 10^{-8} mol IAA. (e) A difference in the responsiveness to exogenous IAA between top and bottom halves of the graviresponding pulvini is about 1000 times.

(3) ***Kinetic induction of invertase mRNA in graviresponding oat shoot pulvini:*** Northern blot analysis was performed using the partial-length invertase cDNA (PCR product) as a probe. Results show that a 1.90 kilobase (Kb) invertase mRNA is detected from the pulvini of oat plants (Figure 4A). The level of this 1.90 Kb mRNA is very low at time zero, but it is significantly induced 1 hour after initiation of gravistimulation treatment, after which time it decreases. In order to examine the induction pattern of the gravistimulated invertase mRNA in more detail, mRNA was separately isolated from top and bottom halves of oat-shoot pulvini after different times of gravistimulation. As shown in Figure 4, there is a clear pattern of kinetic changes in invertase mRNA level during the gravitropic response of the respective halves of oat shoot pulvini. The 1.90 Kb mRNA was detected at a relative low level in the top halves of the pulvini (Figure 4B). Interestingly, this mRNA was shown to occur at a very high level in the bottom halves of the pulvini (Figure 4C). The amount of mRNA rapidly increases to a peak at 1 hour after initiation of gravistimulation, and then it gradually decreases afterwards. Using a densitometer, the maximum level in these bottom halves of the pulvini that occurs at 1 hour represents about a five-fold increase above that of the time zero control (vertical pulvini).

(4) ***Genomic invertase gene(s) estimation:*** Genomic DNA from oat plants was purified and digested with seven restriction enzymes to estimate the size of invertase gene(s). Four bands were detected using the partial-length invertase cDNA as a probe (Figure 5). One band showed a very strong hybridization to the probe, indicating that oat contains only one locus corresponding to the partial-length invertase cDNA. The other weakly hybridizing bands may correspond to other members of the invertase gene family.

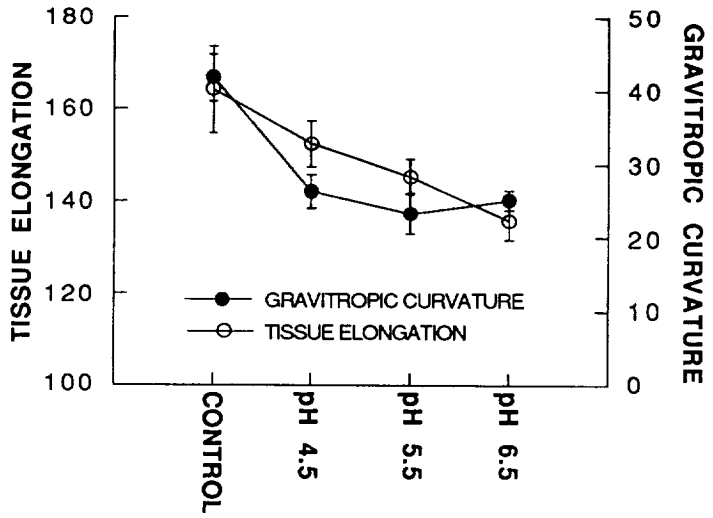

Figure 2. Gravitropic tissue elongation and bending response of the bottom side of oat shoot pulvini under different buffered pH conditions. Initial pH of the control was 6.5.

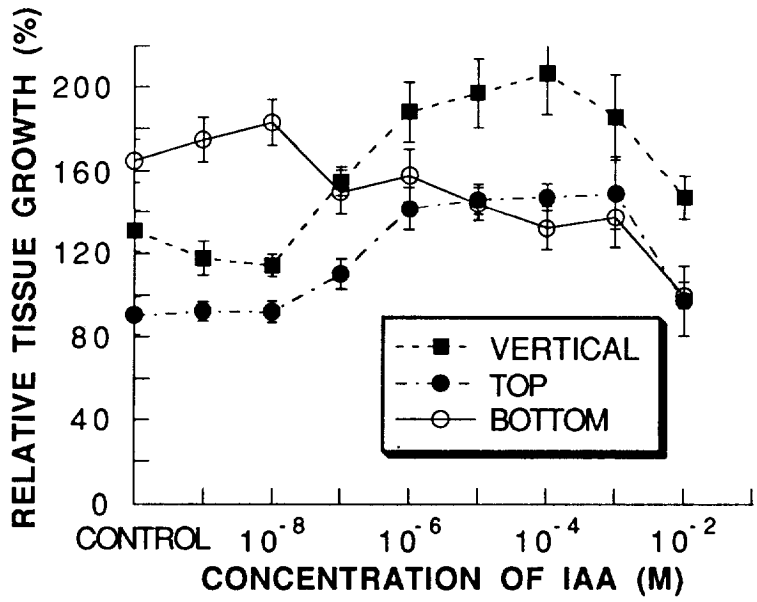

Figure 3. Relative pulvinus growth induced by IAA with or without gravistimulation.

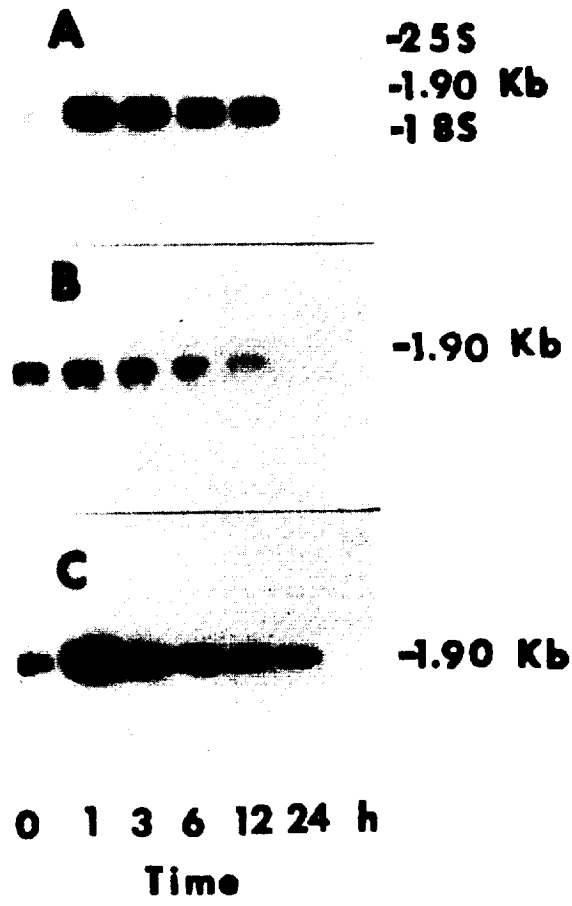

Figure 4. Northern blot analysis of oat shoot invertase mRNA isolated from gravistimulated oat shoot pulvini. Poly(A)+RNA (2 μg/well) was electrophoresed in 1 percent agarose gel, blotted onto a nylon membrane, and hybridized with a-^{32}PdCTP-labeled invertase cDNA (PCR product) as a probe. Figure 4A. mRNA isolated from intact pulvini. Figure 4B. mRNA isolated from the top halves of the pulvini. Figure 4C. mRNA isolated from bottom halves of the pulvini. Lanes 1–6 represent mRNA isolated from oat pulvini during different times of gravistimulation. Lane 1: 0 hour is the vertical control. Lane 2: gravistimulated for 1 hour, Lane 3: 3 hours, Lane 4: 6 hours, Lane 5: 12 hours, Lane 6: 24 hours of gravistimulation, respectively.

Figure 5. Southern blot analysis of oat genomic DNA. DNA (25 µg/well) was digested with restriction enzymes and electrophoresed on a 0.8 percent agarose gel. The gel was denatured, neutralized, and DNA transferred onto a nylon membrane filter. The filter was hybridized to digoxgenin-dUTP-labeled partial-length invertase cDNA as a probe. The bands were detected with Lumi-Phos 530 (Boehringer-Mannheim Biochemicals) and exposed to X-ray film for 30 minutes at room temperature with an intensifying screen. M: DNA molecular weight standard markers. Restriction enzymes used are: *EcoR I*, *BamH I*, *Hind III*, *Kpn. I*, *Pst I*, *Hae III*, and *Sac I*.

Significance of the Accomplishments

Finding 1: Results in accomplishment (1) indicate that the gravitropic tissue elongation of oat pulvini is explained partly by the "Acid Growth Theory." This conclusion is based on the observation that the tissue elongation of the bottom sides of the graviresponding pulvini is enhanced more under acidic buffer conditions as compared to that under neutral buffer conditions. However, the results also demonstrate that a substantial portion of the gravitropic bending response is not affected by the environmental pH, since buffered pH treatment of oat pulvini does not nullify the gravitropic response of the pulvini, regardless of the pH of the buffer solutions.

Finding 2: When oat pulvini are gravistimulated in the presence of IAA, tissue elongation of bottom and top sides of the pulvini shows the different dose-dependent response to IAA. These results demonstrate that the bottom halves of graviresponding pulvini respond to exogenously supplied IAA with 1000 times higher sensitivity than do the top halves of the pulvini and vertically held control pulvini. These results demonstrate that tissue sensitivity changes in oat shoot pulvini elicited by gravistimulation treatment is a very important component of the signal transduction process in the gravitropic response mechanism.

Finding 3: We observed an induction of invertase mRNA by gravistimulation treatment in top and bottom halves of oat shoot pulvini. We report here that there is a very low level of invertase mRNA before the onset of gravistimulation of oat shoot pulvini. However, the mRNA level dramatically increases to a maximum at 1 hour following the gravistimulation treatment and gradually decreases afterwards (Figure 4). This indicates that the induction of invertase mRNA by gravistimulation of oat pulvini is rapid. Particularly, we observed that the level of invertase mRNA

in the lower halves of pulvini was much higher than that in the top halves during the graviresponse, with about three- to five-fold increases observed at the specified times (Figures 4B and 4C). We here demonstrate for the first time that the expression of invertase genes is up-regulated by gravistimulation and is an early, essential molecular step in the cascade of events that occurs in the gravitropic response mechanism.

Finding 4: Southern blot analysis showed that one genomic DNA fragment from oats strongly hybridizes to the cDNA probe, and three fragments show a weak hybridization signal (Figure 5). This indicates that there may be an invertase gene family that exists in oat plants and that the partial length cDNA could be one of the genes in the family encoding for a particular isoform of oat invertase. This is, to our knowledge, the first time that an invertase gene family has been shown to exist in oat plants. Each of these invertase genes could be differently regulated by gravistimulation.

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GRAVITROPISM AND GOLGI APPARATUS FUNCTION IN *CHARA* RHIZOIDS

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Description of Research

The purpose of this research is to characterize the gravitropism pathway in *Chara* rhizoids and to determine the role of the major organelles and structures in each step of the pathway. We have been using rhizoids of the alga *Chara* as a model system since they are strongly gravitropic and since all stages (i.e., perception, transduction, and response) of gravitropism occur in a single cell. In contrast to higher plants which appear to utilize amyloplasts as statoliths in gravity perception, *Chara* rhizoids have single membrane-bound compartments located near the cell apex that function as statoliths. These vesicles settle to the new lower cell wall within minutes following horizontal reorientation.

Our specific goals have been (1) to reappraise the ultrastructure of *Chara* rhizoids with special emphasis on organelles and other structures related to the gravitropic response by using cryofixation and immunocytochemical techniques; (2) to analyze the structure and biosynthesis of statoliths in order to determine their role in the development of gravitropic competence.

Accomplishments

(1) ***Chara rhizoids can be successfully frozen and preserved by cryofixation.*** We can now routinely cryofix rhizoids by high-pressure freezing. Compared to chemical fixation, rhizoids prepared by cryofixation showed a number of significant structural differences, such as improved preservation of the Golgi apparatus and cell wall and improved preservation of antigenic determinants (see Kiss and Staehelin, 1993).

(2) ***Antibodies against complex polysaccharides of higher plants cross-react with Chara rhizoids in a specific manner.*** Results to date show that several antibodies against higher plant pectic polysaccharides cross-react with *Chara* rhizoids. These include a polyclonal antibody to polygalacturonic acid/rhamnogalacturonan I (PGA/RG-I), a monoclonal antibody to methyl-esterified pectin (JIM 7), a monoclonal to de-esterified pectin (JIM 5), and a monoclonal antibody to RG-I (CCRC-M2). The binding of these antibodies is specific, as indicated by controls including preabsorption of a particular antibody with its appropriate carbohydrate. These antibodies bind to the Golgi apparatus, apical vesicles, and the cell wall.

(3) ***The statolith compartment in Chara rhizoids contains carbohydrate and protein in addition to the previously reported barium sulfate crystals.*** We have used cytochemical and immunocytochemical assays to better characterize the statolith compartment. Coomassie Brilliant Blue cytochemistry indicates that statoliths contain protein. In addition, CCRC-M2 (a monoclonal antibody to RG-I) labels the statoliths (Figure 1), which indicates that these compartments contain a carbohydrate matrix.

(4) ***The response to gravity is correlated with the number of statoliths in Chara rhizoids.*** Differential-interference-contrast microscopy demonstrated that rhizoids from soil water (SW) medium grown plants typically contain 50–60 statoliths per cell while rhizoids from artificial pond water (APW) grown plants contain 5–10 statoliths per cell. Rhizoids from SW are more responsive to gravity than rhizoids from APW since (1) SW rhizoids were oriented to

gravity during vertical growth while APW rhizoids were relatively disoriented, and (2) curvature of SW rhizoids was three to four times greater throughout the time course of curvature (Figure 2). The growth rate of APW rhizoids was significantly greater than that of SW-grown rhizoids.

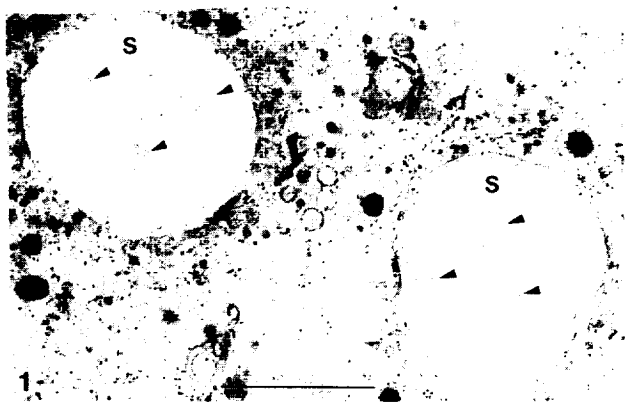

Figure 1. Ultrathin section of a *Chara* rhizoid that was immunolabelled with the CCRC-M2 antibody (against the carbohydrate RG-I) followed by a secondary gold label. The gold particles are localized in the statolith (S) compartment (arrowheads), and label also is present in vesicles. Scale bar = 1 μm.

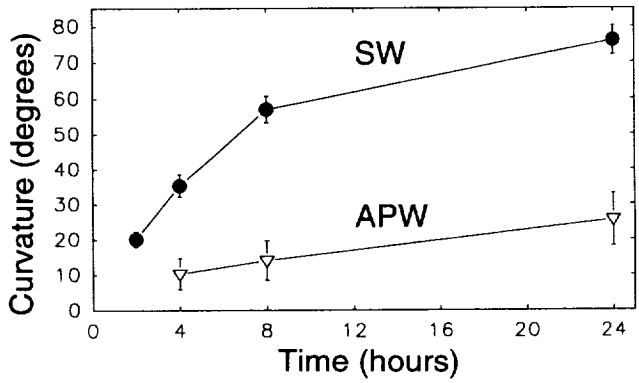

Figure 2. Time course of curvature of *Chara* rhizoids from plants grown in either soil-water or artificial pond water (APW) medium. The downward curvature of rhizoids was measured following placement n a horizontal orientation. Data points are mean curvature ± SE ($15 < N < 24$).

Significance of the accomplishments

Finding 1: In previous studies chemical fixation of rhizoids was employed, which can cause numerous well-documented artifacts. However, our data suggest that the new methodological approach of cryofixation will yield insights regarding the role of the Golgi apparatus and the cell wall in the differential growth in response to gravity. Cryofixation will also allow us to accurately determine antibody labeling patterns in the rhizoids (see below).

Finding 2: The specific binding of higher plant antibodies to *Chara* is consistent with the fact that cell walls in Characean algae are biochemically similar to higher plant cell walls; i.e., they are composed of cellulose, hemicellulose, pectin, and protein in a similar ratio. Our battery of antibodies will allow us to investigate the vesicle blockage hypothesis (for the response

mechanism) since apical vesicles can be divided into several categories depending on size, staining patterns, and antibody labeling.

Finding 3: Previous reports have demonstrated that the statolith compartment contains barium sulfate crystals. Our studies demonstrate that this compartment is more complex in that, in addition to these crystals, carbohydrate and protein also are present. Improved knowledge about the composition of the statolith compartment is important for understanding statolith synthesis and the precise role of statoliths in gravitropism.

Finding 4: While there have been several reports which show a correlation between the presence of statoliths and gravitropic sensitivity, our studies provide strong evidence for the importance of statoliths in *Chara* rhizoids. In addition, this system is in many ways comparable to the studies with higher plant starch-deficient mutants (*Arabidopsis* and *Nicotiana*) which provide support for a statolith-based mechanism of gravity perception in plants.

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ANALYSIS OF THE *LAZY-2* GRAVITROPIC MUTANT OF TOMATO

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Description of Research

We are studying a tomato mutant that appears to provide an ideal system with which to test the theories pertaining to shoot gravitropism. The shoots and petioles of the single gene-recessive, gravitropic mutant *lazy-2* (*lz-2*) exhibit the striking phenotype of downward curvature in response to gravity. That is, curvature of *lz-2* shoots is in the same direction as the gravity vector (positive gravitropism), whereas the normal response of shoots is upward curvature (negative gravitropism). The roots of *lz-2* are wild type in their response to gravity. The abnormal downward curvature of the shoots is manifested only in the light. When *lz-2* plants are grown in the dark, the shoots exhibit a wild-type gravitropic response, and they revert to the wild-type phenotype if returned to the dark after illumination. Other than the gravitropic response of the shoots and petioles, *lz-2* plants appear completely normal; they are vigorous and healthy, are easily crossed, and produce many fruit and plenty of seed.

Since only the gravitropic response of the *lz-2* shoots is affected and there are no other apparent phenotypic abnormalities, a genetic lesion specific to some aspect of the shoot gravitropic pathway is indicated. There are many other documented agravitropic or sluggishly gravitropic mutants which are generally caused by starch deficiencies or are of a pleiotropic nature. However, we know of no other mutant which can actually carry out gravitropism, but in a direction reversed from wild type. We have shown[1] that *lz-2* plants can perceive gravity and also have the ability to respond with asymmetric growth but that some component of signal transduction appears to be reversed. These studies also demonstrated that the light-induced switch from negative to positive gravitropism in *lz-2* is potentiated by red light. This indicates the involvement of the photoreceptor phytochrome.

We have now established that phytochrome is indeed the pigment involved in regulating the *lz-2* phenotype.[2] The red light induction of downward growth can be reversed by a far red light pulse, and downward curvature reaches a maximum 16 hours after a 1-hour red light pulse, after which dark reversion is observed. Fluence response curves indicate that this response has characteristics of both low-fluence and high-irradiance phytochrome responses.

The physiological characterization of *lz-2* has allowed us to test the sufficiency of the Cholodny-Went theory for plant gravitropic responses. The Cholodny-Went theory would predict that in plants which have been exposed to red light, the IAA asymmetry during gravitropism is reversed in *lz-2* as compared to "wild type". This would lead to a growth asymmetry resulting in greater cell elongation on the upper side relative to the lower side, thus causing downward curvature. In order to test this hypothesis, we have measured the relative growth rates on either side of both *lz-2* and wild-type seedlings, either gravistimulated or not. These studies have shown that, as compared with "wild type", red light stimulates growth on both sides of the stem, with the highest growth rate on the upper side.[3] This results in the downward curvature of the *lz-2* stems. The increase in growth rate on both sides of *lz-2* stems after light induction indicates that the reversed curvature is not a result of increased concentrations of IAA inhibiting growth on the lower side. Rather, it suggests that either increased IAA is transported to the upper side or an inhibitor is removed.

During this granting period, we also began genetic studies designed to both map the *lz-2* lesion to its chromosome and to study the effect of creating double mutants with other tomato gravitropic and phytochrome mutants. For mapping, we created an interspecific cross between *lz-2* (in *Lycopersicon esculentum*) and a wild tomato species, *Lycopersicon pennellii*. The F_1 progeny from this cross has been grown to maturity and allowed to self. Genomic DNA from the resulting F_2 plants will now be used with restriction fragment length polymorphism (RFLP) probes to determine the chromosomal linkage of the *lz-2* lesion and to place the *lz-2* gene on the extensive tomato RFLP map.

To study the interaction between two distinct mutations in the gravitropic mechanism, we constructed a double mutant between *lz-2* and *diageotropica* (*dgt*), a tomato mutant which responds sluggishly to gravistimulation and which is insensitive to exogenously applied auxin. The resulting *lz-2/dgt* double mutants were both selfed and backcrossed to the respective parents to establish that they are truly double mutants. Interestingly, the plants carrying both mutant genes have an *lz-2* type shoot gravitropic response (positively gravitropic) and a *dgt*-like root response (agravitropic), slower shoot gravitropic response, and auxin insensitivity. This indicates that the two genes do not complement each other (and are, thus, not the same) and that one is not dominant over the other. Since *dgt* is affected in its auxin responsiveness, these results indicate that auxin is involved in the reversed gravitropic response of *lz-2* and that the *dgt* gene is likely to lie downstream of *lz-2* in the gravitropic signal transduction pathway.

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MECHANICAL STRESS REGULATION OF PLANT GROWTH AND DEVELOPMENT

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Description of Research

This project has two main objectives: (1) To characterize plant responses to mechanical stress; and (2) To determine the physiological mechanisms of the responses.

Mechanical stresses, forces, or stimuli are natural occurrences in the growth and development of higher plants on earth. Whether these forces occur as gravity, wind, or seedling movement through the soil, they affect the growth, development, and orientation of seedlings and mature plants. These physical forces not only inhibit growth and development, but also strengthen plants against subsequent physical and environmental stresses. The dark-grown soybean (*Glycine max* Merr. cv. 'Century 84') seedling has been selected as a model system for characterizing and analyzing events involved in the inhibitory response because of its rapid growth rate and extreme sensitivity to mechanical perturbation. Thigmic (contact rubbing) stress is used as the model for seedling movement between abrasive soil particles during emergence. A 10 second application of thigmic stress is accomplished by 20 upward strokes with the thumb and forefinger to the apical hook and subtending 5 mm of the hypocotyl. The typical response to a single application of thigmic stress is a temporary inhibition of hypocotyl elongation by the dark-grown seedling. Understanding the early events and controlling factors of the growth response will better enable us to provide proper growth conditions in a space environment.

Accomplishments

Characterization of seedling responses to a single episode of thigmic stress has been a primary focus. We have defined several characteristics and have determined several factors affecting the response:

(1) The response of dark-grown seedlings to a single (10 seconds) application of thigmic stress is very rapid (seconds) and dramatic (up to 90% inhibition of elongation). The initial response is followed by a long (hours) recovery period which is responsible for the cumulative effect of reducing hypocotyl length. After 24 hours, thigmo-stressed seedlings are as much as 30% shorter than unperturbed control seedlings.

(2) The cumulative response to thigmic stress by seedlings of the same starting height and equivalent rates of elongation was affected by the time it took for seeds to germinate (Figure 1). Seedlings were selected for planting 24, 48, or 72 hours after imbibition, when the radicle length reached 1 cm. Seedlings in the earliest germinating group (radicle length of 1 cm, 24 hours after imbibition) had the greatest response to thigmic stress; seedlings had 30% shorter hypocotyls than did control seedlings 24 hours after stress application. Seedlings grown from later germinating seeds, 48 or 72 hours after imbibition, had a diminished response to thigmic stress resulting in seedlings 25% or 15% shorter, respectively, than unperturbed control seedlings in each germination group.

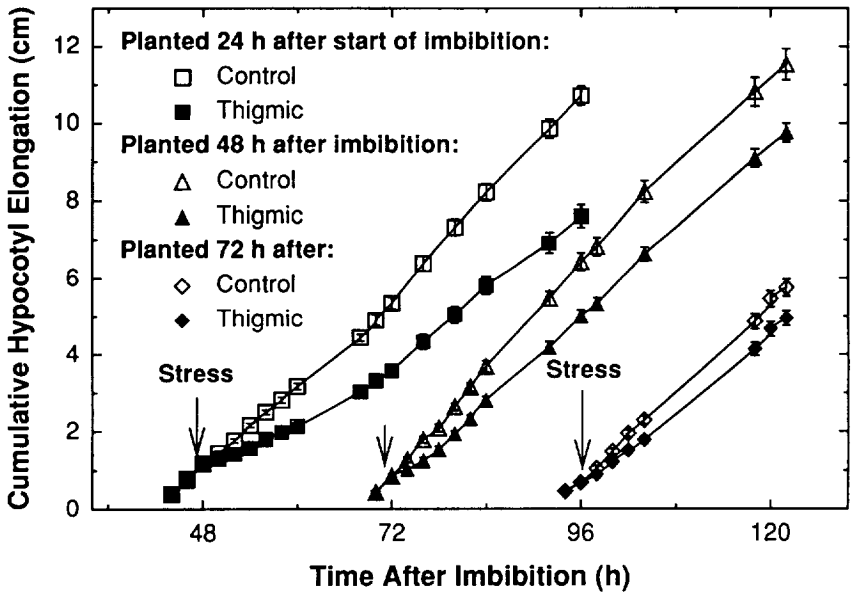

Figure 1. Response to a single episode of thigmic stress (arrow) by dark-grown soybean seedlings differing in age (time after imbibition to reach a radicle length of 1 cm) at time of planting. Once emerged, all three undisturbed control groups elongated at the same rate and were at equivalent heights when stress was applied. Bars represent ± the standard error of the mean of 32 to 36 seedlings.

(3) Although the initial, rapid response to thigmic stress was consistent among all treatments, recovery time was variable and dependent on nutritional status of the seedling. Increased Ca^{2+} in the growth medium from 1 to 5 mM increased hypocotyl elongation rate from <1 $mm \cdot h^{-1}$ to >2 $mm \cdot h^{-1}$. Recovery time following a single episode of thigmic stress decreased significantly from >9 hours to <3 hours by increasing Ca^{2+} from 1–5 mM in the growth medium. Supplementing the medium with 5 mM K^+ further enhanced recovery time.

(4) After elongation rate had recovered for thigmo-stressed seedlings, the solute potential of solution expressed from the elongation zone of the hypocotyl was significantly less than that of unperturbed control seedlings (-0.96 vs. -0.80 MPa).

Significant of Accomplishments

Finding #1: The initial response of soybean seedlings to a single episode of thigmic stress is so rapid that we hypothesize a turgor collapse of cells in the elongation zone of the hypocotyl may be occurring. We currently are investigating this possibility using a pressure microprobe.

Finding #2: The rapid recovery from thigmic stress by seedlings selected for long (>48 hours) germination time suggests that any environmental stress prior to, or during, germination which slows the germination process, may render the seedlings more resistant to the long-term effects of mechanical stress.

Finding #3: The variability of recovery time can be accounted for by the pre-stress physiological condition of the seedling and the nutrient composition of the culture medium. Time for recovery to occur can be enhanced by increasing the Ca^{2+} level, as well as by including 5 mM K^+ in the growth medium.

Finding #4: The decreased solute potential in the elongation zone following growth rate recovery may indicate that the stressed seedlings were adjusting osmotically in order to regain and maintain the pre-stress elongation rate and turgor potential.

These findings not only aid our basic understanding of stress-induced growth inhibition, but also will help in controlling the effects of a stressful environment. Potential application to the field of gravitational and space biology is to provide information to assist in plant culture in the space environment and to understand the mechanisms by which plants respond to physical forces such as gravity and mechanical stress.

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TRANSDUCTION OF THE GRAVITROPIC STIMULUS

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Description of Research

The short-range goals of our recent research, funded partly by the Space Biology Program and partly by the Innovative Research Program of the Office of Space Science and Applications, were (1) to identify modulators, promoters, and inhibitors of an ion channel that putatively serves as a gravitropic sensor for higher plants; (2) to utilize such modulators and modifiers for biochemical characterization of the channel; and (3) to study diverse roles of the channel.

The long-range goals of our research program, now several years old, have been (1) to characterize the primary transducer of the gravitropic stimulus; (2) to test the hypothesis that this mechanotransducer acts as a signal integrator in plant development and maintenance; and (3) to work out the structural, biochemical, and physiological features of the control system in which we believe the transducer is embedded.

Accomplishments

In our last report, we described a stretch-activated ion channel and provided evidence that it is a primary gravitropic signal transducer. Activity of this channel was shown to be strongly modulated by transmembrane voltage and by temperature, so the channel can serve as a multimodal signal integrator.

Acid and the regulation of growth. We have now shown that acid in the free space of the cell wall also strongly modulates channel activity. Since one of the effects of gravitropic stimulation is often a spatially patterned shift in wall pH, this modulation suggests the occurrence of a variety of feedback loops in which the channel may be critically involved.

In particular, we have developed a model purporting to explain how mechanical and electrical stimuli could regulate the rate and the vectors of cell expansion during straight growth, as well as control the net direction of the mediational hormone auxin during the asymmetric growth required for gravitropic curvature.

The feedbacks of this model involve many steps, as would be expected of complex cellular regulation, and are summarized in a paper devoted to the topic (Pickard and Ding 1993) Briefly, the model suggests that Ca^{2+} passing inward through the sensory channels results, via a "signal transduction chain," in pumping of H^+ into the wall, loosening it, and allowing it to yield to cell turgor and expand. But expanding the wall around a set of channels creates a weak zone around them; and, therefore, mechanical stress such as is always experienced by the turgid cells will be focused on that group of channels, leading to a positive feedback between channel opening and wall loosening. Damping of channel activity by increasing wall acidity, however, could replace such runaway behavior with a regulatory negative feedback and allow continual adjustment of the local rate of wall expansion so that reasonable uniformity of the wall is maintained and so that neighboring cells maintain their fit in the tissue.

Other responses to Ca^{2+} and H^+ are postulated to address the vectorial control of expansion. When Ca^{2+} in the cytosol adjacent to the activated channels reaches a certain threshold,

microtubules are possibly released from certain proteins that moor them to the cell membrane. At the same time, acid at the other side of the membrane may free the mooring proteins from association with transmembrane proteins that normally fix their orientation with respect to the cell wall. Now, assuming that the mooring proteins contain an appropriate electrical dipole and that they are clustered to increase net dipole strength, they are free to orient in the axial electrical field which normally prevails in growing tissue. As is well known, the orientation of the microtubules controls the orientation of newly forming cellulose fibrils, and these in turn control the direction in which the cell wall can be stretched if loosened. Finally, as the elevation of mural acid reduces channel opening, the microtubules are locked in place again, ready to maintain allometry by participating in the next cycle of local expansion. Or, if there has been a change in orienting conditions, the microtubules are ready to shift the vectors of growth.

As introduced in our last report, we are currently working with fluorescent tags and a computational optical sectioning microscope in order to observe not only the individual components of the model but also how their arrangements and activities can change with time.

Aluminum toxicity. Over the past year, we have shown that the channel is inhibited by low levels of aluminum ions, suggesting that blockage of this or a closely related stretch-activated channel might play a key role in the agriculturally and sylviculturally detrimental acidic soil syndrome.

Figure 1. illustrates such inhibition by aluminum ions. It is a temporally compressed patch clamp recording of an excised, outside-out, plasmalemmal patch from onion bulb scale epidermis. It shows (see pointers) the absence of channel currents in the absence of membrane tension, the presence of large currents when suction is applied, and the immediate inhibition of currents when a large amount of dissolved aluminum chloride is perfused past the patch. At the pH of the perfusate, only a little of the aluminum is in the trivalent ionic form commonly thought to be the major toxic aluminum species. Experimental conditions are specified in the fourth reference listed below; of particular note is the provision of K^+ rather than Ca^{2+} ions to support the currents, since in the absence of Ca^{2+} the conductance for K^+ is higher than that for Ca^{2+}.

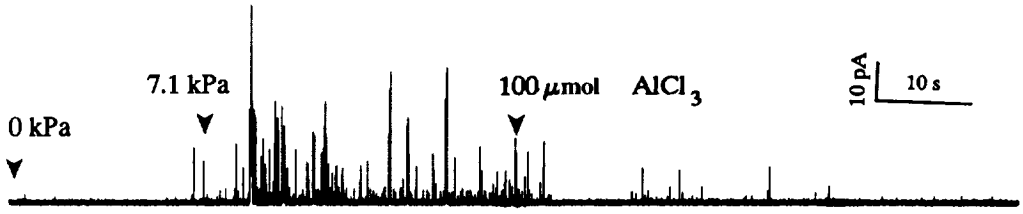

Figure 1. Inhibition by aluminum ions.

Target of a fungal toxin. A potent fungal toxin recently described as a gravitropic inhibitor (A. Sievers and M.B. Busch, *Planta* 188:619, 1992) has an immediate enhancing effect on stretch activation of the channel, and after a lag begins to activate the channel in the absence of externally imposed tension. It is possible that these effects are due to insertion of the molecule in the bilayer. Regardless of how the stimulation is achieved, it suggests a possible mechanism by which cytosolic Ca^{2+} might be elevated during causation of certain plant rots. We previously predicted that the channel would be a target for a variety of pathogens, and we hope to check to what extent the action of this and other toxins may partly account for various disease syndromes. Meanwhile, in view of the use of the toxin by plant physiologists as a putatively specific agent to

interdict a rather different cell function, as discussed by Sievers and Busch, the data serve warning that previous "pharmacological" studies need to be reevaluated.

Turgor regulation. We have tested that a moderately low concentration of the channel inhibitor gadolinium conspicuously and reversibly delays the response of protoplasts to osmotic shifts in the surrounding medium. Evidently, the putative gravitropic receptor can also serve in osmoregulation. There is a growing sense that turgor sensors play diverse roles in the plant, ranging from control of syntheses to partitioning of photosynthate. Thus, extension of channel inhibitor studies to such processes might prove highly profitable.

Significance of the Accomplishments

Finding 1: If, as we propose, the alleged gravitropic transducer is also a primary target of toxic aluminum ions in acidic soils, it should now be possible to make faster progress in understanding why many crop and forest plants do not thrive in these soils. In the very long range, it might be possible to work out mechanisms by which the effects of acidic soils may be ameliorated.

Finding 2: More generally, the data on channel modulation by acid in the wall space extend the concept presented in the preceding report that the alleged gravitropic transducer is a multimodal sensor with a second messenger output. Because of the integrational capabilities of this channel, and because of the accumulating evidence that it may play a role in control of turgor and of the rate and vectors of cell growth, our statement in the preceding annual report seems even stronger: "...systematic expansion of the gravity sensor program into the arena of molecular architecture and function should greatly broaden understanding of the plant cell and of the developmental processes and maintenance activities of plants." As exemplified in a publication by Pont-Lezica, listed below and discussed in the preceding report, the new data on the multimodal sensory channel can be combined with high-resolution optical sectioning microscopy, molecular biology, and antibody staining to further our understanding of how responses and feedbacks of this system help to integrate cell behavior.

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Ding, J.P. and Pickard, B.G. 1993. Mechanosensory calcium-selective cation channels in onion epidermis. *Plant Journal* 3: 83-110.

Ding, J.P. and Pickard, B.G. 1993. Modulation of mechanosensitive calcium channels by temperature. *Plant Journal* 3: 713-720.

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CALCIUM MESSENGER SYSTEM IN GRAVITROPIC RESPONSE IN PLANTS

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Description of Research

The primary goal of our research on gravitropism is to determine the mechanisms involved in the plant's detection of the gravity signal and resulting differential growth of the upper and lower halves of roots. Signal-induced change in free Ca^{2+} concentration in the cell has generally been portrayed as a switch regulating various cellular activities. Plant responses to external signals are believed to be achieved by changes in intracellular free concentration; which, in turn, modulate the activity of Ca^{2+} and Ca^{2+}/calmodulin-dependent protein kinases. Figure 1 shows a schematic diagram that illustrates how elevated levels of cytosolic Ca^{2+} affect various enzymes and proteins in the cell.

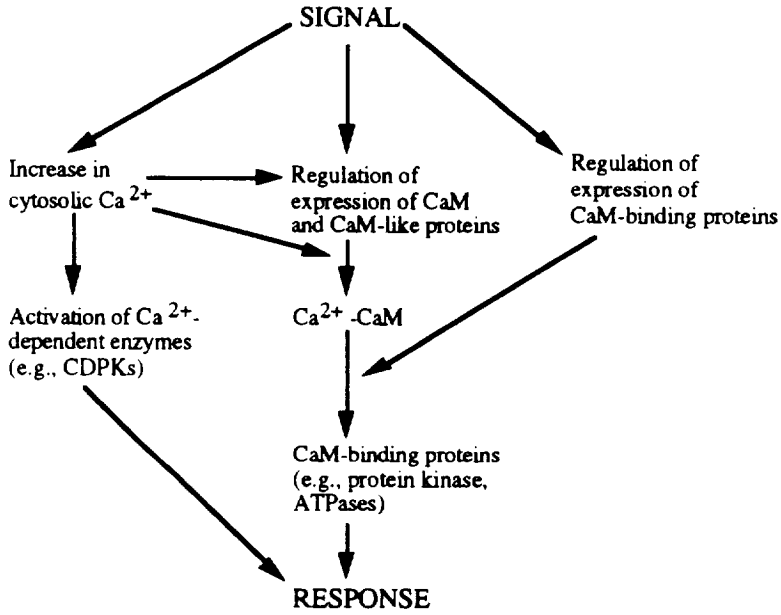

Figure 1. Schematic illustration of the proposed events involving Ca^{2+} in signal transduction.

Many difficulties exist in making free Ca^{2+} measurements in intact plants and cells. Precise measurement of changes in the level of cytosolic Ca^{2+} concentration in response to external stimuli is crucial in establishing the role of Ca^{2+} in signal transduction. Aequorin, the best known of many Ca^{2+}-activated photoproteins, emits a blue light with an intensity proportional to the Ca^{2+} concentration. This is a photoprotein isolated from the jellyfish, *Aequorea foskalea*. We are using a molecular approach to quantitate gravity-induced changes in free Ca^{2+} concentration by introducing aequorin gene into plants. Our ultimate goal is to study gravity-induced changes in intracellular free Ca^{2+} concentration and its relationship to the regulation of Ca^{2+} and Ca^{2+}/calmodulin-dependent protein kinases in plants.

Accomplishments

(1) During the past year, we have produced transgenic plants carrying the aequorin gene that can report changes in cytosolic free Ca^{2+} concentration. To accomplish this, the apoaequorin-coding region from complementary DNA clone was fused to the cauliflower mosaic virus (CaMV) 35S promoter and transferred to plants using the *Agrobacterium tumefaciens* binary vector system. Northern analysis revealed that we have a set of transgenic plants expressing different levels of apoaequorin. F_1 progeny from the transformant expressing the highest levels of apoaequorin were selfed and homozygous plants were produced.

(2) Two approaches have been taken to detect and characterize Ca^{2+}-regulated protein kinases in plants. In the first approach, two degenerate oligonucleotide primers corresponding to the highly conserved regions, GGELFD and DLKPEN of serine/threonine protein kinases were used. These primers were used to amplify a DNA fragment from a corn root tip cDNA library by polymerase chain reaction (PCR). The PCR-amplified product was used to screen the library to obtain corresponding cDNA clones. Sequence analysis of one of the isolated cDNA clones revealed that it represents a Ca^{2+}-dependent protein kinase. This is a unique protein kinase which has both a kinase domain and an adjoining calmodulin-like domain with four Ca^{2+}-binding EF-hand motifs in a single polypeptide. The junction domain between the kinase and the calmodulin-like domains has a putative calmodulin-binding region. In situ hybridization studies indicate that the expression of this kinase is higher in the root tip. The genomic Southern revealed that there are other related protein kinases. Two other PCR products show very high homology to Ca^{2+} and Ca^{2+}/CaM-dependent protein kinases.

By screening the corn root tip cDNA expression library with ^{125}I and 35S-labeled calmodulin as probes, several cDNA clones were obtained. Two partial cDNA clones (CBP-1 and CBP-5) have been characterized. Comparison of the deduced amino acid sequence of both the clones showed 100 percent conservation of the 34 amino acid stretch at their carboxy-terminal ends. The highly conserved 34 amino acid stretch contained putative calmodulin-binding domain, a basic amphiphilic alpha helix. Results indicate that mRNA for one of the clones (CBP-5) increases when the seedlings are subjected to mechanical perturbations.

(3) To study the regulation of calmodulin gene expression, transgenic plants carrying a chimeric fusion between the calmodulin promoter and chloramphenicol acetyltransferase (CAT) reporter gene were produced. Depletion of Ca^{2+} by EGTA and ionomycin resulted in decreased expression of the CAT activity. Plants carrying CaMV 35S promoter with the reporter gene CAT were used as controls. Ca^{2+} depletion did not affect the CAT expression driven by the CaMV 35S promoter. Furthermore, verapamil, a Ca^{2+} channel blocker, also decreased the expression of the reporter gene driven by the calmodulin promoter. These results suggest a role for Ca^{2+} in regulating the transcription of calmodulin gene.

Significance of the Accomplishments

Finding 1: Our results suggest that free Ca^{2+} concentration in the cell and the changes in protein phosphorylation that are under the control of Ca^{2+} and Ca^{2+}/calmodulin-dependent protein kinases are closely linked. A better understanding of the interrelationships between Ca^{2+}-regulated protein kinases and signal-induced changes in free Ca^{2+} concentration would be of immense value in understanding the Ca^{2+} messenger system and its role in gravity signal transduction.

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MECHANISM AND CONTROL OF GRAVITROPISM IN PLANTS

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Description of Research

How plants respond to gravity to produce a predictable pattern of growth is an interesting problem in developmental biology and has important ramifications regarding our ability to grow and utilize plants in the microgravity environment of space. Our research objective is to understand the signal transduction events that lead from gravity perception to asymmetric cell elongation in seedling roots and shoots. To dissect the steps in root and shoot gravitropism, we are using the tomato mutant *diageotropica* (*dgt*).

The *dgt* mutant arose from a spontaneous, single gene-recessive mutation of the parental variety, VFN8. Shoots of *dgt* exhibit a set of morphological and physiological abnormalities including an altered stem gravitropic response, which suggests that these plants may have a defect associated with some primary event in auxin action.

Other than qualitative data showing that *dgt* plants fail to form lateral roots and do not respond to gravity, no information on the *dgt* lesion in roots and its resultant phenotype was available. Here we report on our efforts to characterize the growth and gravitropic behavior of *dgt* roots.

Accomplishments

(1) The gravitropic response of *dgt* roots is abnormal. Although the *dgt* radical has some capacity for positive gravitropism when it emerges from the seed, after reaching a length of approximately 5 mm, the primary roots of *dgt* seedlings become agravitropic. When seedlings are placed horizontally or at various angles relative to the gravity vector, they continue to grow at that orientation, exhibiting no curvature. In contrast, the roots of VFN8, the wild-type tomato from which *dgt* is derived, have a normal gravitropic response.

(2) Relative to wild-type roots, *dgt* roots are less sensitive to growth inhibition by exogenously applied IAA and auxin transport inhibitors (phytotropins) and the roots exhibit a reduction in maximal growth inhibition in response to ethylene.

(3) IAA transport through roots, binding of the phytotropin, tritiated naphthylphthalamic acid ([^{3}H]NPA) to root microsomal membranes, and NPA-sensitive IAA uptake by root segments are similar in mutant and wild-type roots.

Significance of Accomplishments

We speculate that the insensitivity of *dgt* root growth to auxin transport inhibitors and ethylene is an indirect result of the loss of sensitivity to auxin in this single gene-recessive mutant. We conclude that *dgt* roots, like *dgt* shoots, exhibit abnormalities indicating they have a defect associated with, or affecting, a primary site of auxin perception or action.

Publications

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ASSESSING POTENTIAL TARGETS OF CALCIUM ACTION IN GRAVITROPISM

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Description of Research

Light greatly accelerates the gravitropic response of roots, coleoptiles, and stems in a wide variety of plants. This indicates that some cellular response initiated by light affects at least one of the gravity-induced cellular responses necessary for gravitropism. Our research objective is to identify one or more of these responses, thus clarifying the cellular mechanisms regulating gravitropic growth.

Several lines of evidence indicate that calcium ions may play an important role in transducing the stimuli of both light and gravity into growth changes in plants, including the important fact that both stimuli alter the concentration of cytosolic free calcium in the responding cells. However, full confidence in the validity of this hypothesis will require that at least one additional piece of information be obtained, namely, the identification of specific targets of calcium action that have a clear and significant impact on growth.

In most well-described cases, the immediate target of calcium action during the transduction of an environmental stimulus is a calcium-binding protein. Such proteins tend to be highly conserved evolutionarily, with very similar types occurring in both plants and animals. Calmodulin and calcium-dependent protein kinases are the best known targets of calcium action in plants. More recently, a new class of calcium-binding proteins called annexins has been discovered in both plants and animals. All animal annexins tested to date exhibit ion channel functions in biological membranes, several of them promote the fusion of membranes in cells and in model systems, and annexin VII shows voltage-dependent channel activity that is highly selective for Ca^{2+}. In our 1990–1991 Summary of Accomplishments, we reported the purification, initial biochemical characterization, and localization of an annexin-like protein in peas, called p35. In this year's Summary, we describe additional biochemical properties of pea annexin and provide initial localization evidence that implicates plant annexins in the control of secretion and growth.

Accomplishments

(1) We have localized p35 in ferns and dicots predominately in cells that are rapidly elongating or actively engaged in secretion. Immunolocalization reveals that p35 is highly concentrated in rapidly elongating cells undergoing xylogenesis or differentiating into phloem sieve tubes, in peripheral root cap cells that are actively secreting mucilage, and at the apical tips of polarly growing fern rhizoids and tobacco pollen tubes. At the ultrastructural level, they are found predominately in association with Golgi vesicles and the plasma membrane.

(2) Using the calcium-sensitive fluorescent dye, fura-2, we have found calcium gradients within polarly growing rhizoids of the fern, *Dryopteris*, with the highest concentration at the growing tip. This work was done in collaboration with Drs. M. Poenie (University of Texas) and Robert Scheuerlein (University of Erlangen) to help develop this fern as a model system for studying gravitropism in single cells (the rhizoid is positively gravitropic). Furthermore, there is a significant literature demonstrating that red-light-activated phytochrome plays a major role in inducing both the germination of *Dryopteris* rhizoids and the subsequent growth of the rhizoids,

so germinating fern spores should be ideal for studying both light- and gravity-induced signal transduction in the same cell.

(3) We have sequenced five peptides generated by lys-protease digestion of p35 and found them to be highly similar to known annexins. The peptides were purified by reverse-phase HPLC chromatography. Their sequences were all at least 40 percent identical to one or more known annexins with conserved substitutions at several of the non-identical positions.

(4) We have found that annexin expression is co-induced with xylogenesis in cultured *Zinnia* cells. Cultured *Zinnia* cells can be induced to undergo xylogenesis by hormone treatment. In collaboration with the laboratory of Dr. C. Haigler (Texas Tech University), we have found that, co-incident with the induction of xylogenesis in these cells, there is a great increase in the immunodetectable level of annexins.

Significance of the Accomplishments

Findings 1 and 2: Both in rapidly elongating cells undergoing xylogenesis and in polarly growing rhizoids and pollen tubes, there is active secretion of wall materials into the newly forming or expanding walls. Calcium is thought to play a major role in this process, but the target of calcium action in polar growth is unknown. Our findings suggest that calcium-binding annexin proteins, which are known to promote the fusion of secretory vesicles with the plasma membrane in animal cells, are highly concentrated in cells active in the secretion of wall materials and are thus major candidates for being a target of calcium action in this process. The coincidence of calcium and annexin gradients in fern rhizoids is consistent with the hypothesis that annexins may play a role in calcium transport in these cells. If annexins play an integral role in delivering vesicles to expanding walls for growth, they should be key agents in the process of differential growth leading to gravitropic curvature.

Finding 3: These structural data further support the conclusion drawn from our earlier functional analyses that p35 is indeed a member of the annexin family. These amino acid sequences are among only a few known for plant annexins at the present time. These data also provide a valuable information base for verifying the identity of cDNA clones that we have selected from a pea cDNA library by antibody screening as ones that encode annexin-like proteins.

Finding 4: These results indicate that annexins are selectively induced by hormones that promote the elongation and differentiation of cultured cells into xylem cells. Hormones are also thought to be key promoters of the differential growth leading to gravitropism. Therefore, learning more about the role of annexins in hormone-induced growth should provide valuable insights into the cellular mechanisms of gravitropism.

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CELLULAR POLARITY AND INTERACTIONS IN PLANT GRAVIPERCEPTION

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Description of Research

Our long-term goal is to understand the mechanisms of gravitropic sensing in higher and lower plants. This involves identifying the cells that sense gravity and determining the cellular mass that gravity acts upon to trigger sensing. We hope to learn which events occur during the transduction of a physical signal into a physiological signal that affects growth.

A second goal is to understand how cells have "used" gravity in orienting and organizing themselves. A related question is what mechanisms have evolved that prevent the stratification of cell components with respect to gravity?

One major focus of our research has been the tip-growing cells of the protonemata of the moss *Ceratodon*, which grow up in the dark. We have shown that the process of upward curvature involves a redistribution of microtubules close to the growing zone.

Accomplishments

(1) Gravitropic roots of the aquatic angiosperm, *Limnobium*, have sedimented amyloplasts in their elongation zone but not in their root cap.

(2) Nuclei as well as amyloplasts sediment in the elongation zone.

(3) Plastids sediment in vertical cells of the moss *Ceratodon*, but sedimentation is incomplete and the number of plastids that sediment and the location and extent of plastid sedimentation is highly regulated in vertical cells.

(4) Microtubules restrict plastid sedimentation in vertical protonemata; i.e., microtubule deploymerizers cause complete plastid sedimentation, whereas microfilament inhibitors have no effect on sedimentation.

Significance of the Accomplishments

Finding 1: These data extend our previous findings from roots of the more primitive plant, *Equisetum*, that amyloplast sedimentation in roots can occur outside the cap. While this does not prove that the elongation zone is capable of sensing, it does provide structural data that warrant a reinvestigation of the question of whether sensing is confined to the cap in all roots.

Finding 2: As far as I know, these cells in roots of *Limnobium* and *Equisetum* are the only cases reported where nuclei sediment at 1-g. Presumably, this demonstrates that nuclei have enough mass to sediment in other systems as well but do not do so, perhaps because of restraint by the cytoskeleton.

Finding 3: Comparison of upright and inverted cells shows that plastids do sediment in vertical cells, not just in horizontal cells. However, heretofore, such sedimentation has been difficult to detect since sedimentation is incomplete; i.e., plastids are present throughout the length of vertical cells and plastids do not fall through the length of these cells. Inversion reveals subtle but real amyloplast sedimentation. Such restricted sedimentation argues strongly for a role for the cytoskeleton in maintaining plastid distribution.

Finding 4: These data establish a central role for microtubules in controlling plastid sedimentation in these moss cells. This uncovers a second gravity-related role for microtubules in these cells, since microtubules also become redistributed prior to and during upward gravitropic curvature. The finding that microtubules restrict sedimentation demonstrates the importance of the cytoskeleton in maintaining organelle position and cell organization against the force of gravity. Although it has been proposed that the cytoskeleton evolved, in part, to prevent the stratification of cell components with respect to gravity, there are few direct proofs of this role in the literature.

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DEVELOPMENTAL STUDIES OF WHEAT IN MICROGRAVITY

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Description of Research

Since 1987, we have been preparing for flight experiments with a superdwarf cultivar of wheat on the U.S. Shuttle and the Russian Space Station *Mir*. During 1992, it became apparent that the Shuttle experiments might be indefinitely delayed; but, in December of 1992, two of our experiments carried out in cooperation with personnel at the Institute of Biomedical Problems in Moscow were incorporated as part of the Shuttle/*Mir* rendezvous (called Spacelab *Mir-1* or SLM-1) scheduled to take place in 1995. These exercises will include a seed-to-seed experiment for up to about 120 days followed by a second crop for about 30 days, at which time the U.S. Shuttle will arrive. Fixed and dried samples from the first crop and frozen samples from the second crop will be returned for analysis. Virtually all previous long-term experiments with plants in microgravity have resulted in plants with retarded developmental stages and greatly reduced yields; however, these experiments have never been done with environmental conditions known to produce normal plants on Earth. Thus, the goals of our experiments include the improvement of growing conditions plus the diagnosis of reasons for observed growth responses.

The Russian plant growth chamber, *Svet*, is being provided with environmental sensors to monitor various aspects of the plant environment, including substrate moisture levels, temperatures, irradiances, pressure, oxygen, atmospheric humidity, and carbon dioxide during growth of both crops. Gail Bingham in the Space Dynamics Laboratory at Utah State University is the project engineer who is developing equipment for this monitoring, which will allow calculation of photosynthesis, dark respiration, and transpiration. During the seed-to-seed crop, plants will be sampled and chemically fixed at five stages: prefloral (6 days after planting), early floral (14 days), boot (head surrounded by leaf), anthesis, and seed filling. All remaining plants will be harvested at maturity, and the resulting seed will be tested for viability and ability to produce normal plants. Samples will be studied by William Campbell (anatomy, histology) and John Carman (a spectrum of plant hormones, other parameters), both at Utah State University; and David Bubenheim (various biochemical parameters) at NASA Ames Research Center. Boris Yendler, also at Ames, will examine the returned root module in cooperation with Igor Podolsky in Moscow and other investigators in Utah. Leaf area measurements will be made on collected samples at each sampling time.

In addition to hardware development, ground-based research emphasizes growth of plants under conditions comparable to those that will be encountered in the space experiments. These include relatively low light levels (125—250 mmol m^{-2} s^{-1}, photosynthetic photon flux) and high CO_2 levels (up to about 1.0 percent before canisters are changed). The root module appears to be an especially challenging problem. The substrate *(Balkanine*, an ion-exchange material charged with plant nutrients) may be saturated at some points and dry at others, judging by previous experience with this system. We can only monitor this situation this time but hope to achieve better control in future space studies.

Accomplishments

Our studies with superdwarf wheat (25—30 cm tall) have so far emphasized responses to the low light levels available in space-rated plant growth chambers. We have grown plants in a controlled

environment (temperature, CO_2 levels) under a tilted bank of fluorescent lamps. Plants closest to the lamps receive about 350 μmol m^{-2} s^{-1}, while plants farthest from the lamps receive about 50 μmol m^{-2} s^{-1}. Plants under the lowest irradiances are about at the compensation point; photosynthesis producing about the same amount of fixed carbon as respiration uses; hence, there is virtually no growth (although a few leaves form using food stored in the seed and a sterile head might eventually form). Plants under the highest irradiances are essentially normal although their yields are reduced compared with plants under even higher light levels (e.g., full sunlight = 2000 μmol m^{-2} s^{-1}). To summarize the results of several experiments: All developmental stages (e.g., formation of heads) are delayed at lower irradiances compared with higher light levels, but final yields rise sharply with irradiances above the compensation point and then level off. The leveling is because plants at lower irradiances take much longer to mature and thus have a longer time to accumulate light energy. In addition to our studies with light, we have tested various substrates. Our future work will place more emphasis on substrates, CO_2 levels, temperatures, photoperiods, and measurement of leaf areas with photographic techniques. We will soon have a facility with twelve *Svet* mockups, in which we can vary these factors.

In September of 1990, two of our Russian colleagues (Alexander Mashinsky and Galina Neichitailo) visited us in Utah, and we gave them a few seeds from superdwarf wheat. In late April of 1992, these were sent up to *Mir* and grown in *Svetoblock M*. An attempt was made to complete a life cycle with the plants although light levels were low (about 135—175 μmol m^{-2} s^{-1}), and plants were exposed directly to the cabin atmosphere. Water was added manually to the substrate *(Balkanine)*. Plant growth was substantially delayed on the 40th day and then actually ceased. But on the 90th to 100th day, new shoots developed and three pairs of leaflets formed. Three heads appeared in the boot (i.e., each surrounded by a leaf) in two plants, while the plants were still in *Mir*. After return to Earth on October 10, 1991 (167th day), three heads from these developed after 10 to 32 days under laboratory conditions (somewhat higher light). One head was sterile, but 28 seeds formed in the other two heads and most of these seeds produced healthy plants that, in turn, produced viable seed. On Earth, wheat matures in 60 to 100 days.

Mashinsky and Neichitailo visited Washington, DC in late October of 1991, where further plans were made and a protocol was signed. Results of the preliminary *Mir* experiment were presented to us at that meeting. They (with Elena Shubina, a translator) visited Utah again in August of 1992 for further planning. In July of 1992, I visited Krasnoyarsk and then Moscow, where another protocol was prepared. In November of 1992, our team (Salisbury; Bingham; Steven Brown, an electrical engineer; Linda Gillespie, Salisbury's technician; Scott Jones, graduate student working on substrate problems; and David Bubenheim, plant physiologist from NASA Ames) spent a week at the Institute of Biomedical Problems in Moscow. We took the monitoring equipment that had been developed and were able to interface it with the Russian growth chambers in which superdwarf wheat and dwarf pea were being grown. Since then, various members have made several visits to Moscow, and our Russian colleagues have made several visits to Utah State University and to NASA Ames. We are now cooperating with a team under the direction of Vladimir N. Sychev, Head of Laboratory.

Significance of the Accomplishments

Few studies have investigated plant responses to the low irradiances available in current growth chambers for space experiments. Our results emphasize the importance of increasing the light in such chambers. Although plants may appear relatively normal when grown close to the compensation point, it is important to realize that development is greatly delayed and yields are reduced, although yields are somewhat higher than expected because of the longer exposure to the low light. These adverse effects make it very difficult to evaluate the effects of microgravity. High CO_2 and substrate-moisture problems compound these difficulties.

Although not described in detail in this report, hardware development has progressed nicely. Excellent equipment exists to monitor such environmental factors as CO_2, O_2, light, air and leaf temperatures, pressure, and substrate moisture. This will make it possible to diagnose and better understand the observed plant responses. The newly developed instrumentation could be of considerable value in future space experiments with plants by others as well as ourselves.

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GRAVITY-INDUCED POLARITY IN SINGLE CELLS OF *CHARA*

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Description of Research

The overall goal of our research program is to elucidate the gravireceptor and the biophysical events that the activated gravireceptor initiates in order to understand how cells perceive and respond to a gravitational stimulus. In order to accomplish our goals, we have studied the effects of gravity on the polarity of cytoplasmic streaming in the single internodal cells of characean algae.

We have proposed a model for gravisensing in which the entire protoplast experiences the force of gravity and settles within the extracellular matrix. Consequently, proteins connecting the plasma membrane to the extracellular matrix at the ends of the cells experience a differential compression or tension as a result of gravitational pressure. This leads to the activation of certain classes of Ca^{2+} channels, localized at the ends of the cells, and subsequently a change in the polarity of cytoplasmic streaming.

Accomplishments

(1) ***Hydrostatic pressure mimics gravitational pressure.*** In order to test the hypothesis that cells sense a differential pressure on the top and bottom of the cell induced by gravity (i.e., gravitational pressure), we subjected horizontal cells to a unidirectionally applied pressure. We find that a unidirectionally applied hydrostatic pressure mimics gravitational pressure in inducing the physiological response. Both stimuli produce physiological responses that are identical in terms of ionic requirements, inhibitor sensitivity, etc.

(2) ***Gravireceptor may be an integrin-like protein.*** In order to characterize the mechanoreceptor responsible for sensing gravitational and hydrostatic pressure, we treated cells with a variety of impermeant enzymes and peptides, including Arg-Gly-Asp. These experiments demonstrate that the receptors are localized at the plasma membrane-extracellular matrix junction at the ends of the cell and that the mechanoreceptor for gravitational and hydrostatic pressure is an integrin-like protein.

(3) ***Gravireceptor is distinct from touch-receptor in same cell.*** In order to test the hypothesis that a single mechanoreceptor is involved in all responses to mechanostimulation, we characterized the mechanoreceptor involved in the touch-induced action potential in characean internodal cells. We find that the touch receptor is distinct from the receptor involved in sensing gravitational and hydrostatic pressure. The two classes of receptors vary in their spatial localization, their sensitivity to proteases and peptides, their energetics, their ability to perceive direction, etc.

(4) ***A change in membrane potential is not involved in the signal transduction chain involved in sensing gravitational or hydrostatic pressure.*** In order to test the hypothesis that a change in membrane potential contributes to the signal transduction chain involved in gravitational or hydrostatic pressure sensing, we measured the membrane potential of cells subjected to either gravitational or hydrostatic pressure. The resting membrane potential measured with intracellular electrodes is approximately -0.22 V. The membrane potential at the

ends of the cells, measured with intracellular electrodes, varies by less than 0.001 V when cells are subjected to hydrostatic pressure. The difference in membrane potential at the ends of the cells, measured with extracellular electrodes, varies by less than 0.001 V when cells are subjected to gravitational pressure. These variations in membrane potential are too small to be involved in signaling and may be the result of changes in the partial membrane conductance.

(5) ***Two distinct classes of Ca^{2+} channels are involved in gravisensing, a third is not.*** Pharmacological and localization experiments indicate that two distinct classes of Ca^{2+} channels are localized at the ends of the cells, are sensitive to organic channel blockers, and are involved in gravisensing (Classes I and II). A third class of Ca^{2+} channel is found throughout the plasma membrane, is insensitive to organic channel blockers, and is involved in sensing electrical stimulation and touch (Class III). These conclusions are supported by flux measurements using Sr^{2+} as a tracer.

(6) ***Characterization of the structure of the Ca^{2+} channels.*** We have used the lanthanides to probe the structures of the three classes of Ca^{2+} channels. We find that Class I is an elastic peristaltic channel that has three binding sites within the pore for Ca^{2+}. Likewise, Class III is an elastic peristaltic channel with three bindings sites for Ca^{2+}; however, the binding sites are definitely multidentate. By contrast, Class II channels have an external vestibule that can only be blocked by large cations. Lanthanide flux studies show that the lanthanides go through at least Class I and Class III channels and kinetically inhibit channel activity. We are currently testing whether or not they go through Class II channels or remain in the external vestibule.

Significance of Accomplishments

Finding 1: We have established an artificial system to mimic the effect of gravity and test the gravitational pressure hypothesis.

Finding 2: We have determined that the gravireceptor may be an integrin-like protein.

Finding 3: We have demonstrated that the mechanoreceptor involved in gravisensing is distinct from the mechanoreceptor involved in touch sensing in the same cell, indicating that there are multiple classes of mechanoreceptors.

Finding 4: We have found that a change in membrane potential is not involved in the signal transduction chain involved in sensing gravitational pressure in characean cells.

Finding 5: We have established that two distinct classes of Ca^{2+} channels are involved in gravisensing, a third is not.

Finding 6: We are characterizing the structure of the Ca^{2+} channels in order to determine how the structure of a channel relates to the function in which that channel is involved.

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ANIMAL PROJECTS

COMPARATIVE TRANSDUCTION MECHANISMS OF VESTIBULAR OTOLITH HAIR CELLS

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Description of Research

Using *in vitro* preparations,[5-7] we have previously shown that hair cells in different regions of the bullfrog sacculus and utriculus differ markedly in hair bundle morphology and physiological response properties. More recently, using the aminoglycoside antibiotic gentamicin sulfate, we have demonstrated our ability to induce *in vivo* the degeneration and subsequent regeneration of saccular and utricular hair cells in the sacculus and utricular striola.[1,3] Hair cells in these organs are differentially sensitive to gentamicin, with saccular hair cells and hair cells in the utricular striola being damaged at lower gentamicin concentrations than hair cells in the utricular extrastriola. Regenerating hair cells in these studies were easily distinguished from their mature counterparts by their hair bundles, which were distinctly smaller and shorter than those of more mature hair cells. These cells were also classified into a number of types using the same morphological criteria used to identify their mature counterparts.

More recently, we have developed *in vitro* explant cultures of the bullfrog saccular and utricular maculae to extend our earlier *in vivo* studies of hair cell regeneration in the vestibular otolith organs. These preparations have allowed us to control the concentration and duration of gentamicin application and to continuously follow the fate of individual regenerating hair cells. In morphological studies, we have studied the formation and migration of individual regenerating hair cells, documenting changes in their cellular and hair bundle morphology. Ultimately, we hope to document changes in the physiological response properties of hair cells, correlating these changes with the time of acquisition or changes in the amplitude or gating kinetics of specific membrane currents.

Accomplishments

(1) ***In vivo studies of cellular proliferation.*** The results of earlier *in vivo* studies suggested that hair cell regeneration in the vestibular otolith organs might involve the mitotic production of new hair cells. To more closely examine the role of mitotic division in hair cell recovery, we injected bullfrogs with 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue that is incorporated into mitotic cells. We were then able, using immunocytochemical methods, to measure ongoing and gentamicin-induced cell proliferation in the vestibular otolith organs. The primary aim of this study was to determine if mitotic division in gentamicin-treated animals was upregulated from the ongoing rate, if any, of mitotic division in normal animals. We also wished, by examining the spatial and temporal distribution of BrdU-labeled cells in gentamicin-treated animals, to compare the patterns of cell proliferation and hair cell regeneration in these organs, and to identify the progenitor cells of new cells in the vestibular otolith organs.

BrdU labeling was seen in normal and, to a greater extent, in gentamicin-injected animals. Labeling in the saccular macula was more extensive than in the utriculus, consistent with the greater damage caused by gentamicin to the former organ. BrdU-labeled cells were initially seen in the peripheral macular margins and, within the maculae, in a subset of supporting cells immediately adjacent to the basement membrane. These supporting cells had spherical cell bodies and, unlike typical supporting cells, had little or no apical projections. At later times, hair cells and supporting cells

were also located further from the basement membrane, suggesting that mitotic cells and their progeny had migrated to more apical positions. The macular distribution of BrdU-labeled cells was not correlated with the pattern of gentamicin-induced cellular damage. First, the macular distribution of BrdU-labeled cells was similar in both normal and gentamicin-injected animals. Second, proliferating cells were not restricted to only those macular areas damaged by gentamicin treatment. Proliferating cells in the sacculus, for example, were largely restricted to the abneural half of the sensory macula. In the utricular macula, proliferating cells were seen in both the striolar and extrastriolar regions.

Cellular proliferation was insufficient to explain hair cell recovery seen in the vestibular otolith organs. Moreover, proliferating cells had a different macular distribution from that of regenerating hair cells. In addition, the great majority of regenerating hair cells in the vestibular otolith organs were not BrdU-labeled, suggesting that hair cell recovery in the vestibular otolith organs is primarily determined by non-mitotic mechanisms. In less damaged areas, such as the utricular striola, hair cell recovery appeared to be largely carried out by hair bundle repair. In more heavily damaged regions, unlabeled supporting cells with immature hair bundles and cellular morphology transitional between that of hair cells and supporting cells were often seen, suggesting that hair cell recovery in these regions was primarily determined by the transdifferentiation of undamaged supporting cells into hair cells. Supporting cells have long apical projections which have extensive surface contact with hair cells. We hypothesize that the loss of this contact due to local hair cell death may trigger intercellular signals which initiate the transdifferentiation of supporting cells into hair cells.

The results of these studies have been presented in abstract form at the Annual meeting of the Society for Neuroscience [4] and are now being written up in manuscript form.[8]

(2) ***Phalloidin labeling of filamentous actin.*** Phalloidin labeling was used to label filamentous actin in the cell bodies and hair bundles of hair cells. This technique has significantly improved the visibility of immature hair bundles. In addition, the cellular distribution of filamentous actin in regenerating hair cells, particularly at early developmental times, differs from that of normal hair cells, enabling us to identify new hair cells before the appearance of their hair bundles.

In normal animals, phalloidin strongly labeled the intercellular adherens junctions at the apical epithelial surface and the stereocilia array of hair bundles. The distribution of filamentous actin in intercellular adherens junctions revealed a continuous reticular lamina throughout the confines of the former sensory surface. Little or no labeling, by contrast, was observed within hair cells or supporting cells. Twenty-four to forty-eight hours after intraotic gentamicin sulfate injection, little or no labeling was seen above the macular surface, confirming the rapid loss of hair bundles throughout the saccular macula and within the utricular striola. In heavily damaged regions, the apical surfaces of supporting cells had expanded in size to fill spaces normally occupied by hair cells.

Hair cell recovery in the sacculus and utriculus of gentamicin-treated animals proceeded in a fixed spatial order. In the sacculus, new hair bundles were first seen in the peripheral macular margins adjacent to the entrance of the saccular nerve, indicating the presence of new hair cells in the saccular macula. Similar bundles were observed in the abneural portion of the maculae only at later times. By 7-9 days after gentamicin injection, new hair bundles were observed in both the neural and abneural regions of the saccular macula, although hair bundles in the abneural region were often smaller than those in the neural region. The line of reversal of hair cell polarization in the saccular macula remained largely devoid of hair bundles. Large epithelial holes, surrounded by supporting cells, were also observed in the abneural, but not the neural, region. In the utricular macula, new hair bundles were seen only in the striolar region. Hair cells repopulated the striola in

a fixed manner, with new hair cells appearing first on the outer striolar rows and only later appearing on the innermost rows of the striolar region.

The cellular distribution of filamentous actin in regenerating hair cells, particularly at early developmental times, differed from that in mature hair cells. In normal animals, little or no phalloidin labeling was seen within hair cells or supporting cells. By contrast, new hair cells were diffusely labeled just below their apical surface, indicating that their cuticular plates contained an appreciable amount of filamentous actin. This labeling was particularly obvious in the utricular striola and at the line of reversal of hair cell polarization in the sacculus. In these regions, many cells were observed with cuticular plate labeling but no hair bundles. These cells were assumed to represent new cells which were in the process of creating or repairing their sensory hair bundles.

The results of these studies have been presented in abstract form at the annual meeting of the Society for Neuroscience [4] and are now being written up in manuscript form.[9]

(3) ***Cell proliferation and hair cell regeneration in explant organ cultures.*** We have created explant organ cultures by removing saccular and utricular maculae from the membranous labyrinth in cold, oxygenated HEPES-buffered saline, trimming these organs of excess nervous and connective tissue to improve the visibility of hair bundles and removing the otolith membranes of these organs with proteolytic digestion and gentle mechanical agitation. Excised organs were then incubated in Wolfe-Quimby incubation media (GIBCO) and placed, hair bundles upward, in lab-built culture chambers. Cultured organs were maintained for 7-14 days, replacing half of the culture medium with fresh culture medium every 2 days.

The extent of cellular damage in normal cultures was assessed using Nomarski optics and vital stains with light (Trypan blue) and fluorescent (Ethidium bromide) microscopy. Normal cultures displayed little or no cellular damage and exhibited normal morphology for periods up to 14 days. Exposing organ cultures for 6-18 hours to varying concentrations of gentamicin sulfate (100-400 μmol), we have induced *in vitro* patterns of hair bundle and cellular damage similar to those seen *in vivo*. Hair cell recovery in the saccular and utricular maculae, as measured by hair bundle and nuclear density, was also similar to that seen in earlier *in vivo* studies. We have also cultured normal and gentamicin-treated organ cultures with varying concentrations of BrdU. Sacrificing cultures at varying times subsequent to gentamicin application, we have then been able to monitor the number, macular location, and cellular morphology of both proliferating and regenerating hair cells. These preliminary studies have demonstrated that our *in vitro* organ cultures are a good model system for the study of cell proliferation and hair cell regeneration following gentamicin ototoxicity.

In recent studies, we have demonstrated that hair cell regeneration takes place even in the absence of cell proliferation. This was verified by maintaining organ cultures in aphidicolin, a blocker of mitotic division, subsequent to gentamicin treatment. Under these conditions, little or no cell proliferation was seen, but hair cell regeneration occurred at close to its normal rate. The results of these preliminary studies, when complete, will be written up for publication, acknowledging the support of NASA Grant 2-651.

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EFFECT OF SKELETAL UNLOADING ON BONE FORMATION

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Description of Research

Skeletal unloading, whether a result of bedrest, immobilization, or space travel, leads to a reduction in bone mass. In a remodeling skeleton this is due both to a decrease in bone formation and an increase in bone resorption. However, in a modeling skeleton such as in the growing rat the relative loss of bone is thought to be due primarily to a decrease in bone formation. We have evaluated the response of bone formation to skeletal unloading in a rat model in which the animal's hindlimbs are unweighted by elevation of the hindquarters while the forelimbs remain weightbearing. We observed that bone formation in the unloaded bones ceases after 5 days of hindlimb elevation but resumes after an additional 5 days despite continued unloading. Bone mass reaches a new and lower steady state, which is then maintained. Upon normal weightbearing, bone formation is accelerated until bone mass is recovered.

Serum $1,25(OH)_2D$ levels parallel the changes in bone formation during skeletal unloading; falling during the first week of skeletal unloading, returning to control by 2 weeks, and increasing upon normal weightbearing. However, infusion of $1,25(OH)_2D$ to prevent its fall during skeletal unloading did not prevent the fall in bone formation. Following this observation, a significant question to be investigated was whether the locally produced bone growth factor IGF-1 was involved. Surprisingly, the levels of IGF-1 mRNA tended to increase during the initial stages of unloading. Infusions of IGF-1 or growth hormone (which increases IGF-1 production by bone) increased bone formation in normally loaded bones but did not increase bone formation in unloaded bones. These results are consistent with the hypothesis that skeletal unloading tends to counteract the bone-growth-promoting actions of IGF-1 (and GH) at several levels including IGF-1 production.

We are currently testing this hypothesis by evaluating the level at which skeletal unloading blocks the ability of IGF-1 and GH to regulate events involved in bone formation. In particular, the effect of skeletal unloading on GH and IGF-1 receptors, IGF-1 production, osteoblast function (osteocalcin, collagen, and alkaline phosphatase), and the effect of skeletal unloading on the ability of GH and IGF-1 to regulate these functions are being determined. We expect to be able to determine at what level resistance to these bone regulating hormones is encountered during skeletal unloading.

Accomplishments

(1) Demonstrated the changes in the mRNA and protein levels for IGF-1, IGF-2, and their receptors as a function of development from 5 days before birth to 28 months.

(2) Determined the mRNA levels for IGF-1, IGF-1 receptor, alkaline phosphatase, collagen, and osteocalcin as a function of skeletal unloading.

(3) Demonstrated that the potent bisphosphonate, alendronate, reversed the relative loss of bone incurred during skeletal unloading.

(4) Established the *in situ* hybridization method for IGF-1 mRNA using fetal bone.

Significance of the Accomplishments

The striking changes in mRNA levels for IGF-1, IGF-2, and their receptors during fetal through adult development served to validate our methods and provided important insight into the relative contributions of IGF-1 and IGF-2 in bone development. Surprisingly, the mRNA levels for IGF-1 and IGF-1R are much lower in bones from post-weaning and early adolescent rats than in bones from the fetal or adult rats. Such observations suggest that, during this period of rapid growth, IGF-1 may not be the dominant regulator of bone growth. In light of the failure of IGF-1 mRNA to change during skeletal unloading, this possibility needs to be considered further. In this regard, the collagen and osteocalcin mRNA levels fell with skeletal unloading, but the IGF-1 and IGF-1R mRNA levels did not. Measurements of IGF-1 protein are in progress, but these data indicate a surprising dichotomy between parameters of bone formation and the production of a factor thought to be responsible for bone formation, i.e., IGF-1. We are examining the localization of the mRNA for IGF-1 by *in situ* hybridization as affected by skeletal unloading, since changes in the mRNA levels in cells critical for bone formation and affected by skeletal unloading may be obscured by the IGF-1 mRNA levels in other cells not responding to skeletal unloading, yet contributing to the total mRNA signal in whole bone extracts.

Alendronate, a potent inhibitor of bone resorption, was evaluated for its ability to affect bone mass during skeletal unloading as a test of the hypothesis that bone resorption contributes little to the relative bone loss in the growing rat subjected to skeletal unloading. The results indicated that the contribution of bone resorption was higher than expected. Alendronate resulted in increased bone in the metaphyseal region in both controls and unloaded rats, loss of contact between osteoclasts and the underlying bone, and inhibition of bone formation in the tibia-fibula junction. Thus, protection against bone loss during skeletal unloading is not without some potentially serious side effects. Nevertheless, these data indicate that bone resorption contributes substantially to the relative loss of bone during skeletal unloading even in the growing rat and suggests a potential means of reducing bone loss during space flight.

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PARATHYROID HORMONE STIMULATES *c-fos* AND *c-jun* mRNA ABUNDANCES IN RAT OSTEOBLASTIC CELLS

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Description of Research

Exposure to microgravity results in hypercalciuria and loss of skeletal mass. The cellular and intracellular events which mediate these physiologic changes remain unclear. We hypothesize that in a weightless environment bone cells, specifically the osteoblasts, may have an altered sensitivity to certain resorptive factors like parathyroid hormone (PTH). The objective of my NASA-supported research was to define further the intracellular mechanisms which mediate PTH-altered gene expression in the osteoblast. We were specifically interested in analyzing the immediate-early genes, *c-fos* and *c-jun*. This work was designed to establish a model which could serve as a foundation for future experiments conducted at zero gravity.

We utilized the rat osteoblastic osteosarcoma cell line, UMR 106-01, which is commonly used as a model of the osteoblast. The effects of PTH and other agents of interest were measured at the mRNA (Northern blot analysis) and transcriptional (nuclear run-on assay) levels. We studied the immediate-early genes *c-fos* and *c-jun* which are proto-oncogenes that respond to, and mediate, the effects of many extracellular stimuli. These genes encode the protein products *fos* and *jun* which are major components of the activator protein-1 transcription complex.

Accomplishments

Our work clearly demonstrated that in the UMR 106-01 osteoblastic cell line PTH stimulated a marked, transient increase in *c-fos* (50 fold) and *c-jun* (5 fold) mRNA levels. Both transcripts were maximally elevated approximately 30 minutes after hormone treatment. Nuclear run-on assays demonstrated that PTH also stimulated a transient increase in the rate of *c-fos* and *c-jun* transcription which was maximal 20 minutes after hormone exposure. Additional experiments were performed to further define the signal transduction pathways involved in these responses. We demonstrated that the protein synthesis inhibitor cylcloheximide had no effect on the PTH-induced increase in *c-fos* and *c-jun* mRNAs. Second messenger analog experiments showed that PTH stimulates *c-fos* and *c-jun* mRNA abundance via a mechanism which is partially mimicked by protein kinase A activation and is independent of PMA-sensitive protein kinase C pathways.

Significance of Accomplishments

Parathyroid hormone is a potent stimulator of bone resorption and could be involved in the process of microgravity-induced bone loss. Nevertheless, little is known about the intracellular events which mediate PTH-altered gene expression. The osteoblast is the PTH target cell in bone; and, therefore, we analyzed the effect of PTH on immediate-early gene (*c-fos* and *c-jun*) expression in this cell type. Our work identified and characterized two immediate-early gene responses which may be fundamental to PTH-altered gene expression in the osteoblast. These data have enhanced our understanding of the intracellular events which mediate the action of PTH. By further understanding the "normal" cellular response to PTH, we can formulate experiments to determine

whether these responses are altered at zero gravity and whether they play a role in microgravity-induced bone loss.

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BASIC GRAVITATIONAL REFLEXES IN THE LARVAL FROG

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Description of Research

Little is known about how vertebrates are able to sense gravity and how they process this information to generate appropriate motor responses. This investigation is designed to determine how a primitive vertebrate, the bullfrog tadpole, is able to sense and process gravitational stimuli. Because of the phylogenetic similarities of the vestibular systems in all vertebrates, the understanding of the gravitational reflexes in this relatively simple vertebrate should elucidate a skeletal framework, on an elementary level, upon which the more elaborate reflexes of higher vertebrates may be constructed.

The tadpole manifests a powerful counter-rolling response of the eyes to static tilt of the head. In addition, the fact that amphibians are cold-blooded means that their cellular metabolism is much lower than that of mammals. Consequently, the entire head can be maintained *in vitro* with the brain, sensory structures, and eye muscles exposed. The strong gravitational reflexes are still evident under these conditions and persist for several days. Such conditions permit detailed and reproducible electrophysiological and anatomical investigations of this reflexive behavior.

The purpose of this study is to understand how the nervous system of the larval amphibian processes gravitational information. This study involves predominantly electrophysiological investigations of the isolated, alert (forebrain removed), bullfrog tadpole head. The focus of these experiments is threefold: (1) to understand from whole extraocular nerve recordings the signals sent to the eye following static gravitational tilt of the head; (2) to localize neuronal centers responsible for generating these signals through reversible pharmacological ablation of these centers; and (3) to record intracellularly from neurons within these centers in order to determine the single neuron's role in the overall processing of the center. This study will provide information on the mechanisms by which a primitive vertebrate processes gravitational reflexes.

Accomplishments

(1) ***Quantification of the behavior.*** A software event detection technique, which has been developed over the last 15 years, has been finalized and published (Cochran, 1993). This technique allows for analysis of spontaneously occurring voltage deflections over time. Electrophysiological recordings digitized (at 50 kHz) over many minutes can be rapidly and automatically analyzed. The publication illustrates this technique for spontaneously occurring synaptic potentials recorded intracellularly from VIIIth nerve afferents that are consequent from convergent hair cell innervation of the afferents. This technique allows for tens of thousands of excitatory post-synaptic potentials (EPSPs) to be analyzed during this period. With respect to the experiments in this study that involve whole nerve recordings from the trochlear and medial rectus nerves, this same technique can be applied to detect and quantify the extracellularly recorded nerve action potentials. Since these whole nerve recordings are more stable than the intracellular recordings of EPSPs, digitized periods are often over an hour in duration, allowing for continuous quantification of hundreds of thousands of spike potentials that occur during the recording period. Since the amplitude (as well as other parameters) of each spike potential is quantified, whole nerve-recorded spike potentials can be parceled according to size and their size can then be related to the time of occurrence during a tilt stimulus.

(2) ***Responses to static tilt of the head.*** Trochlear motoneurons increase their frequency of firing with nose-up tilts and decrease it with nose-down tilts. Motoneurons

innervating the medial rectus muscle respond reciprocally; i.e., they increase their activity with nose-down tilts and decrease their activity with nose-up tilts. For both the trochlear and medial rectus motoneurons, the larger sized units are phasic and adapt more rapidly than the smaller sized units, which appear to have a larger tonic component and adapt less rapidly. Typically larger units fire only during a movement (relating them more to velocity), while smaller units appear to be modulated both by the table movement as well as by the absolute position of the table (relating them both to table velocity and position).

(3) ***Pharmacological studies.*** Iontophoresis of the kynurenic acid, an antagonist of the excitatory transmitter glutamate, into the extraocular motor nuclei reversibly abolishes spike activity in the extraocular motor nerves. Intracellular recordings from oculomotor neurons show complex mixtures of EPSPs and inhibitory post-synaptic potentials (IPSPs) following electrical stimulation of the vestibular nerves and afferent brainstem pathways to this of nucleus. Bath application of kynurenic acid reversibly blocks over 95 percent of the EPSPs. Both of these findings are consistent with the hypothesis that the principal excitatory transmitter to the oculomotor nucleus is glutamate or a related compound.

Significance of the Accomplishments

In general, the above accomplishments provide a step toward understanding how these primitive vertebrates sense gravitational stimuli and how they process these stimuli and integrate them into reflex control.

The development of the quantification technique is an important step in acquiring the ability to investigate how gravity can influence eye movements. Moreover, this technique is generalizable to any spontaneously occurring events that can be represented as voltage deflections over time, indicating that it has a potential use other than its strict application in this study.

The similarity of the responses from the trochlear versus medial rectus motoneurons (although their receptive field properties are different) suggests that different inner ear end organ signals converge upon these motoneurons but that the quality of the processing of these signals is similar. The contribution of individual end organs and specific vestibular nuclear complex neurons in channeling the static and dynamic signals remains to be determined.

The finding that glutamate is the main excitatory transmitter to the oculomotor nucleus indicates that glutamate is the principal excitatory transmitter of the entire vestibulo-ocular reflex. Glutamate (or a related compound) is the hair cell transmitter, it is the transmitter of VIIIth nerve afferents, it is the transmitter of excitatory commissural vestibular neurons, and it is the transmitter of vestibulo-oculomotor neurons.

These findings provide basic information as to the organization of gravitational-ocular reflexes in a relatively simple lower vertebrate. These studies can provide a basic framework for understanding how these reflexes are organized and elaborated upon in the more complex vestibulo-ocular behaviors exhibited by higher vertebrates.

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A GRAVITY-SENSITIVE PERIOD DURING FROG OOCYTE MATURATION

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Description of Research

The unfertilized amphibian egg appears to be radially symmetric about its animal-vegetal axis. Establishment of the embryo's bilaterally symmetric body plan, referred to as dorsal-ventral (DV) axis specification, requires a 30° rotation of the vegetal yolk mass relative to the egg surface during the first cell cycle. The direction of this rotation reliably determines the DV axis orientation and usually reflects the sperm entry position (SEP). Thus, the SEP is often used to predict the plane of bilateral symmetry. However, the spatial relationship between the SEP and the DV axis is actually quite variable, suggesting that cues other than the SEP also bias the rotation mechanism. One well-known external influence is gravity; i.e., when fertilized eggs are tilted from their usual orientation, gravity-driven internal rearrangements result in rotation directions different than that specified by the SEP. Endogenous cues to direct rotation are also present in the egg prior to fertilization; for example, activated eggs exhibit a normal, directed rotation, even though they have not been fertilized. We observed that, in eggs of the frog *Xenopus laevis* tilted 90° off axis during *in vitro* maturation, the maturation spot appears about 15° from the center of the animal hemisphere closest to the point of the equator that was positioned upward. When such eggs are activated, the vegetal yolk mass rotates toward this point. The fact that normal spawned eggs behave similarly suggests that off-axis influences also occur under *in vivo* conditions. As in oocytes matured *in vitro*, the yolk mass of spawned eggs rotates toward the maturation spot, confirming the presence of an endogenous gravity-sensitive cue in the egg. The goal of this project is to identify the gravity-sensitive mechanism by which eggs direct the assembly of the parallel microtubule bundles that constitute the cortical rotation apparatus.

Accomplishments

We demonstrated a strong functional correlation between maturation spot position and yolk mass rotation direction in activated eggs. Rotation direction was recorded by time-lapse confocal fluorescence microscopy of fluorescently labeled subcortical mitochondria, a method that gives an accurate overall view of cortical/cytoplasmic rearrangements occurring over the entire vegetal surface. Both spawned eggs and eggs that had been experimentally manipulated to produce an eccentric maturation spot showed a strong bias to rotate toward the maturation spot meridian, supporting the hypothesis that the orientation of oocytes undergoing maturation affects the polarity of the cortical rotation machinery. Significantly, in fertilized eggs, the direction of yolk mass rotation is detectably influenced by this gravity-based cue, acting in cooperation with the sperm aster. Because the rotation direction ultimately establishes the orientation of the dorsal-ventral axis, the wide variability in the relationship between the SEP and the dorsal side probably stems from the random orientations experienced by individual oocytes during maturation. These results suggest two possible mechanisms by which gravity could cue the rotation direction: (a) the cortical cytoskeleton of the maturing egg becomes entrained in a way that promotes directed microtubule assembly in the vegetal cortex; or (b) a microtubule organizer, perhaps one associated with the female pronucleus, becomes displaced from a concentric axial location.

Significance of the Accomplishments

We have established that obliquely applied gravitational force experienced by *Xenopus* eggs during meiotic maturation affects the rotation-determining cortical cytoskeletal machinery. It is well

known from work in various laboratories, including our own, that the vegetal yolk mass rotation direction can be experimentally redirected by simply tipping the egg for a few minutes at the beginning of the rotation phase of the first cell cycle. This sensitivity to gravity implies a positive feedback relationship between rotation itself and the directed growth of cortical microtubules in the shear zone that develops between the cell surface and the deep cytoplasm. Recently, it was revealed that many, if not most, cortical microtubules are continuous with radial microtubules emanating from the cell center.

A plausible scenario for the sequence of events leading to directed rotation would begin with the growing microtubule tips arriving at the egg surface early in the first cell cycle. Once at the surface, they could easily respond to shear-generated alignment that would result from any relative movement between cortex and inner cytoplasm. Their subsequent parallel growth would, in turn, reinforce continued motion in the same direction by enabling (+) end-directed transport of vesicular components of the yolk mass, for example, the endoplasmic reticulum (ER). Thus, a preexisting rotation-fixing bias might take the form of asymmetries induced in the yolk mass during maturation.

Alternatively, non-microtubular components of the vegetal cortex might have become aligned by shear during meiotic maturation. One candidate for such alignment would be the network of intermediate filaments (cytokeratin) in the cortex. It is known that cytokeratin filaments undergo disassembly and reorganization during maturation. However, whether the organization of the reassembled network reflects directional shear experienced during maturation has not been investigated.

Finally, there is the possibility that the microtubule organizer at the cell center becomes displaced from its original location toward one side during maturation. Following parthenogenetic activation, microtubules emanating from this eccentric cell center would arrive at the egg cortex on one side more quickly than elsewhere. This initial asymmetry might then be sufficient to promote a limited yolk mass movement relative to the cortex that would then entrain the growth of microtubules arriving at the surface in other regions.

We are presently carrying out experiments to address these various possibilities.

Publications

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MECHANICAL MODULATION OF STRIATED MUSCLE PHENOTYPE

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Description of Research

Mechanical load has been documented to regulate the mass of the heart and skeletal muscles. This investigation employs cultured adult cardiac myocytes and skeletal muscle myotubes to evaluate how mechanical load controls the expression, turnover, and assembly of contractile proteins and to determine whether specific cytoskeletal proteins are crucial to the maintenance of the striated muscle phenotype and its contractile properties. The hypothesis being presently tested is that microgravity alters the forces that the heart and skeletal muscles must overcome to conduct meaningful work and that a reduction in mechanical workload alters cytoskeletal-myofibrillar interactions in these tissues. Experiments described briefly in the progress report reveal that the cytoskeletal-associated proteins, α-actinin and vinculin, appear to have a pivotal role in maintenance of myofibril structure. The α-actinin appears to be important in the early phases of myofibril assembly, whereas vinculin redistribution may be associated with the ability of myofibrils to withstand force generation. Regulation of cytoskeletal protein expression, turnover, and incorporation into myofibrils will provide new information on the subcellular mechanisms that mediate mechanical load-dependent myofibril assembly and potential signal pathways that may be modulated in a microgravity environment, where gravitational forces are markedly reduced and mechanical work is believed to be diminished proportionately.

The principal objective of year 1 was to determine how activation of beating altered the turnover of contractile cytoskeletal proteins in cultured adult cardiac myocytes and whether such changes modulated growth or atrophy of the heart cell. The specific aims of year 1 were (1) to monitor the redistribution of the cytoskeletal-associated proteins α-actinin and vinculin in beating heart cell preparations by immunofluorescence microscopy, (2) to determine the turnover rates of contractile/cytoskeletal proteins, and (3) to explore the potential pathways of proteolysis and their contribution in regulating contractile/cytoskeletal protein composition.

Accomplishments and Significance of the Accomplishments

The experiments conducted in the past year were designed to distinguish the direct actions of catecholamines from beating (i.e., mechanical loading) on the turnover and reorganization of contractile proteins and the maintenance of myofibrillar structure. Observations derived from non-beating, passively loaded rabbit heart cells indicate that catecholamines can maintain and even promote the fractional rate of heart cell growth and modulate the turnover of contractile proteins, but adrenergic activation is incapable of inducing the assembly of these nascent proteins into myofibrils in nonbeating rabbit cardiac myocytes (Decker et al., 1993a). Norepinephrine enhanced contractile/cytoskeletal protein synthesis to a greater degree than either the β-agonist, isoproterenol, or the α-agonist, phenylephrine. However, these catecholamines provoked myofibrillar disruption, suggesting that α- and/or β-adrenergic stimulation, in the absence of contractile activity, may enhance protein turnover but fail to promote myofibrillogenesis in these heart cells.

In cultured adult feline myocytes however, adrenergic agonists with β_1 activity induced contractile activity. The β_1-agonist, isoproterenol, stimulated the synthesis of cytoskeletal proteins, and

myofibrillar reorganization was apparent within 24 hours after the induction of beating (Simpson et al., 1993). If beating was blocked by membrane depolarization with KCl, then myofibril reassembly was prevented even though changes in contractile protein synthesis were not altered by potassium. The results of these experiments support the hypothesis that catecholamines stimulate cytoskeletal protein synthesis, but changes in mechanical loading (i.e., beating) are required to assemble these proteins into functional myofibrils (Decker et al., 1993c).

To further explore the role of mechanical load in regulating the turnover and assembly of contractile proteins in the absence of adrenergic stimulation, feline myocytes were either passively stretched 10 percent of their rest length or subjected to electrical field stimulation at 1 Hz (60 beats per minute). Both experimental paradigms enhanced the rate of contractile protein synthesis, although field stimulation elevated the fractional rate of protein synthesis about 55 percent within 4 hours of electrical activation, whereas passive stretch induced an increase of only 35 percent over the same interval (Decker et al., 1993b). Both passive stretch and field stimulation induce myofibril reassembly, which appears to be preceded by the appearance of primitive α-actinin/vinculin positive focal contacts. New myofibrils are probably assembled at these sites, but future experiments will be required to verify this assumption (Decker et al., 1993e).

The extent to which heart and skeletal muscles maintain the mass of their contractile proteins must reflect the difference between the amount of protein synthesized and the amount degraded. Experiments designed to explore proteolytic mechanisms have uncovered two pools of protein in cardiac myocytes. The first is a small pool accounting for 10-15 percent of total myocyte protein (Simpson et al., 1993). This pool has a half-life of about 22 hours and is composed of nascent contractile proteins that may represent precursors for myofibrillogenesis. The second pool consists of predominantly contractile proteins whose half-lives approach 2 weeks. Isoproterenol significantly slows the degradation rate in the small, rapid-turnover pool. Since myofibrillar proteins (e.g., myosin, actin, tropomyosin, etc.) can be found in both pools, changes in pool size and the turnover rate within these pools may provide some insight into how mechanical load regulates the composition of the contractile apparatus in cardiac and skeletal muscle.

Publications

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OTOLITH-CANAL CONVERGENCE IN VESTIBULAR NUCLEI NEURONS

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Description of Research

The primary objective of the current investigation is to determine how information regarding head movements and head position relative to gravity is received and processed by central vestibular nuclei neurons in the brain stem. Specialized receptors in the vestibular labyrinths of the inner ear function to detect angular and linear accelerations of the head, with receptors located in the semicircular canals transducing rotational head movements and receptors located in the otolith organs transducing changes in head position relative to gravity or linear accelerations of the head. The information from these different receptors is then transmitted to central vestibular nuclei neurons, which process the input signals and then project the appropriate output information to the eye, head, and body musculature motor neurons to control compensatory reflexes. Although a number of studies have reported on the responsiveness of vestibular nuclei neurons, it has not yet been possible to determine precisely how these cells combine the information from the different angular and linear acceleration receptors into a correct neural output signal. However, by utilizing a mechanical stimulation technique for the semicircular canals, along with electrical polarization of afferent fibers and traditional rotational and linear acceleration stimuli, the current project will be able to discretely activate the different receptor systems and thereby directly examine these questions.

Accomplishments

Vestibular afferent and nuclei neuron responses elicited by electrical stimulation. Before the synthesis of information from the different linear and angular acceleration receptors by the central vestibular neurons was examined, it was first necessary to determine if selective types of afferents could be differentially activated by their thresholds to electrical stimulation. Previously, it had been demonstrated in monkeys that small electrical currents applied to the labyrinth can selectively either silence or excite afferent fibers that have irregular discharge rates, while afferents with more regular firing patterns are little affected. It is currently believed that these regular and irregular firing afferents converge upon vestibular nuclei neurons in unique patterns to control different types of muscle movements. For example, there is evidence to suggest in monkeys that (a) regular afferents are more involved in the control of eye movements, while (b) irregular afferents primarily are involved in the control of head and/or postural movements. As an added tool in the current investigation of synthesis of converging linear and angular acceleration inputs onto vestibular nuclei neurons, electrical stimulation of the labyrinth was used to examine the effectiveness of selectively stimulating the regular and irregular afferent fibers in pigeons.

Electrical stimulating electrodes were implanted into the perilymphatic space of the labyrinth, while extracellular electrophysiological recordings were made from the ipsilateral semicircular canal and otolith afferent fibers. Both anodal and cathodal electrical direct currents were utilized, with current magnitudes ranging between −100 and +100 μA. In addition, for many neurons, sinusoidal rotational (semicircular canal afferents) or off-vertical axis rotational (OVAR or otolith afferent) stimulation was applied with and without simultaneous electrical stimulation, in order to determine if the responsiveness of the fiber changed during the electrical stimulation. To date, 67 afferents have been studied using electrical stimulation, with effects observed in both semicircular canal and otolith afferent fibers.

Similar to the results reported in monkeys, pigeon afferent fibers can be selectively affected based upon their discharge regularity. All afferent fibers were inhibited by cathodal (positive) currents and were excited by anodal (negative) currents. Irregular firing afferent fibers had substantially lower thresholds to electrical currents applied to the labyrinth than did regular afferent fibers. In fact, irregular afferent fibers can be selectively silenced with low amplitude (5–25 μA) anodal currents. Regular firing afferents, however, could never be silenced even when administering large amplitude (100 μA) currents. The afferents' response magnitudes were correlated with the electrical stimulus magnitude, with the sensitivity of the afferents to electrical stimulation being proportionally related to the discharge regularity. When electrical stimulation was applied to the afferent fiber during either rotational or OVAR stimulation, the discharge rate and the sensitivity of the neuron to the acceleration stimulus was altered for irregular firing afferents but not for regular afferents with low amplitude (25 μA) electrical currents, as shown in Figure 1. Irregular firing semicircular canal fibers could be ablated (silenced) with electrical stimulation, even during sinusoidal rotations at 20° or 30° per second peak velocity. Similarly, irregular firing otolith afferent fibers could also be ablated during off-vertical axis rotations of 36° per second constant velocity. However, with regular firing afferents, either no effect or only a slight change was observed in the sensitivity of the fibers to OVAR or angular rotation.

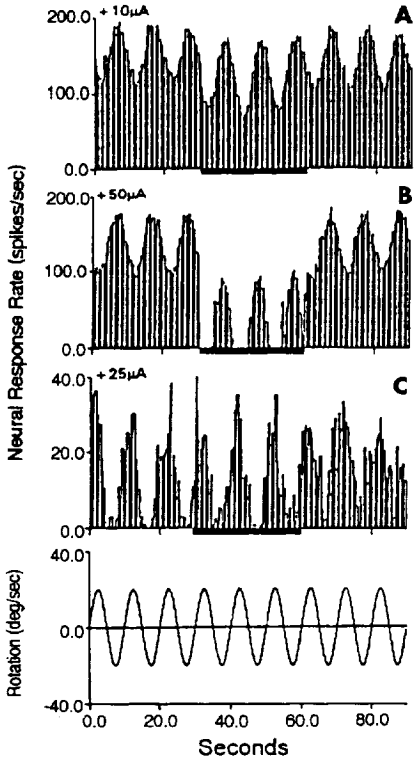

Figure 1. Neural response histograms of vestibular nuclei neurons to sinusoidal rotational and simultaneous electrical current stimulation. **A** and **B** = responses from a vestibular nuclei neuron that received probable monosynaptic input from the ipsilateral ear (1.3 msec latency) and was classified as a horizontal canal Type I neuron. The neuron did not respond to rotations in the vertical canal planes. **C** = responses from a second horizontal canal related vestibular nuclei neuron that received polysynaptic input from the ipsilateral ear (4.0 msec latency). Rotational stimuli (*bottom trace*) were applied in the horizontal plane at a frequency of 0.1 Hz and a peak velocity of 20¤ per second. Electrical stimulation (*dark bar*) consisted of 30 sec +25 μA of current applied bilaterally.

Now that the response characteristics and sensitivity of the afferent fibers to electrical stimulation have been established, studies of the irregular and regular afferent fiber inputs to the vestibular nuclei neurons can be conducted. Recordings are currently being obtained from these central neurons while electrical ablation of the irregular afferent inputs is utilized. The responses of the central neurons to both rotational and linear accelerations are obtained, first, without electrical stimulation to determine the response sensitivity, and then, with electrical stimulation while simultaneously stimulating the labyrinth with either rotational or linear accelerations. Differences in the response sensitivity can then be correlated with the types of afferent input (regular or irregular) to the neuron, as well as the amount of convergence between semicircular canal and otolith organ afferents.

Significance of the Accomplishments

During manned space flight, acute vestibular disturbances often occur, leading to physical stress and a loss of performance. The purpose of the current project is to determine how vestibular nuclei neurons synthesize information from the different linear and angular acceleration receptors in the labyrinths into an integrated output signal. However, it was first necessary to delineate the different types of afferent fibers, since it is thought that regular and irregular firing afferents differ in their input patterns onto vestibular nuclei (VN) neurons and their subsequent control over eye, head, and postural musculature. Using electrical polarization of the labyrinth while obtaining extracellular recordings from afferent fibers, it was found that both semicircular canal and otolith fibers could be selectively affected based upon the cell's discharge regularity. Irregular-firing afferent fibers are much more sensitive to the electrical stimulation and can be reversibly ablated with low-amplitude stimulation. Thus, while delivering electrical stimulation to ablate the irregular firing afferents, simultaneous rotational and linear acceleration stimuli are now being applied to study the effect of convergence of these different types of inputs onto vestibular nuclei neurons. It is possible that information regarding linear and angular accelerations of the head are encoded differently in the central neurons, since muscle groups concerned with compensatory motor reflexes produce differing responses. Investigations during the next year will determine how the central vestibular nuclei neurons synthesize the different irregular and regular firing afferent inputs along with their varied linear and angular acceleration signals. In this way, we hope to discover how the vestibular system is affected by the changes in linear acceleration that occur during manned space flight.

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MODULATION OF BONE REMODELLING VIA MECHANO-SENSITIVE ION CHANNELS

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Description of Research

One of the most negative physiologic effects resulting from extended periods of weightlessness is a rapid loss of bone mass as well as total body calcium. This condition is similar to a pathology which results from prolonged immobilization, termed disuse osteoporosis. Conversely, chemical observations noted during *in vivo* studies suggest that physical exercise or applied mechanical load has a positive effect on bone formation. Continuous or intermittent compressive force applied to bone or bone cells has been shown to increase a number of parameters which are associated with increased osteoblast activity. While mechanical force has been shown to modulate the second messenger levels in the osteoblast, such as cyclic AMP, inositol phosphate, and intracellular calcium, the signal transduction mechanism by which physical force is converted to chemical message is still unclear. We have characterized a mechanically sensitive cation selective channel in the osteoblast-like osteosarcoma cell line UMR-106.01, which we hypothesize may be the signal transducer for mechanically induced osteogenesis. The goal of this research is to determine the effects of mechano-sensitive ion channel activity in osteoblasts and to find the effects of these channels on osteoblast function. In addition, we will characterize the effects of hormonal factors on these channels and whether hormonal factors will potentiate the response of the osteoblast via these mechano-sensitive channels. To attain these goals, we will combine the patch-clamp technique to study ion channel activity with methods from cell biology. We will characterize the effects of stretch on this mechanically sensitive channel and determine their role in formation and secretion of bone matrix proteins, such as collagen, osteopontin, and osteocalcin. We will determine if second messengers such as cyclic AMP, intracellular calcium, diacylglycerol, or inositol phosphate are involved in the translation of biophysical stimuli in the biochemical event. We will also determine if hormones such as the parathyroid hormone (PTH) and glucocorticoids have an effect on this channel activity and whether these hormones have an additive effect on stretch or mechanical stimulation in bone. During this reporting period, the focus of this research centered on the modulation of these channels in response to PTH and the second messenger system by which PTH modulates this channel.

Accomplishments

(1) Patch-clamp analysis of UMR-106.01 cells demonstrated that PTH modulates the mechanically sensitive cation channel in two distinct ways. PTH produced a two to fourfold increase in channel activity during stretch and also increased the conductance of the channel.

(2) Application of membrane-permeable second messengers to the UMR cell indicated that cyclic AMP and an increase in intracellular calcium could mimic only one of the effects of PTH, which was an increase in single-channel conductance.

(3) Patch-clamp studies demonstrated that this mechanically sensitive channel was dependent on attachment to the cytoskeleton of the cell. When cytochalasin D, which cleaves actin filaments, was added to the cell, the mechanically sensitive ion channels increased their channel activity in the same way in which PTH modulated the stretch-activated cation channel. These data

would indicate that PTH modulates osteoblast behavior possibly through affecting the cytoskeleton.

Significance of the Accomplishments

PTH is a calciotropic hormone which is released in response to low plasma calcium. PTH, however, has paradoxical effects on the osteoblast. Acutely, PTH raises serum calcium by stimulating the osteoblast to release signaling messengers to stimulate osteoclast activity. However, long-term PTH stimulation appears to induce the osteoblast to increase bone formation. Since these channels are a likely candidate for the signal transduction of mechanical stimulation, the stimulation of this channel by PTH would suggest this channel is an important site of convergence of two distinct osteoblast activator pathways.

The significance of the observations described in Accomplishments (2) as well as (3) would indicate that PTH modulates this mechanically sensitive cation channel through two distinct mechanisms. One is mediated through the classical second messengers of PTH, which are increases in cyclic AMP in the cell as well as increases in the level of cytosolic calcium. These second messengers stimulate this channel by increasing single-channel conductance. By increasing single-channel conductance, the channel is kinetically changed so that more ions can traverse the channel in the same open times. PTH has also been shown to effect the shape of the osteoblast, causing a shrinkage of the osteoblast into a stellate shape. This shrinkage would appear to be mediated through the actin cytoskeleton, as evidenced by the effects of cytochalasin D, which produces similar morphologic changes. Alteration of the cytoskeleton also affects the stretch-activated cation channel, but in a different way. Data acquired by holding the channel open for a longer period of time for each channel opening indicate that effects of PTH being modulated through two different mechanisms would increase the amount of ions going through the channel, resulting in a much more rapid signal for the PTH response. Since we hypothesize that these channels are responsible for the signal transduction of the mechanical strain into chemical events in the cell, these findings would suggest that these channels may be responsible for the osteogenic activity of PTH.

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NEURONAL PROTEINS BEFORE, DURING, AND AFTER VESTIBULAR SYNAPTOGENESIS

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Description of Research

The long-term objective of our investigation is to determine, at the light and the electron microscopy level, the anatomical changes that cells and tissues of the inner ear undergo during embryonic development. The expression of neuronal proteins in the axons and neurons is quantitated with color thresholding. In previous work we showed that ultrastructural modifications of cells in gravity detecting organs of the chick are very similar to those modifications reported for mammals, reinforcing the usefulness of the chick embryo (*Gallus domesticus*) model in space experimentation. Previous analyses of the otoconia and cells of the gravity detecting organs at various stages of development emphasized the importance of understanding the modifications occurring at 1g before we can properly evaluate any change occurring in microgravity. The neurons under study carry sensory information about the body position from the inner ear to the brain. In this report the 8th cranial nerve is emphasized. The peripheral neuron body with nuclei are called perikarya and their grouping form a ganglion. Projections from the perikarya toward the hair cells of gravity detecting organs, and toward the brain stem form the vestibular nerve which carry information from the inner ear to the brain (see diagram below).

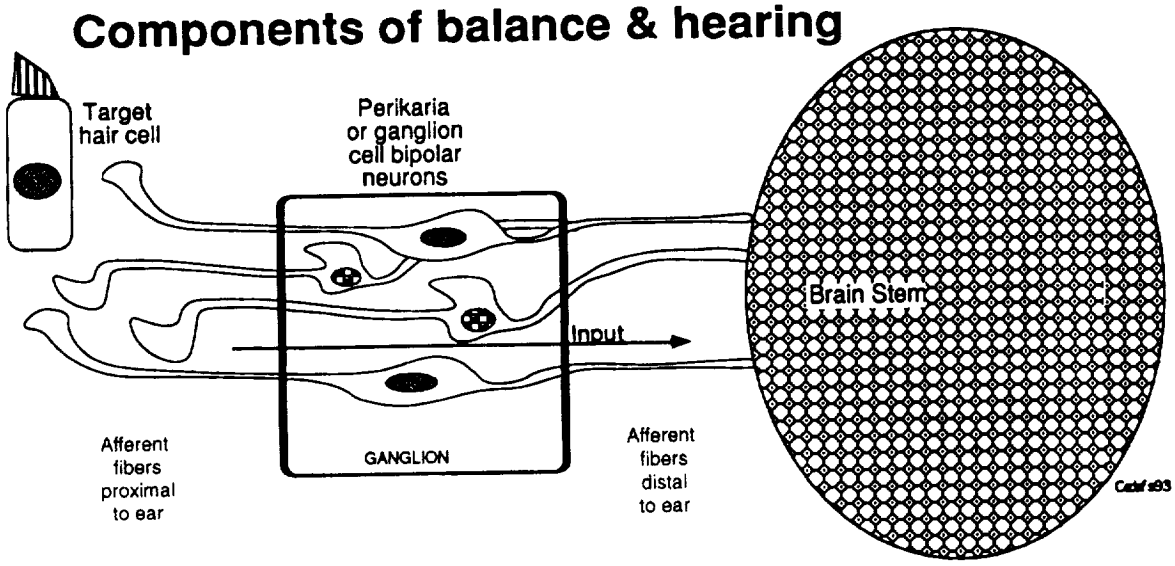


Figure A. The peripheral neuron's body with nuclei are called perikarya, and their grouping forms a ganglion. Projections from the perikarya toward the hair cells of the gravity-detecting organs and toward the brain stem form the vestibular nerve, which carries information from the inner ear to the brain.

Modified gravity induces changes in gravity sensors of the inner ear during embryonic development. Such modifications range from alterations of vestibular brain stem responses to ultrastructural modifications of cells and tissues including the branching of afferent fibers that connect the inner ear to the brain. At each level, morphological and biochemical manifestations of the changes at critical periods of development can be studied and the significance of the changes characterized.

We stated in last year's report that, in addition to neurofilaments and microtubule associate proteins, which are both expressed differentially during the development of neurons, the expression of S-100 protein also increased in the inner ear gravity-detecting organs of the chick. We mapped the distribution of this protein in neurons of the 8th cranial nerve that connect the inner ear to the brain and showed that S-100ß is found in the nuclei of the neuronal body (perikarya) in addition to the axons and Schwann cells. The reaction product is restricted to perikarya in different parts of the vestibular ganglion. Until our work, S-100 was found mainly in support cells of peripheral and control origin. A few reports indicated that S-100ß may have neurotrophic properties needed for guiding axons to find hair cells.

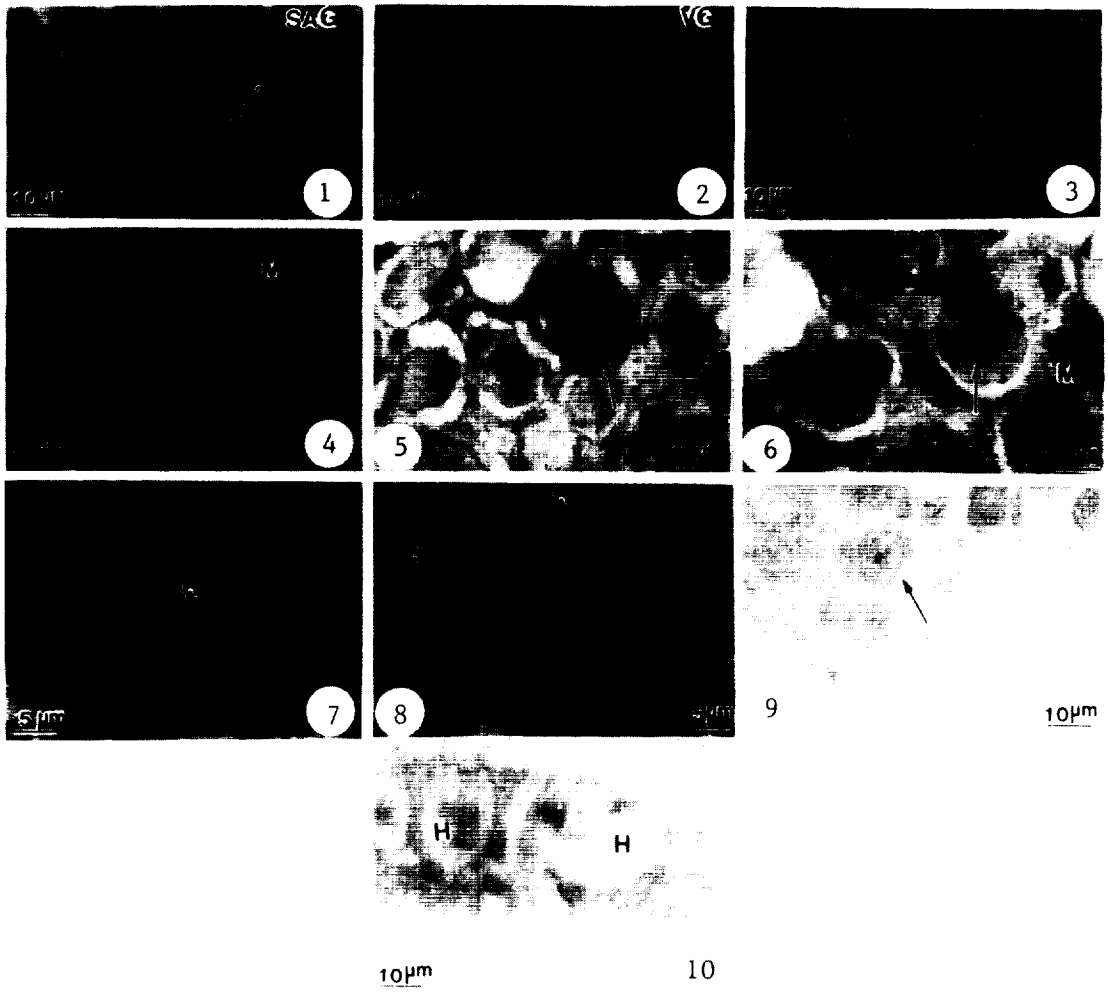
Accomplishments

We investigated the reactivity of S-100α and S-100ß in the 8th cranial nerve of the developing chick. In the vestibular portion of the nerve, afferent bipolar neurons and their nerve fibers are covered by myelin of Schwann cells. Schwann cells of the peripheral 8th nerve serve the equivalent function of cerebral glial cells. S-100ααßß was originally isolated from brain glial cells, and it was later shown to react with Schwann cells and other ectodermic derived cells. We determined (a) that expression of S-100ß in the chick inner ear vestibular afferents is time dependent, and (b) that expression of S-100ß in the vestibular, afferent, nerve terminals contacting hair cells changes before and after synaptogenesis, indicating that the period between embryonic day 9 (E9) and E13 of incubation may correspond to a critical stage of development during which S-100ß expression is up regulated concomitantly with myelination of axons.

A color-thresholding computerized analysis of the peroxidase and the alkaline phosphatase reaction products showed increased concentration of S-100ß in the afferent axons from E9 to hatching E21. The increased stain of afferent axons was most pronounced in the chalices around hair cells type I, where the nerve endings have no myelin. Thus, expression of S-100ß around hair cells was probably not contributed by Schwann cells inside the epithelia.

The expression of S-100ß in the neurons of the vestibular ganglion (VG) appeared simultaneously (Figs. 1 and 2, arrows) in both VG and auditory or statoacoustic ganglion (SAG) of the 8th nerve at E9. However, more perikarya nuclei expressed S-100ß earlier in the VG than the SAG, indicating that the expression of S-100ß follows the well-known accelerated development of the vestibular organs when compared to the auditory organs. By E13 when myelination is under way, expression of S-100ß increased in the nuclei of perikarya in both ganglia (Figs. 3-4, arrow), but the number of positive perikarya varied in each ganglion. Some nuclei remained negative (Fig. 3, arrowhead), indicating that there is a differential expression of this protein in the perikarya of the vestibular and auditory ganglion. Table 1 shows the percentage of stained neurons in each ganglion at different stages. Note that the expression of S-100 is not the same in each ganglion of the same age. By E16, after myelination is more than half completed and synapses are established, S-100 is distinctly present in the axons and Schwann cells (arrowheads), whereas nuclear expression decreased (Figs. 5-6, arrows), suggesting that increased expression of S-100ß is probably related to myelination and synapse formation.

Expression of S-100ß in lateral crista is absent from the terminal endings around hair cells (H) and minimal (arrows) of fibers at E13 (Fig. 7), but is prominent at hatching in the chalices (arrows) of hair cells (H) at P5 (Fig. 8), suggesting an association between the expression of S-100ß and maturation of afferent neurons. Specifically, the antibody reaction was demonstrated by preabsorbing the anti-S-100ß with pure S-100ß protein. Both neurons (Fig. 9) and hair cells (Fig. 10) did not react.



Figures B1-10. The expression of S-100ß, in the neurons of the VG appeared simultaneously (Figs 1-2, arrows) in both VG and SAG of the 8th nerve at E9. However, more perikarya nuclei expressed S-100ß earlier than in the SAG, indicating that the expression of S-100ß follows the well known accelerated development of the vestibular organs when compared to the auditory organs. By E13 when myelination is under way, expression of S-100ß increased in the nuclei of perikarya in both ganglions (Figs. 3-4, arrows), but the number of positive perikarya varied in each ganglion. Some nuclei remained negative (Fig. 3, arrowhead) indicating that there is a differential expression of this protein in the perikarya of the vestibular ganglion. By E16 after myelination is more than half completed and synapses are established, S-100ß is distinctly present in the axons and Schwann cells (arrowheads), whereas nuclear expression decreased (Figs. 5-6, arrows), suggesting that increased expression of S-100ß may be related to the process of myelination and synapse formation.
Expression of S-100ß in lateral crista is absent from the terminal endings around hair cells (H) and minimal (arrows) in fibers at E13 (Fig. 7), but is prominent at hatching in the chalices (arrows) of hair cells (H) at P5 (Fig. 8), suggesting an association between the expression of S-100ß and the maturation of synaptic contacts. Specifically the antibody reaction was demonstrated by preabsorbing the anti-S-100ß with pure S-100ß protein. Both neurons (Fig. 9) and hair cells (Fig 10) did not react.

Table 1. Percentages of S-100 Positive Perikarya in the 8th Nerve

DAY	VG	SAG
E9	74	59
E11	29	31
E13	39	45
E16	9	13
E21	5	15

Significance of the Accomplishments

The growth cones (growing tips) of nerve fibers use different cues for searching and contacting their targets, and S-100ß may be one of several factors expressed during synaptogenesis and myelination of afferent terminals onto hair cells. S-100ß may also assist in the maintenance of neuronal function, consistent with its expression in the 8th nerve fibers and terminals contacting hair cell type I after hatching. Another possibility is that S-100ß acts as a cofactor needed for the function of cytoskeletal proteins required for neurite outgrowth; this is because of the parallel expression of S-100ß with microtubule associate protein and neurofilament protein observed by us (1990-1991 progress report) and by other investigators.

Inner ear structures (e.g., utricle, cochlea, etc.) develop according to gradients that determine hierarchy of end organ maturation. The hierarchy ensures multiple levels of response in the mature organism and, most importantly, gradual maturation of specific areas of those organs. There are five vestibular end organs and an auditory organ in the chick inner ear, where each organ receives afferent nerve terminals from the 8th nerve. Since each end organ is located at different distances from the ganglion, nerve endings probably arrive to those organs at different times. In the cochlear or SAG fibers, more soma stained far away from the vestibule (distal end of basilar papilla) than close to the gravity organs, suggesting that neurons at the distal end may contact younger hair cells.

A transient expression of S-100ß in portions of the ganglion (Table 1) suggests that S-100ß expression is related to a gradient of maturation of afferent neurons. While further proof of this is required, S-100ß could serve as a selective marker for assessing the relative maturation of certain neurons.

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MICROGRAVITY *IN VITRO* MODEL OF BONE CELLS: FLOW EFFECTS

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Description of Research

It is well established that vascularization is required for effective bone healing. This implies that blood flow and interstitial fluid (ISF) flow are required for healing and maintenance of bone.

ISF flow in bone results from transcortical pressure gradients produced by vascular and hydrostatic pressure and mechanical loading. Conditions observed to alter flow rates include increases in venous pressure in hypertension, fluid shifts occurring in bed rest and microgravity, increases in vascularization during the injury-healing response, and mechanical compression and bending of bone during exercise.

It is the objective of our research to characterize mechanisms that regulate ISF flow and the effect of ISF flow on bone cell physiology.

Accomplishments and Significance of Accomplishments

(1) ***Endosteal hydraulic conductivity.*** By modeling the endosteum and periosteum of bone as a monolayer of osteoblasts grown on filters, we have been studying their ability to act as a regulated barrier to the transosteal flux of solvent. Our findings are: (a) Osteoblasts represent a significant barrier to flux of fluid. (b) The hydraulic conductivity can be regulated by calcitropic hormones, calcitonin, and parathyroid hormone. Changes in cellular morphology in response to hormonal action has been documented by morphological analysis on a scanning laser cytometer (Meridien).

(2) ***Osteoblast differentiation in response to flow.*** Osteoblasts grown on macrocarrier beads (Verax) were maintained in a fluidized bed and on a slowly rotating shaker. By assaying for mineralization, as determined by a method based on von Kossa staining, we demonstrated that within 10 days there was significant mineralization by the osteoblasts on the bioreactor beads as opposed to the slowly shaken beads. This suggests that fluid flow can increase differentiation.

(3) ***Nitric oxide production by sheared osteoblasts.*** Osteoblasts subjected to shear stress for 12 hours or longer produce significant amounts of nitric oxide. Nitric oxide synthase is not present in cultured primary rat osteoblasts. There is apparently up-regulation of the enzyme with fluid flow.

NEURAL MECHANISMS BY WHICH GRAVITATIONAL STIMULI AND STRESS AFFECT THE SECRETION OF RENIN AND OTHER HORMONES

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Description of Research

The long-term goal of this research, which has been supported by NASA since August 1, 1983, is delineation of the brain pathways and neurotransmitters that mediate gravitational and stress-induced changes in the secretion of renin and other salt- and water-regulating hormones. One of our goals has been determination of the specific parts of the hypothalamus and related areas that affect circulating renin and angiotensin. A second of our goals has been determination of the pathways from this region to the renin-secreting cells in the kidney. A third goal has been analysis of the importance of this region in the physiologic control of renin secretion. To study the role of the brain in the regulation of renin secretion, we have used stimuli which increased renin secretion in diverse ways. Emphasis has been placed on the gravitational stress of 45° and 60° head-up tilt in rats. In past research, we have demonstrated that discharge of serotonergic neurons in the dorsal raphe nucleus increased renin secretion, so we have also tested the effect of the serotonin-releasing drug p-chloroamphetamine (PCA). Additional standardized stimuli have included the psychological stress of immobilization, the chronic volume depletion stress of a low sodium diet, and the acute volume depletion stress of nonhypotensive hemorrhage. We have added to this the acute volume depletion produced by extravascular administration of polyethylene glycol, a hypertonic substance which draws fluid from the vascular system in a reproducible way. We are also studying the role of vasopressin-secreting neurons that connect the hypothalamus to the brain stem and spinal cord. A cumulative list of publications that report research supported by the grant is attached.

Accomplishments

(1) ***Further research on the role of brain stem vasopressin and oxytocin in the regulation of renin secretion.*** In a previous study (Golin, et al, 1989), we demonstrated that in homozygous Brattleboro rats, which have no vasopressin in their brains or circulating blood, renin secretion is chronically increased and there is a chronic increase in sympathetic discharge. The increased sympathetic discharge does not appear to be peripheral in origin, since chronic subcutaneous infusion of vasopressin failed to restore renin secretion to normal. Vasopressin does not cross the blood-brain barrier, so it seemed likely that the increased sympathetic discharge was due to vasopressin deficiency in the brain. During the year, repeated injections of vasopressin directly into the brain in Brattleboro rats failed to inhibit renin secretion. However, one is never sure with such injections that the dose and timing are right. Therefore, we have embarked on other approaches to the same question. One of these, in collaboration with Dr. Lanny Keil of the NASA Ames Research Center, is measurement of oxytocin and vasopressin in the brain stem under conditions in which it is known that there is increased sympathetic discharge producing renin secretion. An experiment on the effects of a low salt diet has been completed, but results are not yet available.

(2) ***Further study of the unique effect of stress and certain anesthetics on circulating angiotensinogen.*** Some years ago, we made the chance observation that 24 hours after surgical stress, plasma angiotensinogen was elevated with little, if any, change in plasma renin activity. Since this effect is blocked by hypophysectomy or paraventricular lesions, we explored the possibility that it was due to increased secretion of a hormone from the anterior pituitary, which

was controlled by the paraventricular nuclei. No increase in ACTH, thyroid hormones, luteinizing hormone, or prolactin has been observed in animals in which angiotensinogen is increased. Xylazine, one of the anesthetics which produces this effect, increases plasma oxytocin and vasopressin, but injection of these hormones or blocking their effects does not produce relevant alterations in plasma angiotensinogen. Current evidence indicates that permissive levels of glucocorticoids are needed for the response, and this is why hypophysectomy blocks it.

(3) ***Further analysis of the role of the ventromedial nuclei in the regulation of renin secretion.*** In previous experiments, we demonstrated that bilateral destruction of the ventromedial nuclei does not lower circulating angiotensinogen but prevents the increase in renin secretion produced by PCA, head-up tilt, immobilization, and a low sodium diet (Gotoh, et al., 1988). We then tested the effect of electrical stimulation of the ventromedial nuclei and found that this increased renin secretion. However, microinjection of the excitatory amino acid dl-homocysteic acid failed to increase renin secretion. Since this amino acid stimulates cell bodies but fails to stimulate fibers of passage, it appears that the ventromedial nuclei are not integrating centers *per se*, but part of a descending pathway. Destruction of cell bodies with excitatory amino acids confirmed this hypothesis. To look for the pathways feeding into the ventromedial nuclei, we electrically stimulated areas of the hypothalamus and forebrain rostral to the ventromedial nuclei and found that stimulation in the so called AV3V area and a descending ventral pathway from it increased renin secretion. We also investigated the pathways caudal to the ventromedial nuclei and found that bilateral lesions just behind these nuclei inhibited the renin response to immobilization.

(4) ***Use of immunocytochemistry for c-fos products to determine the parts of the brain activated by stimuli to renin secretion.*** We recently set up an immunocytochemical method for visualizing the products of the *c-fos* immediate-early gene, with the goal of using it to identify the cells in the central nervous system that are "turned on" by stimuli which increase renin secretion. The method was used in a pilot experiment employing the stress of injection of bacterial lipopolysaccharide and a paper by Drs. Tkacs and Strack on the results is now under editorial review for publication in the *Journal of Neuroscience*.

Significance of the Accomplishments

The experiments described above and the grant-supported experiments conducted in previous years have done much to elucidate the role of the brain in the regulation of renin secretion. This has important significance for NASA because both postural changes and stressful stimuli increase renin secretion, and renin via angiotensin II stimulates the secretion of the salt-retaining hormone aldosterone, affects water balance, and maintains blood pressure. In addition, our demonstration that brain lesions lower circulating angiotensinogen via a neuroendocrine mechanism involving thyroid hormone secretion is important because it demonstrates another way the brain can influence the amount of circulating angiotensin II and, consequently, its effects.

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MUSCLE PROTEIN LOSS IN WEIGHTLESSNESS ENVIRONMENTS: ROLE OF THE ATP-UBIQUITIN-DEPENDENT PROTEOLYTIC SYSTEM

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Description of Research

The goal of this research proposal is to learn more about the cellular mechanisms responsible for the marked loss of muscle mass in weightless environments. In particular, we hope to delineate the roles of different proteolytic systems in the rapid loss of muscle protein. Recent studies indicate that the most important pathway for protein degradation in skeletal muscle wasting is the cytosolic process involving ATP, ubiquitin, and the proteasome particle. The biochemical details of this pathway have been elucidated only recently. We have developed methods for analyzing this pathway in incubated rat muscles and are studying its regulation in different models of muscle atrophy that appear relevant to the wasting seen in weightless environments. Our recent studies indicate that the activation of the ATP-ubiquitin-dependent pathway for protein breakdown is primarily responsible for the muscle atrophy and loss of myofibrillar components induced by a large variety of pathological stimuli (disuse, stress, cortisol infections, hyperthyroidism, fasting). Therefore, we are trying to clarify the physiological factors that lead to its activation or its suppression. One goal of this research program is to find simple approaches that may suppress overall protein breakdown in muscle by dietary or perhaps pharmacological interventions. Such treatments might represent a novel method for combating muscle wasting in space.

Accomplishments

During the past year, under grant support from the National Aeronautics and Space Administration, my laboratory pursued a variety of studies along the lines proposed earlier. A number of interesting and important findings were made that provide a basis for the work we proposed to carry out in the coming year. As proposed earlier, the main goal of these studies is to understand the mechanisms underlying the excessive protein breakdown that leads to negative nitrogen balance and muscle wasting in a variety of catabolic studies, such as in individuals exposed to the space flight environment. Particular emphasis has been placed on further clarifying the mechanisms by which glucocorticoids, the major adrenal stress hormones, may be important in enhancing muscle proteolysis. These studies and our earlier ones had suggested that activation of the ATP-ubiquitin-dependent proteolytic pathway was responsible for muscle wasting under a variety of conditions. This past year, particular emphasis has been placed on obtaining stronger evidence that this pathway is the primary cause of the excessive proteolysis in atrophying rat muscles. We have shown that adrenal steroids not only suppress muscle protein synthesis, but also enhance the level of ubiquitin gene expression and ubiquitin conjugation to proteins. Moreover, adrenalectomy can block the enhancement of muscle that is seen in food deprivation. We and our collaborators have also obtained evidence that this ATP-ubiquitin-dependent system is activated in a large variety of conditions where muscle atrophy is seen, including acidosis, denervation, disuse (hindlimb suspension), infection, fasting, and hyperthyroidism. Thus, there appears to be a final common cellular mechanism which is induced by diverse stimuli and causes muscle wasting.

In addition, we have been pursuing promising studies to understand the physiological mechanisms that can suppress intracellular protein breakdown and that might possibly be of benefit for treatment or prevention of muscle wasting. We have investigated the metabolic and biochemical effects of dietary protein deficiency, which suppress protein breakdown and preserve muscle mass (unlike fasting, infections, glucocorticoids, and denervation). We have found that decreased

protein intake (with adequate calories) leads to a reversible reduction in both the lysosomal and ATP-ubiquitin-dependent proteolytic pathways and a fall in the muscle's content of the critical enzymes (i.e., lysosomal proteases and the proteasome particle). We have also defined the mechanisms of the very rapid buildup of muscle mass ("catch-up growth") during restoration of normal protein intake.

Significance of the Accomplishments

These studies have provided strong evidence that the ATP-dependent proteolytic system involving ubiquitin and the proteasomes is the major degradative pathway and is activated by a variety of physiological stimuli that lead to muscle wasting, including stress (e.g., cortisol) and disuse. We have also discovered a dietary regimen (reduced protein intake) that suppresses this degradative process and the lysosomal degradative system in skeletal muscle, and that may thus be useful for the maintenance of muscle mass in various stressful states.

Publications

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GRAVITY INFLUENCE ON SIGNAL TRANSFER AND DIFFERENTIATION

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Description of Research

Goal: The goal of the Cellular and Molecular Biology Laboratory at NASA Ames Research Center is to understand the effects of microgravity as part of the larger dynamic of physical stress response in mammalian cells. Our research seeks to integrate the study of physical force interplay, extracellular matrix interactions, and gene expression events.

Objectives: The architectures of living systems perceive both load-bearing and tension/contraction stresses. Gravity has played a fundamental role in the ontogeny of these systems and acts as a constant input modifier. In the body, many cell types experience either constant or periodic tension and compression. Dermal (skin) fibroblasts undergo both stretch and flexure, endothelial (blood vessel lining) cells are exposed to strong blood flow pulses, osteoblasts (bone cells) sense both structural and load-bearing forces, and muscle cells provide both contraction and support strength. One of our objectives is defining the role of external forces on cell behavior.

Cell populations are organized with extracellular matrix molecules to form functional tissue. The matrix is the glycoprotein mortar that binds the cellular bricks together. Cells may be embedded in the matrix or form layered sheets on a lining of matrix. The integral attachments of cells to matrix molecules is part of the mechanism that allows cells to transduce and interpolate physical force information from the environment. Accordingly, our research is directed toward defining the role of matrix materials in modifying the stress responses of cells.

Specific membrane receptors for circulating protein growth factors deliver (when occupied by their ligands) signals to the intracellular response machinery. Matrix links contribute to the total informational content of these signals. One of the observed effects of microgravity is a failure of signal transfer and appropriate response in exposed cells. This is particularly striking in studies of immune effector cells, but is also seen in other cell types. The defect in response is selective, affecting some intracellular pathways and not others. Our laboratory is engaged in teasing apart relevant signal transfer processes in the response of cells to platelet-derived growth factor (PDGF). We study several aspects of this growth factor response, in a range of dermal cell strains that differ by degree of senescence. Senescent cells are very similar in the reactions to growth factor treatment, as are cells exposed to microgravity.

The communication pathways initiated by growth factor binding at the cell membrane consist of two related networks: the chemical mediator cascade and the cytoskeleton. The cytoskeleton provides three-dimensional structure to the cell, distributing forces through tensional elements. An architecture of highly ordered protein polymers, it is both a structure of morphology and a transducer of information. An important objective of our work centers on examining the cytoskeletal changes induced by external stress, or absence of stress (microgravity).

Accomplishments

We have taken two routes to the evaluation of cellular behavior under conditions of varying physical force.

First, in collaboration with NASA life sciences engineers, we have developed a prototype clinostat. This instrument supports the cultivation of our cells under conditions of vectorially changing gravity. There is a constant (small) force perpendicular to the direction of rotation, and the vector of the 1-g gravitational force is constantly shifting. The cells are grown adherent to one side of a flask in a monolayer. We have adapted the cells to growth in media that are independent of constant CO_2 exchange for buffering, because the rotating culture vessels must be sealed. Figures 1 and 2 show cells grown in this medium in the traditional orientation and kept for 1 week on the clinostat (respectively). The gravity vector-shifted cells show a marked alteration in intercellular contacts and colony morphology. We have successfully maintained these cultures for 8 weeks on the clinostat. Improvements and modifications to our prototype unit have led to design changes, and two new units incorporating these changes (and providing a rotation-only control) will be available this summer.

Figure 1. Young fibroblasts grown in CO_2 independent medium with 10% fetal bovine serum, under normal 1g conditions

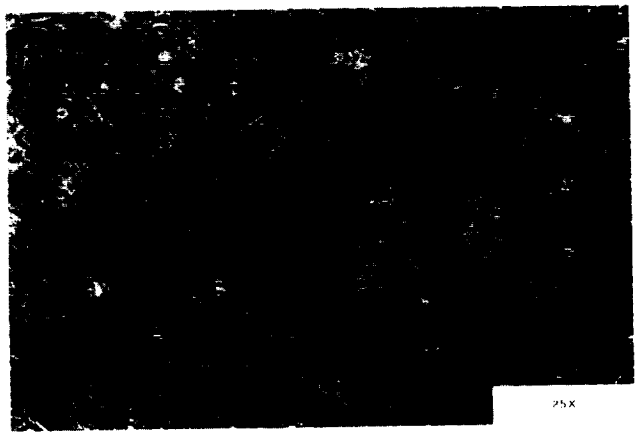


Figure 2. The same cells and medium described in the legend to Figure 1, but grown under conditions of continuously vectorially shifted gravity. Color differences are due to the greater volume of media in the sealed rotated flasks.

Our second series of experiments in force variation have utilized the Flexercell Strain Unit. This instrument transmits a two-dimensional force over a 24 mm flexible membrane. The amplitude and frequency of the stress can be controlled precisely. Monolayers of cells are grown on the flexible membrane support and subjected to predetermined flex regimens. Figures 3 through 7 reflect the behavior of senescent dermal fibroblasts to selected regimens. Cellular response was measured by incorporation of tritiated thymidine (which indicates DNA synthesis) and by direct cell counts (not shown).

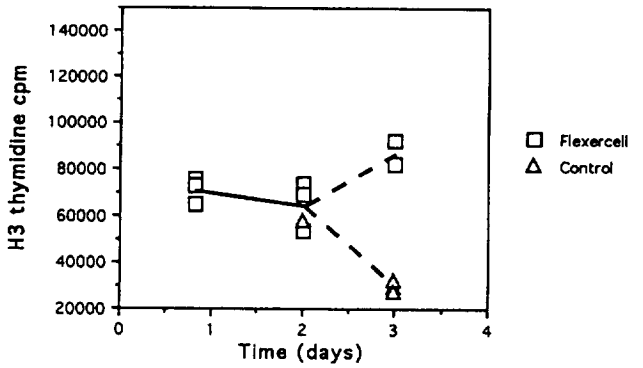


Figure 3.

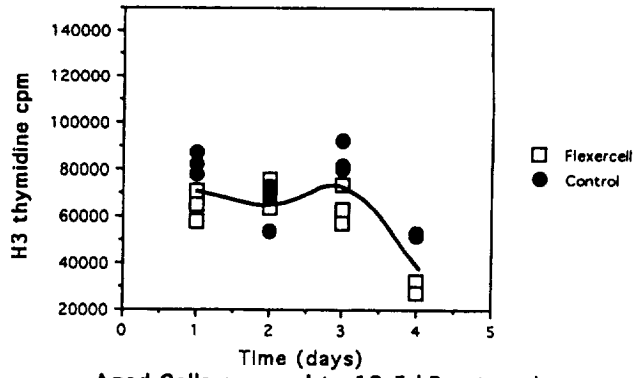


Figure 4.

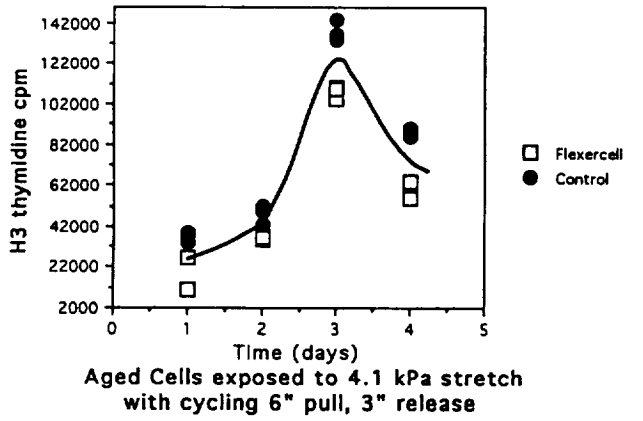


Figure 5.

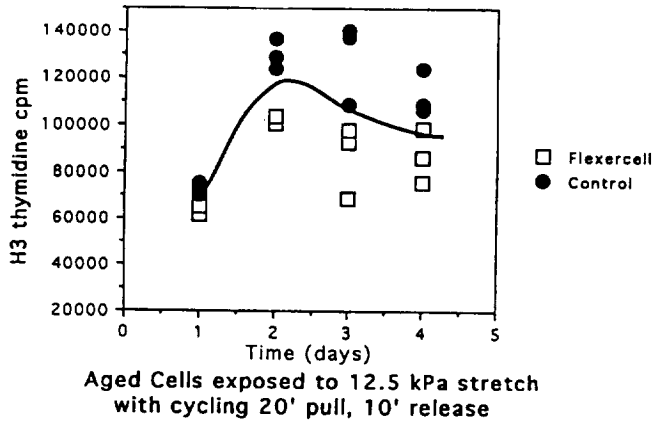


Figure 6.

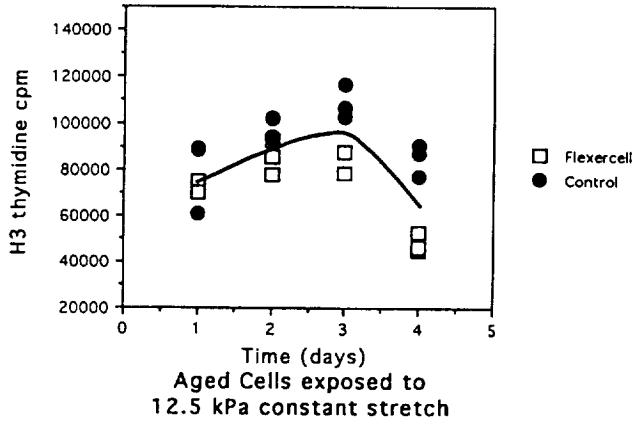
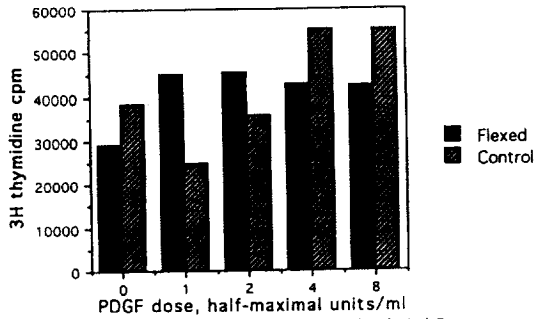


Figure 7.

These cells, and other dermal cells we have tested, show that many levels of stress can be accepted. Notice that the curves representing the flexed, or stressed, cells parallel those of the controls but show generally lower values. We interpret these lower values as indicating that responses other than proliferation are occurring in response to the applied stress. We are currently assessing the responses of flexed cells in terms of induced cell injury (induction of stress proteins, release of lactate dehydrogenase) and apoptosis, or programmed cell death (DNA fragmentation).

Our experiments over this time period have utilized a trio of dermal fibroblast cell strains. These strains were selected to represent a range of proliferative capacities. They could also be described as providing reference points along a timeline that starts with fetal growth and ends with senescence. They are (1) young cell strain (AG4431), (2) chronologically aged cell strain (WU77220), and (3) Werner's syndrome cell strain (AG780). Werner's syndrome (WS) is a genetically determined advanced aging disorder. Many of its clinical symptoms involve the skin and connective tissue. In culture, dermal cells from WS patients fail to respond to growth factors such as platelet-derived growth factor (PDGF), indicating a fundamental defect of signal transfer. These cells also overexpress the enzyme collagenase I, a gene product whose transcriptional regulation is well studied. All dermal fibroblasts produce this enzyme, which plays a central role in remodeling and removing collagen type I, the major constituent of both mineralized and non-mineralized matrix. The induction defect in WS is a conveniently assayable end point for effective signal transfer to the nucleus. The WS cells also demonstrate the mitogenic anomalies characteristic of senescence. Similarities between the generalized response failures of senescent cells and of those exposed to microgravity are the basis for our choice of this "age range" model.

Using the flexible membrane Flexcell plates, we have analyzed the behavior of AG4431 (fetal) cells on untreated or collagen type I coated membranes. While both strains of old cells grow reliably on the untreated membranes, the young cells form only tenuous attachments to the supporting material in the absence of a collagen coating. This is particularly evident when force is exerted on the membrane, and is irreparably damaging to the cell monolayer if the continuity of the cell sheet is disrupted before the application of stress. We see an altered program of signal transduction events in those young cells grown on collagen and an interaction between the applied stress and PDGF responses. Maximal stimulation of stressed cells is achieved by a 1 unit/ml PDGF, and does not show a dose dependence relationship. Control cells demonstrate this dose dependence of response (Figure 8). In combination with the results observed in the ± stress, ± PDGF studies, we see evidence of cooperative but separate pathways for the transduction of physical force and growth factor response. Other evidence (now shown) indicates that these fetal cells are producing and responding to their own growth factors. To clarify the experimental picture, we will be adding neonatal and adult dermal fibroblasts to our studies this year.



Young cells exposed to PDGF and 12.5 kPa stretch with cycling 6" pull, 3" release

Previously, we evaluated the regulatory region of the collagenase type I gene in WS cell strain AG 780 and demonstrated that its sequence was normal. The transcriptional regulatory protein complex AP-1 interacts with this DNA region. The FOS protein is one of the "immediate early" response factors. Encoded by the *c-fos* gene, its message transcript is transiently induced following stimuli such as PDGF binding. We analyzed *c-fos* and collagenase type I transcripts ± stress and ± PDGF addition. Both the young and the WS cell strains produced similar overall amounts of *c-fos*. Induction of *c-fos* transcripts by stretch alone was observed in the fetal cells at the 30-minute time point, and the response was sustained at 24 hours. In control, unflexed fetal cells, *c-fos* transcripts were induced by PDGF only transiently. WS cells experiencing stretch in the Flexercell Strain Unit unexpectedly induced *c-fos* transcripts in the presence of PDGF. The WS cells produced copious amounts of collagenase type I transcripts under all conditions tested. We have also examined the production of transcripts of the *c-jun* gene. Both the product of *c-jun*, the JUN protein, and FOS are required for the induction of collagenase type I. Thus far, we have been unable to detect *c-jun* transcripts in the senescent or WS cells using an oligonucleotide probe. We postulate that this may indicate these aged cells are producing another member of the *jun* family of related genes. We are pursuing this finding.

We have used photomicroscopy to examine our samples. We acquired a phase-contrast inverted microscope with camera attachment for this work, and have documented the observations on growth characteristics and healing of an experimentally produced simulated wound. Preliminary samples are being analyzed by NASA-Joint Venture (JOVE) fellow, Prof. M.V. Brown', using fluorescent-antibody stains for the localization of cytoskeletal components. In addition to this work, she is examining the arrangement of the intermediate filaments and tubulin compartments of the cytoskeleton using transmission electron microscopy and immunogold labeling. This year Christine Sawyer, the laboratory's Research Associate, completed an intensive training course in immunocytochemistry and photomicroscopy. We will continue to expand our capabilities in this area.

Significance of the Accomplishments

The importance of physiologic tensional stresses has been demonstrated both *in vivo* and *in vitro*. The cytoskeleton receives and transmits environmental stimuli, including growth factor responses and matrix interactions. It is both a structure of morphology and a transducer of information. As a component of structural integrity, this architectural complex is ideally suited to sense the physical changes associated with altered gravities. At the same time, there are clearly other pathways that contribute significantly to the transmission of growth factor signals such as that delivered by PDGF. These intracellular chemical second messenger cascading pathways are probably the most important determiners of cellular responses to growth factors, with cytoskeletal/matrix interactions acting as modifiers or educators. We are linking these three fields (tensional force integration/matrix biology/growth factor response) to more fully understand gravity modulation of cellular response. The similarities between aged and microgravity-exposed mammalian cells can be exploited in productive experimentation geared towards a fuller understanding of the basis of cellular "unresponsiveness" in spaceflight.

Perhaps our most significant accomplishment lies in technology and hardware development. Determining the specifications and response parameters for the continuous vectorially shifted clinostat apparatus and performing preliminary experiments on cells cultivated in the prototype unit, are essential to our pursuit of further gravitational biology studies. In addition, we have tested the capabilities and nuances of the Flexercell Strain Unit and Flexcell plates. These permit us to alter the dynamics of the physical forces that compose the cells' environment.

Living organisms develop and grow in an environment where external stimuli are unbounded and unpredictable. Understanding the cell/environment interface, in terms of information acquisition and cellular response, is a broad goal of cellular and molecular biology. Cytoskeletal architectures are fundamental to this communication. Having evolved under the influence of gravity, they are clearly designed as tension/compression systems. The integration of perceived force is a summation of the gravitational vector, with constant magnitude and direction (unless manipulated in a clinostat) and applied environmental force of varying magnitude and direction. We believe that these force components are integrated at the level of the cytoskeleton and that the integration is impacted in part by connections to the matrix which educate the cell as to its local environment and homeostatic function. We hope that the intracellular conversation between force transduction and signal transduction can be revealed by our methods.

SKELETAL MUSCLE GLUCOSE TRANSPORT DURING SIMULATED WEIGHTLESSNESS

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Description of Research

The long-term goal of this research project is to characterize on a cellular and molecular level the responses of the skeletal muscle glucose transport system to alterations in muscle activity state. The focus of the current research is to identify how the regulation of the skeletal muscle glucose transport system is altered when the muscle undergoes a period of reduced weight bearing (also known as unweighting). Skeletal muscle glucose transport is a major regulator of glucose removal from the blood and, therefore, will be a primary factor in blood glucose homeostasis. Glucose transport into skeletal muscle is stimulated by two independent pathways, one sensitive to insulin and insulin-like factors, and the other sensitive to muscle contractile activity and other interventions which activate Ca^{2+} release from the sarcoplasmic reticulum. Numerous studies have demonstrated that the maximal effects of both pathways on the stimulation of *in vitro* glucose transport are completely additive, indicating that the stimulatory effects of insulin and contractions are mediated by different mechanisms and, as such, may respond differently to alterations in muscle activity.

To study the regulation of glucose transport in skeletal muscle independently of the influence of muscle blood flow, the isolated soleus strip preparation has been developed. Soleus strips of equal size are incubated *in vitro* and can be stimulated by a variety of factors and manipulations under highly controlled conditions. Glucose transport activity is then assessed by measuring the uptake into the muscle cells of a non-metabolizable glucose analog, 2-deoxyglucose (2DG). Reduced weight bearing of the soleus, a lower leg muscle used in locomotion, is induced by the tail-cast suspension model and results in rapid (within 3 days) and significant atrophy of this muscle. This is a widely used model of simulated weightlessness and mimics many aspects of actual microgravity. A major finding of previous research by this lab and others is that unweighting of the soleus leads to an enhanced action of insulin on glucose transport activity, likely a result of increased insulin binding capacity and enhanced intracellular levels of glucose transporter protein. How unweighting of the soleus can affect the response of the glucose transport system to insulin-like factors such as insulin-like growth factor I (IGF-I), vanadate, or phospholipase C (PLC) and to contractile activity, such as occurs during exercise, was not previously known. This line of research can provide important information on the adaptative response of skeletal muscle in a microgravity environment and also during recovery from an unweighting period. In addition, this line of research can be used to better understand how the skeletal muscle glucose transport system functions under normal conditions and in such pathophysiological conditions as diabetes and denervation.

Accomplishments

(1) Whereas soleus unweighting leads to an enhanced response to insulin for maximal stimulation of glucose transport activity, this intervention does not alter the contraction-dependent pathway for activation of this process. The hindlimbs of juvenile (~100 g) male Wistar rats were suspended by a tail-cast system for 3 or 6 days. Glucose transport activity was then assessed in isolated soleus strips (~18 mg) using 2DG uptake. Insulin (2 mU/ml) had a progressively enhanced effect on 2DG uptake after 3 and 6 days of unweighting (+44 and +72

percent vs. control, both $p<0.001$), in agreement with previous findings. In contrast, at these same time points, there was no difference between groups for activation of 2DG uptake by maximally effective treatments with *in vitro* contractions (10 tetani). The response of glucose transport activated by stimuli that work through the contraction-pathway but do not induce sarcolemmal membrane depolarization, namely hypoxia (60 min) and caffeine (5 mM), also was not significantly different from the control. Collectively, these results indicate that the enhanced capacity for stimulation of glucose transport following soleus unweighting is restricted to the insulin pathway, with no apparent enhancement of the contraction-dependent independent pathway.

(2) Soleus unweighting leads to an enhancement of the stimulatory effects of IGF-I, vanadate, and PLC on glucose transport activity. This study used a novel approach to further evaluate the potential role of post-receptor binding mechanisms in this enhanced insulin effect following unweighting. IGF-I, vanadate, and PLC were used to stimulate glucose transport activity independently of insulin receptor binding. Soleus glucose transport activity was evaluated *in vitro* using soleus strips (~18 mg). Progressively increased responses to maximally effective doses of insulin or IGF-I were observed after 3 and 6 days of unweighting compared to weight-matched controls. Enhanced maximal responses to vanadate (6 days only) and PLC (3 and 6 days) for 2DG uptake were also observed. The results of this study (1) provide evidence that post-insulin receptor binding mechanisms also play a role in the enhanced response of the insulin-dependent pathway for stimulation of glucose transport in unweighted skeletal muscle, and (2) indicate that IGF-I action on glucose transport is included in this enhanced response in unweighted muscle.

Significance of the Accomplishments

Finding 1: These results indicate that unweighting of the soleus muscle by tail-cast suspension causes a marked increase in insulin-stimulated glucose transport activity, with no enhancement of the insulin-independent pathway for glucose transport. These findings support the hypothesis that in muscle there are two separate intracellular pools of glucose transporters, one recruited in response to insulin (or insulin-like factors) and another recruited in response to contractions or hypoxia. The present results would be compatible with the idea that unweighting causes an increase in only the insulin-recruitable pool of GLUT-4 protein, with no expansion of the contraction- or hypoxia-recruitable pool. This model of altered muscle usage, with its discordant adaptive responses for insulin-dependent and insulin-independent glucose transport activity, could be effectively utilized in future research endeavors to individually examine the cellular mechanisms regulating these two pathways for stimulation of glucose transport.

Finding 2: These results provide evidence that soleus unweighting by hindlimb suspension induces an enhancement not only in the action of insulin, but also in the action of IGF-I on the glucose transport system. Furthermore, the enhanced effects of insulin-mimickers in unweighted muscle suggest that multiple sites of adaptation that include post-receptor mechanisms, must be involved in the increased effect of these hormones on glucose transport activity. Therefore, this model of altered muscle use may be used in the future to help justify the intracellular factors that are important in the action of insulin and insulin-like factors on the skeletal muscle glucose transport system.

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STRUCTURAL DEVELOPMENT AND GRAVITY

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Description of Research

The goal of this research is to understand the role of gravity in skeletal growth and development. To achieve this goal, we must first learn (a) if gravity turns bone cells on or off, (b) if/how these cells communicate with each other and with their environment, (c) if/how secretory products are altered by different gravity levels, (d) how alterations in organic matrix affect mineralization and material properties, and (e) the role of local and systemic factors (including endocrine, blood flow, and fluid shifts) in gravitational responses. To accomplish these studies, both ground-based and flight experiments are essential.

Gravity is a major factor determining the amount of structural support required by Earth organisms. The hypotheses of this research effort are: (1) Skeletal support structures will change during spaceflight and/or unloading. (2) The magnitude of change will be dependent upon the modeling or remodeling activity in each bone and the length of exposure. (3) Changes will be manifested primarily through altered matrix formation and/or mineralization. (4) Changes in both quantity and quality of bone will occur. (5) Systemic and local factors will be involved in the changes. Most ground-based research involves growing rats exposed to simulated space flight, and flight experiments in whole animals or primary bone cell cultures will allow gathering of more information to support or negate the hypotheses. During this report period, ground-based studies focused on the response to skeletal unloading, with emphasis on the role of bone resorption in the response. Flight activities included preparation for experiments on the first and second dedicated Spacelab Life Sciences missions and middeck rat and cell culture experiments.

Accomplishments

(1) Alendronate (AHBP), a bisphosphonate inhibitor of bone resorption, was tested in growing rats to determine if it could inhibit the defect in mineralization during skeletal unloading. Young male rats were injected subcutaneously with the drug (experimental group given 0.1 mg AHBP/kg or 0.4 mg AHBP/kg), or with saline (control group) on each of the 2 days preceding skeletal unloading. The hindquarters of half the animals in each group (n=6) were unloaded by tail-traction. Bone markers were given before and during the experiment. The unloading lasted 9 days. The average weight gain during the experiment was approximately 33 g in each group. Tibia mass was significantly different in all groups (basal=209±4 mg, unweighted vehicle = 229±16 mg, weighted vehicle = 261±9 mg, unweighted AHBP = 286±8 mg, and weighted AHBP = 303±7 mg) with the major difference being in the cancellous (proximal tibia) rather than cortical (tibia diaphysis) bone. Calcium concentration in the proximal tibia increased significantly in the AHBP treated rats compared to basal and vehicle treated groups while unloading did not change the concentration of calcium at this bone site. The bisphosphonate caused a striking increase in the number of osteoclasts in cancellous bone in all animals compared to saline controls. However, the osteoclasts were apparently inactive, as the cells did not appear to be firmly attached to the bone surface and they had minimal ruffled borders; also, the amount of bone was increased significantly. The decrease in osteoclastic activity may be the reason for the significant increase in cancellous bone in the proximal tibia of the bisphosphonate treated rats. Interestingly, cortical bone formation at the tibiofibular junction was still decreased at the periosteal surface in all unloaded rats compared with their appropriate loaded control; and, furthermore, AHBP controls showed a significant suppression of bone formation at this site compared to vehicle treated controls.

(2) Data from rats flown on STS-40 (Spacelab Life Sciences 1) suggest that housing (individual vs. group) influences the response to flight with fewer changes and a more rapid recovery from flight noted in the group-housed animals as compared to individually housed rats. Also, the bone changes varied not only from bone to bone but at different sites within the same bone.

(3) A mineralizing primary fetal rat osteoblast system was tested for suitability as part of the Space Tissue Loss (Walter Reed Army Institute of Research) hardware flown on STS-45 and STS-53. Cells grown on collagen-coated beads were evaluated after incubation in the newly developed tissue culture hardware which uses hollow-core fibers and continuous-flow medium. Under normal temperature (37° C) and CO_2 (5 percent) conditions, the osteoblasts thrived during exposure to a 10-day flight after 10 days of preincubation on STS-45. Although mineralization was not evident in the STS-45 flown cells, no ground controls were available for comparison. During the STS-53 flight, the ground control unit malfunctioned.

Significance of the Accomplishments

Finding 1: suggests that this bisphosphonate selectively protects the filamtentous cancellous bone found at the end of long bones and in vertebrae during unloading in growing rats but does so by adversely changing bone chemistry, bone growth, and bone remodeling.

Finding 2: suggests that housing (group vs. individual) impacts the bone response, possibly through differences in activity, and that the apparent bone response to space flight depends on the bone site studied.

Finding 3: provided valuable information on flight cell culture hardware and its ability to support primary bone cells during space flight.

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GRAVITATIONAL FIELDS AND NEURAL SIGNALING IN THE HIPPOCAMPUS

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Description of Research

Our research has an ongoing, long-term objective of elucidating the effects of altered gravitational fields on neural networks. This past year our experiments dealt with the effects of serotonin (5-HT), a neuromodulator, on hippocampal neurons. The hippocampus is an area of the central nervous system associated with several types of neural activity including sleep, temperature regulation, and, perhaps most importantly, memory and learning. Our experiments were based on the observation that rats flown on Spacelab-3 showed a 48.6 percent increase in hippocampal 5-HT_{1A} receptor number after 7 days exposure to the microgravity environment, whereas rats at 2-g for 7 days showed a 27 percent decrease in receptor number.

In our experiments, we first exposed animals to a 2-g hypergravity field for 7 days. Previous studies showed that during this time the number of serotonergic receptors in the hippocampus decreased, a down regulation in receptor number. Within 1 hour after the animals were removed from the hypergravity field, the tissue was prepared for recording electrical activity. Because it takes days for receptor numbers to change appreciably, the electrical activity reflected neural activity of a down-regulated system. We measured electrical activity in the dentate and CA1 pyramidal cell layer in the hippocampal slice preparation before, during, and after perfusion with serotonin and 8-OH-DPAT. Serotonin binds with several receptor types whereas 8-OH-DPAT is a specific agonist that preferentially binds to one serotonin receptor type, the 5-HT_{1A} receptor. We interpreted data in terms of altered electrical activity over a trisynaptic hippocampal neural network.

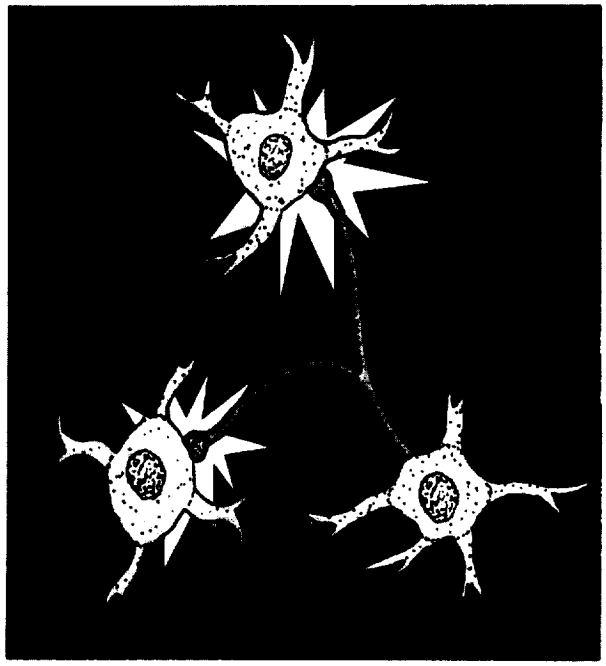

Figure 1. Sketch showing synaptic transmission. (Prepared by C.J. Howard).

Accomplishments

(1) The hippocampus is a laminar neural structure containing a trisynaptic neural circuit, and we examined two key locations (areas receiving major serotonergic inputs) on this pathway. Due to the sequential nature of information transfer through the hippocampus, if serotonin affects any one part of the pathway, it affects the output of the entire neural system. We found that two sites along the neural pathway (the dentate and the CA1 pyramidal cell layer) retain a robust serotonergic response in animals exposed to 2-g for 7 days.

(2) We focused on characterizing the effectiveness of a particular class of serotonergic receptors on dentate and CA1 pyramidal cells, 5-HT_{1A} receptors, by using 8-OH-DPAT. This set of experiments confirmed and extended observation (1) above; namely, that cells at two critical sites along the pathway retain responsiveness to a 5-HT agonist in animals exposed to 2-g fields. Thus, signals from the serotonergic Raphe nuclei in the brain stem, which anatomically have been shown to project out over the dentate and CA1 pyramidal cell layer, can modulate the flow of information over the trisynaptic network.

(3) Based on these observations (together with receptor number studies), we are developing a model for serotonergic modulation of information transfer over the trisynaptic pathway in animals exposed to 2-g fields. An essential feature of the model is that modulatory effectiveness is maintained as shown by measurements of electrical activity in animals exposed to 2-g conditions even though there is a down regulation of overall receptor number.

Significance of the Accomplishments

We are developing a model that accounts for a wide variety of experiments we have completed on the effects of altered gravitational fields on the effectiveness of serotonin to modulate neural activity. At this stage our most significant finding is that modulation of hippocampal cell activity is maintained in animals that have been exposed to hypergravic fields. Thus, even though anatomical evidence shows that the number of receptors for serotonin is decreased, there appears to be a surplus of receptors so that neural information processing is maintained and electrical activity is not altered.

The overall significance of this project is that information processing over a trisynaptic network as modified by a neuromodulator has now been more fully elucidated. The circuit compensates for the loss in the number of receptors so that information processing appears unchanged. The 2-g experiments were conducted on animals that were exposed to a hypergravic field for exactly as long as animals were on Spacelab-3 so that, in addition to defining effects in hypergravity, information is acquired that will provide baseline data for future experiments in space. Parallel experiments can relatively easily be carried out to test changes in a microgravity environment, since hippocampal 5-HT receptor numbers increase in a microgravity environment.

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MECHANISM OF CONTROL OF BONE GROWTH BY PROSTAGLANDINS

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Description of Research

The growth and repair of bone is a complex and poorly understood process. Over the past three decades, it has been noted that astronauts lose large amounts of calcium and bone when exposed to the microgravity of space flight. The mechanism by which bone growth is regulated is not known; however, clinical observations have demonstrated that increases of endogenous cortisol as seen in Cushing's syndrome are associated with bone loss and osteoporosis. Treatment of asthma and rheumatoid arthritis with glucocorticoids is also associated with poor bone formation in these patients. The bone loss associated with glucocorticoids involves the trabecular bone, and examination of patients treated with the synthetic glucocorticoid prednisone shows a reduction in bone formation, which is probably due to a direct inhibition of osteoblast function during space flight. In the Skylab missions, urinary cortisol of the nine crewmembers increased almost two-fold. These data suggest that the glucocorticoids as well as microgravity play a role in the loss of bone during space flight.

This laboratory has previously shown in osteoblast cultures that glucocorticoids cause a decrease in endogenous prostaglandin E_2 synthesis accompanied by a decrease in osteoblast growth and mineralization. Taken together, these observations suggest that glucocorticoids may regulate bone growth by inhibiting endogenous PGE_2 synthesis. The direct cause of glucocorticoid-induced bone loss is not known, and an understanding of the relationship between bone growth and the eicosanoid synthetic pathway may help elucidate the regulation of bone growth. Using a cloned osteoblast cell line as a tool to study the cellular changes in growth, we have tested the hypothesis that endogenous prostaglandin E_2 directly plays a role in regulation of cell growth by perturbing endogenous osteoblast synthesis of prostaglandins and by testing for alterations in growth and morphology. This year we have found that the prostaglandins are mediating early oncogene signals much like the polypeptide growth factors.

Accomplishments

Ground based experiments: Our bone model is the MC3T3-E1 cell, a cloned osteoblast line that retains synthetic functions of normal bone tissue, including production of alkaline phosphatase, prostaglandin E_2, and mineralized matrix-containing collagen type I and hydroxyapatite. In these studies, we have serum deprived cloned osteoblasts and added back PGE_2 alone to test the ability of prostaglandin *per se* to stimulate osteoblast growth.

Prostaglandin E_2 acts as bone cytokine.

(1) Prostaglandins act as autocrine cytokines to stimulate osteoblast growth, normal growth is inhibited when prostaglandin synthesis is blocked by glucocorticords or nonsteroidal anti-inflammatory drugs (NSAIDS), and this inhibition of growth is reversed by addition of exogenous prostaglandin. When prostaglandin synthesis is slowed by serum depletion, growth is also inhibited. When prostaglandin is added to the serum deprived osteoblasts, cell growth, as measured by DNA synthesis, was increased from 2–3 times the control. These data show that prostaglandin synthesis plays a pivotal role in bone growth.

(2) Prostaglandins induced oncogene message synthesis. Within 30 minutes, prostaglandin causes a 20-fold increase in expression of *c-fos*; addition of cyclohexamide caused a super induction of *c-fos* suggesting that this up-regulation does not require new protein synthesis. In addition, the *c-fos* response of the osteoblast is proportional to the amount of PGE_2. The rise in *c-fos* expression is followed a few hours later by a 4-fold increase in *c-jun*. Messages for ß-actin and γ-actin were also induced within 4 hours of the addition of prostaglandin.

(3) In addition, we found that PGE_2 causes a rise in intracellular calcium within 2 minutes of addition of PGE_2. When the L-channel blocker nifedipine (10 µmol) was added to the osteoblast cultures, the signal was abolished; and removal of the blocker restored the PGE_2 mediated calcium signal, suggesting that the prostaglandins regulate calcium flux through L-type calcium channels.

(4) Using epi-fluorescent microscopy, we noted changes in the *F*-actin cytoskeleton of the control and prostaglandin treated osteoblasts, with the control osteoblast having little stress fibers in their actin architecture. Addition of prostaglandin not only stimulated actin message, but also caused striking increases in *F*-actin stress fibers that were associated with increased growth of the prostaglandin treated cells.

(5) Since PGE_2 is the major eicosanoid synthesized by the osteoblast, we asked the question if it was also an effective mitogen by testing cell growth stimulation by various eicosanoids. We found that PGE_2 is by far the most effective growth stimulatory eicosanoid we have tested.

Space flight Experiments: Using a cloned osteoblast cell line as a tool to study the cellular changes in growth, we have tested a second hypothesis that space flight plays a role in growth regulation by directly affecting prostaglandin synthesis, growth, and morphology of bone in space flight. My collaborator, Dr. Marian Lewis, University of Alabama, Huntsville, and I have selected the MC3T3-E1 cell, a cloned osteoblast line that retains synthetic functions of normal bone tissue, including production of alkaline phosphatase, prostaglandin E_2, and mineralized matrix-containing collagen type I and hydroxyapatite as our bone model. Using the CMIX apparatus, we are able to add sera while in flight, thereby activating the osteoblasts in microgravity and then fixing them while still in microgravity, thus enabling us to study the real-time effects of space flight on osteoblasts. In these studies, we started with serum-deprived, cloned osteoblasts and then added serum during space flight to test the ability of serum to stimulate osteoblast growth in microgravity.

(6) Dr. Lewis and I have developed and perfected the technique for bone cell growth on coverslips in microgravity on Consort 5 and STS-52.

(7) We have flown the MC3T3-E1 cells on STS-56 and found that mammalian osteoblasts have a significantly reduced glucose utilization in space. These results agree with the work completed on Skylab with the Hayflick WI-38 cells in 1973.

(8) Using microscopic analysis, we have also found reduced osteoblast cell growth in microgravity conditions.

(9) In addition, the actin cytoskeleton of the flown osteoblasts has a reduced number of actin stress fibers and drastically changed cell morphology.

(10) We are currently measuring prostaglandin synthetic capacity of the flown osteoblast as compared to its flight profile controls.

(11) These observations suggest reduced growth and DNA synthesis in bone osteoblasts grown in microgravity. We spent over a half-year in 1993 perfecting new molecular biology methods to allow us to quantitate specific messenger RNA content in osteoblasts grown in microgravity conditions. Quantitative reverse transcriptase polymerase chain reaction (RTPCR) methods are now in place to allow us to assay very small quantities of material flown in space.

(12) Looking at liver samples from rats (flown and ground controls), Dr. Tom Dietz and I have measured several genes, including heat-shock, *c-fos* and others, to establish what, if any, differences in gene expression are found in flown animals. These findings will be ready in abstract form for the 1994 ASGSB meetings in San Francisco.

Significance of the Accomplishments

Biomedical studies of space flight crews have consistently indicated a loss of weight-bearing skeletal bone. Various lines of evidence from humans and animals suggest that this loss is due to a lack of bone formation in the absence of gravity. Biomechanical properties of both cortical and trabecular bones show strength deficits that appear to represent failure of normal increases in growth rather than an accelerated loss of bone. Joint U.S./U.S.S.R. biosatellite flights of small animals have shown clear morphological evidence that bone formation is reduced during flight.

The cause of this loss of bone has been postulated to be related to the lack of biomechanical stimulation during flight, to increases in systemic hormones associated with space flight, or to a direct effect of microgravity on the cell. We have begun studies in microgravity, testing the effect of weightlessness on the growth response of the osteoblast. Even in the absence of elevated glucocorticoids, microgravity reduced the growth of the osteoblasts, and caused changes in morphology and actin stress fiber expression. We are currently testing the prostaglandin synthetic capacity of cells in microgravity. These studies suggest a primary role of microgravity in growth regulation of osteoblast cells in space flight.

Urinary cortisols of crewmembers during the Skylab missions increased from a preflight value of 54±4 μg/day to 94±5 μg/day during flight, with individual crewmembers increasing 1.2- to 2.8-fold during flight. Osteoporosis seen in Cushing's syndrome patients is associated with elevated cortisols (2- to 5-fold control values), which suggests that the elevated cortisols seen in space flight may contribute to decreased bone mass. Since cortisol and the other glucocorticoids like dexamethasone cause a decrease in endogenous prostaglandin synthesis, prostaglandins have been implicated in the regulation of bone loss both in Cushing's syndrome and in long-term space flight. We have recently reported our observation of the glucocorticoids on osteoblast growth and prostaglandin synthesis.

In order to test further our hypothesis that prostaglandins play a direct role in the regulation of bone growth, we have used a serum-deprived osteoblast model to test the mitogenic signaling of the prostaglandin E_2. In this publication, we report that eicosanoids increase osteoblast growth, DNA synthesis, and *c-fos* oncogene message. Our results agree with studies by Web Jee and others, who have demonstrated that prostaglandin E_2 causes significant increases in bone cell growth *in vivo*. Also, our epifluorescent microscopic studies have demonstrated an alteration of actin cytoskeleton resulting in changes in cell morphology when prostaglandins are added to osteoblasts. These data add to the growing evidence that prostaglandin E_2 is a natural regulator of osteoblast growth and that PGE_2 directly plays a pivotal role in bone cell growth and formation.

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MECHANOCHEMICAL TRANSDUCTION ACROSS EXTRACELLULAR MATRIX

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Description of Research

The general goal of this project is to characterize the molecular mechanism by which mechanical signals, such as those due to gravity, are transduced into changes in cell form and function. The more specific objective is to analyze the process by which mechanical forces are conveyed by extracellular matrix (ECM) molecules, transmitted across the cell surface, and transduced into a cytoskeletal response. This approach is based on the concept that cell shape and, thus, the form of the cytoskeleton depend on a dynamic balance between tensile forces that are generated within contractile microfilaments and resisted by both internal cytoskeletal struts and ECM adhesion sites at the cell periphery. If this type of tensional integrity or "tensegrity" mechanism is used by cells, then externally applied mechanical loads may alter the cellular force balance and change cytoskeletal and nuclear structure by inducing internal cytoskeletal rearrangements as well as changes in cytoskeletal filament polymerization inside the cell. These changes in cytoskeletal organization would then alter the distribution and, hence, the function of associated elements of the cell's metabolic machinery. Cells normally generate tension within their contractile microfilaments and apply these forces on their points of attachment to ECM. Thus, one specific aim of this proposal is to determine whether transmembrane ECM receptors, which are known as integrins, mediate mechanochemical transduction. For the purpose of this grant, we initially focused on the biomechanical mechanism by which actin microfilament assembly is regulated inside the cell. However, during the past year, we have also measured the effects of varying cell ECM binding on microtubule polymerization as well as nuclear structure.

Accomplishments

Demonstration that Cell Shape Depends on a Mechanical Force Balance. A system was developed in which cell shape control could be studied in membrane-permeabilized cells. Endothelial cells adherent to fibronectin-coated dishes were permeabilized in saponin using a buffer that retains the functional integrity of the actin cytoskeleton. Addition of ATP was shown to induce retraction and rounding of permeabilized cells over a period of approximately 10-30 minutes. ATP-induced rounding was also accompanied by nuclear retraction as well as redistribution of actin microfilaments. All ATP-driven changes in cell and nuclear structure could be inhibited using a synthetic myosin peptide that prevents mechanical tension generation within contractile microfilaments. In addition, both the rate and degree of cell and nuclear retraction could be augmented by using soluble arginine-glycine-aspartate (RGD)-containing peptides to dislodge immobilized fibronectin from cell surface integrin receptors at the same time that internal cytoskeletal tension is raised using ATP. In contrast, the same peptides had little effect in the absence of ATP (i.e., in the absence of cytoskeletal tension).

Altering the Cellular Force Balance Controls Tubulin Monomer Levels. Changes in tubulin monomer and polymer mass were measured in hepatocytes that were cultured on different densities of immobilized ECM ligands that differ in their ability to resist cell tractional forces. Increasing the laminin coating density from low to high (1 to 1000 ng/cm) promoted cell spreading and resulted in formation of greatly extended microtubule networks, as determined by immunofluorescence microscopy. Nevertheless, the steady-state mass of tubulin polymer was similar at 48 hr, regardless of cell shape or ECM density. In contrast, round hepatocytes on

low-density laminin contained a three-fold higher mass of tubulin monomer when compared with spread cells on high laminin. Ongoing experiments are designed to analyze how these changes in tubulin monomer levels are controlled in cells that likely exhibit autoregulation of tubulin protein synthesis.

Probing the Molecular Basis of Force Transfer across the Cell Surface. Studies were carried out using a prototype magnetic twisting device to apply controlled mechanical stresses directly to cell surface receptors in the absence of a large-scale cell shape change. In this system, adherent cells are allowed to bind to spherical ferromagnetic microbeads (5.5 μm) that are coated with specific cell surface receptor ligands. The beads are magnetized in the horizontal direction, and then magnetic stress (torque) is exerted on the adherent beads by applying a twisting magnetic field in the vertical direction. A sensitive in-line magnetometer allows us to measure changes in the rate and degree of bead rotation in real time and, thus, to quantitate bead deformation (angular strain) in response to a defined applied stress. Preliminary results suggest that the ECM receptor, integrin, can support a force-dependent stiffening response, whereas nonadhesion receptors (e.g., acetylated LDL receptors) cannot. Studies are currently underway to further dissect the molecular basis of transmembrane force transfer and to confirm that the cellular response to stress is mediated by the cytoskeleton.

Significance of the Accomplishments

Our studies with membrane-permeabilized cells clearly demonstrate that large-scale changes in cell, cytoskeletal, and nuclear shape result from the action of mechanical tension that is generated within the cytoskeleton via an actomyosin filament-sliding mechanism transmitted across integrin receptors and physically resisted by immobilized adhesion sites within the ECM. Rapid and coordinated changes of cell, cytoskeletal, and nuclear form result when this cellular force balance is altered. In other words, ECM molecules do not control cell and nuclear form exclusively via chemical mechanisms; rather, they largely function as mechanical anchors which resist cell tractional forces, as suggested by the tensegrity paradigm. Thermodynamic analysis of tubulin polymerization predicts that increasing the mechanical load on a microtubule will result in microtubule disassembly if the tubulin monomer set point is held constant. This would occur because compressing a microtubule increases the critical concentration of tubulin that is required to maintain tubulin in a polymerized form. Results from our experiments on microtubule metabolism are consistent with this type of mechanochemical regulatory mechanism in which changes in ECM contact formation alter local thermodynamic parameters inside the cell. Apparently, cells have evolved the ability to maintain a constant mass of microtubule polymer in the face of these thermodynamic alterations by producing coordinate changes in the total mass of tubulin monomer, as the critical concentration for tubulin is mechanically altered. These results are, therefore, consistent with the mechanotransduction mechanism, in which cytoskeletal filament assembly is modulated by changes in the balance of mechanical stresses that are distributed across transmembrane ECM receptors and through the cytoskeleton. Our preliminary results suggest that we are now in a position in which we can analyze the molecular path of force transmission across the cell surface as well as the molecular basis of mechanochemical transduction. These initial findings are consistent with our hypothesis that integrins provide a molecular path for transmembrane force transfer and, hence, act as cell surface mechanoreceptors. The ability to measure stress/strain relationships in living cells should allow us to begin to identify molecular elements (e.g., different cytoskeletal filament systems) that are critical for mechanotransduction and thus, to test the tensegrity hypothesis more directly. If the specificity of force transfer can be confirmed using the magnetic twisting device, then it may also provide a novel method for mechanically stimulating cells as well as for measuring a cellular response.

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THE EFFECTS OF GRAVITATIONAL LOADING AND VIBRATION ON VESTIBULAR ONTOGENY

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Description of Research

The principal aim of this research is to examine the role played by gravity in controlling or influencing the ontogeny of peripheral and central vestibular function. Ultimately, an in-depth comparison will be made of how vestibular function develops and matures under the influence of gravitational fields having strengths less than 1-g (hypodynamic); i.e., equal to 1-g, and greater than 1-g (hyperdynamic); i.e., the natural gravitational field strength of Earth. Studies will be undertaken to examine how the gravitational vector strength and direction may modulate or influence normal responses to transient stimuli. Efforts will also be directed to investigate how vestibular function may adapt to changes in gravitational fields and to evaluate the vestibular system's ability to adapt as a function of the age of the organism. Through these kinds of studies, we may begin to appreciate the limits to, and determinants of, physiological adaptation in the vestibular system under a variety of gravitational environments.

The research program has led to the development of a new vestibular functional test for gravity receptors. The test consists of recording short latency vestibular responses to linear cranial acceleration. Salient features of these responses include short latencies (4 to 7 peaks occurring within 8 msec following the stimulus) and resistance to intense auditory masking (96 to 104 dBSPL). Moreover, the responses are abolished upon complete bilateral destruction of the labyrinth but are little affected by selective bilateral destruction of the cochlea and lagena. The research has demonstrated that responses to pulsed linear acceleration in birds are vestibular in origin and represent compound action potentials of the vestibular nerve and central relays. Similar responses have been recorded in rats and have been shown to be resistant to intense auditory masking and depend on the eighth nerve bilaterally. The effects of selective cochlear destruction are currently under study in rats. Among other things, the responses allow us to measure the sensitivity of gravity receptors (i.e., vestibular thresholds) and whether or not sensory information is processed at normal rates through vestibular relays. These functional attributes of gravity receptors can be tested and characterized throughout development.

New research summarized in the current report sought to (a) begin the evaluation of normal vestibular development and factors that may alter development including microgravity and drugs; (b) evaluate the potential for functional recovery following vestibular injury (c) estimate the statistical power of vestibular response testing, if threshold shifts are very near the step size for stimulus intensity; and (d) better define the adequate stimulus for vestibular responses.

Accomplishments

Normal Development. Vestibular thresholds to linear acceleration were studied for ages ranging from E19 (embryos at 19 days of incubation) to 4 weeks post-hatch. Thresholds fall markedly during the first 10 days of this period and undergo much less change thereafter. Similarly, latencies shorten and amplitudes tend to increase as the vestibular system matures. These findings suggest that the developing gravity receptors systematically become more sensitive to acceleration stimuli as development proceeds on Earth. Moreover, the neural circuits transmitting vestibular impulses operate progressively faster and are better able to follow repetitive stimulation as they mature.

Effects of the microgravity environment. Vestibular responses were measured in chicks exposed late in development to 5 days of space flight (E9-E14, STS-29). Vestibular response latencies and amplitudes were the same in all groups studied. However, thresholds were significantly higher in animals incubated in space compared to synchronous, but not vivarium, controls. These mixed results were due to grossly abnormal responses found only in a fraction of the flight animals (38 percent).

In this fraction of flight animals, did the sensitivity of vestibular neurons fail to increase normally during maturation? The findings leave open the question of what caused the abnormal responses. We cannot rule out space flight effects; and, therefore, the results underscore the importance of doing additional space flight studies using bird embryos.

Effects of drug exposure. One advantage of using birds in developmental studies is that embryos may be studied without concerns about a potentially confounding maternal response to the experimental conditions. This is especially useful when testing drug effects on the developing embryo. Our experiments evaluated the effects of nicotine on embryonic vestibular development. The study revealed that despite our expectations of a regulatory response there was no change in receptor densities or in vestibular function as a result of 9 days of nicotine exposure. These results suggest that embryonic regulatory mechanisms serving nicotinic receptor populations may operate differently than their mature counterparts.

Can gravity receptors recover from serious injury? Until recently it was thought that destruction of gravity receptor hair cells of warm-blooded vertebrates would produce permanent functional loss. It is now apparent that vestibular hair cells may be capable of regeneration. It was not known, however, whether or not regenerated hair cells would actually work. To better understand the recuperative abilities of gravity receptors, we measured vestibular responses before and after producing severe drug-induced lesions. Streptomycin (an antibiotic that can destroy gravity receptor hair cells, 600 mg/kg/day) virtually eliminated vestibular responses over a period of 7 days in chicks. Within 2 weeks following drug treatment, thresholds recovered to normal levels. Response latencies and amplitudes returned to normal slowly within 70 days following cessation of drug treatment. These results clearly demonstrated that a virtually complete recovery of function can occur following severe hair cell destruction. These findings, and those of others, prompt new hope for the discovery of ways to restore hearing and balance senses in patients suffering from hair cell lesions.

What is the statistical power of vestibular tests when threshold changes are very near the step size of stimulus intensity? The vestibular response threshold is a valuable quantitative means of identifying changes in the sensitivity of gravity receptors. It is important to determine the resolving power of this vestibular test and, therefore, estimate the smallest threshold shift that can be detected using vestibular responses. Given a normally distributed sample, a sample size of 5, an acceptable statistical power of 0.8, a traditional α-level of 0.05 and the mean and variance of 27 randomly chosen control animals from our laboratory, the minimum detectable threshold shift would be 6.6 dB when 6 dB intensity steps are used. These theoretical exercises suggest that even with small samples we can expect to discern threshold changes having magnitudes near the test measurement step with some confidence. The smallest intensity step used currently is 3.0 dB.

Are embryonic responses really vestibular? To demonstrate that responses to pulsed linear acceleration were in fact vestibular in the embryo, new studies were completed. Bilateral destruction of the labyrinth eliminated the response, but bilateral destruction of the cochlea did not. These results serve as definitive evidence that responses cannot depend on the cochlea and thus are vestibular in the embryo.

Is the adequate stimulus for vestibular responses linear acceleration (g) or linear jerk (time derivative of acceleration dg/dt, g/msec)? A new stimulus paradigm was tested in detail. Results

show that response thresholds, latencies and amplitudes may be dependent upon jerk magnitudes (units of g/msec) and not simply acceleration amplitudes. These results provide new insight into the neural generators of the vestibular responses as well as the characteristics of natural stimuli for gravity sensors A detailed study of the effects of stimulus rise time on vestibular responses was carried out. The results provide support for the hypothesis that vestibular responses are elicited principally by linear jerk rather than linear acceleration.

Significance of Accomplishments

The results presented here suggest that the gravity receptors of developing birds are dynamic in that they exhibit a sharp increase in sensitivity in late embryos and early hatchlings. There may be as yet undetermined factors that can alter maturational changes. One such factor could be related to space flight. Ongoing studies are evaluating the influence of substrate vibration and hypergravity fields on receptor function. Gravity receptors in maturing chicks are resilient and are capable of complete recovery following severe injury. The discovery of mechanisms controlling recovery could lead to new clinical strategies for the deaf and dizzy patient. Vestibular responses to pulsed linear acceleration likely reflect a subset of gravity receptor neurons, in particular those signaling linear jerk. This knowledge further improves our understanding of vestibular responses and our ability to characterize the developing vestibular system. We continue to study changes in vestibular function during ontogeny using a new physiological test. It is critical that we clearly define the nature of the test and establish that it is in fact a vestibular test for all ages studied. The results summarized here add significantly to our understanding of the origins and nature of vestibular responses and, ultimately, to our understanding of vestibular ontogeny. Moreover, these studies provide important insights that may lead to the successful application of the new vestibular test in the diagnosis of the dizzy human patient.

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MOLECULAR BASIS OF TENSION DEVELOPMENT IN MUSCULAR ATROPHY

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Description of Research

The long-range goal of our research has been to assess the effects of immobility and skeletal muscle unloading on skeletal muscle function—in particular, the effects of exercise on skeletal muscle following prolonged atrophy. It is the aim of this project to describe the role calcium ions and calcium-mediated physiologic mechanisms play in conjunction with gravity during decreased weight bearing.

Our work has focused on the atrophy-associated cellular changes that might predispose skeletal muscle to injury during reuse and recovery. Preliminary studies in this laboratory have shown that exercising atrophied skeletal muscle following unloading produces myofibrillar damage and degeneration. A major finding was the appearance of type IIC, or transitional fibers, and extensive fiber damage coincident with training during exercise recovery from atrophy. The damage appeared in the form of necrotic fibers, central nuclei, and fiber debris in the intrafascicular and intrafibrillar spaces of the soleus.

Initial studies have sought to (1) characterize the occurrence and distribution patterns of control, transitional, and damaged fibers that occur in atrophied soleus muscle during the course of recovery, (2) determine the contribution that the cytoskeletal element titin makes in the production of skeletal muscle damage during the recovery of skeletal muscle from atrophy, and (3) determine the role that nuclear control domains play in skeletal muscle.

Accomplishments

(1) ***Titin isoform adaptation in atrophied skeletal muscle.*** Initial studies by this laboratory have quantitatively compared the location and relative amount of the cytoskeletal protein titin in single fibers of a known myosin type from control, atrophied soleus, and plantaris muscles. Limited data have also been obtained from tibialis anterior and gastrocnemius muscles exposed to 5.3 days of microgravity [STS-48, Physiological and Anatomical Rodent Experiment 1 (PARE 01)]. Our preliminary findings have been: (a) There are differences in titin electrophoretic mobility and antibody location between muscles, but not in the relative amount of titin associated with myosin type. (b) There is a decrease in the relative amount of titin, as well as changes in titin electrophoretic mobility and antibody location in atrophied skeletal muscle. (c) Atrophied single muscle fibers normalized for fiber size are significantly more fragile than control fibers at resting lengths.

Titin in Control and Atrophied Muscle. We examined titin antibody localization in single muscle fibers of adult rat soleus and plantaris using immunofluorescent microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The first fiber segment was used for demonstration of myosin type: either fast or slow as demonstrated by SDS-PAGE or typed with slow myosin heavy chain (MHC) monoclonal antibody (S58). Fiber segments used for gel electrophoresis were also analyzed for relative amounts of titin protein and titin electrophoretic mobility. The second fiber segment was processed for titin antibody staining with 9D10 (M. Greaser, University of Wisconsin) specific for titin under fluorescent microscopy. The distance of titin antibody localization to the Z-band and sarcomere spacing were determined. We found that fibers from control soleus muscle demonstrated a significantly different antibody

to Z-disc distance (Ab-Z) from control plantaris fibers (Table 1), irrespective of myosin type. This difference was maintained when the Ab-Z was normalized by sarcomere length. However, the relative amount of titin and myosin was not significantly different between muscles.

The ability of titin to adapt in response to altered muscle function has not been previously demonstrated. Therefore, a study was performed to examine the effects of 28 days of hindlimb suspension (HK) on titin (Table 1). Atrophied single fibers demonstrated a significant loss of relative titin weight between muscles and between groups within the same muscle.

Table 1. Pooled data from all fibers irrespective of myosin type (N = 207) ± SEM

Fiber	Soleus		Plantaris	
	Control	HK	Control	HK
N	34	37	60	76
Ab-Z (μ)	0.459±0.017	0.402±0.013*	0.406±0.009†	0.427±0.007*†
Ab-Z/SL	0.173±0.005	0.164±0.004	0.159±0.003†	0.178±0.003*†
Titin Wt.	0.040±0.003	0.029±0.003*	0.048±0.003	0.043±0.003
Myosin Wt.	0.160±0.011	0.116±0.015*	0.185±0.014	0.179±0.012

* = Significantly different from control, $p \leq 0.05$
† = Significantly different from soleus, $p \leq 0.05$
SL : Sarcomere length
Wt. : Relative weight of protein

Titin in Slow and Fast Single Fibers from Soleus and Plantaris. Additional fibers from control and atrophied muscles were analyzed for myosin type and Ab-Z distance in order to determine if a portion of the data variability could be accounted for by myosin type. We found that Ab-Z distance is characteristic of myosin type. However, while fast fibers in control and atrophied fibers are similar irrespective of muscle of origin, type I fibers are different between muscles. In atrophied type I fibers from soleus, titin Ab-Z distances decrease by 17 percent compared to control values, whereas distances increase by 8 percent in soleus type II fibers compared to controls. These Ab-Z distances are not significantly different from control values of the opposite type. These data would indicate that atrophy may drive an adaptation in titin isoform to accommodate an altered resting tension matched to fiber size and force-generating capacity. The dramatic decrease in the relative amount of titin in soleus fibers during hindlimb unloading may account for the selective degeneration of type I fibers during reloading of atrophied soleus.

Effect of short term microgravity on titin. Samples of muscle obtained from the STS-48 mission (PARE.01, Sept. 1991) demonstrated that removal of weight-bearing by weightlessness was sufficient to cause significant changes in titin Ab-Z distances and a significant loss of relative titin weight in the gastrocnemius. As both the tibialis anterior (99 percent type II) and the mixed gastrocnemius (93 percent type II) are "fast muscles", changes in titin were not anticipated in this time frame. It is possible that the titin molecule is very sensitive to gravitational forces.

(2) ***Effect of short-term microgravity on myonuclei.*** The purpose of this investigation was to examine the adaptation of myonuclei, and cytoplasm-to-myonucleus ratios, to short-term microgravity. The fast-twitch tibialis anterior (TA) and gastrocnemius (G) muscles from young SPF (S/D) female rats (N=8/group) were exposed to 5.3 days of microgravity (STS-48 mission, PARE.01, M. Tischler, PI). The flight group (S) was compared to ground-based controls (C) and hindlimb unloaded controls (HK). Single skeletal muscle fiber segments were dissected from TA and G muscle and stained with acridine orange (AO) to enable counts of myonuclei. Fibers were viewed with a Leica laser confocal scanning microscope, and fiber volume and myonuclear

number were calculated. Fibers were also analyzed with gradient SDS-PAGE for MHC isozymes and measured with laser densitometry. Mean ± SD cytoplasmic volume/myonucleus ratio was significantly decreased in the S groups (1.95±0.07 TA; 2.13±0.09 G) when compared to control (2.25±0.19 TA; 2.56±0.13 G) and HK (2.31±0.19T A; 2.34±0.15 G) groups, respectively. C and HK groups were not significantly different. The number of sarcomeres per myonucleus was also significantly less than both C and HK groups; while, myonuclei/mm in these group was significantly greater than C and HK groups. The increase in myonuclei and shift in fast to slow MHC prior to an increase in fiber volume is consistent with an early adaptation of fiber type. These results demonstrate that 5.3 days of microgravity resulted in (1) a significant decrease in single fiber volume; (2) a significant increase in myonuclear density when compared to either ground-based controls or HK groups; (3) an increase in myonuclear density coincided with an early shift in MHC expression; and (4) no change in the spatial arrangement of myonuclei.

Significance of the Accomplishments

Finding 1: Titin is the largest protein in skeletal muscle and forms a third filament system for the myofibril, which assists in sarcomeric alignment and elastic recoil. These data correlate with altered cell size and a shift in titin isoform expression in unloaded muscles and may reflect the ability of the muscle to modulate elasticity and compliance by the regulation of titin size isoforms. Given that titin "holds" the contractile machinery in alignment during contraction, its ability to adapt during microgravity and periods of atrophy could predispose skeletal muscle to damage during reuse during periods of recovery. Further investigations could determine the optimal time frame to conduct rehabilitation and training on patients recovering from bed rest and atrophy.

Finding 2: The decrease in the cytoplasm-to-nuclear ratio in atrophied muscle fibers could suggest that, when lower cytoplasmic levels are maintained, the availability of myonuclei are no longer a modulatory factor in controlling fiber size.

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ORIENTATION OF CELLS BY GRAVITY, ILLUMINATION, AND SHEAR: QUANTITATIVE RELATIONSHIPS

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Description of Research

Introduction. All life began with microorganisms, and they are still today the majority of living beings. Their existence depends on, and is organized by, gravity, light, their fluid habitat, and by interactions with one another. These influences are somehow integrated by the cells into an overall behavior pattern. In the case of swimming algal and bacterial cells, this complex and nonlinear integration of environmental factors is expressed as directional swimming. The probability that a given cell swims in a certain direction and at a certain speed is thus a function of these influences. It is one major overall objective of this research to measure the probability distribution of cell swimming velocities with particular attention to the role of gravity and the way in which this mechanical influence combines with fluid shear and light or oxygen, which affect cell metabolism. It is another overall objective to analyze how the individual cell's motile behavior combines into gravitationally mediated population dynamics.

Objectives/Goals.

(1) To develop quantitative computer-assisted microscopy for measuring the probability distribution of swimming velocities of microorganisms subjected to gravitational and other guiding influences.

(2) To develop apparatus and associated methods by which swimming cells can be exposed to fluid velocity gradients (shear) plus additional orienting influences, the resulting cell trajectories being measurable, as in objective (1) above. The additional influences are gravity, acceleration, molecular concentration gradients, illumination (variable incident direction, wavelength, intensity).

(3) To develop a rotating cuvette system for (a) supplying uniform shear, and (b) for use as an unbiased statistical sampler of motile populations.

(4) To develop quantitative methods for investigating bacterial bioconvection patterns and their significance for gravity-mediated microbial ecology.

Accomplishments

(1) ***A "Motion Analysis" computer system was purchased.*** This system processes in parallel many simultaneously acquired cell swimming trajectories. It calculates their direction and speed and provides sophisticated statistical analysis of the results. The original data can consist of hundreds of tracks, acquired during 1 or more seconds at frame rates up to 60^{-1}s. Typical statistical analysis takes a few minutes per run. This system is central to the research method. It provides sufficient statistics rapidly enough to guide experiments and to yield results. However, extensive programming and trouble shooting have been required. Because of its quantitative output, the system has delivered important cautionary information concerning systematic sampling errors, which has resulted in the development of Objective (3).(b) cited above. This motion analysis apparatus has been used to determine the gravity-oriented swimming

distribution function of *Pleurochrysis carterae* and other cells. It has been used to investigate the effect of shear on swimming cells. *P. carterae* is particularly relevant to gravitational biology because of the $CaCO_3$ coccoliths which it produces.

(2) ***Two operationally distinct shear-producing apparatuses were designed and built during the previous grant period.*** As of the date of this report, one of these has been tested with swimming algal cells and the motion analysis system. With it, shear and gravity can be parallel or orthogonal. The second apparatus allows only orthogonal gravity and vorticity vectors, but it is more flexible than the first with respect to the possible directions of illumination. It has been found that cells in the shear apparatus are unharmed by the moving components of the mechanism.

(3) ***A rotating cuvette (called "Clinovar") was constructed and tested.*** Since the cell sample in the cuvette is always returned to the field of view, rotation eliminates the disappearance of fast and accurately directed cells that swim toward one end of the measuring cuvette. Uniform rigid rotation also introduces a spatially constant vorticity equal to twice the rotation rate, and so provides shear. The amount of shear thus varies with rotation but not with location in the cuvette. When rotation by 180° is intermittent, it produces unbiased, bimodal sampling but no extra shear. Programming the motion analysis system for conversion between rotating and stationary reference frames was accomplished. It was then found that continuous rotation requires better bearings because of the mechanical jitter, which becomes apparent at high magnification. The theory of particle sedimentation within the rotating cuvette system was developed, and experiments were performed to show its accuracy, even when the rotation rate is varied.

One important result of this experimental/theoretical development is the very serious doubt that it casts on clinostat experiments: It was shown conclusively that free organelles within fluid compartments of cells move, and, presumably, tend to disrupt the cytoplasm.

(4) ***Gravity-mediated functional self-organization of bacterial cell populations.*** The swimming direction of individual algal and bacterial cells is partly random and partly guided by environmental factors. The environmental influences include gravity, chemical concentration gradients, and illumination. For algae, gravity is often the major influence that orients the swimming path of single cells. For the case of the bacteria studied during this research, *Bacillus subtilis*, we have so far found no evidence that gravity has any direct influence on the swimming of individual organisms. However, cells do swim in the direction of increasing oxygen concentration. In large bacterial populations (e.g., 10^9 cells/cm^3), gravity and oxygen consumption cause regular convective motion of large, spatially recognizable subgroups of the population (Figures 1 and 2). The entire dynamical system, which is powered by directional cell swimming, functions as an autonomous entity that supplies, transports, mixes, and consumes oxygen. This spontaneous development of macroscopic ordering of a vast population of independent undifferentiated organisms that do not interact directly with one another results from (1) their consumption of oxygen; (2) gravitational forces that place the air/fluid interface so that oxygen is supplied directionally, from above, thereby inducing upward swimming trajectories; (3) gravitational forces, once more which produce convection in anisotropic fluids; and (4) the laws of fluid mechanics, which provide dynamic long-range coherence.

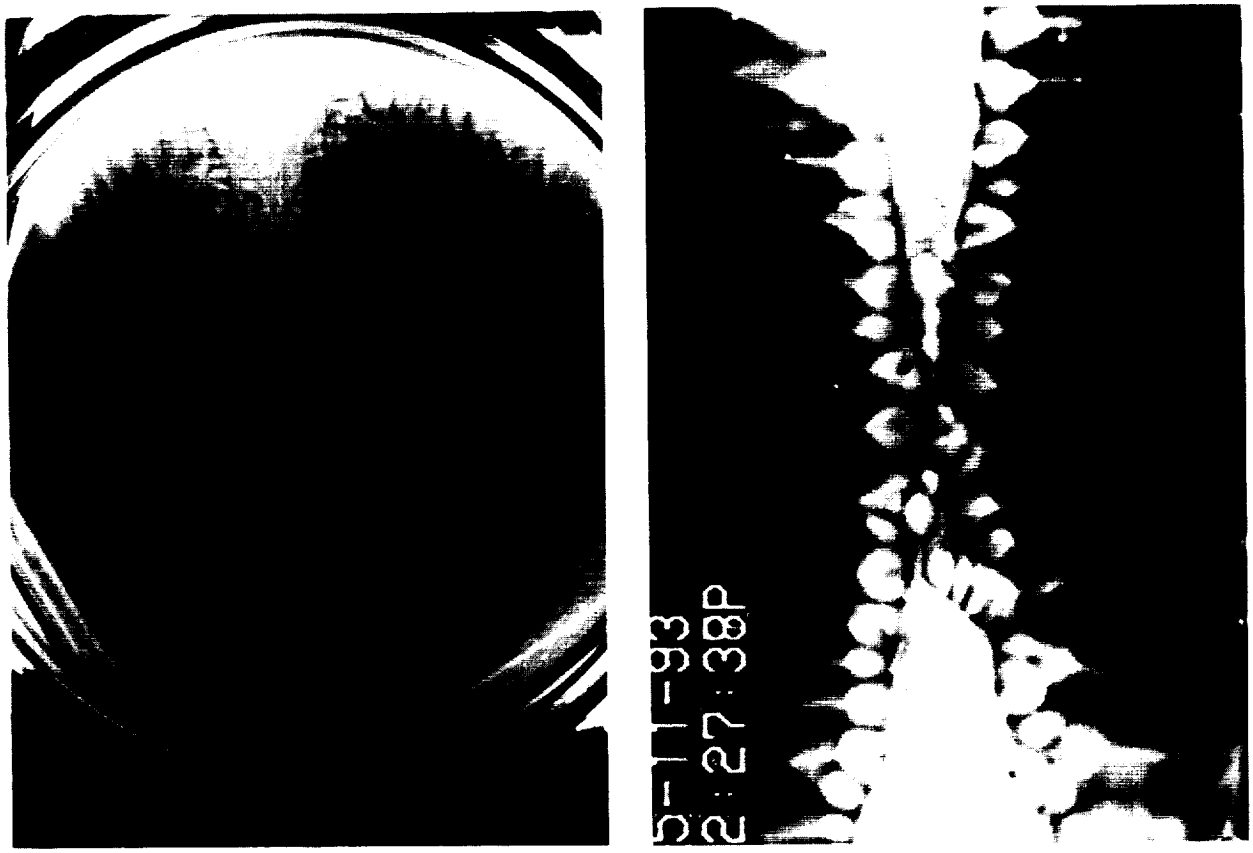

Figure 1 (a) Top view of bacterial convection patterns. A 2-mm deep fluid layer contains ~10^9 cells/cm^3 of *Bacillus subtilis*. The petri dish is 8 mm in diameter. Illumination is "dark field"; i.e., regions containing many cells are bright, and, conversely, bacterial respiration depletes oxygen. Diffusion from the air interface above the fluid replenishes the oxygen. Consumption and supply in the -g direction generate a gradient up, in which the cells swim. The upward accumulation of cells generates a dense upper layer of fluid (i.e., mean fluid density is proportional to local cell concentration). This situation is unstable. Convection rolls, and columns appear and supply oxygen to those lower regions of bacterial population which are otherwise beyond the reach of a sufficiently rapid diffusive supply from the surface. The cell-swimming-driven convection, of course, also mixes both the oxygen and the bacterial cells. The entire living fluid mechanical system functions concurrently as a transporter and consumer of oxygen. It should be remembered that these remarkable dynamical structures are shaped by consumption of oxygen, directed by oxygen gradients, swimming, and the force of gravity. There is no direct communication among the cells. Figure 1(b). The central portion of Figure 1(a), slightly later, enlarged and copied from a time-lapse video film. The entire structure is in slow aperiodic motion.

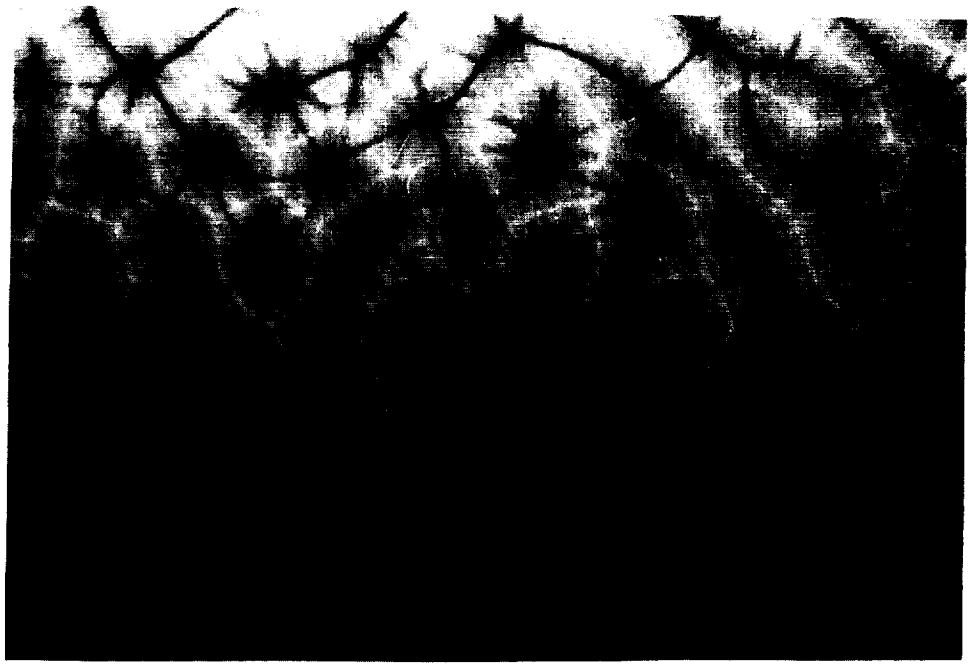

Figure 2. General description is the same as for Figure 1, except that the fluid layer is slightly shallower and data were acquired with a different cell culture.

Gravity is basic for the generation and stable development of this macroscopic ordering, which functions to improve survival by augmenting diffusive transport of a dissolved metabolite with convective supply and mixing. The ordering also creates diverse microenvironments, characterized by various levels of oxygen concentration and various cell residence times.

The development of life forms clearly proceeded through stages, starting from molecular mechanisms. Very little is known about the coalescence of individual microorganisms into associated complex specialized groups and structures. The observations and insights gained during the grant period suggest that one route for the development of multicellular organisms, from an original sea of simple cells, may well have included the sort of catalysis by gravitational forces and symmetries that is modeled by these experiments.

Significance of the Accomplishments

The integration by motile cells of environmental influences is a totally unsolved problem which forms the core of gravitational biology/physiology for free cells. Cells which are normally part of large organisms may obey similar laws. Some aspects of the larger biota which comprise many cells are analogous to the patterns into which dense populations of survivors organize themselves. This research quantitatively investigates problems of gravitational biology that relate to individual and collective behavior. Accurate, statistically significant methods of measuring complex individual cell behaviors are being developed. Results that concern single cells can then be confidently integrated into theories of population structure. Applications will include insights into the dynamics of large populations of microorganisms. For macroapplications, there will be new information concerning individual and group behavior of phytoplankton. On a smaller scale, this research presents the growth and self-maintenance of concentrated populations of bacteria in an entirely new light. Areas of application include bacterial invasions of small liquid pools as in air conditioners, sick room humidifiers, and in enriched open environments, such as waste disposal sites. The central role of gravity in the generation of bioconvection also implies that the dynamics of waste recycling systems may differ markedly, relative to expectations, when they are placed in a microgravity situation.

Experimental Observations and Implication

Swimming cells of *Pleurochrysis carterae*, an alga that is a prime example for biomineralization ($CaCO_3$), are gravitactic. During the first 4 days after inoculating into new culture medium, the population is bimodally gravitactic: one population segment swims up, the other down. After 7 days or more, the entire population swims upward. There is no significant change in the swimming speeds.

These experiments suggest the new research area of Motility-Based Taxonomy. Are the observed bimodality and its disappearance associated with explicitly observable morphological changes? Are such hypothetical changes involved with passive versus active gravity sensing?

Gravity-Mediated Biological Self-Organization

Bacterial concentration/convection patterns that are organized by the swimming population of *Bacillus subtilis* resident in dense cultures were discovered by the PI and associates. These dynamic patterns transport and mix oxygen. They are driven by the force of gravity and by swimming asymmetry deriving from oxygen consumption by cells. To understand these systems deeply, one requires the velocity probability distribution function of the bacteria swimming in an oxygen gradient. The first usable measurements of this oxygen taxis of *B. subtilis* were performed during this grant period using the computerized microscope system.

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COLLAGEN SYNTHESIS, ASSEMBLY, AND MINERALIZATION IN CHICKEN OSTEOBLAST CELL CULTURES AND OTHER CALCIFYING VERTEBRATE SYSTEMS

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Description of Research

The research of this laboratory concerns describing the basic molecular biology and biochemistry, morphology, and ultrastructure of a bone cell culture system to be utilized for future flight experiments under zero gravity conditions. Results are correlated with investigation *in vivo* of other calcifying vertebrate systems including bone, cartilage, and tendon. The bone culture system is currently characterized under normal gravity. Cultures are derived from osteoblasts of chicken calvaria, and the growth and development of the bone cells and the onset and progression of mineralization in the culture extracellular matrix are studied. We are interested in defining (1) the mechanisms of collagen gene expression, (2) the temporal relationship between collagen cross-links and collagen ultrastructure, (3) the assembly of collagen fibrils into orthogonal arrays, (4) the precise interaction between collagen fibrils and mineral deposition in the cultures, and (5) the effects of non-collagenous proteins on mineralization in the system. Data from the culture system are compared with results obtained with bone, cartilage, and tendon particularly with respect to collagen-mineral interaction deduced from electron microscopy and novel three-dimensional image reconstruction techniques.

Our most recent previous report had shown that the mineral from our bone cell cultures is similar in its size, shape, surface texture, crystallographic properties, and relation with collagen as that determined from bone tissue *in vivo*; and that strontium may be used as a reliable and critical marker for the determination of mineral accumulation associated with collagen in the cultures. In addition, the interaction of collagen and mineral crystals in both tissue *in vivo* and the bone culture system had been examined in initial studies utilizing high-voltage electron microscopy and three-dimensional computer graphic image reconstruction techniques. Further, we had developed a cell culture system of chondrocytes as an extrapolation of our work with bone cells, and we had documented that this culture mineralizes as does its counterpart, normal growth plate cartilage. The chondrocyte system then would be useful as a model describing growth plate developmental events.

Accomplishments

The studies we have undertaken in the previous year and a half have continued with further characterization of our bone culture system in terms of extracellular matrix formation and regulation and with the elaboration of the interaction between collagen and mineral during calcification of model vertebrate systems *in vivo*. We have also initiated experiments to determine the atomic surface structural features of mineral species important in biological calcification. A summary of the respective results follows:

(1) We have studied the pre- and post-translational mechanisms controlling extracellular matrix formation in our cultured chicken embryonic bone cells. Gene expression of specific proteins of bone (type I collagen, osteopontin, and osteocalcin) was measured in one series of experiments, and collagen cross-link analysis was correlated with microscopic structure in another series of investigations. The results of the work showed two aspects of the regulation of

extracellular matrix formation by one cell. The first, that osteoblast gene response to 1,25 $(OH)_2D_3$ appears to be dependent on the embryological or developmental stage of the osteoblast; and the second, that extracellular matrix assembly is post-translationally controlled, mediated by polarized secretion of collagen and by collagen cross-link stabilization.

(2) We have determined a number of structural features and events related to the development and mineralization of our bone cell cultures, as well as a model calcifying vertebrate tissues. The work has been accomplished through the novel use of high-voltage electron microscopy and three-dimensional computer graphic imaging. We have found in the osteoblast cultures, in the normally calcifying leg tendons of turkeys, and in the long bones of chicks that the respective extracellular matrices are similarly composed of collagen fibrils strictly organized and assembled so that their hole and overlap zones are in contiguous registration. This assemblage creates channels for mineral accommodation and deposition at independent sites throughout the collagen arrays. Crystal nucleation may occur along the surface and within the channels of the fibrils, and a fusion process appears to be responsible for the formation of larger crystals from smaller ones. Ultimately, the growth and development of crystals in association with collagen forms parallel sheets of mineral in the extracellular matrices. These results are important for elaborating the basic means by which mineralization occurs among vertebrate tissues.

(3) We have studied a 66-kDa phosphoprotein which comprises a major non-collagenous protein in our cultured chicken embryo osteoblasts. We have characterized its expression and tissue distribution during osteoblast differentiation and culture development. A cDNA clone for the protein was isolated from an expression library made from embryonic chicken bone mRNA and a complete primary protein sequence of 264 amino acids was determined. Sequence comparison, hybridization analysis, and gene product induction by phorbol ester suggest that the 66 kDa phosphoprotein is the avian homologue to the mammalian protein, osteopontin. The regulation of avian osteopontin appears to be under transcriptional control during osteoblast development.

(4) As a new approach to characterizing the mineral deposition in vertebrate tissues, surface structures of hydroxyapatite and brushite, two crystals important in mineralization, were determined for the first time by atomic force microscopy. A method of suspending the minerals as powders in ethanol was used to isolate small crystal clusters on glass. Measured atomic spacings corresponded to the (001) and (110) planes for hydroxyapatite and the (110) crystal plane for brushite. These initial surface structural data are important for further work defining precise interaction between crystals and organic matrix components such as collagen, fundamental in the calcification of vertebrate tissues.

Significance of the Accomplishments

The current results are significant in describing a number of physical (structural), chemical, and biological events occurring during the mineralization of a bone cell culture system which very closely resembles the mineral deposition of normal calcified vertebrate tissues, some of which have been independently studied in correlative investigations. Our data provide details of calcification which are new in some instances and which cannot be obtained with other methods or model systems. Our most recent results (1) demonstrate differences in hormonal response to 1,25 $(OH)_2D_3$ by embryonic osteoblasts isolated at different developmental ages; (2) indicate that extracellular matrix assembly is controlled at a post-translational level through polarized secretion of collagen and collagen cross-linking; (3) provide fundamental details of the ultrastructural interaction between mineral and collagen: (4) isolate and characterize the first non-mammalian form of osteopontin; and (5) report the first atomic surface structural features for vertebrate mineral crystals. These data help expand information which is fundamentally important in understanding bone cell growth and development, extracellular matrix organization and assembly, and mineralization. They provide the comparison for data obtained from experiments in other systems.

including future bone cell cultures flown in zero gravity. From such results, the basis of space flight skeletal mass loss and related effects may be more readily understood.

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GRAVITATIONALLY SENSITIVE MOLECULAR REARRANGEMENTS INVOLVED IN ESTABLISHING EARLY EMBRYONIC AXES IN AMPHIBIANS

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Description of Research

Shortly after fertilization, amphibian embryos rotate so that the presumptive posterior, or vegetal portion, is down relative to gravity and the presumptive anterior, or animal portion, is up relative to gravity. The orientation of the anterior-posterior axis is established during oogenesis and is reflected by a gradient distribution of yolk platelets. The dorsal-ventral axis of amphibians is normally determined by the sperm entry position (SEP); the dorsal axis forms on the side of the embryo opposite the SEP and the ventral side of the embryo normally forms on the same side.

However, tipping the animal-vegetal axis obliquely relative to the gravitational force vector results in repositioning the dorsal-ventral axis so that the dorsal side forms on the gravitationally "up" side of the embryo, irrespective of where the sperm enters. These results suggest that some cytoplasmic components are becoming rearranged within the single-cell embryo such that information leading to the formation of the dorsal axial structures become localized, or activated, on only one side of the embryo. This hypothesis is further corroborated by centrifugation studies showing that the dorsal axial structures will form on the centripetal side of the embryo (Black and Gerhart, 1985).

Our long-term goal is to identify the cytoplasmic components involved in dorsal axial formation and those which become rearranged during the first cell cycle. The second portion of this study is to understand the mechanisms responsible for the rearrangement and to understand better how gravitational forces influence these mechanisms.

Accomplishments

We have addressed two issues relative to dorsal-ventral axis formation, both as it forms normally here on Earth and the role gravity plays in establishing axial information. All experiments addressed here were performed on embryos of *Xenopus laevis*. The first series of experiments addressed the issue of when the presumptive dorsal animal and presumptive ventral animal cells acquire, or are able to maintain, different identities. Using a monoclonal antibody directed against non-neural epithelium (Epi 1), we were able to show that isolated and cultured dorsal animal blastomeres from the eight-cell stage will not express the Epi-1 antigen, whereas the cultured ventral animal blastomeres from the eight-celled stage will express the Epi-1 antigen at the correct developmental time (London, Akers, and Phillips, 1988). These results suggest that dorsal and ventral blastomeres have received significantly different instructions by at least the third cleavage. We went on to show that the portion of the embryo which inherits the "determinants" for establishing dorsal axial structures is responsible for sending molecular signals which modify and control the final pattern of Epi-1 expression (Savage and Phillips, 1989). We have subsequently shown that the signal(s) responsible for controlling spatial patterns of gene expression travel(s) through the plane of the ectoderm (Savage and Phillips, 1989) and that they affect the spatial patterns of expression for several other neural specific molecules as well (Doniach, Phillips, and Gerhart, 1992).

These data suggested that determinants for several developmental events relating to eventual axis formation might become localized during the first cell cycle following fertilization. We also have preliminary evidence that the localization of many such determinants is gravitationally sensitive movement. Therefore, we have constructed and screened cDNA libraries of maternal RNA to isolate clones for spatially localized RNA. The first dorsally enriched maternal RNA isolated from these libraries coded for the small ribosomal subunit of mitochondria (clone 49). *In situ* hybridization probes were prepared from this clone and we have shown that mitochondria become heavily enriched on the presumptive dorsal side of the embryo during the first cleavage cycle (Yost, Phillips, and Danilchik, 1993). We have also demonstrated that the mechanism for the localization of mitochondria is gravitationally sensitive. Embryos, whose animal-vegetal axis is obliquely oriented relative to the gravitational vector, will co-localize both the enriched concentration of mitochondria and the position of the dorsal axis (Phillips and Danilchik, 1993).

The polymerase chain reaction (PCR) display method for comparing various related populations of RNA has been applied to the problem of isolating clones for localized RNAs in the early animal blastomeres. In this technique, either dorsal or ventral animal blastomeres are isolated at the end of the eight-cell stage. RNA was extracted either immediately or after the blastomeres were cultured to the equivalent of early gastrula stages. A series of three prime primers were used in the PCR reaction with each type of RNA. The PCR reactants are separated on a sequencing gel, and bands of interest are cut from the gel, reamplified, and cloned into a suitable vector (Figure 1). We have identified a number of RNAs which become localized to either the dorsal or ventral animal blastomeres during the first cell cycle, or isolated clones for RNAs are regionally synthesized in response to some localized components. To date, we have isolated eight clones for ventral maternal RNAs and four clones for dorsal maternal RNAs (Table 1). In addition, we have begun to isolate clones for regionally expressed RNAs and have obtained clones for four ventral and five dorsal zygotic RNAs. Of particular interest is the number of clones which appear to be localized to the ventral animal blastomeres. This finding suggests that ventral cells may be more highly specialized than previously believed. These clones are now being sequenced and used to prepare probes for *in situ* hybridization analysis. Preliminary data suggest that there are several possible mechanisms of localization. Clones representing localized RNA are being used to study a number of issues relating to both the mechanisms of localization and the effects of the localized RNA on the development of the dorsal axial structures.

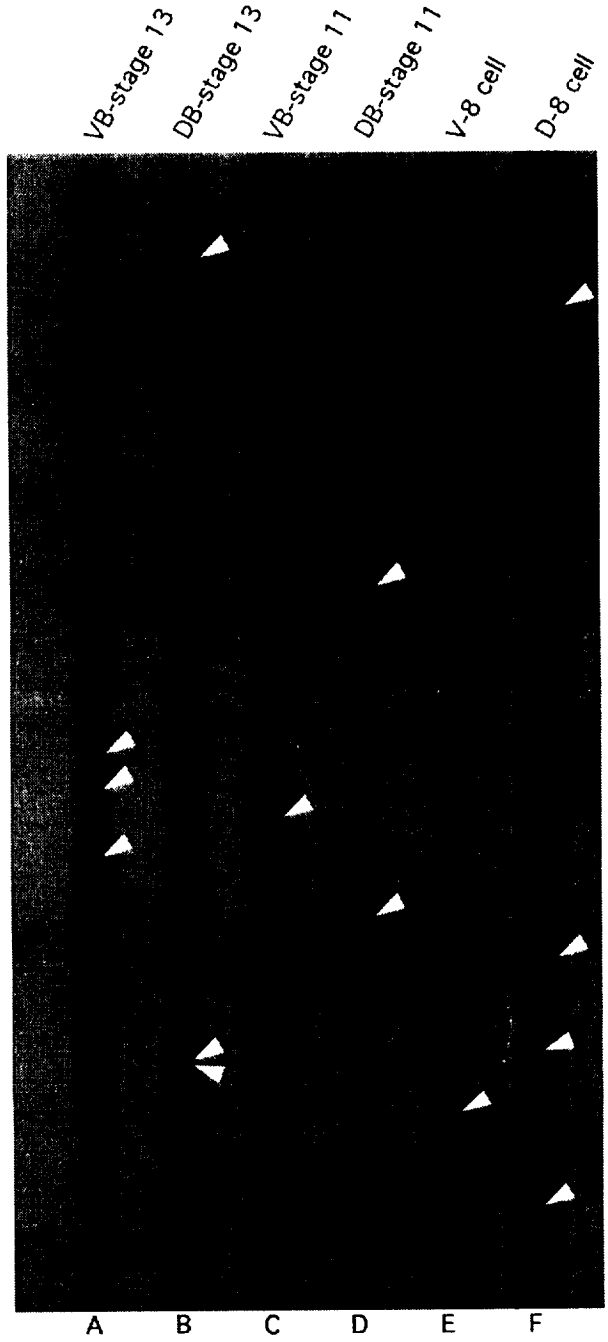

Figure 1. Dorsal and ventral animal blastomeres were removed at the eight-cell stage. Lanes E and F represent PCR products from RNA isolated immediately after dissection. The arrows mark the bands which were cloned from this section of the gel. Lanes C and D show the PCR products when RNA was extracted from cells cultured to the equivalent of stage 11 (mid-gastrula). Arrows mark the bands which have been cloned. Lanes A and B indicate the PCR products from cells incubated to the equivalent of stage 13 (early neurula).

Ventral Maternal	Ventral Zygotic	Dorsal Maternal	Dorsal Zygotic
vmcc11	vzga5	dmga1	dzga3
vmga3	vzca1	dmca5	dzgg2
vmga4	vzcg1	dmcg1	dzca2
vmcg100	vzgc1	dmgc6	dzcg1
vmcg101			dzgc2
vmcg1			
vmca1			
vmcg1			

Table 1. PCR products of RNA isolated from different portions of the early *Xenopus* embryos were displayed on a sequencing gel (Figure 1). The bands of interest were excised from the gel, reamplified and cloned into a Bluescript vector. Each clone was used to prepare probes for a Northern analysis against RNA isolated from the dorsal and ventral regions of the embryo. The clones listed above represent the selected clones which met all the criteria for representing localized transcripts. The nomenclature indicates the three prime primers used to obtain the clones. Each three prime primer consists of 11 Ts and two bases (ga, ca, cg, etc.) listed within the clone designation. The clones are separated relative to the time of their synthesis, either as stored maternal RNA or newly synthesized zygotic transcripts.

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EVOLUTION OF GRAVITY RECEPTORS IN THE EAR

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Description of Research

The vestibular system of the inner ear includes mechanosensory cells that respond to external signals such as position and sound. Such cells in amniotes (reptiles, birds, mammals), have been divided into two types; type I and type II. These differ in terms of ultrastructure, innervation, and (presumably) physiology. The locations of type I and type II cells differ from one another in each of the otolithic end organs of the ear. It has been suggested that the two types of hair cells have functional differences.

Although type II hair cells are considered to be ubiquitous among all vertebrates, it has been argued that type I hair cells are found only in amniotes and not in anamniotes (jawless fish, cartilaginous fish, bony fish, amphibians). Our recent work, however, shows that sensory hair cells very much like the type I hair cell are present in the otolithic end organs of the ears of a fish. These findings suggest to us that there is a complex organization to the otolithic receptors of the fish ear and that this organization may have significant similarities to the organization of mammalian otolithic end organs. Thus, it may be possible to use the otolithic end organs of the ears of fishes to gain better insight into the sensory cells of mammals and other amniotes.

The thrust of our research is to investigate the types of sensory hair cells in the ears of anamniotes and to test the hypothesis that multiple hair cell types evolved very early in the origin of the octavolateralis system, rather than at the time of the evolution of amniotes. The work also involves determining the structural diversity of hair cells in various anamniotes, as well as determining whether different otolithic end organs vary in their hair cell composition.

Accomplishments

(1) ***We have used ultrastructural techniques to analyze the structure of sensory hair cells in the saccular end organ of the goldfish.*** We found that two types of hair cells are found in the teleost species *Carassius auratus* at very distinct locations on the sensory epithelium. These results correlate closely with physiological studies of hair cells done in other laboratories and provide a potential correlation between hair cell structure and physiological properties. Moreover, this finding suggests that the different hair cell types are potentially involved with different responses of the ear. While we do not yet fully understand the function of the saccule in the goldfish, there is evidence that it is an auditory receptor and that the regions of the saccule containing different types of hair cells have different frequency response characteristics. Thus, it is possible that the ultrastructurally and physiologically distinct hair cells of the saccule may have evolved to respond to different frequency characteristics of sound stimulation.

(2) ***Multiple Hair Cell Types in the Lateral Line.*** The lateral line is located on the body surface of fish and amphibians and provides for detection of hydrodynamic signals impinging upon an animal. The lateral line system contains sensory hair cells that are very similar to those found in the ear of fish. Moreover, there is reason to believe that the ear and lateral line share some common ancestor structure in the early evolution of vertebrates. Thus, if the lateral line has two hair cell types, as does the ear, the implication is that the presence of multiple hair cell types and multiplicity of function arose far earlier in the evolution of vertebrates than even indicated by their presence in the ears of fishes.

In order to test this hypothesis, we have started to examine the lateral line system of the oscar, *Astronotus ocellatus*. In the first part of this study, we treated the lateral line using gentamicin sulphate, an ototoxic drug that destroys type I-like hair cells but that does not appear to damage type II hair cells. Fish were treated with very low doses of gentamicin and the hair cells of the lateral line were examined from 1 to 12 days later using scanning electron microscopy (SEM). Our results demonstrated that the hair cells found in neuromasts of lateral line canal organs were totally destroyed within 1 day of treatment, while the hair cells in free neuromasts were undamaged after 12 days of treatment. The results strongly imply that there are differences in the hair cells of the canal and free neuromast parts of the lateral line.

Significance of the Accomplishments

Finding 1: The results with goldfish, when combined with our earlier investigations of the oscar (*Astronotus ocellatus*) demonstrate the possible ubiquitousness of multiple hair cell types in fish. These two species are taxonomically very distinct from one another. Consequently, the finding that both species have basically similar multiple hair cell types leads us to hypothesize that multiple hair cell types may be an important component of otolithic end organs in most fishes.

Finding 2: Our discovery of there possibly being multiple hair cell types in the lateral line is the first demonstration of differences in hair cells of this end organ. Our hypothesis is that the gentamicin-sensitive cells of the canal neuromasts are type-I-like and those of the free neuromasts are type II. If this proves to be the case, it would mean that the two hair cell types evolved very early in the origin of vertebrates. Such results would also have profound implications on questions pertaining to lateral line function, since it would mean that different lateral line regions have evolved to respond to signals of a very different nature.

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EFFECTS OF VECTOR-AVERAGED GRAVITY ON SINGLE CHANNEL PROPERTIES OF THE ACETYLCHOLINE RECEPTOR IN CULTURED MYOCYTES

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Description of Research

The objective for the investigation reported herein was to measure single acetylcholine receptor (AChR) channel amplitudes and kinetics after clinostat rotation at different rotation paradigms by means of the cell-attached patch clamp technique.

Our laboratory has previously reported that co-cultures of nerve and muscle cells from *Xenopus* embryos show specific sensitivity to altered gravity conditions as generated by rotation in a slow-rotating clinostat. The alterations in muscle cells included changes in cell shape and nucleus size, as well as a reduced accumulation of AChRs at the junction between the nerve and the muscle cell. This led us to test if clinostat rotation also alters the properties of single acetylcholine channel molecules. During a recent space flight experiment, we were able to verify qualitatively the reduced incidence of AChR aggregates. In addition, we found marked changes in the distribution and organization of actin filaments in space-flown myocytes, which were in accordance with clinostat experiments performed in parallel.

Accomplishments

AChR single-channel activity is expressed as sequential opening and closing events which yield information on how much current is passed through the ionic pathway and the kinetics of channel transitions between the closed and open state. We found that the conductance and channel mean open time were not statistically different from controls after clinostat rotation. However, the values for junctional AChR channel open time showed a consistent trend toward large values. This observation may suggest an event of cellular adaptation to clinostat rotation.

Significance of Accomplishments

Our results show that excitable cells are sensitive to alterations in the perceived gravity field. The function of AChR channels, however, is not drastically affected, possibly due to a process of cellular adaptation. Taken together, these results indicate that the development of the neuromuscular junction may be affected during exposure to microgravity. This conjecture needs further investigation because of the little knowledge and small data base presently available regarding the effects of microgravity on the function and development of excitable tissues at a cellular level.

STRUCTURE AND FUNCTION OF MAMMALIAN GRAVITY RECEPTORS

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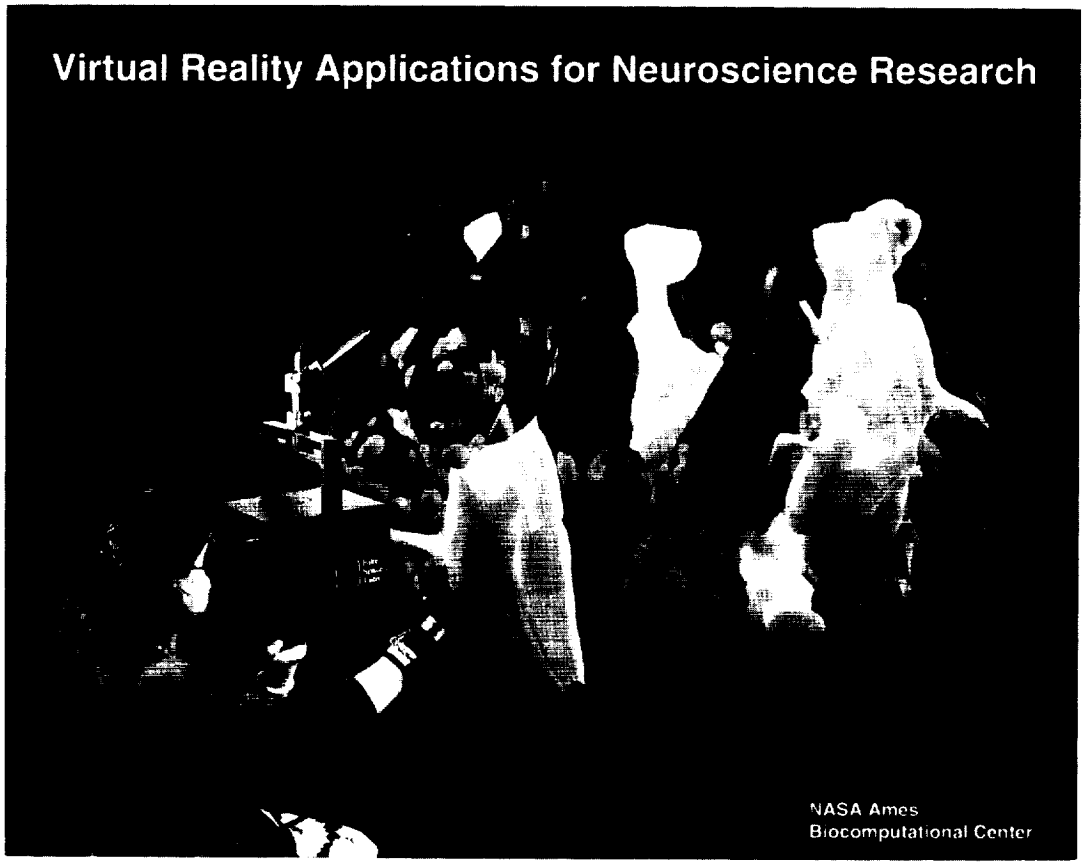
Description of Research

The long-term goal of this research is to understand how information is processed in gravity receptors on Earth and in space, with special reference to understanding adaptive responses to altered gravitational environments. To help achieve this goal, my laboratory carries out ultrastructural studies of gravity sensor organization in ground control, space flown, and centrifuged rats. Since the basic organization of all mammalian gravity sensors is similar, the rat can serve as a model species. The ultrastructural research is carried over to computer reconstruction work so that, eventually, nerve terminal/receptive fields of maculas from both ground control and space flown rats can be compared for possible differences in size and number of neuronal processes. For the past year, effort has concentrated on a study of synapses in utricular maculas of ground control and flight rats of the SLS-1 mission, flown in 1991.



Accomplishments

The accomplishments continue to fall naturally under two headings: Results and Theoretical Interpretations.

Results.

It is necessary to introduce this section by considering a theoretical interpretation of macular organization that will be taken up again in the following section. This concept is that vestibular maculas consist of two main circuits, highly channeled and distributed modifying. Type I cells feed into the highly channeled circuit while type II cells are part of the distributed modifying circuit. On the basis of this organization, it was predicted that type II cells of the distributed modifying circuit would exhibit more synaptic plasticity in an altered gravitational environment than would type I cells of the highly channeled circuit.

The primary findings of the space flight experiment, analyzed over the past year, are: (1) the adult mammalian gravity sensor retains the potential for neural plasticity; (2) synaptic changes are greater in the distributed modifying circuit than in the highly channeled circuit; (3) microgravity reveals synaptic plasticity in type I cells not observed otherwise; (4) most of the new synapses are located at new sites; and (5) stress may also cause changes in synaptic number.

These results were obtained by statistical analysis of more than 6,800 synapses in more than 1,000 hair cells. Synapses were counted in blocks of 50 section series obtained from the right utricular maculas of 3 rats euthanized on launch day (LD), 3 ground control and 3 flight rats euthanized on the day of Shuttle landing (R + 0), and 3 ground control and 3 flight rats euthanized 9 days after landing (R + 9). Nine days are allowed for a recovery period (equivalent to mission length). All animals were shared and, therefore, were subjected to handling and manipulations unrelated to the vestibular experiment. This caused variabilities in responses that were interesting but not necessarily related to flight, as will be discussed briefly below.

LD rats exhibited a slight but statistically insignificant rise in the mean number of synapses in type II hair cells. This was attributed to the modest stress of injections, blood withdrawals, and weighings conducted before Shuttle launch. R + 0 rats experienced 9 days free of experimental handling, with the chief difference between flight and control rats being the exposure to space flight conditions, including Shuttle ascent and descent. At R + 0, synapses were increased by ~55 percent in type II cells and by ~41percent in type I cells in maculas of flight rats. There was a shift in the kind of ribbon synapse from the rod-like to the sphere-like form in both types of hair cells. Additionally, pairs of synapses nearly doubled and there was a twelve-fold increase in groups of 3 to 6 synapses in type II cells. All these changes were statistically significant at the $p \geq 0.0001$ level. Further analysis of the number of synapses distributed as groups in type II cells of flight rats indicated that only a small fraction (9–12 percent) of them could be accounted for in this way. Thus, most of the new synapses were located at new sites. The findings collectively indicate that space flight induced a re-weighing of synapses in hair cells of utricular gravity sensors, although the effects of a possible initiation of readaptation to Earth's 1-g cannot be discounted at this time.

Surprisingly, at R + 9, synapse counts in type I and type II hair cells were scarcely diminished from those at R + 0. Additionally, counts in type II cells of controls rose to nearly match those of the flown rats, although counts in type I cells remained essentially unchanged. These findings are interpreted to indicate that the stress of the many manipulations conducted postflight (injections, blood withdrawals, weighings) resulted in the synaptic changes observed. While it is of interest to note that stress may affect synaptic counts in vestibular maculas (another sign of neural plasticity in an adult mammal), the findings negate any conclusions regarding macular readaptation to Earth's 1-g following space flight.

The questions raised by the present results are: (1) What is the influence of stress on macular synapses? (2) Are rats already readapting to Earth's 1-g by the time they are euthanized postflight? These important questions should be answered totally or in part following the flight of SLS-2 when rat tissue will be collected in space as well as postflight and when no procedures will be carried out on the rats to be used for the vestibular study.

Theoretical Interpretations.

The findings continue to support the theory that vestibular maculas are organized similarly to other regions of the nervous system such as the retina, olfactory bulb, and cortex. Our most recent publications have stressed the point that the vestibular maculas seem to represent a prototypic neural organization. That is, the maculas have a simple, highly channeled/distributed modifying circuitry that becomes more complex in the retina and other parts of the brain as more layers are added. In effect, there is much to learn about more complex neural tissue organization and functioning by research on vestibular maculas. In this effort, space and centrifugation studies will continue to play important roles.

Significance of the Accomplishments

Finding 1: Vestibular macular organization is now shown to be comparable to the fundamental architecture of other more complex neural tissue, such as the retina, olfactory bulb, and neocortex. This means that maculas are worthy of study as more simple examples of neural parallel processors of information. The results of my space experiment are the first results to demonstrate that neural plasticity is a property of the adult mammalian peripheral macula. So far as I am aware, they are also the first examples of increased neural plasticity in the distributed modifying circuitry compared to that in the highly channeled circuit. This finding may later find applications in the understanding of memory and learning, particularly in more complex neural systems. This is because the distributed modifying circuits, also referred to as "local" or "intrinsic," are coming to the forefront in neuroscientific research because they help to shape the output of the system. Finally, according to a recent Deafness Research Foundation Report, it is estimated that more than 90 million Americans over the age of 17 experience some form of dizziness or balance disorders. Problems with balance are responsible for more than one-half of the accidental deaths in the elderly (because of falling). Additionally, it is estimated that, in the United States alone, more than 500,000 individuals suffer from Ménière's disease, a disorder of the inner ear that affects the balance organs as well as hearing. The number of children experiencing some form of motion sickness or inner ear distress that impairs travel and physical activity is doubtless large but was not included in these estimates. The results of my space research could eventually help explain some of the causality of these serious afflictions, especially if further research should indicate that stress and changes in mobility are factors influencing synaptic efficacy in the peripheral organs of balance.

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OSTEOBLASTIC RESPONSE IN TRANSGENIC MICE

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Description of Research

Although it is intuitive that the skeleton has evolved mechanisms to sense changes in mechanical stress and to transduce these physical signals into a biological response that alters the structural properties of bone, this complex process has evaded analysis at the molecular level. The effect of mechanical strain on bone has been resistant to molecular analysis because it can be assessed only in intact animals. This grant will develop an *in vivo* model to delineate molecular mechanism for the transduction of mechanical signals in bone to a transcriptional event in osteoblasts. It will explore the feasibility of using transgenic mice harboring DNA constructs that reflect the activity of the α1(I) collagen (COL1A1) promoter in bone as a method to probe molecular mechanisms that alter type I collagen production in response to varying degrees of mechanical stress. The rationale is based on the increased realization that molecular techniques of promoter analysis in a setting of a highly differentiated cell, such as an osteoblast under mechanical stress, is not accurately mirrored by cells cultured from intact tissue. Instead, the molecular tools that have been used in cultured cells must be modified to approach questions that can be asked only with respect to the intact animal.

In our first series of experiments, we wanted to demonstrate that osteoblasts carrying the transgene would reflect increased activity in an animal experiencing increased weight. Because the models for bone loading in normal mice required development in our laboratory, we first turned our attention to murine model of the human disease, osteogenesis imperfecta mouse (OIM). The OIM produces a weakened bone matrix that does not provide adequate resistance to mechanical loading. In this context, the OIM would exemplify chronic weighting of bone. The collagen chloramphenicol acetyl transferase (CAT) transgene was bred into the OIM. Much to our surprise, mice of 8 days of age did not show increased CAT activity relative to normal wild type litter mates. At one month of age, the same observation persisted. However, by 3 months of age when the rapid phase of somatic growth had ceased, CAT activity was 2-3 times higher in both calvarial and humeral bone extracts relative to the normal mice. The relative increase in CAT activity was paralleled by the content of COL1A1 mRNA extracted from the tissue. This work was reported in a plenary session at the Space Biology meetings this year and is being assembled for publication.

Accomplishments and Significance of the Accomplishments

The analysis of the OIM made a number of useful points. First, as predicted by Frost, factors that control bone formation during rapid growth are probably different from those which control bone remodeling due to mechanical loading. I believe that the osteoblasts are maximally stimulated during the rapid phase of somatic growth and that further activity due to mechanical loading is obscured. Because of these studies, all our subsequent work examining the effect of mechanical loading will be carried out in mice that are over 3 months of age.

Analysis of bone taken from normal transgenic animals loaded by sciatic or patellar tendon scission failed to show a significant difference in CAT activity between the weighted and unweighted side, even after the animals were examined two weeks after the manipulation. Reports by others have shown that in rats it is the cortical bone cells which respond to mechanical loading. Based on that observation, we have started to examine enzymatic digest of bone for CAT activity. This has

shown dramatic difference in CAT activity between the weighted and unweighted side (ten-fold) when cortical cells are examined. Differences in the residual bone or cells of the trabecular compartment have not shown such dramatic changes. At this point, we do not know if the increased activity is due to more osteoblastic cells or increased CAT activity in the same number of osteoblastic cells.

An important aspect to this project, particularly as related to the population of bone cells that are responding to mechanical loading, is the histomorphometric analysis using CAT immunofluoresence. The initial histological work that formed the body of the project was carried out in fetal rat calvaria which had not yet undergone significant calcification. When applying the immunostaining to adult bone, significant background problems were encountered. This appears to preclude the ability to do quantitative assessment of CAT activity. However, recently this problem appears to be successfully resolved by Dr. Gronowicz, when she changed to a newly developed fluorochrome.

Important progress has been made by members of our bone research group in other aspects of the collagen promoter analysis that is relevant to the success of the current proposal. Finer deletions of the collagen CAT construct have now delineated a region of about 40 bases that is required for activity of the transgene in osteoblastic cells. This and other experiments have lead us to believe that a bone enhancer element exists that is distinct from elements in other type I collagen producing cells (e.g., tendon and periosteal fibroblasts and vascular smooth muscle cells) and is active only when the osteoblast has assumed its most differentiated state *in vivo*. Even more exciting has been the identification of a nuclear factor that binds to this bone element. The factor is present only when obtained from freshly isolated, highly differentiated bone cells. It is not present in cultured bone cells. If this new line of work continues to bare positive results, it will eventually provide a marker for a highly differentiated bone cell and be a likely candidate for transducing mechanical loading to an osteoblastic response.

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MECHANISMS OF ALTERED LYMPHOCYTE ACTIVATION IN SIMULATED MICROGRAVITY AND HYPERGRAVITY

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Description of Research

Lymphocytes have been repeatedly studied during space flight and have consistently exhibited an apparent gravitational influence on *in vitro* activation by mitogenic lectins. An unresolved issue concerning the interpretation of the currently available flight data is whether the observed effects are a result of the direct effect of gravity on cellular systems or indirect effects, such as changes in nutrient transfer or cell contact. Direct inhibition of the proliferative response may occur at any one of the many events regulating the progression through the activation cascade. In addition, *in vitro* lymphocyte activation is sensitive to the specifics of the cell culture environment including such aspects as the effective cell concentration and biochemical transfer. Some of these characteristics may be altered during culture in non-unit gravity. The objective of this investigation is to examine these alternatives and determine the mechanisms responsible for the gravity sensitivity of *in vitro* lymphocyte activation.

We hypothesize that gravity influences lymphocyte activation through one or both of the following general mechanisms: (1) alteration of the cell culture environment (e.g., cell-cell and cell-substrate contact, mass transfer), or (2) changes in the specific elements of the activation cascade (e.g., signal transduction, transcriptional regulation). To test this hypothesis, lymphocyte activation will be characterized as a function of gravity and specific aspects of the cell culture environment. Lymphocytes will be activated under static unit gravity, hypergravity (in a cell culture centrifuge), hypogravity (in a clinostat), and unit gravity suspended culture (gentle stirring). Response to each altered gravity condition will be assessed as a function of cell density, cell aggregation and mitogen type (surface or transmembrane active). The progression of activation events will be determined as a function of gravity and culture condition. The activation sequence will be characterized by assessing the production of second messengers, transcription of cellular oncogenes, production of cytokines, and expression of specific cell surface receptors.

Accomplishments

(1) A major concern in the interpretation of space flight experiments with cultured cells is the influence of indirect environmental effects on the alterations observed. Factors such as changes in the effective cell concentration and an altered degree of cell contact can have major effects on the proliferative response at specific cell concentrations. Exposure to altered gravity levels will cause similar effects in that microgravity will support the continued dispersion of cells, while hypergravity will promote cell and surface contact and an increase in the apparent cell concentration. These factors may contribute significantly to the observed gravity sensitivity of *in vitro* lymphocyte function.

(2) Previous reports have documented a significant decrease in mitogen activation of lymphocytes during culture in clinostats. These results have been attributed to a reduction of the apparent gravity on the cells to approximately 10^{-2} g or less by the clinorotation at appropriate speeds. An alternative explanation is that clinostats evenly disperse the cells and interfere with the cell-cell and cell-surface interactions required for mitogen-induced activation. To test whether simple dispersion was sufficient to inhibit activation, we treated lymphocytes with a mitogen while

cells were either static, in a clinostat, or maintained in suspension on a hematology rocker. The hematology rocker provided gentle suspension without the microgravity simulation of clinorotation. Activation was monitored by the appearance of the CD69 activation marker which appears within a few hours after stimulation with mitogens and is not expressed on resting peripheral blood lymphocytes. Suspending the cells caused a 60–80 percent decrease in the percentage of T cells expressing the CD69 marker 24 hours after addition of mitogen (Figure 1). Tritiated thymidine uptake at 72 hours was also decreased by 65–80 percent compared to the static control (Figure 2). No decrease in the viability of the suspended sample was observed as determined by propidium iodide staining and flow cytometry. These results suggest that suspension/dispersion of the cells is a significant element in the decreased responsiveness observed in clinostats. In addition, these data indicate that early events in the activation sequence are affected by changes in culture environment when surface-active mitogens are used. Overall, these data are consistent with the observation that very few lymphoblasts (enlarged cells which have progressed to the G1 phase of the cell cycle) are observed after stimulation with mitogens during space flight.

Figure 1

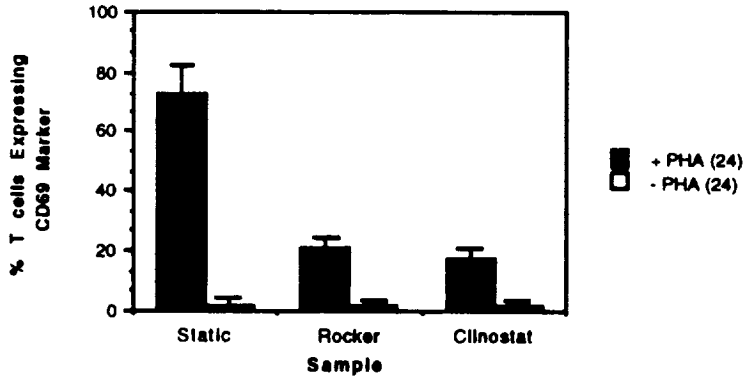

Figure 1. Effects of suspension on activation of peripheral lymphocytes. Lymphocytes were incubated with a mitogen (PHA) for 24 hours in static culture, suspended in a hematology rocker, or in a clinostat. The black bars represent the activated samples and the open bars the control (without mitogen). Data are represented as the percentage of T cells expressing the CD69 activation marker.

Figure 2

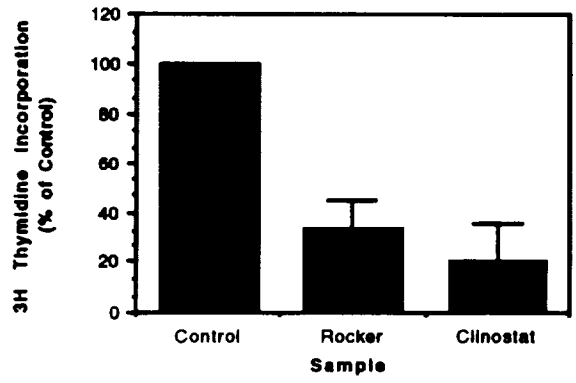

Figure 2. Effects of suspension in lymphocyte proliferation. Lymphocytes were treated with mitogen for 72 hours and pulsed with radio-labeled thymidine. The data for the rocker and clinostat are represented as a percentage of control (static) incorporation.

(3) Phorbol esters function as direct activators of protein kinase C and, in conjunction with appropriate accessory signals, are effective activators of peripheral lymphocytes. These compounds bypass the initial cell surface-mediated signaling events and provide an alternate means for activating the cells. Our preliminary studies have shown that treatment of lymphocytes with phorbol myristate acetate (PMA) induced similar expression of CD69 on the T cells, whether static or suspended. The lack of sensitivity to the degree of cell dispersion observed with PMA indicates that bypassing the receptor-mediated signaling mechanisms allows the initiation of early activation events in the suspended state. These results further support the hypothesis that the early surface-mediated events may be critical determinants in the apparent gravity sensitivity of lymphocyte activation. Mitogens which bypass these events are equally effective at inducing specific early activation markers whether in suspended or static culture. Therefore, these mitogens will be useful tools for dissecting the gravity sensitive steps in activation sequence. These data appear to contrast with space flight experiments which indicated that PMA was ineffective at inducing IL1 and IL2 production in monocyte and T cell lines during microgravity culture. This disparity may be due to (1) differences between peripheral lymphocytes and the cell lines, (2) specific inhibition points distal to those regulating CD69 expression but prior to interleukin production, or (3) additional direct effects in actual microgravity. Further experimentation will be necessary to resolve these differences.

Significance of the Accomplishments

From the above results, it is apparent that environmental or indirect effects have substantial impact on mitogen activation and may account for a significant portion of the observed effects in clinostats or microgravity. These effects can be observed at very early points during the sequence of activation events and are different for surface-active mitogens and transmembrane-active mitogens. Overall, the preliminary data confirm the validity of examining the activation sequence with different mitogens and culture conditions to determine specific cellular mechanisms which are potentially responsive to direct or indirect effects of altered gravity.

This investigation will improve the understanding of environmental and gravitational influences on cells in culture. In addition, it will provide basic data to permit the isolation of indirect gravity effects from those cellular responses which are directly altered or influenced by gravity. This information will be generally applicable to other cell systems and will provide basic information concerning the effects of gravity on living organisms. Results obtained from these studies will provide a firm scientific basis for the design and interpretation of future flight experiments and the development of more detailed hypotheses concerning gravitational biology.

DEVELOPMENT, MATURATION AND PHYSIOLOGY OF THE BRAIN-PITUITARY-GONADAL AXIS OF FISH IN THE CEBAS/AQUARACK SYSTEM

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Description of Research

Our project identifies with NASA's Space and Gravitational Biology Research Program goal to determine the effect of long-term space flight and exposure to reduced gravity on the regulatory mechanisms controlling and affecting the structure and function of endocrine systems. It is an integral part of the German space program's Closed Equilibrated Biological Aquatic System (CEBAS)/Aquarack (C/A) Scientific Frame Program. C/A is a long-term, multigeneration experimental system for aquatic organisms which is used in a frame program that involves German and United States Universities and the DLR Institute of Aerospace Medicine.

The long-range goal of our project is to study the effects of space flight conditions, including microgravity and space radiation, on the development, function, and regulation of the reproductive system, specifically the brain-pituitary-gonad (BPG) axis in aquatic vertebrates of different age and gender in the frame of the C/A project. The BPG axis is the primary internal vertebrate control system for reproduction. The biology of the reproductive system will be analyzed by evaluating changes in the synthesis, storage, and release of neurotransmitters, neurohormones, and pituitary hormones in late embryonic, neonatal, immature, and adult animals using immunocytochemistry, *in situ* hybridization, and morphometry. Additionally, specific sensory organs will be studied because they are the first-line contacts between the organism and its environment. They can monitor the environmental conditions of space and transmit information back to centers in the brain which regulate the BPG axis. This relationship is depicted in Figure 1. The swordtail (*Xiphophorus helleri*), a freshwater teleost, has been designated as the experimental vertebrate model in the C/A Scientific Frame Program.

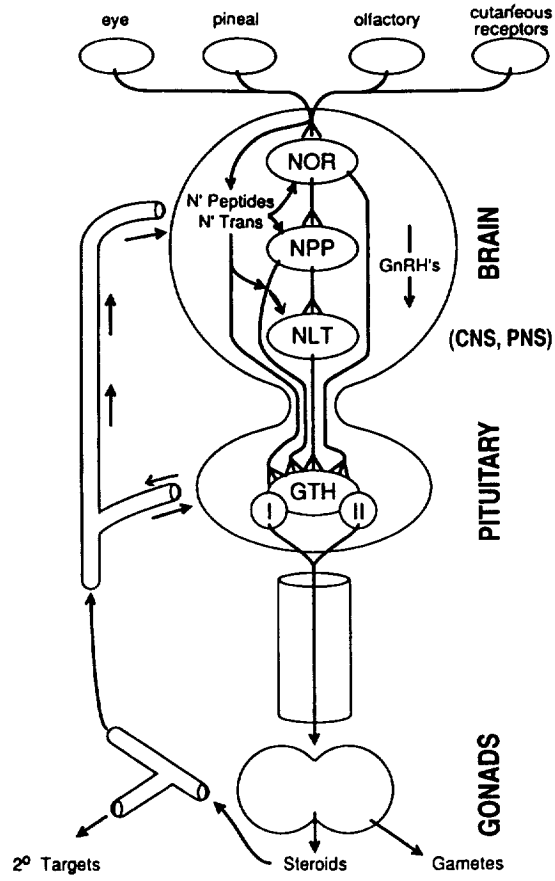

Figure 1. This figure depicts the structure and functional relationship between sensory organs, the monitors of environmental signals, and the neuroendocrine system that regulates reproductive organ development and function.

This project is part of a broad, joint international program of zoological research dedicated to the study of reproduction, among other topics. Our studies collect essential, fundamental information on the reproductive biology of *Xiphophorus* so as to establish a comprehensive data base that will permit the correlation of physiological and morphological parameters for proper evaluation of future space flight and space station experiments.

Accomplishments

(1) We have made considerable progress in preparing a data bank for the times of appearance and sites of localization of a variety of neuropeptides and neurotransmitters in two closely related species of *Xiphophorus, X. helleri* (swordtail) and *X. maculatus* (platyfish). We have reported previously the localization of some two dozen neuroregulatory peptides and neurotransmitters in the brain and pituitary gland of *Xiphophorus*. More recently, we have selected five of these neurohumors (dynorphin, FMRF-amide, neurotensin, neuropeptide Y, galanin) for detailed analysis during the developmental period that spans late embryonic development to sexual maturity. The data derived suggest that specific neurotransmitters and neuroregulatory peptides are involved in the maturation of the reproductive system and that they may possibly play important roles in communicating information between peripheral sensory organs and the control systems for reproduction. This is suggested by their appearances during key developmental periods in important anatomical sites in the brain and pituitary gland.

(2) We have also localized, by immunocytochemical methods, various forms of gonadotropin releasing hormone (GnRH) [mammalian (m), chicken II (chII), salmon (s) and lamprey (l)] in the brain and pituitary gland of *Xiphophorus* at various developmental stages from birth to sexual maturity. GnRH is responsible for controlling the synthesis and release of gonadotropin (GTH) from the pituitary gland, a hormone that regulates gonadal development and function. There are now two recognized forms of GTH, Beta I and Beta II. We have found that GTH I and sGnRH are present together early in *Xiphophorus* development and that the onset of sexual maturation is marked by the appearance of GTH II and the chII form of GnRH. Our results demonstrate that variant forms of GnRH and GTH are present at defined stages of development in specific regions of the brain and pituitary gland and suggest that different forms of GnRN and GTH regulate different aspects of reproductive system development and physiology.

Significance of the Accomplishments

The identification of neuroregulatory peptides, neurotransmitters, and pituitary hormones at key stages of development is critical to our fundamental understanding of reproductive system maturation and function, the control systems that orchestrate these processes, and how they may be affected by conditions in space.

The data we are gathering are absolutely essential if we are to recognize and correctly interpret the influence of hypogravity and other environmental conditions of space flight on the neuroendocrine regulation of the reproductive system. This data base of morphological events will enable us to evaluate and understand the effects of microgravity and other conditions of space flight.

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EFFECTS OF HINDLIMB SUSPENSION ON SKELETAL MUSCLE GROWTH

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Description of Research

Studies are focused on how the weightless environment, induced by hindlimb suspension (HS), alters satellite cell behavior in immature growing animals. Satellite cells are very important during skeletal muscle growth because they are cells associated with myofibers that are responsible for the increase in myonuclei that accompanies myofiber growth. This myonuclear increase is accomplished by the continued mitotic activity of satellite cells during the postnatal growth period and the subsequent fusion of the cells with the enlarging myofibers. A second important function of the cells is to provide a source of myoblasts during a regeneration response following injury. In this sense they are regarded as the stem cells of muscle regeneration. Each of the described functions of satellite cells in normal or injured muscle is dependent upon mitotic divisions of the cells to provide progeny for growth or regeneration. The long-term goal of our studies is to understand the manner in which the proliferative behavior of satellite cells in immature muscles, either in the intact growing system or during muscle regeneration, is altered by the weightless environment.

Our previous studies indicated that when young animals are placed in HS, muscle growth is markedly retarded. A prominent concurrent feature is a severe reduction in the proliferative activity of satellite cells, which is most pronounced in the soleus muscle. The reduced mitotic activity of satellite cells is sustained and appears to parallel the relative atrophy or reduced growth exhibited by the soleus muscle and the less severely affected extensor digitorum longus (EDL) muscle. The reduction in satellite cell mitotic activity occurs quickly after initiation of HS, suggesting the cells in both the soleus and EDL muscles respond to the HS environment before any morphological atrophic changes are evident in the muscles. The goal of our most recent studies is to examine the early response of satellite cells, focusing on the period of HS when single injections of bromodeoxyuridine (BrdU) indicate that no mitotic divisions are taking place. A second goal is to initiate studies to examine the behavior of satellite cells during a regeneration response in the HS environment.

Accomplishments

Immature growing rats were placed in HS for a total period of 5 days. After 3 days, the animals were anesthetized and removed from HS. An incision was made just proximal to the base of the tail to place an Alzet miniosmotic pump filled with BrdU (25 mg/ml) subcutaneously, parallel to the vertebral column. The pump had been previously incubated in saline at 37 ° C in order to initiate pumping. The animals were returned to HS. At the end of the fifth day, the animals were removed from HS and euthanized. The soleus and EDL muscles were removed and immunostained for the presence of BrdU-labeled nuclei. The continuous infusion paradigm was chosen because it gives a cumulative account of all mitotic activity over the duration of the experimental period. Inspection of transverse paraffin sections for BrdU-labeled nuclei demonstrated that control soleus muscles had abundant labeled nuclei (i.e., 86.4±18.5/unit area), whereas HS soleus muscles had significantly fewer, i.e., 7.3±1.1/unit area ($p < 0.01$). Virtually all labeled cells present in the HS muscles were found in the perimysium while few were found immediately adjacent to muscle fiber peripheries. Labeling in the HS-EDL was also greatly reduced compared to weightbearing control but not to the extent seen in the soleus. These results add further evidence that the unweighted environment has a profound effect on the development

of immature muscles, producing a complete cessation of growth (in terms of nuclear accretion) that starts within hours of initiation of HS. The results of the continuous infusion experiment also shed light on the reduction in the number of satellite cells that was observed in EM studies. After HS is initiated, radial growth of the soleus fibers is reduced, whereas growth in length continues, leading to a small increase in the volume of the fibers. Presumably, the supply of satellite daughter cells is smaller than the demand for myonuclei in the slowly growing muscles. Continued generation of myonuclei from fusion competent satellite cells in the absence of additional mitotic divisions would serve to deplete the population. In the EDL, there is a reduction in the number of satellite cells at 3 days, but the reduction is never as severe as in the soleus because division of satellite cells is never completely stopped in this muscle.

Studies of muscle regeneration have shown that the HS environment also reduces the quality of the regeneration response. A regeneration response was induced by free-grafting the muscle or by injecting the myotoxic snake venom Notexin. The muscles (soleus or EDL) of suspended and weightbearing rats were removed and studied after 10-days. During the 10-day experimental period, the rats were given BrdU injections every 12 hours. The results of these studies demonstrated that muscle weights of the regenerated soleus muscle were significantly less than weightbearing controls at the end of the experimental period. The weights of the EDL muscles of suspended animals were not significantly different than weightbearing controls. The muscle weight results were mirrored by fiber diameters of the muscles and their respective controls; the regenerated soleus myofibers were significantly smaller than the regenerated fibers in weight-bearing animals. We are examining if a change in the proliferative behavior occurs during the early phase of the regeneration response and contributes to the reduction in muscle mass seen at 10 days. An examination of the quantity of labeled nuclei in the regenerated muscles after multiple injections of BrdU is currently being carried out by counting the number of immunostained BrdU positive nuclei in paraffin sections or by measuring total fluorescence. Preliminary results suggest that there is no difference in the number of labeled nuclei in the regenerated muscles of suspended and weightbearing muscles. The tentative conclusion is that although satellite cell divisions are significantly reduced in intact muscles, particularly the soleus, mitoses of satellite cells are not influenced by the weightless environment in regenerating muscles. We speculate that it is not until myofibers are formed and start to grow that differences in the suspended and weightbearing muscles are produced. Thus, any differences seen in regenerating muscles are not related to the early proliferative phase but more likely occur after fiber formation and innervation. Confirmation of this awaits further analysis.

Finally, over the past year, we have developed and described a simple apparatus for suspending rats. The importance of the construction is that all the materials are readily available at any local hardware store, which means the cages can be inexpensively constructed without the need for custom work. The cages can be easily dismantled for cleaning and storage. The cages utilize an apparatus that allows the animal to reach all areas of the cage, yet prevents them from resting their hindlimbs against the sides or end panels.

Significance of the Accomplishments

Our results suggest that unloading a growing muscle has a profound and rapid influence on satellite cell proliferative and fusion behavior within hours of the initiation of HS. How placing a myofiber in an unweighted environment induces such rapid changes in satellite cell behavior is unknown. There are no junctional complexes between satellite cells and myofibers that would permit rapid signaling between them. In addition, behavioral changes of satellite cells in both damaged and HS muscles are so rapid that the mechanism of activation cannot be easily attributed to diffusion-related changes in the levels of regulatory factors from their myofibers. One possibility is that cells in undamaged portions of injured muscles or throughout HS muscles respond to changes in electrical activity of myofibers. Electrical activity of soleus muscles in HS animals undergoes a significant and sudden decrease (-91percent) at the initiation of HS and

exhibits a slow recovery through day 7 of HS, when it achieves approximately 80 percent of control values. The proliferative behavior of satellite cells parallels the electrical changes in soleus muscles, but whether this alteration in electromyographic (EMG) activity of the soleus has an influence on the behavior of dividing satellite cells or the changes are coincidental and independent of one another remains unknown. However, because muscles of the anterior compartment, such as the tibialis anterior (and probably the EDL), do not exhibit a similar decrement in EMG activity but show parallel changes in satellite cell mitotic activity argues against behavioral changes being induced by altered electrical activity patterns.

In summary, changes in the proliferative behavior in satellite cells of intact muscles that occur within hours of the initiation of HS are a good indicator of future atrophic changes that will occur in the muscles. Satellite cell proliferative activity is most severely affected in soleus muscles where all mitoses are stopped between 3 and 5 days, and overall proliferative activity is suppressed for as long as HS is maintained. These results suggest that the ability of growing muscles to recover from even short periods of unweighting may be seriously compromised.

The situation is slightly different in regenerating muscles. The results of our studies suggest the following scenario. When a myofiber is damaged, the ability of satellite cells to initiate a repair response to replace that fiber appears not to be compromised by the unweighted environment. That may be because the levels of mitogens that are thought to activate the cells into a proliferative phase are not reduced in unweighted muscles. The proliferative response of the cells is sufficient to form the appropriate number of myofibers. It is not until the myofibers are formed (with their population of satellite cells) that the unweighted environment influences the course of the regeneration response. Control of further satellite cell proliferative behavior is regulated by the fibers. However, as is seen in intact muscle, because the myofibers are not growing, satellite cells are not maintained in a proliferating mode. The net result is that regeneration is reduced.

In summary, the effect of unweighting on dividing satellite cells in intact and regenerating muscles is not the same. The variance in the response of satellite cells may be related to the manner in which the proliferative behavior of the cells is regulated during these processes.

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GROWTH FACTOR INVOLVEMENT IN TENSION-INDUCED MUSCLE GROWTH

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Description of Research

Long-term manned space travel will require a better understanding of skeletal and cardiac muscle adaptations which result from microgravity. Astronaut strength and dexterity must be maintained for normal mission operations and for emergency situations. Although exercise in space slows the rate of muscle loss, it does not prevent it. A biochemical understanding of how gravity, tension, and exercise help to maintain muscle size by altering protein synthesis and/or degradation rate should ultimately allow pharmacological intervention to prevent muscle atrophy in microgravity.

The overall objective of this research project is to examine some of the basic biochemical processes involved in tension-induced muscle growth. Differentiated avian skeletal myofibers and rat cardiac myocytes can be "exercised" in tissue culture using dynamic mechanical cell stimulator devices which simulate different muscle activity patterns. We have found patterns of mechanical activity which significantly stimulate muscle growth, and organogenesis and alter myofiber metabolic characteristics. With this experimental *in vitro* system, we are examining the interaction of exogenous and endogenous muscle growth factors in mechanically stimulated muscle growth.

Exogenous growth factors found in serum, such as insulin, insulin-like growth factors (IGF), and glucocorticoids modulate muscle protein turnover rates in mechanically induced muscle growth *in vitro*. Endogenous growth factors, such as the prostaglandins, are synthesized and released into the culture medium when muscle cells are mechanically stimulated. This lipid-related family of endogenous factors helps to regulate the rates of protein turnover in the muscle cells.

Studies on the mechanism by which muscle mechanical activity interacts with both exogenous and endogenous growth factors to regulate muscle protein turnover rates in these model systems will be continued.

Accomplishments

(1) Organogenesis of single skeletal muscle cells into functional muscle organs can be mechanically induced in tissue culture (Figure 1).

(2) The glucocorticoid dexamethasone induces atrophy of skeletal myofibers, and mechanical stimulation reduces this atrophic response. Mechanical activity reduces muscle atrophy by stimulating the production of the anabolic growth factor prostaglandin F_{2a}. Prostaglandin production is increased by stretch activation of phospholipases, which provide arachidonic acid for prostaglandin synthesis and by stretch activation of prostaglandin synthase, the enzyme which converts arachidonic acid into prostaglandin (Figure 2).

(3) Neonatal and adult rat cardiac cells can be mechanically stimulated in our tissue culture model systems in a similar fashion to skeletal muscle cells.

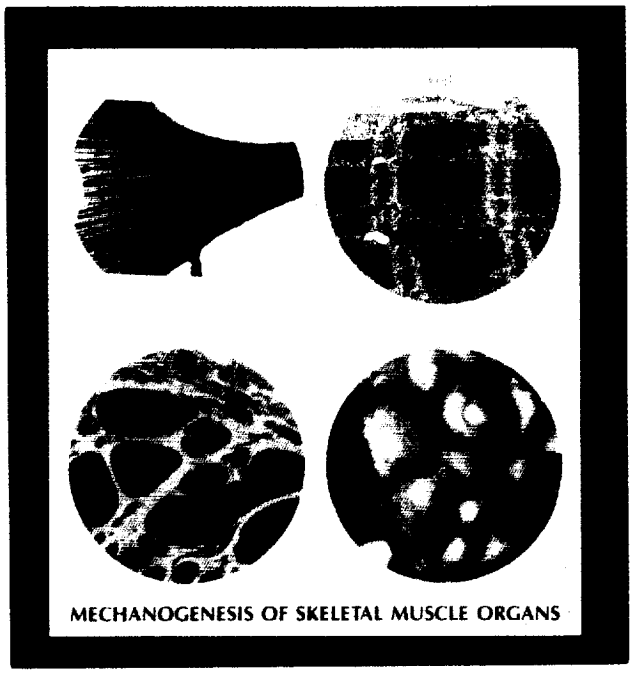

Figure 1. Computer-aided mechanical generation of artificial skeletal muscle organs have *in vivo*-like characteristics including parallel network of long unbranched myofibers (upper left), well-organized sarcomeres (upper right), primary and secondary-like myofibers (lower left), and concentration of the tendon-associated protein tenascin at the end of the organ (lower right). Reprinted from the cover of *FASEB Journal* 5, 1991.

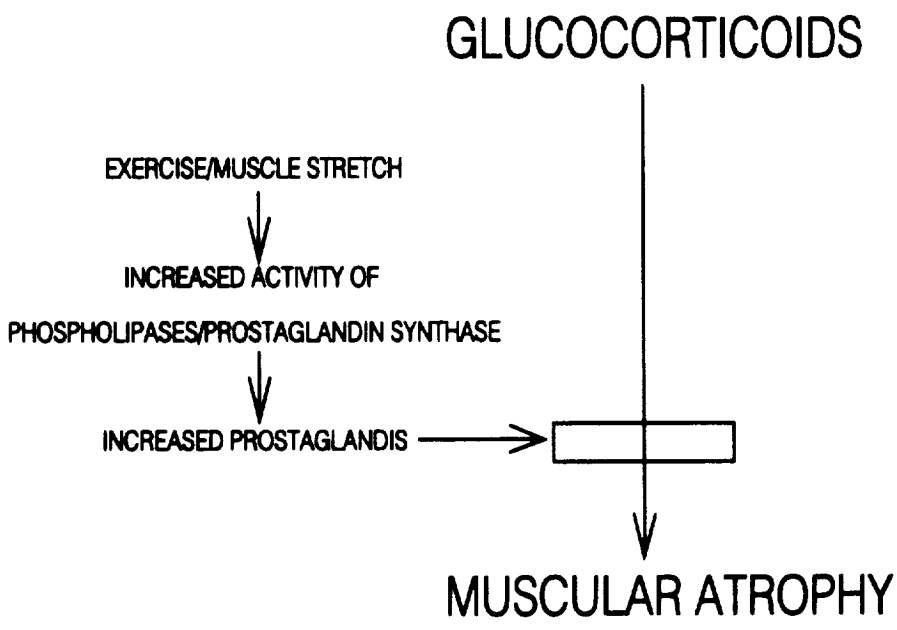

Figure 2. Proposed mechanism by which stretch/exercise attenuates glucocorticoid induced skeletal muscle atrophy.

Significance of Accomplishments

(1) Mechanical/tension/gravity effects on the organogenesis of tissues can be studied with our mechanical cell stimulator devices.

(2) The skeletal muscle atrophying effects of the stress-related glucocorticoids are reduced by exercise/mechanical stimulation, and this occurs by stretch-activation of anabolic prostaglandin production.

(3) Stress/pressure/mechanical regulation of cardiac growth or atrophy can be studied in our mechanical cell stimulators.

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MECHANO-TRANSDUCTION IN MAMMALIAN CILIATED CELLS

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Description of Research

In a broad range of species, detection of gravitational force is based on a motile/sensing organ in which cilia operate as both a sensing transducer and a steering actuator. However, the mechanical response of ciliated cells to changes in hydraulic load is still poorly understood. Our studies have been aimed at investigating the role of Ca^{2+} ions in mechanical stimulus-response coupling in ciliated cells. To accomplish this objective, it was necessary (1) to develop an assay to characterize the response of ciliated cells to mechanical stimulation, and (2) to design an optical system to monitor on-line fluctuations of intracellular $[Ca^{2+}]_i$ as reported by fluorescent probes.

Accomplishments

(1) ***Epitheliary ciliated cells in culture can adjust their mechanical output when challenged by increased viscous load. This response takes place by lengthening the effective stroke of the cilium, at the expense of shortening the recovery stroke, while keeping ciliary frequency and amplitude relatively constant.*** Three forms of mechanical stimulation of ciliated cells were tested in our laboratory: (a) local touching with a piezoelectrically driven microneedle; (b) electromagnetically driven motion of magnetizable microspheres suspended on top of the ciliated cells by a viscous fluid; and (c) exposing ciliated cells to increased viscous load. Ciliated cells were found to respond to all these forms of stimulation. Although these three methods can be precisely calibrated, the responses of ciliated cells to both electromagnetically driven micromotion and poking with a microneedle were inconsistent, making it very difficult to interpret results. Conversely, viscous loading was found to elicit reproducible responses of both beat frequency and $[Ca^{2+}]_i$. Viscous loading also better resembles the mechanical stimulus to which epitheliary ciliated cells are normally exposed, and it is easy to quantify and to deliver.

Additional studies to further characterize the beat cycle dynamics of rabbit respiratory ciliated cells under conditions of increased viscous load (5–180 cp) were conducted using high-speed cinematography (500 frames/sec). They indicate that the autoregulatory response of epitheliary ciliated cells takes place by lengthening the effective stroke of the cilium at the expense of shortening the recovery stroke, while keeping ciliary frequency and amplitude relatively constant.[1]

(2) ***Development of a system to measure changes of $[Ca^{2+}]_i$ at high sampling rates and with high spatial resolution.*** A large number of methods to detect fluctuations intracellular $[Ca^{2+}]_i$ have been developed in recent years. Fluorescent techniques offer the best approach to monitor intracellular $[Ca^{2+}]_i$. However, with the exception of confocal microscopy, most of these methods do not allow one to distinguish precisely where, inside the cell, these changes occur. Also, high-speed confocal microscopy has an extremely high price tag. Other low-speed scanning confocal systems have a rather low sampling rate, which can severely alias the Ca^{2+} signals, particularly in cells where $[Ca^{2+}]_i$ undergoes quick changes.

We have developed the optical and electronic hardware, as well as the software of a powerful and inexpensive system to detect changes of $[Ca^{2+}]_i$ with high temporal and spatial resolution. This system is based on an epi-illumination inverted microscope (Nikon Diaphot), a low-cost digital Peltier-cooled CCD video camera (SpectraSource, M-220, CA) originally developed for astronomical observations, and a 486 IBM-clone microcomputer. The software includes a set of high-speed assembly language instructions to control the communications between the camera and the central processor, to command shutters, and to read/write routines. Another set of instructions implements a program that removes out-of-focus optical noise, which is the main limitation for high-resolution imaging in fluorescence microscopy.[2] The strategy consists of removing all the optical information outside the depth of field of the microscope objective. Thin optical sections inside the cell can then be obtained by using a shallow depth-of-field objective (160–200 nm).[3,4]

(3) ***Our preliminary results indicate that, following ciliostimulation, $[Ca^{2+}]_i$ increases first in the endoplasmic reticulum and then in the cytosol of ciliated cells.*** Calcium has a critical role in stimulus-response coupling in a broad variety of cells. In most cells, $[Ca^{2+}]_i$ remains at micromolar concentrations but can transiently increase following stimulation. These fluctuations of $[Ca^{2+}]_i$ play a central role in triggering the intracellular cascade that couples specific responses of the cell to correspondingly specific stimulus. Calcium ions can reach the cytosolic compartment from either, or both, the extracellular space via Ca-channels of the plasma membrane, or from intracellular sequestering sites via Ca-channels of intracellular membranous compartments. It has been assumed that sequestered calcium found inside mitochondria or in the endoplasmic reticulum (ER), or in the sarcoplasmic reticulum (SR) of myocytes, is in two pools: (a) an ionized pool which is in equilibrium with (b) a bound pool associated to the chelating proteins calsequestrin or calreticulin, which are normally found inside the SR and ER, respectively. It has been assumed that the concentration of the ionized fraction of Ca^{2+} inside the storage compartments remains constant at concentrations much higher than the cytosol.[5] When the cell is stimulated, Ca-channels open allowing the entrance of extracellular Ca^{2+} or its release from the sequestering compartment to the cytosol. In either case, Ca^{2+} flow into the cytosol is driven by a large diffusional gradient, raising the cytosolic $[Ca^{2+}]_i$. Calcium ions then function as a second messenger, coupling the activation of a variety of cellular processes, including phosphorylation of effector proteins via type II Ca-calmodulin-dependent protein kinase (CaM-PKII).

A direct implication of this notion is that the fluctuations of $[Ca^{2+}]_i$ reported by fluorescent probes take place only in the cytosol of the cell. Free Ca^{2+} inside the storage compartments is assumed to be at high concentrations (>μmol). Thus, Ca-probes inside the ER would be saturated with Ca^{2+}, contributing to a large fluorescence signal, but it would not vary in time.[6] This assumption has remained untested because the characteristic fuzziness of fluorescent images cannot resolve the detailed spatial distribution and, hence, the kinetics of $[Ca^{2+}]_i$ at a subcellular level.

We have found that submicromolar changes of $[Ca^{2+}]_i$ play an important function in the coupling of adrenergic, purinergic, and mechanical stimulation in ciliated cells.[7] As in other cells, Ca^{2+} can enter the cytosol of ciliated cells from the extracellular space via Ca-channels, or it can be released from intracellular membranous subcompartments, particularly the ER.

At present, our work in ciliated cells is focused on the detailed analysis of subcompartmentalization of Ca^{2+} in the cytosol and the ER of ciliated cells undergoing viscous loading. Using the high temporal and spatial resolution of our digital video-microscopy system, combined with fluorescent double labeling, we have preliminarily documented that, following

viscous loading at 10 cp, the concentration of $[Ca^{2+}]_i$ (as reported by fluorescent probes), increases in both the ER and the cytosol of ciliated cells. In these experiments, we have used the potentiometric fluorescent probe $DiOC_6$ as a tracer to identify the ER of ciliated cells.[8] We have found that the permeant (AM) fluorescent Ca-probes accumulate in the same compartments as $DiOC_6$, suggesting that the ER of ciliated cells must have the esterases to turn these probes impermeant. This feature is consistent among different AM Ca-probes, including Fura2, Rhod2, and Fluo3 (Molecular Probes, Inc.). The increase in cytosolic $[Ca^{2+}]_c$ is slightly delayed with respect to the Ca^{2+} increase inside the ER $[Ca^{2+}]_{er}$, and both of these increases precede the changes in beat frequency. Thin optical sections (160 nm) of Rhod2 fluorescently labeled ciliated cells, taken every 250 msec, reveal that Ca^{2+} seems to be released from the ER, not ubiquitously across the cell but in very specific places in the cytosolic compartment. Also, when released, the Ca^{2+} does not seem to follow a typical diffusional profile but rather a cooperative pattern resembling a Ca-induced-Ca-release mechanism. These observations suggest a new, previously unrecognized, level of complexity in the mechanisms that control Ca^{2+} release to the cytosol following mechanical stimulation.[9]

(4) We have also found that mucins, which are the principal polymer chains of the polymer matrix of the mucus gel, can spontaneously self-assemble, forming liquid crystalline domains.

References and Footnotes

[1]Our early observations have been published by Johnson, et al., 1991 (*American Review of Respiratory Disease*. 144: 2680-85). The more recent results discussed herein were presented at the 8th Annual Meeting of the American Society of Gravitational Biology.

[2]This approach was originally developed by K.R. Castelman for optical slicing of the heavens in astronomical imaging (*Digital Image Processing*, Prentice Hall, 1979). It was later adapted and validated for microscopic applications by Monk, et al., 1992 (JCB 116: 745), using a Sun-Station-Unix platform; and was further reprogrammed for a low-cost IBM-DOS platform by Mr. Thien Nguyen in our laboratory (Mr. Nguyen is one of our M.D./Ph.D. students, and he will continue with this project).

[3]The total cost of this system is less than $15,000, and it can provide optical sections of up to 160 nm. The maximum sampling rate is 6 images/sec using a standard 60-W mercury burner-excitation source and up to 320 images/sec using a submillisecond chopped 2-W Argon-ion laser excitation source.

[4]This material was recently presented by T. Nguyen, et al., at the 38th Annual Meeting of the Biophysical Society (*Biophysical Journal* 66: A275, 1994), and it is now being prepared for publication.

[5]For review see E.M. Milner, et. al., 1992 (*Molecular and Cellular Biochemistry* 112: 1-13).

[6]See J.P.Y. Kao, 1994 (*Methods in Cell Biology* 40: 155-181).

[7]See the following:
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P. Verdugo, et al., 1980a (*Fertility and Sterility* 33: 193, 176).
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[8]M. Tarasaki and T.S. Reese, 1992 (*Journal of Cell Science* 101: 315-322).

[9]These results were presented at the 9th Annual Meeting of the American Society of Gravitational Biology.

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THE ROLE OF CALCITE SKELETAL MATRIX PROTEINS IN BIOMINERALIZATION

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Description of Research

The ultimate goal of our work is to learn how calcified tissue matrix proteins preside over the process of biomineralization resulting in the formation of hard tissues. Disturbances in bone and calcium metabolism occur in humans and other vertebrates during extended space flight. The basic molecular and biochemical basis of formation of mineralized skeletal elements is poorly understood. Hence, basic information about biomineralization will be required to understand better the complex events that occur in microgravity environments. It is hoped that analysis of simpler model systems to study basic aspects of biomineralization will be of substantial benefit to the Space Biology Program.

With this in mind, our studies have focused on the formation of the calcite spicules of the developing larva of the California sea urchin, *S. purpuratus*. The calcite spicules of the pluteus larva have an integral protein matrix. Our studies center on learning more about the proteins comprising the spicule matrix. Several approaches have been used to study these proteins and their function. First, we have directly isolated the matrix proteins and studied their properties by biochemical methods; these ongoing studies have shown that there are over 3 dozen matrix proteins, all but 4 of which are acidic. There are probably 6 of them that are not glycosylated. Second, we have isolated and cloned the genes of 2 of these proteins, naming them SM30 and SM50. The cloned genes have been used to make specific antibodies against these two well-characterized spicule matrix proteins, and the antibodies have been used to study the function of the proteins in biomineralization. The cells that form the spicule matrix and spicules, the primary mesenchyme cells, have been cultured, and biomineralization has been shown to require an intact extracellular matrix. Finally, a powerful new approach to studying the function, *in vivo*, of the SM30 and SM50 proteins has begun using genetic engineering techniques. Genes that encode counterfeit SM50 protein have been made, and these genes and their products are being introduced into the living embryo in order to discover, by means of the anomalies produced, what the function of SM50 is during mineralization.

Accomplishments

(1) Significant portions of spicule matrix genes have been cloned from DNA of distantly related species. The SM30 cDNA has been cloned from *L. pictus*, a species distantly related to *S. purpuratus*. The promoter region of SM 50 has also been cloned from *L. pictus*. In each instance, both the promoter and coding region of the two species are similar in nucleotide sequence.

(2) Cloned SM30 cDNAs have been used to study the details of expression of the gene by RNAse protection assays. The SM30 gene is activated slightly earlier than was formerly known because of the increased sensitivity of the assay.

(3) Both SM30 and SM50 genes have been cloned into bacteria which express these foreign genes as proteins. The bacterially synthesized proteins have been used to produce monospecific antibodies against the two proteins. Exposure of spicule-producing cells to these antibodies does not interfere with biomineralization.

(4) Cultures of primary mesenchyme cells have been treated with agents that interfere with formation of the extracellular matrix. When the matrix is perturbed, SM 30 and SM50 gene expressions are reduced, and the spicule does not form. This result is in contrast to treatment of whole embryos with such agents, where only SM30 expression is inhibited.

(5) The SM50 gene of *L. pictus* has been engineered to contain the 10 amino acid epitope of c-myc, a vertebrate gene, and the epitope tag has been inserted into 3 different places in the SM50 protein. This allows one to locate the tagged protein, *in vivo*, by using antibodies against c-myc. The presence of the epitope tag, at least in one of its locations in SM50, should compromise the function of SM50 and help one understand SM50 function, *in vivo*. Such tests are underway.

Significance of the Accomplishments

Finding 1: We had previously shown that SM50 coding regions in the two sea urchin species were closely conserved. We can now extend that generalization to the SM30 protein and to the SM50 gene regulatory region upstream of SM50. This lends strong support to the idea that important functions of the protein are embedded in the protein structure and that, by modifying this structure, we can learn how these proteins function in biomineralization.

Finding 2: A continued study of the SM30 genes in *S. purpuratus* revealed slight differences in the mRNAs. A gene-specific probe has been designed that allows detection of only one of the three SM30 genes; and this sensitive detection method, called RNAse protection assay, shows that this form of SM30 is expressed at very low levels somewhat earlier than was heretofore known. Further, this gene may be expressed only in adult spines and not in tube feet. Hence, for the first time, there is a clear indication that the multiple genes of SM30 may subserve slightly different functions in different situations.

Finding 3: Since both SM30 and 50 were subcloned into bacterial expression systems during the past year, substantial amounts of pure SM30 and SM50 can be obtained, something not formerly possible. The pure proteins have been used to make monospecific antibodies against the two different proteins. When such antibodies are added to cultures of embryonic mesenchyme cells that make spicules, there is no effect on spiculogenesis. This is consistent with our hypothesis that the function of these two proteins is intracellular, not extracellular.

Finding 4: Perturbation of the extracellular matrix disturbs biomineralization. We had formerly known that treatment of whole embryos with lathyrogenic agents disturbed spicule formation and reduced SM30 expression. This result has now been reproduced in mesenchyme cell cultures, where the phenomenon is easier to study closely. In contrast to whole embryos, SM50 expression is also affected in cultures. Hence, the proposal that extracellular matrix function is generally important in biomineralization receives strong support.

Finding 5: We have been able to use molecular biology techniques to produce mRNAs that encode epitope tagged versions of SM50 and, furthermore, have engineered the mRNAs in such a way that their function, *in vivo*, should be compromised. This paves the way to examination of SM50 function, *in vivo*, by the "dominant negative" approach, which is proving to be so powerful in other systems.

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EFFECTS OF ALTERED ENVIRONMENTS ON CELLULAR STRESS GENES IN REPRODUCTION AND DEVELOPMENT

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Description of Research

The long-range goal of our research program is to examine the effects of the space flight environment on mammalian development and cellular differentiation. The main aim of this project is to characterize the response of mammalian embryos and germ cells to altered environmental conditions and to determine the stages that are most sensitive to stress during embryogenesis and gametogenesis. While the ideal setting for examining these effects is space flight itself, the opportunities for such experiments are severely limited. Therefore, ground-based studies are crucial as a means of evaluating both the aspects of development most likely to be affected in space as well as providing background information as to potential molecular markers of perturbed development. Our studies focus on one set of molecular markers—the cellular stress proteins. The cellular stress protein or heat shock protein (*hsp*) is synthesized in response to diverse conditions of physiological stress and environmental assaults. Our studies have focused on examining the pattern of expression of these gene families initially at the level of RNA and, subsequently, of proteins in mid-gestation mouse embryos and during spermatogenesis in mice.

Accomplishments

(1) One series of experiments focused on characterizing the expression of the *hsp70.2* gene at the mRNA level in different mouse tissues, including adult brain, reproductive organs, and mid-gestation mouse embryos. *Hsp70.2* had been previously isolated and characterized in our lab as a testis-specific gene of Hsp70 gene family with precise patterns of expression during spermatogenesis. The results of the present studies now reveal that this gene, while expressed at high levels in a specific subpopulation of spermatogenic cells, is also expressed in the adult brain and in mid-gestation conceptuses.

The analysis utilized Northern blot hybridization with RNAs isolated from the various tissues using a probe for the 5' end of the *hsp70.2* gene, which did not cross-hybridize with other Hsp70 genes. Low levels of expression of *hsp70.2* were detected in adult ovaries and prostate, and in placenta at stage 10.5 days of gestation. Higher levels of expression were also found in the adult brain and epididymis, in addition to the very high levels seen in the adult testis. A detailed analysis of *hsp70.2* gene expression in dissected brain structures revealed the abundance of this gene in hippocampus, as compared to other brain regions. Other regions of the brain where *hsp70.2* was expressed included the hypothalamus, thalamus, and cortex.

(2) We also initiated studies to examine the expression of the *hsp70.2* gene in mouse tissues employing the method of *in situ* hybridization. This technique gives the opportunity to observe actual expression of the gene at the cellular level. In the testis, the expression of *hsp70.2* was determined to be restricted to germ cell compartment. The pattern of its expression was shown to be developmentally regulated with the most pronounced signal in pachytene spermatocytes, consistent with previous studies; although some expression was seen in later stages of germ cell development (through to the round spermatid stages, again, consistent with previous observations). The preliminary experiments also showed the expression of *hsp70.2* gene in the

epididymis and further revealed a regional localization of its expression, mostly restricted to caput region.

(3) The developmental regulation of members of the Hsp70 and Hsp90 gene families was examined at the protein level using methods of immunocytochemistry. We have begun this analysis using rat monoclonal antibodies (clone 7.10), which recognized all members of Hsp70 family, and rat monoclonal antibodies (16F1 clone) which recognized mouse Hsp86 and Hsp84 proteins (members of the Hsp90 family of proteins). The expression of Hsp70 and Hsp90 was found in histological sections of testes from adult normal mice. The immunoreactivity was restricted to germ cells from pachytene spermatocytes to elongated spermatids. Since, in our studies at the mRNA levels, we have detected expression of the Hsp70 and Hsp90 gene families in mid-gestation mouse embryos and placenta stages 10.5–13.5 days of pregnancy, we looked at the level of protein as well. Whether the immunoreactivity, which was distributed throughout the tissue sections, represents cross-reacting proteins and/or ubiquitous distribution remains to be determined. Western blot analysis of solubilized mouse tissues and placenta revealed expression of Hsp70 proteins in adult testis, brain, and liver, as well as in placenta at embryonic stages, 10.5–17.5 days. Therefore, our preliminary results show the presence of Hsp70 proteins in different adult tissues as well as in conceptuses, notably in extraembryonic compartments. The results of immunocytochemistry indicated that, at the protein level, the both Hsp70 and Hsp90 in the testis are most abundant in the germ cell compartment. The expression of these proteins during embryonic development was most pronounced at 10.5–13.5 days.

(4) A final series of studies was undertaken to extend to the protein level our previous observations which had established the stage and cellular specificity of expression of Hsp86 and Hsp84 in the murine testis, in the absence of exogenous stress using immunoblot analysis. Hsp86 protein was shown to be present throughout testicular development, and its levels were shown to increase with the appearance of differentiating germ cells. Hsp86 was most abundant in the germ cell population and was present at significantly lower levels in the somatic cells. In contrast, the Hsp84 protein was detected in the somatic cells of the testis rather than in germ cells. The steady state levels of Hsp86 and Hsp84 paralleled the pattern of the expression of their respective mRNAs, suggesting that regulation at the level of translation was not a major mechanism controlling *Hsp90* gene expression in testicular cells. Immunoprecipitation analysis revealed that a 70 kDa protein coprecipitated with the Hsp86/Hsp84 proteins in testicular homogenates. This protein was identified as an Hsp70 family member by immunoblot analysis, suggesting that Hsp70 and Hsp90 family members interact in testicular cells.

Significance of the Accomplishments

Finding 1: The *hsp70.2* gene is testis-abundant, not testis-specific, as originally thought. It is expressed at lower levels in other adult murine tissues, most notably in the brain, epididymis, prostate, seminal vesicles, ovaries, and uterus, and in predominantly extraembryonic tissues in the mid-gestation conceptus. These observations raise the question of whether its expression in these tissues will be altered during exposure to environmental stress and changes in hormonal levels.

Finding 2: At the level of *in situ* hybridization, we confirmed the developmentally regulated pattern of expression of *hsp70.2* during mouse spermatogenesis. The strongest signal was observed over meiotically active, pachytene spermatocytes. Furthermore, no other sites of expression in the adult testis were observed. In the brain, *in situ* hybridization revealed that the highest levels of expression were in the hippocampus.

Finding 3: The expression of Hsp70 and Hsp90 in the adult testis was confirmed, at the protein level, to be most abundant in mouse germ cells from pachytene spermatocyte to round spermatid stages.

Finding 4: Hsp90 protein was found to co-immunoprecipitate with a protein of 70 kDa, which appears to be a member of the Hsp70 family. It will be interesting to observe if this association is altered under conditions of altered environmental stress.

Finding 5: Studies on the expression of *hsp70.2* have revealed the surprising observation of transcripts in both the sense and antisense orientations from this area of the genome. In the adult mouse brain, the expression of the antisense mRNAs was highest in the brain stem, cortex, thalamus, and the cerebellum in particular. The prostate also expressed significant levels of this antisense transcript. These observations bring to attention new levels of complexity with regard to the regulation and function of the *hsp70.2* gene during normal development and, ultimately, in response to environmental changes including those encountered during space flight.

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SPACE FLIGHT EXPERIMENTS

PLANT GRAVITATIONAL PHYSIOLOGY

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Description of Research

Goals: Generally stated, the long-range goal of our research is to understand the ways in which gravity is important to plants and, by extension, to the use of plants on Earth and potentially in extraterrestrial situations. Specifically, we want to understand the fundamental mechanisms which enable plants to detect, measure, and respond to gravitational influences. Consequently, it is axiomatic that in our experiments we must be able to vary the gravitational information that test plants receive from their environment. These experimental conditions have been achieved by increasing the G-force by centrifugation or decreasing it by performing experiments in near-Earth orbit.

All of our recent research objectives have pertained directly or indirectly to the support of space experiments. Support from the NASA Space Biology Program enabled us to make ground-based studies which led to the proposal of two space experiments that were flown on IML-1 in 1992. Those experiments, even though only partially successful due to technical difficulties, provided quantitative answers to five different scientific questions involving the kinematics of how plants detect and respond to G-forces. Also obtained were certain serendipitous findings that set the stage for asking new experimental questions. IML-1 research results are now being written for publication. Within the confines of limited space, four of those questions/answers can be described briefly.

Accomplishments and Significance of the Accomplishments

Question 1: How well does clinorotation (a simulation of weightlessness) mimic the effects observed in space flight? If scientists could count on the validity of some kind of simulation of weightlessness, we would be able to get answers to some scientific questions by performing ground-based experiments without doing experiments in space. This would increase our scientific productivity by more than an order of magnitude and decrease the cost by at least 3 or 4 orders of magnitude.

On IML-1 we were able to make nine different kinds of quantitative comparisons between the results we obtained in space and those we obtained on Earth using the same apparatus. For five of the nine kinds of comparison, our results showed no significant differences between space vs. clinorotation experiments. For the other comparisons, the differences were great. Our results only reinforced the cautious judgment that clinorotation experiments on Earth may serve as a guide for what may be the plants' behavior in weightlessness, but we cannot describe the clinostat as a dependable substitute for tests in space.

Question 2: Does the plant obey the "Reciprocity Rule"? The rule predicts that a plant's responses will be the same for any stimulus (G) times duration of its application (t) even though G and t are varied reciprocally, thus maintaining the same G x t product. Superficially, that may sound reasonable, but there is no compelling theoretical reason why the rule must apply.

For technical reasons the data for testing the rule are less ambiguous when responses are measured in space. The results of our IML-1 experiment provided a NO answer; the Reciprocity

Rule was not obeyed. That result is important because to plan and to interpret results of many ground-based experiments over the past several decades the experimenters have taken the Reciprocity Rule for granted. Now we know that the Reciprocity Rule is not valid in all cases; and, therefore, such a generalization is not dependable. As a result of our IML-1 experiment results, we have been motivated to develop an explanation for the failure of the Reciprocity Rule. This is being prepared for publication.

Question 3: What is the threshold G-stimulus for plants responding in microgravity to a range of tropistic stimulations? "Threshold" means the weakest stimulation that elicits a detectable response. By knowing the threshold we can set limits on any kind of mechanism that could possibly explain how the plant detects (and measures) gravity or other acceleratory force.

Measured in two different ways on Earth, experimenters have published threshold data that differ by more than an order of magnitude—far more than what could be attributed merely to experimental error. The supporting data were good, and neither method can be discredited on theoretical grounds.

We made the same kind of measurement in space but by a different method. One was in principle unambiguous, because no assumptions were made with regard to the validity of clinorotation. Our results agreed very well with one set of ground-based results but not with the other.

Question 4: Will oat seedling coleoptiles circumnutate in weightlessness? Circumnutation is a growth oscillation displayed by elongating plant organs. It is absolutely growth dependent. Like any other widespread and possibly universal phenomena, it has attracted experimental attention and has been studied using several different methods for well over a century.

Circumnutation had been attributed to an effect of gravity; therefore, it should not occur in weightlessness. We tested that in the SLS-1 mission and observed that sunflower shoots circumnutated in space—a result that was the driver for the development of a new theory which can explain the plant's circumnutational mechanism independent of gravity and also can account for its consistent occurrence. Much of the work that led up to the development of that theory was supported by Space Biology Program funding.

During the IML-1 mission we had the opportunity to observe growth behavior of wheat and oat seedlings. One species (wheat) circumnutated; the other (oat) did not. Obviously, two out of three fall short of proof of ubiquity. Observations on more species, flown by various investigators in future space experiments, may establish a statistically more adequate basis for generalization.

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AN ATTEMPT TO LOCALIZE THE GRAVITY SENSING MECHANISM OF PLANTS: THE ELECTRICAL IMPEDANCE BETWEEN VASCULAR AND CORTICAL TISSUES

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Description of Research

A plant stem when placed horizontally responds to gravity by growing back into a vertical orientation. For the stem to grow more on the lower than the upper side, more ions, hormones, and solutes required for growth must be transported out of the central vascular stele into the lower side of the horizontal stem (Figure 1). It was the objective of this research to determine how the gravitational stimulus is transduced into a selective leakage of solutes from the central stele into the surrounding cortical tissues. The working hypothesis developed by this laboratory is called the Potential Gating Theory and attempts to link the internal bioelectric fields of the plant to the transport of the growth hormone, indole-3-acetic acid (IAA). Briefly the theory states that:

(1) The growth hormone is in the vascular stele of the plant and to promote growth it must reach target cells in the cortex and epidermis.

(2) The transport of IAA from the vascular stele to the cortical cells is through plasmodesmatal connections and is regulated by the bioelectric potential difference between the stele and cortex.

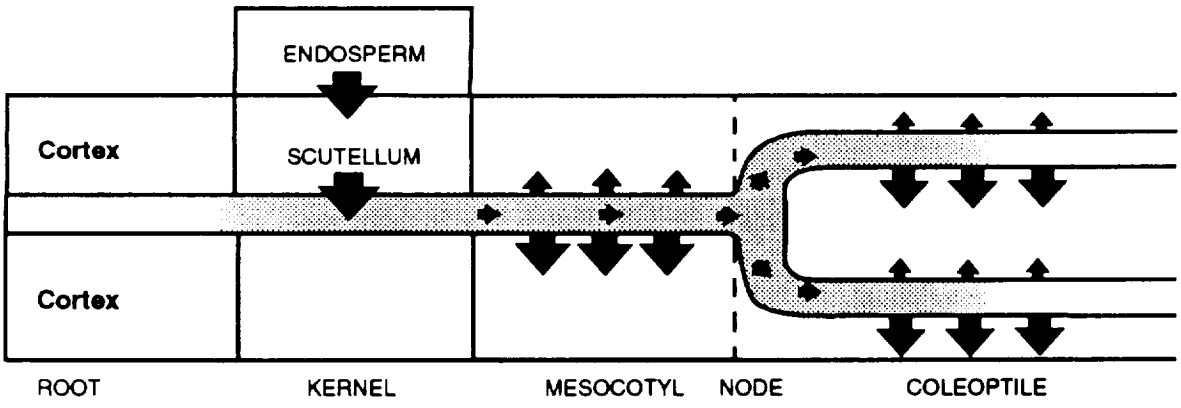

Figure 1. Diagram of IAA transport from the kernel to the growing mesocotyl in young corn seedlings as proposed by the Potential Gating Theory. The size of the arrows represent relative magnitudes of IAA flow.

Gravity, as a stimulus, affects the system by inducing a lateral electrical gradient across a horizontal shoot. We postulate it is this gravity-induced electrical gradient that opens the plasmodesmatal connections between the stele and the cortex on the lower side. Consequently, in a horizontal shoot, more IAA would flow from the stele into the lower cortex and induce growth such that the plant would grow back to its normal upright position. (See Figure 1. The arrows represent the direction and magnitude of IAA transport.)

Previously we have shown that the potential differences (see Figure 3) between stele and cortex are responsive to gravity and stated that the gravity-induced change in the potential difference could function as a regulator of IAA efflux from the stele because a) it is rapid, b) it is sufficiently large in magnitude, and c) it has the proper geometry to produce IAA flow from stele to cortex. The next step in testing the Potential Gating Theory was to measure physical responses of the plant to gravistimulation, which might be a triggering mechanism for alteration of the symmetric bioelectric gradients. We report this has been accomplished.

Accomplishments

Gravistimulation should induce a change in the distribution of charges in the cells. During redistribution of charges, the mobility of these charged structures will change. This can be detected by impedance spectroscopy. We found that the mobility of charges as measured by impedance spectroscopy (mobility is inversely proportional to impedance) undergoes an immediate transient response following gravistimulation. Figure 2 shows a plot of impedance as a function of time and frequency.

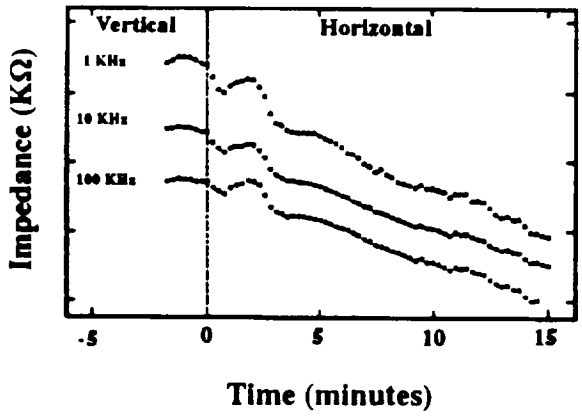

Figure 2. Impedance as a function of time and frequency in a sample at 4 mm below the mesocotyl node.

Significance of the Accomplishments

An early event in gravity detection in plants was observed. The mobility of the charges present in a cell changed immediately following changes in orientation. This is significant because 1) the mobility can change due entirely to physical mechanisms and hence be prior to any biochemical responses, and 2) the fact that the gravity-induced impedance change is of limited duration emphasizes the short-lived nature of this early event. Any gravity-induced charge redistribution should return to equilibrium quickly.

The second step of the Potential Gating Theory states that the charge redistribution induces the biochemical cascade. If the gravity-induced impedance change is due to physical mechanisms, then it will occur prior to the biochemical response and can serve as the trigger for the biochemical cascade which results in and maintains the bioelectric potentials observed following gravistimulation.

Figure 3 summarizes the chemical, biological, and electrical changes occurring in the mesocotyl of a corn shoot following a gravity stimulus. The initial event, following the gravity stimulus, is the change in AC impedance and DC conductance between the two tissues. This is followed rapidly by the change in electrical charges which occurs before the IAA asymmetry, and that may be the causative factor that leads to the increased amounts of IAA on the lower side of the shoot. Finally there is a shoot growth on the lower side of a horizontally placed shoot with the result that the plant grows back into a vertical position.

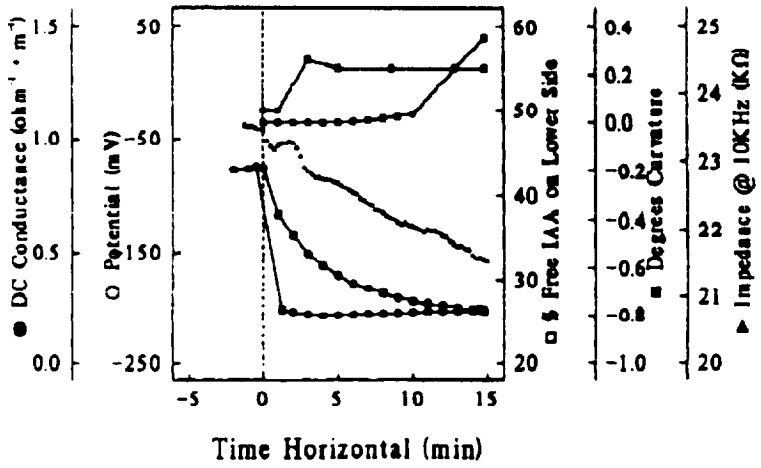

Figure 3. A summary of the known changes in the mesocotyl of Zea mays seedlings following a shift from a vertical to a horizontal orientation. The parameters measured are the IAA concentration in the lower side of the mesocotyl, the mesocotyl curvature and the endogenous voltage, and DC conductance and impedance between stele and cortex tissues.

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STUDIES OF INTERCELLULAR COMMUNICATION AND INTRACELLULAR RESPONSES BY BONE CELLS TO SIMULATED WEIGHTLESSNESS AND SPACE FLIGHT

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Description of Research

Non-weight bearing and space flight cause a reduction in new bone formation, especially in the weight bearing portion of the skeleton. The cellular mechanisms responsible for this reduction are unknown although the bone-forming cells appear to be the cells most affected. Our present hypothesis suggests that the cytoskeleton of these cells could be the cellular element most sensitive to changes in gravity or microgravity. The cytoskeleton is responsible for cell shape, cell movement and attachment, and cell secretory activity. For cells involved in collagen synthesis, the secretory granules which contain pro-collagen are moved from the Golgi region of the cell to the cell membrane and then released to the extracellular space. This activity requires an intact cytoskeletal system. It has been shown that, in bone-forming cells, when the cytoskeleton is disrupted, collagen synthesis and secretion are reduced or inhibited. Previous studies have shown that the collagen-containing granules in these cells following space flight are reduced in number.

We are studying the bone-forming activity of isolated cells in order to simplify and manipulate bone formation and mineralization during space flight. This method involves growing isolated rat bone-forming cells in a three-dimensional culture system. These cells produce significant collagen matrix which will mineralize in culture. The mechanism of cell attachment, the cytoskeletal components, and the collagen secretion mechanisms are much easier to study and manipulate in this system compared to the whole animal studies discussed in the previous paragraph.

We are also continually developing new methods to localize and quantitate specific enzyme activities associated with bone-cell activity. We are quantitating the ability of bone cells to form new matrix and making these measurements at specific sites within bone samples. The technique involves our standard histochemical procedures coupled with a new imaging system and video enhancement of the sample.

Accomplishments

The most recent whole animal studies involved analysis of long bones from Space Life Sciences-1, a 9-day flight onboard Space Shuttle *Columbia*. The findings from these analyses indicated the following: (a) Stromal cells (pre-bone-forming cells) showed significantly more alkaline phosphatase activity in the flight animals than in their controls. This was the first time for this finding and was the reverse of what was found in the differentiated cell population. (b) Secretory granules in the bone-forming cells were significantly increased when the flown animals were given 9 days to recover from their flight. This suggested a reduction in secretory granule formation during the flight itself. (c) The vascular supply to the long bones did not show the extreme degenerative changes we found previously in the Cosmos series of flights.

The bone-forming cells grown in three-dimensional culture will form mineralized bone in the laboratory. We have recovered cells from three Shuttle flights (STS-45, STS-53, and STS-55) and found that they produce a collagenous matrix but have not yet found any mineralization.

This result may be partially explained by the difficulty of maintaining optimal culture conditions during flight. However, if the absence of mineralization, which is also what we found to occur in the whole animal studies, is a consistent finding, then we have the basis for future studies of cellular mechanisms.

By combining our histochemical methods with the video image analysis system, we are quantitating alkaline phosphatase activity (a measure of bone formation) on a site specific basis in each bone obtained from space flight or non-weight-bearing experiments. The results indicate that the total alkaline phosphatase activity in trabecular bone is greater than in compact bone; however, the individual cells in compact bone show greater activity than those same cells along trabeculae. Thus, site specificity becomes important in the understanding of how bone responds to space flight and non-weight bearing.

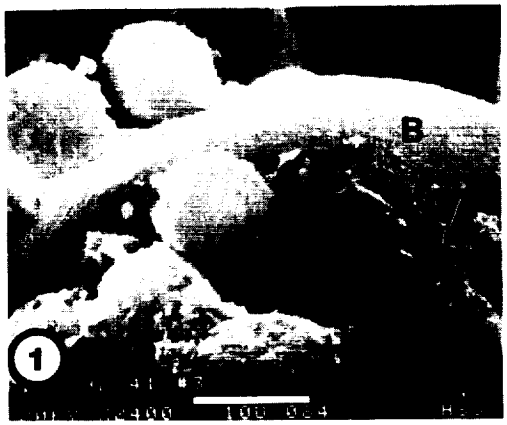

Figure 1. A scanning electron micrograph of a single bead (B) partially covered with bone cells. The cells have two general morphologies; a rounded plump cell (arrowhead) or flattened cells with numerous cytoplasmic projections (arrows). Magnification: 2,400X.

Figure 2. This scanning electron micrograph shows how the cells cover the beads (B) and how the beads and cells mixed together form a mat-like structure in the background. Magnification: 670X.

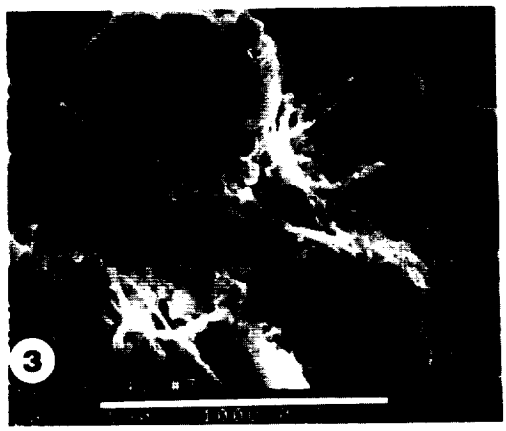

Figure 3. This scanning micrograph shows collagen strands (arrows) stretching between beads as well as attached to them. Magnification: 590X.

Significance of Accomplishments

The whole-animal studies validate our assumption that the bone-forming cells and their cytoskeletal elements should be the major focus of our continuing studies. Quantitation of the secretory granules and analysis of the cytoskeleton components will continue to be related to other data collected from biochemical and biomechanical studies.

The ability to manipulate and study in detail the cellular mechanisms involved in bone formation is greatly enhanced by the use of bone cell culture. In culture conditions, cell attachment, cell secretion, and matrix formation and mineralization can be selectively inhibited or enhanced. The cellular responses to these changing activities can then be compared to the response found in the whole-animal studies following space flight.

The quantitation of enzyme activities, especially for the alkaline phosphatase activity, is especially important for analysis and description of the mechanism of matrix mineralization. This analysis will help to separate the mineralization process from the collagen secretion process and thus improve our understanding of how space flight or non-weight bearing affects new bone formation.

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ALTERED CHONDROCYTE DIFFERENTIATION IN RESPONSE TO ALTERED G-LEVEL

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Description of Research

Changes in loading, including those resulting from changes in the gravitational field, produce corresponding changes in connective tissue (bone, cartilage, tendon). Our laboratory uses pre- and postnatal exposures to altered gravity to determine the effects of such exposures on skeletal development, with an emphasis on cartilage formation and endochondral ossification.

Many defects of the skeleton are due to defects in the chondrogenic process. The use of gravitational fields to modulate cartilage development and, thus, to study its regulation are enormously important, not just to development and human health in space, but also to the understanding and eventual elimination of the myriad chondrodysplasias that occur on Earth.

Accomplishments

(1) Micromass cultures of embryonic mouse limb mesenchyme were flown aboard the first International Microgravity Laboratory (IML-1). Both scanning and transmission electron microscopy studies of flight culture show that little, if any, matrix was produced during space flight. Moreover, until late in the mission (4–5 days), the flight cells were abnormally smooth, having no matrix associated with the cells.

(2) Continuing histomorphometric studies of growth plate found that: (a) Histological changes in growth plates of rats exposed to 14 days of continuous centrifugation (Cosmos 2G experiment) were reduced in a manner similar to that seen in rats of comparable age and weight flown aboard Spacelab 3. (b) Growth plates of older rats flown on Cosmos 2044 were found to be less responsive to unloading by space flight or suspension than those of younger rats from previous missions. (c) Collagen fibrils in both flight (Cosmos 2044) and control 15–week–old rats, when viewed by electron microscopy, often appeared to be unraveling.

(3) Rats flown on Spacelab 3 and on Cosmos 2044 were found to have more metaphyseal vessels at the level of the primary trabeculae than their controls. Rats flown on Cosmos 1887 did not differ from controls in number of metaphyseal vessels, probably because of the long postflight time at 1-g.

(4) At the level of the epiphyseal-metaphyseal junction, most differences in vascularization were found to be due to the age of animals rather than to space flight. Younger rats (Spacelab 3 and 1887 basal controls) were found to have more cells in the last row of the calcification zone, an estimate of column number, and more vessels that appeared to be actively penetrating a lacunae. In both Spacelab 3 and Cosmos 1887, the cell number in the last row of the calcified zone was increased, indicating a slowing of normal development. There was slight increase in the number of vessels in the process of invasion of lacunae in all space flight rats, but this was significant only for Cosmos 2044.

Significance of the Accomplishments

Finding 1: The "CELLS" experiment flown in January of 1992, was the first culture of skeletal cells flown in space and remains the best. This experiment had the largest number of medium changes and fixations ever carried out in space, and the hardware developed for "CELLS" is being used to grow other cell types and even small organisms. The lack of chondrogenesis in cell cultures is similar to that seen in studies of growth plate, and involves both direct and indirect effects.

Finding 2: This was the first study to compare growth plates of centrifuged rats with growth plates of flight rats and demonstrates the response of growth plate to an increase in baseline loading, which, according to orthopedic principles, will slow growth plate activity. The similarities in direction of changes in a microgravity environment and the hypergravity tested demonstrate the need to look for a g–threshold that will maintain normal growth of the epiphyseal plate.

Our electron microscopy observations are of great significance because they constitute the first set of ultrastructural observations of the aging growth plate. Also of major significance for developmental studies is the fact that older growth plates respond less to unloading, either by suspension or microgravity.

Finding 3: The effects of space flight on the metaphyseal vasculature had not been previously studied. Our results demonstrate the relationship between vascularization and trabeculae number in the metaphyseal region of the tibia.

Finding 4: This first study of neovascularization at the metaphyseal epiphyseal junction of the tibia indicates that space flight slows normal development.

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SYNAPSE DEVELOPMENT IN ALTERED GRAVITY: A MODEL FOR THE STUDY OF THE EFFECTS OF SPACE MICROGRAVITY ON EMBRYONIC DEVELOPMENT

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Description of Research

Our continuing research program is designed to accomplish the following goals:

1. Examine specific cell processes that are affected by perturbed gravity on Earth using the clinostat paradigm.

2. Examine the feasibility of using primary co-cultures of embryonic nerve and muscle cells in space flight to determine whether the reduced gravity of space affects cellular processes.

3. Develop a model nerve cell culture system suitable for flight experiments in which to verify findings from preliminary flight data and from clinostat results.

We have previously reported (Gruener & Hoeger, 1990 and 1991) that co-cultures of nerve and muscle cells from *Xenopus* embryos show specific sensitivity to altered gravity after rotation in a slow clinostat. The alterations in cell properties included changes in cell shape, size of the nucleus, reduced accumulation of acetylcholine receptors at the junction between the nerve and the muscle cell, and the formation of neuritic swellings. We interpreted our results to suggest that synapse development will be altered if it were to proceed in the microgravity of space. We hypothesized that our results may be explained by invoking a common pathway—the cytoskeleton—as the cellular organelle through which mechano-information is indirectly or directly transduced. To test this possibility, we have examined cytoskeletal markers (tubulin, actin filaments, vinculin) in both neurons and myocytes after clinorotation, and more recently after space flight. Our preliminary results strongly suggest that consistent changes in cytoskeletal structure may correlate with the changes we have observed both in the clinostat and after space flight. Taken together, our data lead us to believe that specific cellular processes can be examined in space flight and that findings from space flight experiments can be used to test the validity of the clinostat paradigm. To the extent that the clinostat paradigm is verified, it would appear prudent, cost effective and scientifically compelling to use this Earth-bound simulation in order to define, refine, and optimize experiments to be carried out in space flight where access is restricted and where the cost of carrying out experiments is considerably higher.

Accomplishments

(1) *Effects of Vector-Averaged Gravity on Bead-Induced Receptor Accumulation in Embryonic Muscle Cells.* In preparation for flight experiments, where the objective is to obtain maximum critical data from a simplified model system, we used polystyrene beads to replace neurons as the trigger agent for receptor accumulation in muscle cells. Beads act like neurons in that they induce receptor aggregation at the point of contact between the bead and the myocyte. We have previously shown that nerve-induced receptor aggregation is defective after clinostat rotation. Using beads served two purposes: First, it would narrow down the source of the defect, furthermore, it would simplify the cell culture system (only one cell type would be needed) as a model in which to test synapse development in the microgravity of space. Our results indicate that bead-induced receptor accumulation is significantly reduced; and, therefore, we conclude that in the altered gravity of the clinostat the myocyte processing of the signal (from either the nerve or the

bead) is defective. Second, our results encouraged us to use the bead-myocyte culture system in space flight experiments *(vide infra).*

We have repeated this experiment in space flight (STS-54) and found a reduction of about 50 percent in the number of acetylcholine receptor clusters associated with bead contacting myocytes. Further, we found that the size of the clusters from flight cells, where they did aggregate near the beads, was significantly smaller than in the ground control cells. These data are in complete concordance with our reported findings from clinostat simulations examining either nerve-associated clusters or bead-associated receptor clusters. These results are of significant importance as they validate the use of the clinostat as a model for examining the effects of altered gravity on cellular processes.

(2) *Effects of Vector-Averaged Gravity on Single Channel Properties of the Acetylcholine Receptor in Cultured Myocytes.* As a followup on our earlier findings of reduced receptor accumulation after clinostat rotation (vector-averaged gravity), we tested the possibility that not only was receptor accumulation at the neuromuscular synapse defective, but that the apparent disconnection of receptors from their anchoring cytoskeleton also produced changes in single receptor channel behavior. To test this possibility, we examined the AChR channel properties after clinostat rotation of myocytes with, and without, neurons. We found that there was a reduction in the channel open time in receptors located close to the nerve. Figure 1 shows single channel events recorded from a control (1A) and a rotated myocyte (1B). The reader should note the overall reduction in open-time event duration in the rotated myocytes. A compilation of all open times into a frequency histogram (Figure 2) shows that open-time events from a rotated cell cluster at shorter times than in a control cell (the time constant is shortened from 4.0 to 2.2 msec). There was, however, no effect on channel properties in non-junctional receptors, and channel conductance was unaffected. These data further support the idea that vector-free gravity produces specific alterations in cellular properties which may significantly affect synapse development and efficacy in the microgravity of space.

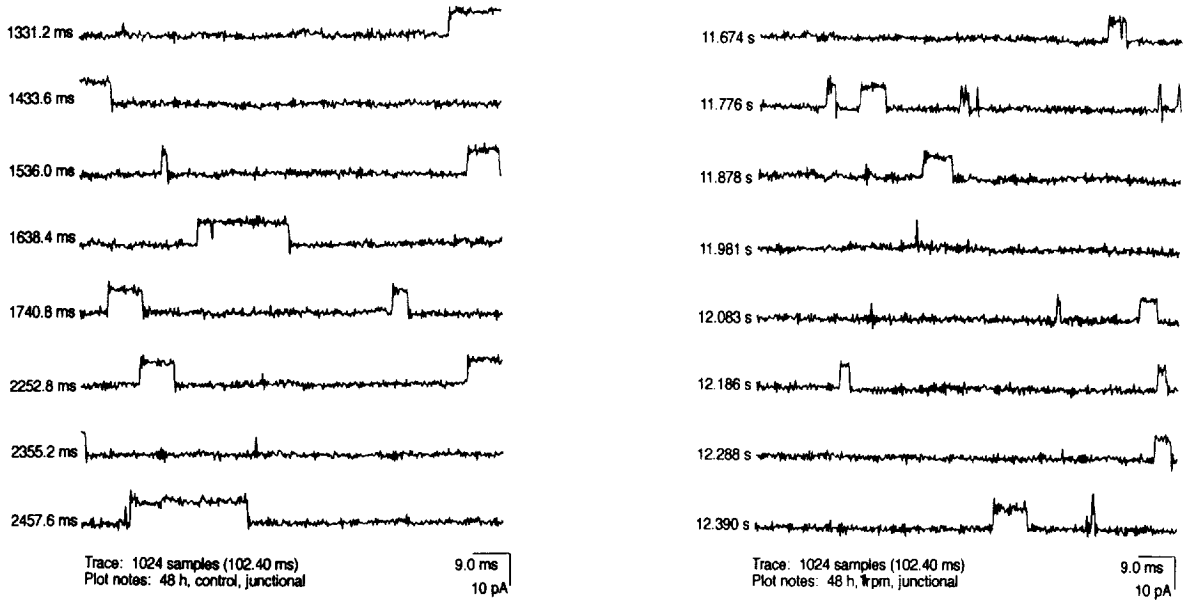

Figure 1 Junctional AChR single channel activity from a control (A) and a rotated myocyte [48 hr at 1 rpm] (B)

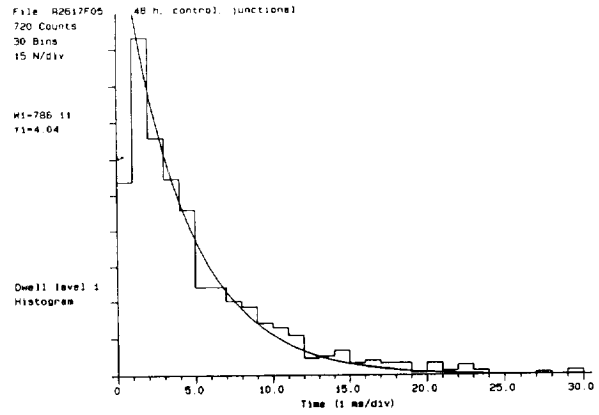

Figure 2.A

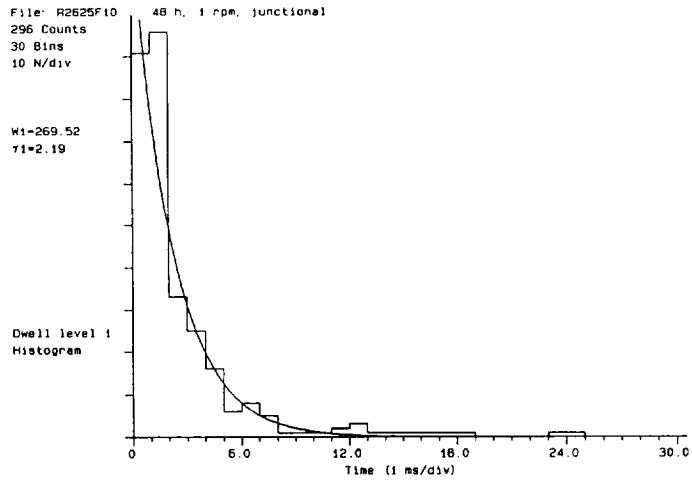

Figure 2.B

Figure 2 Open-time histograms for junctional AChRs from a control (A) and a rotated myocyte [48 hr at 1 rpm] (B)

(3) *Effects of Space flight on Cultured Myocytes*. We had an unprecedented opportunity to participate in the STS-52 and STS-54 Shuttle flights through the courtesies of Mr. John Cassanto (ITA Inc.) and Dr. Marian Lewis (University of Alabama, Huntsville). Our myocyte cultures were placed in the Material Dispersion Apparatus (MDA) hardware wells (ITA, Inc.) and fixed onboard the Shuttle 10 days after launch. The cells survived the flights very well and provided a wealth of information on the behavior of these cells in the microgravity of space. This information can now be correlated with the changes from clinostat simulations as we previously reported. Preliminary analysis of flown cells, when compared to synchronous ground controls, shows that flight cells had a significantly smaller surface area than any of the controls (Figures 3 and 4). In addition, flight cells also had the smallest nuclei compared with all controls. Because of fixation, we were unable to measure cell thickness and therefore cannot estimate cell volume. Therefore, to determine whether the change in surface and nuclear areas are actual, we calculated the nuclear-to-cell-area ratios. This calculation shows that the ratio for any of the cells, including the flight cells, remained constant. This implies that the changes we observed in flight cells are due primarily to reduced cell spreading.

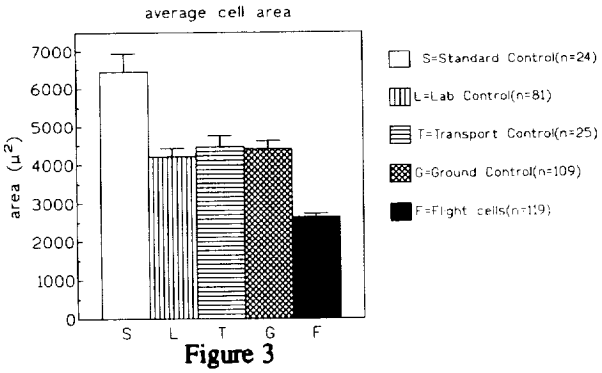

Figure 3

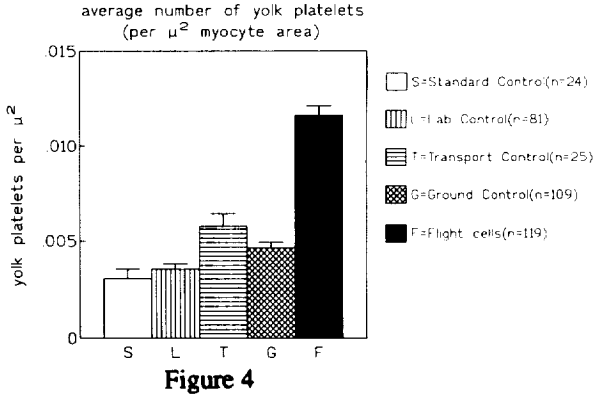

Figure 4

Next, we estimated the metabolic and developmental status of the myocytes based on the number and surface area of yolk platelets in myocytes. The yolk platelets are the source of metabolic energy of the cell. They are consumed as a function of development. Initially, the cells are packed full with yolk platelets; their number decreases steadily with development, and normally they are almost absent after 6–8 days in culture. Flight cells had a larger area occupied by yolk platelets.

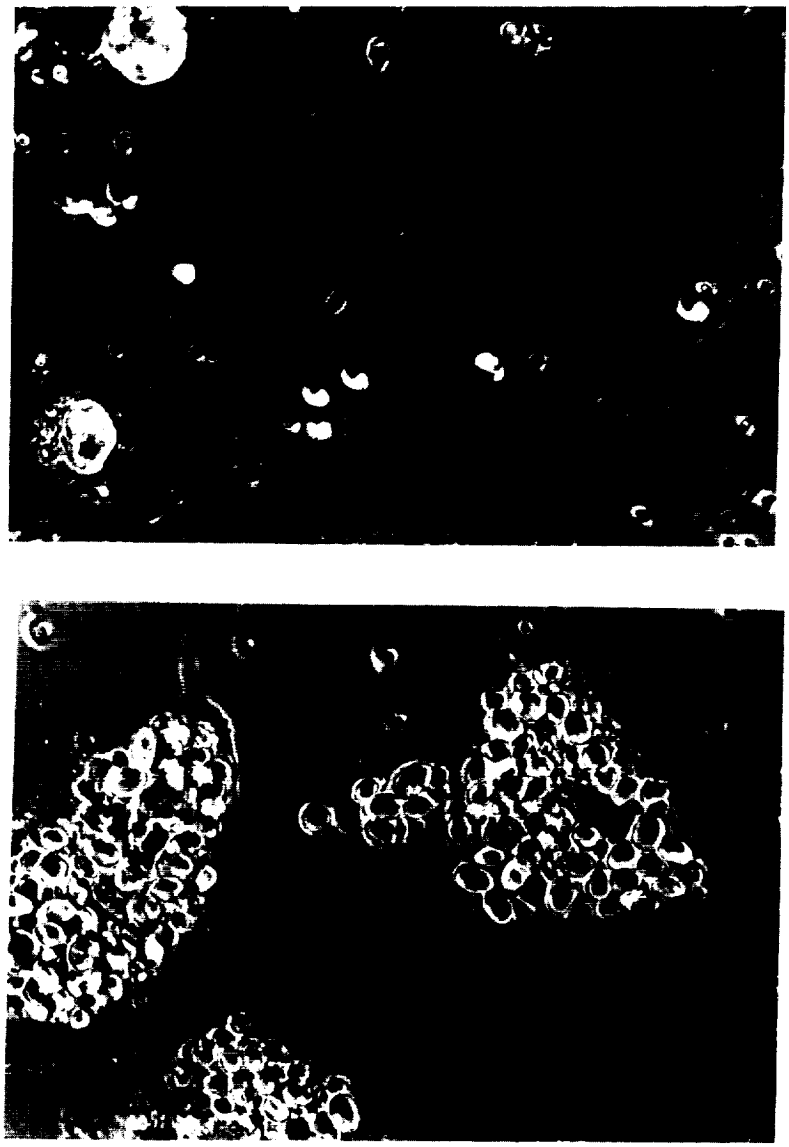

Figure 5: A typical Control (top) and space-flown (bottom) Xenopus muscle cell. Note the smaller size of the flighted cells and the over-abundance of yolk platelets (phase bright bodies) that indicate a significant slowing of development in space-flown cells.

This suggests that the flight cells' development was arrested at a stage equivalent to ca. 2 days. Further confirmation of this finding comes from the analysis of the number of yolk platelets which we found to be significantly higher in flight cells than any of the control cells (Figures 3 and 5). This finding is further substantiated by our finding that the absolute number of yolk platelets calculated per square micron area of myocyte is the highest in flight cells.

Further, we measured cell shape and correlated this shape to the length and linearity of actin filaments, a major component of the cytoskeleton. We found that flight cells were shorter than all control cells and the length of actin filaments was also shorter than controls. The relative linearity indicates that control cells had a more linear array of actin filaments. Finally, flight cells were found to have a significantly reduced number of vinculin clusters (a cytoskeletal marker associated with the infrastructure of acetylcholine receptors) and that the average size of the clusters was smaller than normal.

The results of the NERMUS experiment on the STS-52 Shuttle flight has demonstrated that the MDA hardware is most suitable for cell-attached cells. Cell recovery was adequate. Cells appeared to have remained viable but showed a number of changes consistent with the effects of microgravity (reported also for other cell types and for our cells in the clinostat). From that point of view, the experiment was a significant success.

Significance of Accomplishments

Our results to date indicate that nerve and muscle cells are sensitive to alterations in the gravity field whether through use of the clinostat or in actual space flight. Our preliminary results from the two space flight experiments indicate that the clinostat is a very useful paradigm through which experimental design can be focused and through which cellular processes likely to be affected by altered gravity may be defined. Thus, the clinostat is a useful tool for preparing rigorous experiments for space flight. In addition, our results show that there is considerable concordance between results obtained in the clinostat and those from actual space flight. Specifically, the retardation of cellular development (measured in the density of yolk platelets), and the alterations in cytoskeletal organization (presence of vinculin clusters and linearity of actin filaments) appear to be affected in a similar manner in the clinostat and in space. On the other hand, cell size and nuclear size showed the opposite effects, becoming larger in the clinostat and smaller (than the appropriate controls) in space-flown cells.

Taken together, these results provide further support for our earlier conclusion that synapse development and the development of excitable cells (nerve and muscle) are likely to be significantly affected by the microgravity of space. Further flight experiments, in which specific cellular processes are rigorously examined, are therefore warranted. Our preliminary results show that such experiments are highly feasible even under suboptimal conditions (e.g., no intervention during flight, unexpected problems such as launch delays, and temperature variations). We hope to have the opportunity to continue with these experiments in the near future.

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MICROGRAVITY-INDUCED EFFECTS ON PITUITARY GROWTH HORMONE CELL FUNCTION: A MECHANISM FOR MUSCLE ATROPHY IN MANNED SPACE FLIGHT

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Description of Research

Growth Hormone (GH) secreted from the anterior pituitary gland has a number of effects on body tissues. These include, but are not limited to, increased protein synthesis in the liver, increases in muscle mass, increased fat metabolism, stimulation of immune tissues, and increases in blood cells. The rationale for this research stems from a series of experiments which indicate that the production or release of GH is inhibited in microgravity (μg). The first indication that GH is inhibited in space occurred in 1983 on a cell culture experiment on the STS-8 mission. This was followed by a series of experiments utilizing whole animals flown on the U.S. Space Shuttle and the Russian COSMOS biosatellites, all of which continued to demonstrate a deficiency in the secretion of biologically active GH after space flight.

Since growth hormone has so many important metabolic effects on organ systems of interest to NASA, our goals were to: (a) definitely establish whether or not a secretory defect does indeed exist, (b) determine the mechanisms involved, and (c) attempt to determine if the effect is reversible.

To accomplish the goal of identifying the nature of the secretory defect, a pituitary cell culture experiment was designed that utilized 165 cell culture vials that would be incubated in a middeck locker aboard the space Shuttle. The large number of vials would allow us to examine many different variables at one time, perhaps allowing us to identify which of the variables were important in the observed hormone reductions.

Accomplishments

(1) The experiment was flown on the STS-46 mission in July-August 1992. This experiment utilized 165 culture vials inoculated with rat pituitary cells in a middeck incubator and the 165 corresponding ground controls. Tested were pituitary cell fractions enriched in cells which store a highly active form of GH, fractions depleted of this cell type, cells from various regions of the pituitary which demonstrate different activities, and several types of cell culture media. After flight, the cell cultures were returned to the laboratory and processed. This processing included hormone analysis on all the flight and ground control media, extended cell cultures for some of the cells to test the time needed for the cells to recover, morphometric analysis of recovered cells from the culture vials, and biological stimulation of GH release. This experiment yielded over 1800 separate samples to be analyzed.

(2) A separate experiment was conducted several months later to duplicate the temperature profile of the flight experiment as recorded by flight instruments. This experiment again resulted in over 1800 separate samples to be analyzed.

(3) Preliminary analysis of all the samples has been completed. These tests included assay of growth hormone and prolactin (PRL) levels on over 3600 samples, cell response to growth hormone releasing hormone (GHRH), determining the biological activity of GH and PRL, morphometric analysis of the cells that were recovered, and the distribution of hormone within the cells.

Significance of the Accomplishments

Finding 1: The significance of the experiment lies in the fact that this was the first cell culture experiment flown that specifically targeted the cellular basis of hormone reduction seen in intact animals. It may be possible to begin to identify the molecular changes which result from exposure to microgravity. Any future design for treatment of the hormone reduction in animals will obviously require knowing where the defect is. This may have importance in future long-term manned flights.

Finding 2: The cell culture system worked. This system allows large numbers of variables to be tested while minimizing the space and power needed, an important consideration in space flight. This system should be adaptable to other cell-culture-based experiments.

Finding 3: The analysis of the experiment is still underway, and the significance of the experimental results has not yet been determined.

ELECTROPHORETIC SEPARATION OF CELLS AND PARTICLES FROM RAT PITUITARY AND RAT SPLEEN

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Description of Research

This experiment is designed to explore the kinetics and molecular basis of endocrine deficits which occur in microgravity (μg). The first part is a pituitary cell culture experiment to determine changes in pituitary cell function in μg as a function of time. The second part utilizes the Japanese-designed Free Flow Electrophoresis Unit (FFEU) to separate pituitary cells into functional subtypes with subsequent cultures. The third part utilizes the FFEU to separate subcellular components obtained from pituitary cells cultured in μg.

In man, both Growth Hormone (GH) and Prolactin (PRL) are multifunctional hormones produced by the pituitary that are essential for life. While their existence has been known for 25-50 years, the biochemical details and functional significance of molecular events involved in their synthesis and release from pituitary cells are still incompletely understood. Moreover, the body targets for these two hormones are numerous, including bone, muscle, fat, blood, and the immune system. Since GH and PRL affect a number of organs in the body that are known to change in μg, there is a strong rationale for evaluating functional changes in these hormones.

Rats and rat pituitary cells have been flown under both μg and μg-mimicked conditions. The results of these experiments demonstrate repeatable and statistically significant changes in the pituitary GH and PRL cell systems. While most of the studies have utilized whole animals flown in space, an experiment conducted on STS-46 in July 1992 utilized cultures of rat pituitary cells. While analysis of this experiment is still underway, key findings to date show that μg-induced changes occur within the pituitary GH/PRL cell, and strongly support the idea that the changes seen in pituitaries of space flown rats are explicable by changes within the pituitary cells themselves.

The value of utilizing the FFEU for this experiment lies in the realization that, while flow electrophoresis has been used to separate cells and organelles in the past, there is agreement that gravity limits resolution of separation because of sedimentation during exposure to the electric field, zone sedimentation due to density gradients (droplet effect), and convection due to heating of the sample in the electric field. The only environment where all these instabilities can be overcome while operating with higher sample loads is in μg. This fortuitous circumstance allows us to utilize a research technique uniquely adapted to μg and to examine biological changes that occur in μg.

Accomplishments

(1) We have cultured rat pituitary cells, recovered them from the culture vessel, and electrophoretically separated them utilizing the FFEU. Cells separated in this manner demonstrated a differential mobility, with an enrichment of GH cells in the most mobile fractions, and allows for extended culture of the separated subtypes of cells.

(2) Cells cultured in the vessels have been successfully lysed, the lysate concentrated, and the concentrated lysate separated on the FFEU. This separated lysate can be detected utilizing the FFEU ultraviolet absorption detector. The success of the separation has been documented by assaying both the immunodetectable and bioassayable levels of GH and PRL.

Significance of the Accomplishments

Finding 1: The recovery of the pituitary cells from the culture vessels and subsequent electrophoretic separation indicates that the experiment carries a high probability of success, and that the anticipated enrichment of the GH cells and PRL cells will permit detailed analysis of the functionality of the FFEU. The enriched GH and PRL cell fractions are expected to yield valuable clues as to the cellular source of the hormone disturbances.

Finding 2: The successful separation of the lysate indicates that with this experiment we can begin to detail the changes in the molecular structure and function that occur when the hormone is synthesized and packaged within the cell in μg.

CELLS, EMBRYOS AND DEVELOPMENT IN SPACE

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Description of Research

The problems of development constitute a major part of the objectives of modern biology. One aim of our research effort is to develop and study systems at different levels of initial organization that will enable the effect of microgravity to be tested on the behavior of plants, in contrast or in comparison to their performance at 1-g and in ground controls. While the initial focus is aimed at the broad effects of gravity that operate on systems as they grow and develop, the systems are adaptable to a variety of more specific tests that focus on mechanisms. Any of our test systems can be used to ascertain whether there are differences in the normal rate, frequency, and pattern of cell division or in the fidelity of partitioning of the cell chromosomes during or after exposure to space flight; and, if so, to move toward understanding why these differences have occurred. All this enables us to examine what I call "genome organization and variation in plants in the space environment."

It is clear the plant genome is in a state of flux with many changes occurring during development and reproduction. Variation can occur in response to a range of external and internal influences and stresses. We have encountered examples of these nuclear responses and variations in space-grown somatic embryos, tissue-culture-derived platelets, and seedling-derived materials. The genome changes range from chromosomal perturbations of various extreme sorts such as breakage, translocations, and deletions to ones that are milder, but very significant, such as double nuclei.

A fuller characterization and understanding of the causes of these genomic changes is in order. They may occur in highly repetitive fractions of the genome and are probably limited to a specific subset(s) of DNA sequences at particular chromosomal sites. I believe that a function of these genomic variations is the generation of a radical, but limited, reorganization of the genome in response to stress situations in order to increase the pool of variability from which new types of cells can be selected or emerge. These reorganizations would be of adaptive value in microgravity. The objective in this part of the work is to study these changes in mitotic and, where possible, meiotic cycles in space-grown materials from many viewpoints, including specificity, fate, and, especially, the cause or the mechanism.

Our investigations in the past period have been directed at (1) those dealing with continued analysis and interpretation of flight data, and (2) those dealing with ground-based research. The objectives of the ground-based research include the following:

(a) A better understanding of the induction of active growth and cell proliferation and development in otherwise mature cells as they exist *in situ* and as they become established as embryogenic cultures that are amenable for use in flight experiments; and, further, as a basis for studying how the chromosomes behave, how the genome functions in development, and to examine any gravitational component. The approach we use for establishing cultures has increasingly involved working with short-term pulses of growth regulators or, in the complete absence of exogenously added growth regulators, with increased emphasis on physical factors that significantly affect the induction and progression of embryogenesis.

(b) The multiplication of embryogenic cell cultures and the production in culture of cell units and their contrasted development into unorganized masses, on the one hand, and, on the other, as somatic (non-zygotic) embryos and from these into plantlets.

(c) The growth, morphogenesis, and metabolism of intact plantlets and tissue-culture-derived propagules with their established growing regions of shoot and root, in response to interacting factors which are both environmental (i.e., different regimes of photoperiodicity and changing temperatures) and nutritional cues.

(d) The development of cytological/karyological testing protocols which have a high level of reliability for establishing detailed chromosomal characteristics and profiles for the plant species with which we are working; while, at the same time, seeking to extend the principles so gained to a still broader range of species.

(e) The management of pluripotent cell culture systems from the perspective of being able to use them effectively and with a minimum of human intervention in a space environment setting.

(f) Continued gathering and analysis of data from CHROMEX.

(g) Further analysis of Plant Cell Culture Research (PCR) from our experiment on the Spacelab J mission.

(h) Continued work for the implementation of a cell culture experiment on IML-2 (July, 1994).

Accomplishments

(1) ***CHROMEX-related findings.*** The production and growth of roots in two *in vitro* generated population categories of *Haplopappus gracilis*, which were cultivated for 5 days aboard a NASA Space Shuttle (STS-29) were analyzed in depth. Selective trimming of preformed roots was used in the experimental protocol. This selective trimming allowed production of up to 50 roots per plantlet and ensured availability of roots for study in the event of an unexpectedly curtailed period in space. Asepsis was maintained throughout the flight experiment despite the use of relatively complicated hardware. Roots were generated (a) laterally from pre-existing roots, the tips of which were severed at the time of plantlet insertion into the growth substrate; (b) adventitiously from the basal or cut ends of micropropagated shoots; and (c) *de novo* as a purely space-environment-derived generation of roots, which had its origin from roots that emerged during the period in space. Root growth occurred randomly in all directions in space. In contrast, growth was uniformly positively gravitropic in ground controls. In the spaceflight and ground controls, both population categories produced an equivalent amount of tissue when compared to each other and maintained their characteristic root production patterns. Seedling-derived plantlets produced roots which were numerous but relatively short. Capitulum-derived plantlets produced fewer roots, but they were, on average, longer than those of the seedling-derived plantlets. Thus, the clonal root phenotype was not changed in space, at least for the short duration of the experiment. However, both population categories exhibited total root production values that were 67–95 percent greater than those obtained in their ground control counterparts. We have proposed that microgravity brought about an altered moisture distribution pattern within the horticultural foam substrate that was used as the growing environment, giving a more moist and thereby more favorable environment for root formation. The detailed data for and significance of all this for space flight experimentation is covered in depth in a submitted manuscript.

(2) *The PCR experiment on the Spacelab J mission.* Embryogenic carrot and daylily cells were exposed to space throughout an 8-day flight. The flight was launched Saturday morning September 12, 1992, on the Shuttle *Endeavour*. The middeck experiment, referred to as "Gravity

and Organized Development in Aseptically Cultured Plant Cells" (*PCR*, Plant Cell Research–Experiment No. 54), was launched on Spacelab J. The objectives were (a) to evaluate whether space flight affected the pattern and developmental progression of embryogenically competent daylily and carrot cells from one well-defined stage to another, and (b) to determine whether mitosis and chromosome behavior were modified by the space environment.

The PCR experiment was a passive one and did not involve astronaut intervention. Cultures of embryogenic cells distributed on a semi-solid nutrient culture medium in metal Petri dishes were prepared at Stony Brook and transported to Kennedy Space Center. The methods employed consisted of the following: (a) Embryogenically competent cells (from a 200–400 fraction of carrot and daylily cell cultures) were grown in plant cell culture chambers obtained from NASDA. (b) Two dishes (plant cell culture dishes)—one for each species—were used to grow cells on a semi-solid agar support. (c) Progression to later embryogenic stages occurred in space via the pH-altering metabolic activity of the cells as they grew. The timing of the setup of the experiment thus was very important. (d) Progression to later stages in the case of such embryogenic semi-solid cultures did not need crew intervention. (e) Living somatic embryos were returned for continued post-flight evaluation, development, and *grow-out*.

Results indicate that somatic embryogenesis occurred during the flight. Analysis of data still is in progress. When we study the temperature profile that the cultures experienced during the mission, we will be able to ascertain whether the apparent increase in growth observed was due to the elevated temperature level of the flight environment or whether there was a real increase in rate of development. Significant alterations in the karyology of somatic embryos developed in space were found. The bulk of our analysis has thus far been carried out on the daylily, but the carrot shows similar effects as well. The responses include a substantial number in flight samples of binucleate cells among those that are normally uninucleate. The ground control samples are uniformly uninucleate (see Table 1 with partial data).

Table 1. Effect of Space Flight on Cell Division in Daylily Embryos
[Over 300,000 somatic cells scored]

Phase	Controls, %	Flight, %
Cells in division	2.9	0.5
Interpretable metaphases	32.1	11.36
Double nuclei	0	8.2
Chromosome breaks	0	1.4

Until we receive radiation dosimetry data promised from the National Space Development Agency of Japan (NASDA), it will not be possible to state categorically that radiation damage was not responsible for any of the mitotic disturbances; but, in a personal communication from Dr. S. Nagaoka, radiation levels were said to be in the expected range.

Since our methodology for karyotype analysis involves treatment of cells and somatic embryos with colchicine, we have taken care to eliminate the possibility of an increased sensitivity of the flight-exposed cells to colchicine, leading to a doubling of the nuclei. Serial sampling and examination of flight samples after recovery, beyond what we refer to as sampling 1 (i.e., the first sampling after recovery), indicates that the number of binucleate cells are diminished in some but are not eliminated. We are rearing embryos into plantlets of both flight and control samples to do followup studies. In addition to the condition of double nuclei, various aberrations in chromosome structure have been encountered as well. These will be fully quantified in due course.

An expanded version of the experiment will be carried out on IML-2 scheduled for 1994. It is anticipated that the data from both experiments will permit us to place the events of somatic

embryogenesis and cell division in space on the firm footing that it deserves. I believe that not only will answers obtained from space studies with such systems be of interest to developmental plant biologists but, also, they will have significance for those seeking to use biotechnological procedures and manipulations in space for a variety of reasons, including a Controlled Ecological Life Support System (CELSS). Moreover, the ability to use and manipulate plant cells and other kinds of propagules *in vitro* reliably in space will be a necessary prerequisite to many projected commercialization schemes.

(3) Characterization of embryogenic cells from the perspective of their origin, potential, and behavior continues to be a top priority so that these systems can be used with a high degree of reliability in a space flight context. We have devised and perfected a simple method whereby it is possible to remove a full range of units—both embryogenic structures or cells that may not have responded—non-invasively from an assay system. With the development of this simple but elegant procedure, it becomes possible to remove all the test units for examination with no perturbation whatsoever. The invasive methods used to date have been to remove embryo samples by means of forceps and to manipulate them, either singly or in small groups, depending on how many can be handled at a time. Cells could not be removed very easily.

With our method, after cells are sorted and the appropriate fraction is ready for distribution and testing, embryogenic cells are controllably inoculated onto dialysis membrane by means of a pipette. This spreading of cells on various interfaces, such as polymer membranes, films, carbon and ion exchange papers, etc., and various combinations or sequences of these, has made it possible to follow embryogenic events in much greater detail than has hitherto been possible. For example, cells grown in liquid culture under controlled gas environments have been spread on membranes in Petri dishes on various media for varying periods of time and then removed by lifting of the whole membrane and transferring to a different or challenge environment. The test parameters first scored have focused and will continue to focus on growth rate; level of development; number, size, and weight of organized units (somatic embryos, pre-shoots, etc.); the stage and morphological fidelity of their development; and the level of development of epidermis (or protoderm).

Significance of the Accomplishments

The significance of the flight data is that a clearer understanding of the effects of space flight on plant development, at least in the near-term, is beginning to emerge. Some of the seeming contradictions and disparities in various publications over the years can now be explained on the basis of use of materials that have had a specific cultural history or origin. The biological nature of the test systems, the way in which they are grown, and their predisposition, by virtue of the flight hardware and systems, to respond to the conditions of space flight (both direct and indirect) have a major impact on the outcome. Other discrepancies appear to derive from different strategies that given systems have available to adapt to perturbations from the flight environment. A series of cascading responses initiated from perturbations from the outside, which translate to the internal operations of cells, is manifest.

In the case of the behavior of the nucleus, the story seems to be more complicated, but the implications are farreaching. One can predict that, in the longer term, growth patterns of sensitive systems will be impacted in a major way. The development of plant tissues requires that a strict control of the planes of cell division be in place. The fact that what appears to be a pattern of mitotic waves without daughter cell wall development in somatic embryos indicates that the machinery to put down wall material in the successive pattern of division was perturbed. Yet, two patterns of cell division occurred in the cells of somatic embryos of daylily and carrot that were grown in space. The first is a more random, normal if you will, pattern involving uninucleate cells; the other, a successive pattern within other cells of the same complex wherein, for one reason or another, the wall is not laid down.

As for the chromosomal perturbations, one is tempted to invoke effects on the spindle but this has not yet been directly demonstrated. The role of the cortical microtubules has attracted some interest, and it is known that a cortical system which forms just before prophase (the pre-prophase band) plays an important role in determining the division planes, since later the phragmoplast (wall) grows out exactly to the site of the pre-prophase band. The microtubules cannot "memorize" the planes, since the pre-prophase band disappears before metaphase. During space flight, spindle disturbances lead to the range of nuclear aberrations encountered. The embryogenic cell culture system has shown that daylily and carrot somatic embryos can serve as a model for study of the effects of the space environment on a range of cytological or karyological events. The aseptic experiments we have done in space in NASA's Plant Growth Unit, an apparatus designed to grow small whole plants, are labor intensive to manage. Some environmental parameters, such as air flow and water delivery, so far, are complicated to measure, monitor, maintain, and document with precision. We have shown that it is more convenient and more readily controllable to use developing systems as surrogates for whole plants in certain kinds of studies.

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MECHANISM OF CONTROL OF BONE GROWTH IN MICROGRAVITY

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Description of Research

The growth and repair of bone is a complex and poorly understood process. Over the past three decades, it has been noted that astronauts lose large amounts of calcium and bone when exposed to the microgravity of space flight. The mechanism by which bone growth is regulated in not known; however, clinical observations have demonstrated that increases of endogenous cortisol, as seen in Cushing's syndrome, are associated with bone loss and osteoporosis. In the Skylab missions, urinary cortisol of the nine crewmembers increased almost two-fold. These data suggest that the glucocorticoids could also play a role in the loss of bone during space flight. The laboratory of my collaborator, Millie Hughes-Fulford (MHF), has previously shown, using osteoblast cultures, that glucocorticoids cause a decrease in endogenous prostaglandin E_2 synthesis accompanied by a decrease in osteoblast growth and mineralization. Using a cloned osteoblast cell line as a tool to study the cellular changes in growth, we have tested the hypothesis that space flight plays a role in growth regulation by testing for alterations in prostaglandin synthesis, growth, and morphology of bone cells in space flight.

Accomplishments

My collaborator (MHF) and I have selected as the bone model, the MC3T3-E1 cell line, a cloned osteoblast line that retains synthetic functions of normal bone tissue including production of alkaline phosphatase, prostaglandin E_2, and mineralized matrix containing collagen type 1 and hydroxyapatite. In these studies, we have serum-deprived, cloned osteoblasts pre-flight and added serum during space flight to test the ability of serum to stimulate osteoblast cell growth in microgravity.

(1) We developed and perfected the technique for bone cell growth on small coverslips in microgravity on the Consort 5 sounding rocket and Shuttle flight STS-52.

(2) We have flown the MC3T3-E-1 cells on STS-56 and found that mammalian osteoblasts have reduced glucose utilization in space. These results agree with the work completed on Skylab with the Hayflick WI-38 cells in 1973.

(3) Using microscopic methods, we have noted reduced cell growth in microgravity conditions.

(4) In addition, the actin cytoskeleton of the flown osteoblasts has a reduced number of actin stress fibers, an altered actin cytosketal morphology, and a changed cell morphology compared to ground control cells.

(5) We have also shown altered prostaglandin synthesis capacity of the flown osteoblasts as compared to the flight profile controls.

(6) These observations suggest reduced growth and DNA synthesis in bone osteoblasts grown in microgravity. We are currently perfecting new molecular biology methods to allow us to quantitate specific messenger RNA content in osteoblasts grown in microgravity conditions.

Significance of Accomplishments

Biomedical studies of crewed space flight have consistently indicated a loss of weight bearing skeletal bone. Various lines of evidence from humans and animals suggest that this loss is due to a lack of bone formation in the absence of gravity. Bio-mechanical properties of both cortical and trabecular bones show strength deficits that appear to represent failure of normal increase in growth rather than an accelerated loss of bone. Joint U.S./U.S.S.R. biosatellite flights of small animals have shown clear morphological evidence that bone formation is abnormal and reduced during flight.

The cause of this loss of bone has been postulated to be related to the lack of biomechanical stimulation during flight, changes in cell surface or cytoskeleton and/or due to increases in systemic hormones associated with space flight.

In order to test our hypothesis that microgravity plays a role in the regulation of bone growth, we have used an osteoblast model to test changes in cell metabolism, cell cytoskeleton, and prostaglandin synthesis. In this study, we demonstrated decreased osteoblast growth and reduced stress fibers of actin in microgravity. We are still in the process of measuring prostaglandin synthesis. Our results agree with Skylab studies that demonstrated that microgravity can cause significant decreases in WI-38 cell metabolism. Also, epi-fluorescent microscopic studies have demonstrated an alteration of actin cytoskeleton resulting in changes in cell morphology when osteoblasts are grown in microgravity. These data add to the growing evidence that microgravity directly plays a pivotal role in cell growth regulation.

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PLANT METABOLISM AND CELL WALL FORMATION IN SPACE (MICROGRAVITY) AND ON EARTH

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Description of Research

Variations in cell wall chemistry provide vascular plants with the ability to withstand gravitational forces, as well as providing facile mechanisms for correctional responses to various gravitational stimuli; e.g., in reaction wood formation. A principal focus of our current research is to precisely and systematically dissect the essentially unknown mechanism(s) of vascular plant cell wall assembly, particularly with respect to formation of its phenolic constituents; i.e., lignins and suberins, and how gravity impacts these processes. Formation of these phenolic polymers is of particular interest, since it appears that elaboration of their biochemical pathways was essential for successful land adaptation. By extrapolation, we are also greatly intrigued as to how the microgravity environment impacts "normal" cell wall assembly mechanisms/metabolism.

The following section describes recent progress related to disclosing the effect of gravity on plant growth and development. The first area describes approaches to delineate the fundamental basis of secondary cell wall assembly mechanisms (ground-based studies). The second area examines the effect of microgravity proper (Shuttle flight studies) on plant cell wall formation/metabolism.

Accomplishments and Significance

(1) ***Ground-Based Studies:*** Our most recent research endeavors have addressed development of a model system to investigate early stages of lignin/secondary cell wall formation, the delineation of the sequential steps involved in lignin assembly, and regulation of the pathway itself. The following has been discovered: (a) Using cell cultures of *Pinus taeda* (Eberhardt, *et al.*, *Journal of Biological Chemistry*, 1993), it has been possible to attain a cell line capable of undergoing a developmental-like transition from an unlignified primary wall to a lignified secondary wall (~S_1 deposition). Judicious carbon-13 labeling established that the lignin so formed was a relatively high-fidelity copy of a softwood gymnosperm lignin. We have also discovered that lignin synthesis in these cell cultures can be totally inhibited using H_2O_2 scavengers; e.g., KI (Ncse, *et al.*, *Phytochemistry*, 1994). In such instances, only the monolignols, *p*-coumaryl, and coniferyl alcohols were formed, but without lignin synthesis itself occurring. During lignification/secondary wall synthesis, it was also observed that *p*-coumaryl and coniferyl alcohols had different metabolic fates prior to polymerization (Bernards, *et al.*, *Phytochemistry*, 1994). Thus, coniferyl alcohol underwent facile dimer formation prior to lignin synthesis proper; whereas, *p*-coumaryl alcohol did not react until polymerization was initiated. (b) Using *Forsythia* species, we have shown that three types of phenol-coupling enzymes are present. The most remarkable is a hitherto uncharacterized stereoselective oxidase which catalyzes the coupling of two *E*-coniferyl alcohols to give (+)-pinoresinol (Paré, *et al.*, *Tetrahedron Letters*, 1994); this is being purified to apparent homogeneity. The other coupling enzymes are O_2-requiring lactase(s) and H_2O_2-dependent peroxidase(s), respectively, both of which afford racemic products.

Significance: It has long been known that lignins vary with cell type and species and that gravitational corrections result in altered lignin compositions in the tissues so affected; e.g., a higher *p*-coumaryl alcohol content. Our recent findings now provide a means to systematically define how these compositional changes are regulated; i.e., what factors differentially control

individual monomeric synthesis and how precise mechanisms of assembly are controlled in different cell types. A detailed understanding of such processes is essential if we are to define how lignin synthesis is induced during both "normal" and "gravitational correctional" developmental processes.

(2) ***Space Shuttle Studies***: Previous difficulties in growing plants in space have been partially overcome by development of a nutrient agar pack (Heyenga, *et al.*, 1994) suitable for the uninterrupted growth/development of plants in space (Figure 1). This obviated the need for sporadic replenishment of water/nutrients, the fluid parameters of which have been extremely difficult to control in microgravity.

1. Polypropylene envelope
2. Gas diffusion membrane
3. Seed support matrix
4. Support column
5. Air phase
6. Germination medium
7. Basic nutrient medium
8. Enriched medium

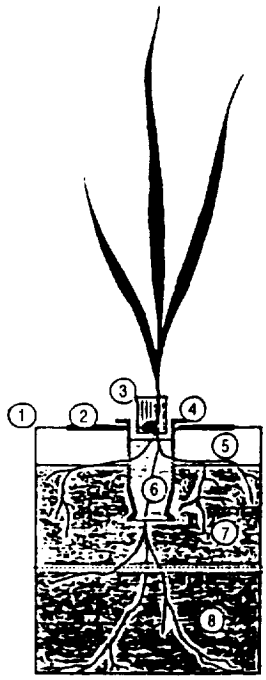
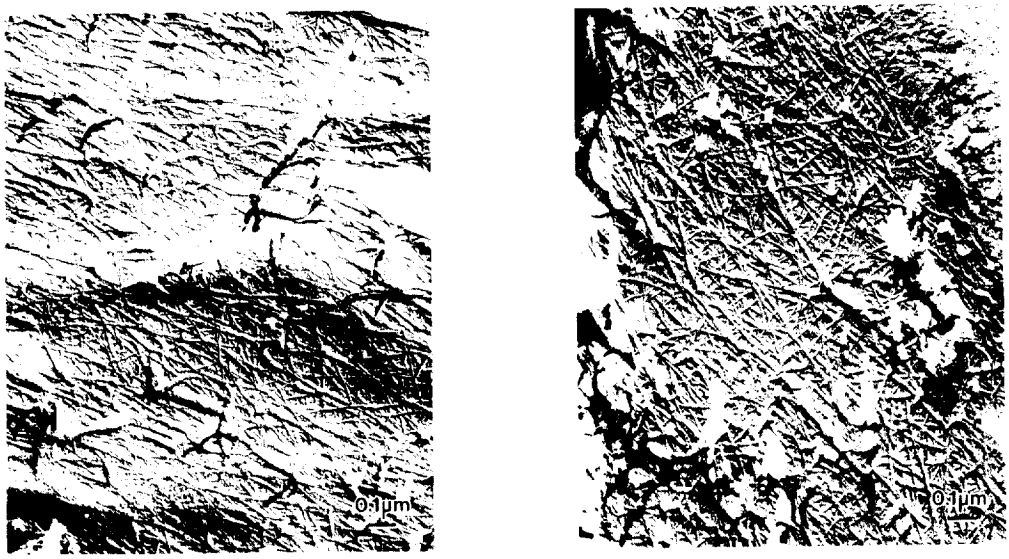


Figure 1. WSU "Nutrient Pack."

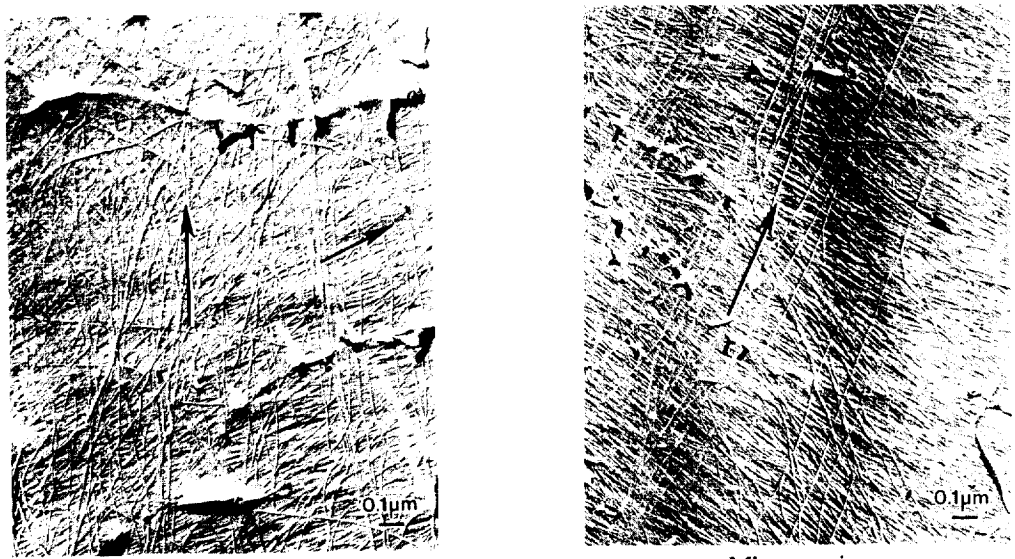
Using this approach, it was found that T. *aestivum* wheat seedlings in the nutrient pack grew very well in space with little differences observed in shoot and root tissue growth/fresh weight over the 10-day flight when compared to the corresponding ground controls. Moreover, electron microscopic examination of cross-sections of serial sections of wheat shoots and roots revealed essentially no differences. That is, the organelles in the cytosol were very similar, and in the root cell walls there were no measurable changes in the cellulose microfibril orientation/cell wall thickness as revealed by freeze-fracture/transmission electron microscopic (TEM)/scanning electron microscopic (SEM) analyses (Figures 2 and 3). This latter observation is particularly important, since it strongly implies that either the process guiding microfibril orientation is fully sensitive to even a microgravity stimulus, or it occurs independent of the g-force experienced.



Panel A: 1-g control

Panel B: Microgravity

Figure 2. Freeze-fractured and deep-etched image of the outer surfaces of the parenchyma cell wall of wheat primary root after methylamine treatment.



1-g control

Microgravity

Figure 3. Inner surfaces of parenchyma cell wall (see Figure 2 for other details). Arrows show orientation of cellulose microfibrils.

Significance: This is the first data set, of which we are aware, where excellent growth, relative to the 1-g controls, was attained in space over a relatively long duration. Importantly, cell wall assembly was not significantly affected; these results are in direct contradiction to previous studies in which plants, it can now be hypothesized, suffered from various stresses in microgravity. Consequently, these results underscore the growing need for development of reliable plant growth systems for the microgravity environment. Note also that the greater significance of these findings must await more detailed chemical and biochemical analyses.

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DEVELOPMENTAL AND PHYSIOLOGICAL PROCESSES INFLUENCING SEED PRODUCTION IN MICROGRAVITY

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Description of Research

To date, the growth of plants in space has been poor. A particularly sensitive time in the life cycle of a plant growing in microgravity seems to be the transition from the vegetative to the reproductive phase. Most plants grown full term in space failed to produce any seed at all; and, in a Soviet experiment in which seeds were produced, the seed quality was poor. Dosimetry readings taken in flight have failed to explain this ubiquitous sterility in terms of a radiation load; thus, some developmental failure seems to be triggered by the microgravity environment itself.

Reproductive events in angiosperms have a number of stages that could potentially be influenced directly by gravity. Pollen production, formation of the embryo sac, pollination, and fertilization are all complex developmental events. No studies exist tracking the success of any of these processes in microgravity.

Aside from the possibility of direct effects of microgravity on seed set events, secondary effects are possible through changes in the space environment which may be brought about in microgravity. For example, poor root zone aeration caused by the lack of directed water movement in microgravity might restrict root respiration to such an extent that metabolites and hormones necessary for successful development during the reproductive cycle would not be available. As a second example, restricted gas exchange in the aerial portion of space flight plants might lead to a buildup of volatile products which could have a negative influence on reproduction. Simultaneous monitoring of developmental and physiological events during the reproductive cycle of plants in space is necessary to determine whether such a relationship might exist.

In this project, microgravity effects on developmental and physiological processes affecting seed set events are being probed with flight experiments. *Arabidopsis thaliana* (L.) Heynh., a cruciferous plant used in molecular biology experiments, has been selected for the flight experiments because of its short life cycle, minimal light and space requirements, and history of utilization in flight experiments by the Soviets. A full life cycle is not possible on a 6-9 day Shuttle flight, so our initial research focuses on early events in seed production. Plants for the flight experiments are loaded into the Plant Growth Unit (PGU) flight hardware at preflowering stage. During exposure to microgravity, pollen and embryo sac development, fertilization, and early embryogenesis occur. The success of these processes in microgravity is determined by comparison of the flight plants with ground control plants using ultrastructural and physiological analyses.

Accomplishments

Work has been closely geared to the successful completion of the first flight experiment, SEEDEX (Chromex.03), which flew on STS-54 in January of 1993. Preliminary observations suggest that flowers were aborting at an early stage of development. Anomalous root tip morphology was also observed.

(1) At Kennedy Space Center, we successfully completed two Science Verification Tests (SVTs) of the procedures to be used in the flight experiments. Material from these studies has served as a preliminary ground control for our flight material.

(2) In January of 1993, we successfully performed the flight experiment, SEEDEX (Chromex.03), on STS-54. Plants flowered during the 6-day flight, and reproductive material was fixed immediately upon return to the ground. This material is now being sectioned and analyzed in our laboratory.

(3) We have adapted our procedures to accommodate a longer duration experiment (9 days) on STS-51, which will be part of Chromex.04. Procedures were also developed which allow for delay and in-flight activation of *Arabidopsis* for a long-duration seed-to-seed experiment planned for *Mir*.

(4) Ground-based studies have investigated embryo sac and pollen development in *Arabidopsis thaliana* and the role of oxygen in pollen development.

Significance of the Accomplishments

In the material collected from the first flight experiment, flowering was apparently aborted at an early stage. Through comparison with ground control material, it should be possible to ascertain which stage(s) of early reproductive development are sensitive to microgravity.

The agar medium which is used in our growth protocol provided intact root systems for postflight observation and analysis. This will be useful for both morphological and physiological studies.

By developing a growing procedure that permits the utilization of preflowering plants in the PGU, we have extended the flexibility of this flight hardware to use in all stages of the plant life cycle. It should now be possible to address microgravity effects on all stages of plant growth and development if the subject plant used is small and possesses a rapid enough life cycle.

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SKELETAL COLLAGEN TURNOVER BY THE OSTEOBLAST

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Description of Research

Among the most overt negative changes experienced by man and experimental animals under conditions of weightlessness are the loss of skeletal mass and attendant hypercalciuria. These clearly result from some disruption in the balance between bone formation and bone resorption (i.e., remodeling) which appears to be due to a decrease in the functions of the osteoblast. In the present studies, the clonal osteoblastic cell line, UMR 106-01, has been used to investigate the regulation of collagenase and tissue inhibitors of metalloproteases (TIMPs). This project has shed some light on the comprehensive role of the osteoblast in the remodeling process and, in so doing, provided some insight into how the process might be disrupted under conditions of microgravity.

Cloning of Rat TIMP-2. We, together with Dr. John Jeffrey, purified the rat collagenase inhibitory activities secreted by the UMR 106-01 cells and showed them to be two TIMP molecules of 20 and 30 kDa. Amino-acid sequence of the inhibitor molecules from medium conditioned by the rat UMR cells showed that one of these is clearly homologous to TIMP-1 in other species. The second, and predominant, inhibitor molecule produced by UMR 106-01 cells has been identified as rat TIMP-2, by virtue of its identity to human TIMP-2 in the amino terminal region. We screened a rat genomic library with several different degenerate oligonucleotides and obtained a positive clone. However, in addition, we obtained a human TIMP-2 cDNA, which successfully hybridized to RNA from UMR cells. This indicated that there was enough sequence homology between the two sequences to use it as a probe to clone rat TIMP-2. We used this to screen our UMR cDNA library and obtained numerous positive clones. These were then sequenced, and it was confirmed that we have obtained a rat TIMP-2 clone which has 98 percent amino acid sequence identity with human TIMP-2.

Regulation of Collagenase Gene. We previously demonstrated that stimulation of collagenase by parathyroid hormone (PTH) involves a substantial increase in collagenase mRNA via transcription. We have also concluded that this effect occurs primarily through the cAMP-dependent pathway in UMR cells and requires the expression of other genes. Our next goals are to identify the regulatory regions of the rat collagenase gene. To do this, we have obtained a genomic clone containing 6.5 kbp of sequence 5' of the transcriptional start site. This region has been subcloned upstream of the reporter gene, and chloramphenicol acetyl transferase (CAT), and processive deletions have been made to determine the minimum amount of sequence necessary for PTH induction. The largest of these constructs has been transfected stably into UMR cells and will be characterized. These cells could be used in flight experiments to assess changes in the transcriptional rate of the collagenase gene under microgravity conditions.

To assess the signal transduction pathway involved in PTH regulation of collagenase gene expression, we investigated changes in immediate early gene expression after PTH treatment. We demonstrated that c-*fos* and c-*jun* mRNA levels are increased 20-fold and 2- to 5-fold 30 min after addition of PTH. These effects precede the changes in transcription of collagenase, 2- to 5-fold suggesting that these transcription factors may be the proteins which induce transcription of the collagenase gene.

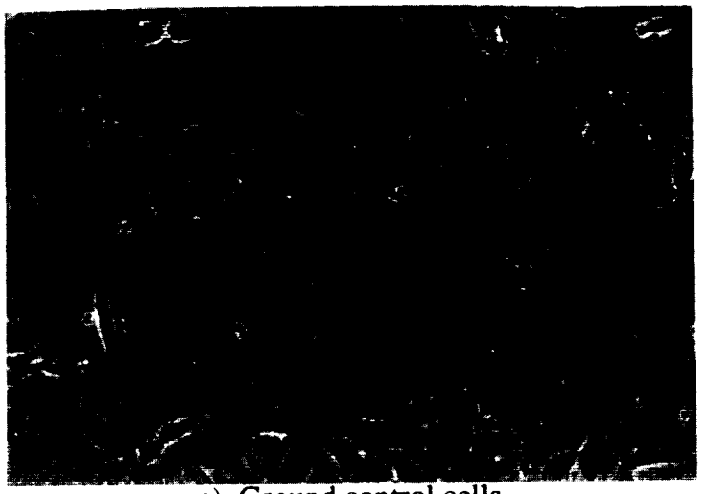
Bone Cell Research (BCR) Experiment on Spacelab-Japanese (SL-J) Mission. We were recruited to Spacelab-J in November 1990 and asked to test whether our rat osteoblastic cells, UMR 106-01, would adhere and proliferate normally in the NASDA cell culture hardware for the Spacelab-J mission. After many preliminary growth experiments, we found that, with some manipulations, cells are viable, and will grow and generally appear normal throughout the period which was planned for space flight using this hardware.

Many other preflight experiments were also conducted in a very short time. For instance, to alleviate the problem of lack of CO_2 in the Spacelab incubator, we tested, and found suitable, a CO_2-independent medium from Gibco. The cells were also tested to determine whether they would resist the effects of vibration due to liftoff and showed no problems. We also demonstrated that the hormone we were to use, PTH-related peptide, is quite stable to freezing, thawing, and incubation at 4° C for an extended period of time. We also examined the stability of collagenase in medium at various temperatures and resolved that the enzyme remains unchanged at 15° C for 4 days, while at 25° C, the enzyme is stable for 24 and 48 h, but is degraded by incubation at this temperature for 72 h. These criteria established our limitations for storage of our collected media samples on the Shuttle. Many Enzyme Linked Immunosorbent Assays (ELISA) were conducted to test each of these parameters. In May 1992, Cheryl Johanns, my technician, was able to participate in a science simulation at Kennedy Space Center (KSC) which involved handover and scrub turnaround procedures. It was only at this time that we learned the protocol for cleaning and seeding the chambers from the Japanese investigators. Before this, we had only been provided with very limited written notes on the use of the chambers, which had led to us developing procedures that may have not been optimal.

At about the same time, the flight chambers were sent to us to bio-condition and were used in a full simulation in the lab. We also sent chambers with growing cells for a crew simulation in which media exchange and photography were done. In the lab we conducted numerous photography sessions to determine the methods that should be followed for good photomicroscopy of the cells.

Launch was set for September 11, 1992. The launch was only a day late (with plenty of notice), and most of the flight went smoothly. The Shuttle landed at KSC, which meant we received our samples within 3 hours of landing and they were in very good condition. The limitations on the amount of flight-certified hardware kept us from conducting the best ground controls. We conducted further ground control experiments in the same flight chambers used on Spacelab-J, since the ones performed during Spacelab-J were not in optimal hardware. One of the astronauts misinterpreted the instructions on the first medium exchange, which led to many mitotic cells being sloughed off the chamber surface. This resulted in far less cells being present than we anticipated. This has made it difficult to measure collagenase produced in the medium, since the cell-to-medium ratio is so low. We have found methods of concentrating the media so that we can still measure the enzyme by our ELISA. We will also measure cellular proteins such as alkaline phosphatase in the cellular lysates.

The photos taken by the astronauts are extremely good and we think there are slight differences in the morphology of the flight cells compared to our ground controls (Figure 1).



a) Ground control cells.

b) Flight cells.

Figure 1. UMR cells cultured in NASDA chambers.

Overall, we would have to conclude that this hardware has severe limitations. The large size limits the number of chambers which can be tested, the syringes are not ideal for media exchanges, and the surface must be treated for use by various cells. Our general experience with flight experiments is that more time is needed between experiment acceptance and flight so that best use of the hardware can be determined sooner, along with complete training of the astronauts.

Accomplishments

(1) Cloned rat TIMP-2.

(2) Used our genomic clones for rat collagenase to make collagenase promoter/CAT constructs.

(3) Isolated UMR cells stably transfected with one of our collagenase promoter/CAT constructs.

(4) Showed that changes in immediate early gene expression, *c-fos* and *c-jun* correlate with changes in collagenase.

(5) Conducted the flight experiment, Bone Cell Research, on Spacelab and showed a change in morphology of osteoblastic cells during flight.

Significance of the Accomplishments

Finding 1: Shows that rat TIMP-2 has close similarity to those identified in other species. The clone will enable us to examine regulation of this gene in osteoblastic cells.

Finding 2: These constructs will allow us to determine the cis and trans elements regulating the collagenase gene.

Finding 3: These stably transfected cells will be characterized and could be used in future flight experiments.

Finding 4: Supports the notion that Fos is the mediator of PTH action causing the induction of collagenase. This will lead us to determine how nuclear proteins activated by protein kinase A (PKA) may regulate expression of Fos.

Finding 5: Suggests that microgravity causes slight retraction of osteoblastic cells from the substratum.

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CELL KINETIC AND HISTOMORPHOMETRIC ANALYSIS OF MICROGRAVITATIONAL OSTEOPENIA

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Description of Research

Past studies of cell differentiation supporting bone formation have shown that osteoblast (bone forming cell) histogenesis is inhibited by decreased skeletal loading and microgravity. To date, analysis of rat maxillary molar periodontal ligament (PDL) from multiple flight experiments (Cosmos 1129, Spacelab-3, Cosmos 1187 and Cosmos 2044) has suggested that the recovery of osteogenic potential following space flight is of high priority. The continued goals and objectives for research in this area are to (1) further define the cellular regulation of osteoblast production, (2) determine how this process is suppressed in microgravity, (3) continue to assess the recovery of osteoblast histogenesis following return to 1-g, (4) begin to assess preosteoblast differentiation *in vitro* using a precisely defined mechanical loading paradigm, and (5) initiate *in vitro* molecular biological studies of mRNA expression for steroid hormone receptors in cultured osteogenic cells.

Using kinetically and/or morphometrically distinguishable cell compartments, we have described the following osteoblast histogenesis sequence: (1) self-perpetuating, less differentiated precursor cells (A type); (2) committed osteoprogenitor cells (A' type); (3) nonosteogenic cells (B type); and (4) preosteoblasts (C and D type). The osteoblast (Ob) histogenesis sequence is AÆ A'Æ CÆ DÆ Ob (Figure 1). The increasing nuclear volume (A'Æ C) is believed to be a morphological manifestation of change in genomic expression (differentiation) indicative of maturation of osteoblast cells. The nuclear volume assay appears applicable to all skeletal sites tested; i.e., periodontal ligament (PDL), tibial metaphysis, mandibular condyle, and mandibular periosteum.

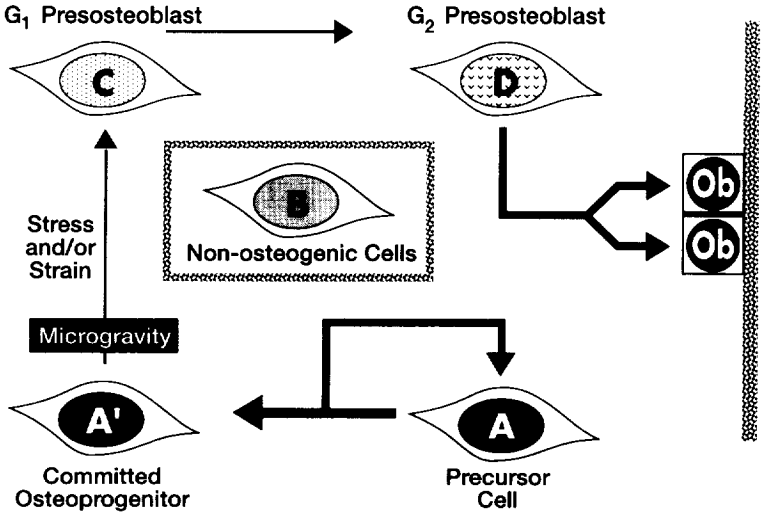

Figure 1. Schematic depiction of osteoblast histogenesis model.

The rate-limiting osteogenic induction step (A'Æ C) is stimulated by mechanical force and appears to be inhibited following exposure to microgravity. To date, the kinetic distinction of these cell populations has required the use of ^{3}H-thymidine, a radioactive tracer for DNA synthesis. During a previous accomplishment period, the ability to utilize 5-bromo-2'-deoxyuridine (BrdU; a nonradioactive marker for DNA synthesis) was initially demonstrated.

Research conducted in the past year has included both flight projects and ground-based preflight experimental development. This work has specifically focused on (1) examination of rat maxillary PDL and mandibular condyle specimens flown aboard the Space Life Sciences-1 mission (SLS-1); (2) verification of 5-bromo-2'-deoxyuridine as a nonradioactive, immunohistochemical labeling technique for cellular DNA synthesis; and (3) assessment of mechanical induction of preosteoblast differentiation, *in vitro*, using a precisely defined mechanical loading paradigm.

Accomplishments

(1) SLS-1 was a 9-day space flight with a 2- to 7-hour recovery period at 1-g. Immediately postflight (R+0), no differences were seen in PDL osteoblast precursor cells (Figure 2) from flight (F) and control (C) rats housed in both the Animal Enclosure Modules (AEM, group housed) and Research Animal Holding Facility (RAHF and Viv-S,[1] individually housed).

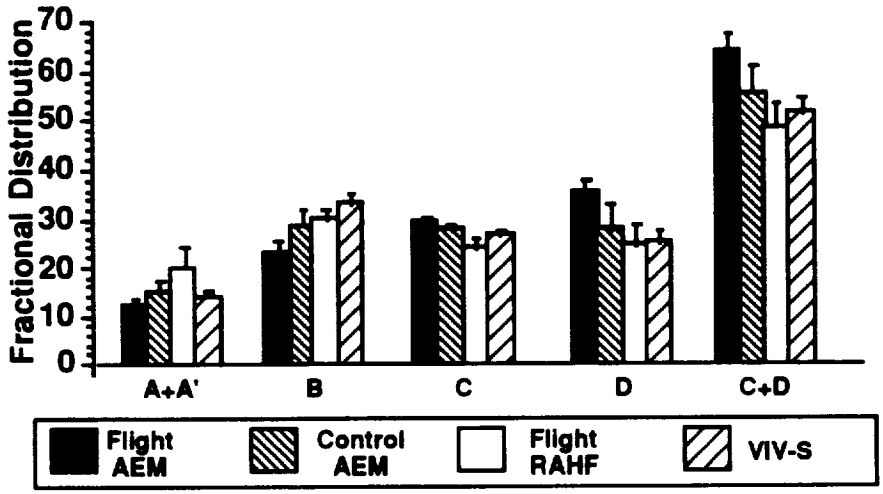

Figure 2. Distribution of PDL osteoblast precursor cells from ground-based controls and SLS-1 flight animals.

(2) PDL osteoblast precursor cell kinetic compartments data from SLS-1 control animal groups do not match previous control results (abnormally depressed A+A' and elevated C+D cells).

(3) Comparison of SLS-1 histomorphometric data (volume percent) from mandibular condyle bone and cartilage likewise revealed no significant differences between flight and ground-based control groups (Figure 3).

[1]Viv-S is the acronym for the vivarium animals that were used as synchronous controls for the F-RAHF.

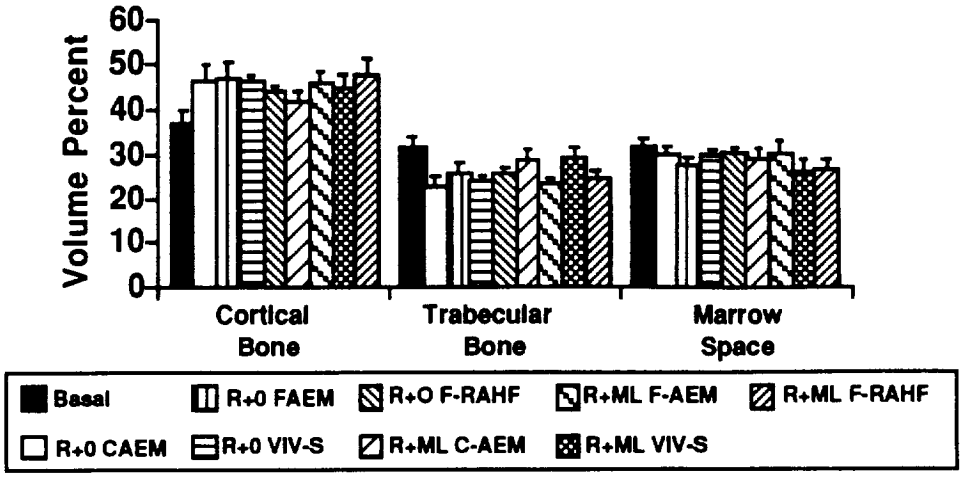

Figure 3. Comparison of SLS-1 mandibular condyle histomorphometric volume percent data for cortical bone, trabecular bone, and marrow space from individually and group-housed flight and control groups. No significant differences were observed.

Significance of the Accomplishments

Findings 1 and 2: The lack of a significant flight effect is disturbing. Both the control data and the apparent lack of a significant flight effect are inconsistent with previous results. The data suggest a block in proliferation of both preosteoblasts and less-differentiated precursor cells in flight and control animals. The reason for this block is not clear. Further data will be gathered from the PARE.03B experiment flown in April 1993.

Finding 3: Previous data describing the effect of microgravity on rat mandibular condyle (Spacelab-3) indicated a diminished magnitude of condylar growth and differences in the distribution of cortical and trabecular bone. The results of the present experiment do not confirm the previous results. Further data will be gathered from the PARE.03B experiment flown in April, 1993.

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[1]Viv-S is the acronym for the vivarium animals that were used as synchronous controls for the F-RAHF.

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MICROGRAVITY EFFECTS DURING FERTILIZATION, CELL DIVISION, DEVELOPMENT AND CALCIUM METABOLISM IN SEA URCHINS

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Description of Research

The long-term goal of this research is to explore the role of microgravity during fertilization, cell division, and early development on cytoskeletal organization and skeletal calcium deposition in a model developmental system: the sea urchin. Gravity has already been shown to affect bone calcium, and it has been reported that it alters fertilization and embryogenesis in several developmental systems. The egg at fertilization performs virtually all the events of any higher cell, many of which involve spatially and temporally separated sequestration of calcium. The metabolic activation of the unfertilized egg is triggered by an intracellular release of calcium which also initiates the secretion of cortical granules leading to a modification of the cell surface. Sperm incorporation is mediated by an eruption of egg microvilli formed by the assembly of microfilaments. Once the sperm has entered the egg cytoplasm, the dynamic assembly of microtubules on the base of the sperm head is responsible for uniting the genomes of sperm and egg. Cell division, involving the chromosomal movements during cytokinesis, also requires the sequential activity of microtubules and microfilaments. Though the precise mechanisms are still unclear, intracellular membranes, competent to sequester and release intracellular calcium, are the primary regulatory systems of the mitotic events. Most striking is the deposit of calcium in the spicules of sea urchin embryos that occurs as a result of the deposition of calcium carbonate on an organic matrix. The utility and speed of this example of the formation of extracellular skeletal array will serve as a significant model in which to evaluate the effects of gravity on calcium deposition, particularly with quantitive analytical scanning transmission electron microscopy. Notwithstanding the global importance of gravity on development, it has only recently been possible to begin to design experiments which will directly investigate the specific effects of this vector. The objectives for these studies are met by developing specific molecular probes, to identify specific structures and analyze their formations under terrestrial and microgravity conditions. Monoclonal antibodies were raised to characterize cytoskeletal components such as microtubules, microfilaments, and centrosomes. The use of these molecular probes, together with valuable collaborations with the NASA space program to design the most suitable conditions for experiments on the Space Shuttle, have enabled us to gain insights into normal developments. By extrapolating our present techniques to the conditions on the Shuttle Orbiter, we will further the understanding of basic events during fertilization, cell division, and development, as well as answer critical questions regarding the space biology of cytoskeletal and skeletal organizations.

Accomplishments

(1) We have used a variety of monoclonal antibodies for the characterization of microtubules (designated E7) and centrosomes (designated Ah-6 and 4D2), as well as polyclonal antibodies raised in rabbits, to characterize microtubules and centrosomes, and human antibodies to characterize centrosomes (designated 5051 and SPJ). DNA could be detected with the commercially available fluorescent dye DAPI. These probes allowed us to document the dynamic microtubule reorganizations necessary for the union of sperm and egg nuclei, and for the formation of the mitotic apparatus, the machinery for cell division. It also allowed us to gain insights in the highly dynamic interactions between centrosomes and microtubules. Centrosomes are the major

organelles in the cell for microtubule organizations. By using inhibitors of microtubule formation, such as colcemid or griseofulvin, we could demonstrate that microtubules, are important for structural changes of centrosomes. In the absence of microtubules, centrosomes were unable to unfold. Additionally, it was demonstrated that chromosomes did not undergo decondensation as required during cell division. Another inhibitor of metabolic processes was used to interfere with calcium metabolism. Membrane fusions require proper calcium concentrations. When dithiothreitol was used (which is suspected to interfere with metabolic processes requiring calcium) membrane fusions as necessary for pronuclear fusion and cell division did not occur.

(2) Major efforts during the past 2 years were devoted to identify the most suitable biologically compatible material and hardware to allow for optimal conditions for the conduct of experiments on the Space Shuttle. Successful collaborations with MPB Technologies, the Canadian Space Agency, and the Kennedy Space Center have led to tests determining the compatibility of constructed materials with sea urchin gametes, ability to maintain life support parameters, proper gamete ratios and volumes to attain fertilization, proper fixation ratios and volumes to preserve specimens for post-flight analysis, and reliability of dilution and mixing hardware components. Several prototypes of hardware were tested. The materials tested for biocompatibility were Silastic, Eccosil 5877 and Teflon. Teflon was chosen to give the most reliable results, and was therefore selected for experimentation on a 0-g KC-135 test flight. The results of the test flight concluded that sperm and eggs do not mix readily under microgravity conditions and devices should be designed which would allow for enforced mixing of both gametes.

Significance of the Accomplishments

The work with the antibodies directed towards microtubules and centrosomes and dyes to detect DNA employing fluorescence microscopy has led to major insights into the complicated and intrinsic interactions of cytoskeletal organizations and reorganization during fertilization, cell division, and further development. Proper calcium concentrations are required for the formation of the cytoskeleton and membrane fusion and for calcium deposition in the spicules during later development. Investigations of these fundamental biological calcium sequestration processes will lead towards understanding the remarkable loss of skeletal calcium by astronauts and cosmonauts, which can amount to up to 460 mg/day. The experiments using sea urchins conducted under microgravity conditions will help us understand problems affecting human health, including osteoporosis. The significance of the research is rather farreaching and may have important implications in understanding the skeletal problems associated with space travel, as well as aging on Earth. New information on fertilization and cell division may well provide useful approaches to solving problems of infertility, contraception, and abnormal cell proliferation during cancer. The development of hardware during the conduct of these experiments will provide improved living conditions for developmental systems. The hardware designs invented in collaboration with MPB Technologies, the Canadian Space Agency, and the Kennedy Space Center, with the goal to culture live cells in space, are reusable and will contribute to further technical developments.

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STL.01 AND STL.02 EXPERIMENTS
IMMUNOLOGY

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Description of Research

The purpose of the STL.01 experiment was to test the ability of the Space Tissue Loss (STL) tissue culture module designed by the Walter Reed Army Institute of Research to support growth of macrophage cell lines in flight. The STL.02 experiment was a repeat of the STL.01 experiment. Our experiments involved the study of the effects of flight on two cell lines of immunologic importance. We wished to determine if space flight using the STL module would result in alterations in immune function of P388D1 cells (a murine macrophage line) and would alter the ability of HL-60 cells (a human stem cell line) to differentiate into macrophage-like cells.

Accomplishments

On the STL.01 experiment, unforeseen technical difficulties were encountered. The temperature on the rail containing only our cells was not regulated properly and remained at 39°C throughout the flight. In addition, medium supplements to stimulate the cells were not released during the flight. As a result of these difficulties, we lost all of our P388D1 cells. Nevertheless, we were able to recover HL-60 cells and tried to stimulate them to differentiate into macrophage-like cells (Table 1). Differentiation was not observed (Table 2), and the nutritional content of the medium was analyzed by Dr. David Epstein of Life Sciences Technologies to ensure there was cell growth during flight (Table 3).

TABLE 1
CELL YIELDS AFTER RECOVERY
(STL.01 FLIGHT)

CELL LINE	INOCULUM	YIELD
P388D1	50,000 cells/cartridge	No viable cells
HL-60	100,000 cells/cartridge	5,000 cells

TABLE 2
DIFFERENTIATION OF HL-60 CELLS FOLLOWING STIMULATION WITH VITAMIN D3 AFTER RECOVERY
(STL.01 FLIGHT)

SAMPLE	NUMBER OF DIFFERENTIATED CELLS	
	(-) VITAMIN D	(+) VITAMIN D
GROUND CONTROLS	0/600 (0%)	382/600 (64%)
FLIGHT SAMPLES	0/600 (0%)	0/600 (0%)

TABLE 3
UTILIZATION OF SELECTED MEDIUM COMPONENTS BY HL-60 CELLS
(STL.01 FLIGHT)

Medium Component	Unused DMEM	Ground Control	Flight Sample
GLUCOSE (g/l)	4.50	3.55	4.08
GLUTAMINE (mg/l)	584.00	433.70	115.10
ARGININE (mg/l)	69.60	73.50	47.20
HISTIDINE (mg/ml)	31.20	29.76	23.60
METHIONINE (mg/ml)	30.00	23.80	24.30
TRYPTOPHAN (mg/ml)	16.00	12.40	11.70
LYSINE (mg/ml)	117.10	97.80	98.80

For the STL.02 experiment, the flight module functioned without major problems; however, an apparent central source of fungal contamination in the media flow path of the STL apparatus caused contamination of both our P388D1 and HL-60 cells, as well as other cell lines that were flown by other investigators. It was apparent that the P388D1 cells and the HL-60 cells had grown and survived in the STL module during flight, but further analysis of the data was impossible because of the fungal contamination. The conclusion from this experiment is that the STL module can support the growth of our cells in flight but still had teething difficulties.

Significance of the Accomplishments

The results of the current study suggest that space flight can inhibit the differentiation of precursor cells to mature macrophages. This result is in accordance with previous human and animal studies which suggest that the macrophage is a key cell in space-flight-induced immune dysfunction. The current study suggests a possible mechanism for the effects seen *in vivo*. It must be noted that this study has been performed successfully only once on STL in an experiment fraught with technical problems with the STL module. Repetition and confirmation are required.

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SKELETAL MUSCLE METABOLISM IN HYPOKINETIC RATS

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Description of Research

This work has concerned the mechanisms of atrophy and metabolic alterations associated with the lack of load-bearing (unweighting), which may occur with prolonged bedrest or weightlessness. For these studies, young rats were used to assess the potential role of gravity in growing muscles.

Our general approach has been to utilize both unweighting and denervation (interrupted nerve supply), to better understand atrophy in general. We have found important differences between unweighted and denervated muscles, and such comparisons have proved to be very fruitful. Other more specific comparisons have led to successful results during this time period. Most important was the opportunity to compare muscle atrophy and glucose transport data from flight and ground-based, hindlimb-suspended animals. Secondly, we have made further use of our ability to compare *in vitro* and *in situ* (intramuscular injections) approaches to evaluate the physiological significance of our findings. Finally comparisons of the myofibrillar (structural) and sarcoplasmic (non-structural) protein responses to unweighting have helped to further complete the picture.

Accomplishments

(1) **Flight experiment.** (a) Soleus (back of leg) of flight animals atrophied to a similar extent as that of hindlimb-suspended animals. (b) Plantaris and gastrocnemius (back of legs) of flight animals showed slower growth, just as these muscles did in hindlimb-suspended animals. (c) Extensor digitorum longus and tibialis muscles (front of the leg) showed no response from flight, just as on the ground. (d) The soleus, but not the extensor digitorum longus muscle, showed increased glucose transport in the presence of insulin after flight, just as after hindlimb suspension on the ground. (e) Space flight increased the interstitial fluid volume of the soleus muscle, as occurred in the soleus of hindlimb-suspended animals.

(2) **Myofibrillar vs. sarcoplasmic proteins.** (a) Loss of protein during unweighting atrophy of the soleus is due to a diminished pool of the structural (myofibrillar) proteins. (b) Disappearance of myofibrillar proteins is due to slower formation and accelerated destruction. (c) The pool of sarcoplasmic proteins remains constant during unweighting atrophy. (d) Sarcoplasmic proteins are spared owing to a slowing of their rate of destruction.

(3) **Physiological significance studies.** (a) Following intramuscular injection with insulin, unweighted muscles take in more glucose than do normal soleus muscles. (b) Insulin-like growth factor-1 has similar effects as insulin on glucose uptake by the unweighted soleus following *in vitro* incubation intramuscular injection. (c) Catecholamines show enhanced effects on the unweighted soleus *in vivo* just as *in vitro*.

Significance of the Accomplishments

Flight Experiment. The most critical significance of the flight experiment was our demonstration the hindlimb unweighting model used in our and numerous other laboratories can reproduce the changes in muscle size and biochemistry which occur in space. The ground-based studies support the notion that the major site of protein destruction with unweighting atrophy occurs in the cytoplasm. The parallel results from spaceflight suggest that a similar mechanism is

true, at least for atrophy of the soleus of young animals. In space, as on Earth, unweighting produces selective effects on muscle size, depending on the particular physiological function of each muscle.

Myofibrillar vs. Sarcoplasmic Proteins. Unlike denervated muscle, which loses all proteins at a constant rate, the unweighted soleus loses only the myofibrillar (structural) proteins. Thus, a significant amount of protein (sarcoplasmic pool) in the unweighted muscle is spared from such loss. These sarcoplasmic proteins include those on the membrane which are responsible for attracting hormones to the cell surface (receptors) or which are involved in transport processes (e.g., for getting glucose into the cell). As the slowing of myofibrillar formation precedes the destruction of these proteins, the two processes are clearly controlled independently by unweighting. It is of further interest that one pool of proteins undergoes accelerated destruction, while the sarcoplasmic proteins show slower destruction. This supports the idea of separate compartments in the cell for protein destruction, which is under independent control.

Physiological Significance Studies. The development of the ability to study biochemical responses by intramuscular injection afforded the opportunity to test the physiological significance of our *in vitro* findings. The discovery of the greater effect of insulin and catecholamines on metabolic processes in the unweighted muscle led to the conclusion that there is a sparing of the receptors on the cell surface, in accord with the sparing of sarcoplasmic proteins. Our ability to demonstrate this phenomenon following intramuscular injection and, therefore, under physiological conditions shows the *in vivo* significance of these results.

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SOFT DENSE FIBROUS CONNECTIVE TISSUE RESPONSE TO HINDLIMB UNLOADING

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Accomplishments

The connective tissue research program has devoted a considerable amount of time in demonstrating that collagen plays a major role in the plasticity of muscle. In particular, the maturation rate and compartmentalization of collagen are affected by load and the rate of muscle growth. Collaboration with the Mayo Clinic (see publications by Gosselin, et al.) clearly demonstrates that load during growth and development plays an important role in affecting the maturation rate of muscle type I collagen, thus effecting passive tensile properties of muscle and supporting the hypothesis that unloading has the potential of reducing passive muscle stiffness, which makes the muscle more likely to tear or damage during eccentric loading.

A second major accomplishment is that the flight studies on COSMOS 2044 report that animal age and endocrine status alter the responsiveness of bone to microgravity. In particular, the skeletally mature rat is less responsive to microgravity as compared to the rapidly growing rat. In addition, there were significant changes in bone microstructure and geometry of growing animals exposed to 2-g. Therefore, animal endocrine status (age) modifies significantly the adaptive potential of bone to various stresses.

Future Goals

The program will continue to study bone plasticity and address adaptive potential to stress by using the dwarf rat model and growth hormone supplementation as a countermeasure. Also, muscle studies are being proposed to actually test the passive material properties at a time where there are significant collagen changes. In addition, we have adapted some microtechniques to adequately determine the resorption rate of type I collagen by measuring the pyridinoline levels in various body fluids of the rat. This marker work has been applied to major clinical studies of primates and humans.

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EARLY DEVELOPMENT OF A GRAVITY-RECEPTOR ORGAN IN MICROGRAVITY

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Description of Research

Adult and embryonic Japanese red-bellied newts, *Cynops pyrrhogaster*, will be flown on the International Microgravity Laboratory-2 Shuttle mission. Our project on this flight concerns the effects of rearing in microgravity (μg) on the development of both the gravity-sensing and angular-acceleration sensing portions of the inner ear. Adult female newts, which were inseminated in the wild the previous fall, will be injected with hormone to induce fertilization and egg laying. Thus, we will be able to observe development of embryos fertilized in μg. Later-stage fertilized eggs will be reared in μg, starting either before the inner ear begins to develop or before the start of production of the stones (otoconia) in the gravity-sensing organs. Our work in the past year has documented the normal developmental sequence and schedule of the inner ear, which had not previously been performed systematically in any amphibian. We have also studied the form and crystal pattern of the otoconia and performed behavioral studies of the reactions of larvae at several developmental stages to transient μg during parabolic flight.

Accomplishments

Published descriptions of the embryonic development of *Cynops*, as well as many other amphibian species, fail to mention the otolith, the collection of stones upon which gravity and linear acceleration act in the inner ear. After experimenting with several techniques, we are now convinced that this is due to the nature of the chemical fixatives traditionally used, which can easily dissolve the otoconia. We have developed rapid fixation and dehydration techniques which do not dissolve the stones but adequately preserve the neural tissue. Using these techniques, we find that the inner ear is first seen at 5 days after the eggs are laid, and the otoconia are first seen at 8 days. The otolith continues to grow throughout development. A relatively mature arrangement of sensory receptor cells (hair cells) and nerve fibers is seen in the two gravity-sensing organs (the utricle and saccule) and the lateral semicircular canal (which detects angular acceleration in the horizontal plane) at 13 to 14 days. The posterior semicircular canal is innervated at about 19 days and the anterior canal another week later. Thus, the basic schedule of development of each component of newt's vestibular system has been established in ground-based control animals.

In several amphibian species it has been reported that the otoconia in the utricle are composed of calcium carbonate in the calcite crystal form, whereas those in the saccule are made of the same mineral but in the aragonite form. We have found this to be true in the adult newt also. However, when the otolith organs first develop, the saccule and utricle cannot be distinguished from one another. Thus, it was difficult to understand how the two crystal forms could be produced in the same organ. Recently, we have shown that in the early larval stages (before 19 days) both the utricle and saccule have otoconia which appear to be of the calcite form. It is only later that the aragonite-appearing otoconia appear. This suggests that the aragonite otoconia might be produced outside of the saccule itself, perhaps in the endolymphatic sac, and transported into the saccule. In adult animals, the entire endolymphatic system, which in amphibians extends to the surface of the spinal cord, is filled with aragonite otoconia. These

have been suggested to serve as a source of metabolic calcium for the animal. Thus, it will be of interest to see if these otoconia are depleted in adult animals which have been in space for 14 days on IML-2.

In conjunction with colleagues in Japan, we have been developing a non-invasive X-ray microfocus tube system, which currently allows an effective beam size of 10 μm. With this system, we have been able to visualize the otoliths in early embryos, as young as 8 days after egg laying. A striking finding with this system is that the otolith appears to be the first system calcified in the embryo. The system may also allow us to quantitate the amount of calcium in the endolymphatic system. It will allow us to follow the growth of the otoliths after space flight without having to sacrifice the animals.

In Japan, we had the opportunity to observe newt larvae of several developmental stages in parabolic flight, to study their reaction to μg. Larvae at stages 42 to 44, in the first three days after hatching, were found to react very little to μg, although they did swim significantly more in the light than in the dark. Larvae at stages 54 to 58, approximately 10 to 14 days after hatching, swam quite vigorously throughout their container, particularly at the onset of μg, in either light or dark. These responses correlate with the development of the newt's vestibular system: at stages 42-44, the otolith organs and lateral semicircular canal are present with well-developed sensory innervation. Both the utricle and saccule are in a horizontal position. Thus, all three organs are suited to sensing movements in the horizontal plane but not upward swimming. In the dark these larvae would have no information about where they had gone if they did swim upward. We have interpreted the results to mean that the animals refrain from making movements which cannot be accurately monitored. At stages 54-58, all three semicircular canals are well innervated and the saccule rises to assume a vertical position. Thus, the saccule, as well as the anterior and posterior semicircular canals, could sense upward and downward movements. With a relatively complete vestibular system, these animals can measure and keep track of any movements and thus are equipped to monitor their exploratory movements in the novel environment of μg. At the early stages (42-44) the limited spatial information provided by the developing visual system allows the larvae to make limited movements at the onset of μg.

Significance of the Accomplishments

Despite several qualitative studies of the vestibular apparatus in animals reared in space, there are conflicting conclusions in the literature. As we contemplate deep-space probes, perhaps lasting many years, we must think about having animals, including humans, conceived and reared in μg. Although there are obvious concerns about how the musculoskeletal system will develop without the constant influence of gravity present on Earth, similar concerns are relevant to the vestibular system, and in particular the gravity-sensing organs. At one level, we know practically nothing about what regulates the production of otoconia to form the otolith. In general, small animals have small otoliths and large animals have large ones. In those species which grow throughout their lives, the otolith continues to grow as long as the animal lives. Thus, there appears to be an ideal mass of otolith for a given size (and probably locomotory style) of animal. If the regulation is based on the weight of the otolith, we would expect larger otoliths to be produced in space. Indeed, this has been found in one of the more quantitative Russian studies. In order to study regulation of otolith production, the studies described here will form the basis of ground-based control studies for comparison with development occurring in space. Other studies we are initiating now will address the mechanisms of otoconial production and assembly.

On another level, even if the otolith were to develop completely normally in μg, the reflex connections in the brain, by which the otolith acts to keep the eyes in a proper orientation during head tilt, will probably develop differently in μg. During development on Earth, the otolith organs always experience the steady pull of gravity, in addition to forces of linear acceleration,

on the mass of the otolith. In space, the steady input of gravity is missing, so the reflexes will develop with only linear-acceleration forces present. We are currently developing tests of the otolith-ocular reflex in newt larvae. With this, we will be able to compare the development of otolith function in space-reared and ground control animals.

Publications

Wiederhold, M.L., Yamashita, M., and Asashima, M. 1992. Development of the gravity-sensing organs in the Japanese red-bellied newt, *Cynops pyrrhogaster*. In: *Proceedings of the Eighteenth International Symposium on Space Technology and Science*, Kagoshima, Japan, 18: 2103-2108.

Wiederhold, M.L., Yamashita, M., Izumi-Kurotani, A., Larsen, K., Koike, H., Nagaoka, S., and Asashima, M. 1992. Developmental changes in the vestibular system of Imori. In: *Proceedings of the Ninth Space Utilization Symposium* , 9: 127-131.

Yamashita, M., Wiederhold, M.L., Izumi-Kurotani, A., Koike, H., Asashima, M., Mogami, Y., Okuno, M., Kawasaki, Y., and Komada, S. 1992. Preparatory work for astronewt experiment. In: *Proceedings of the Ninth Space Utilization Symposium*, Tokyo, 9: 132-134.

SPECIAL ACTIVITY

SPACE BIOLOGY RESEARCH ASSOCIATES PROGRAM

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Description of Program

The Space Biology Research Associates program provides a unique opportunity to train individuals to conduct biological research in areas relevant to NASA's interest. To maximize the potential for Space Biology as an emerging discipline, there is a need to develop a cadre of scientists interested in working in this area. This grant was developed to train young biological scientists by offering them Research Associate Awards. These awards offer opportunities to work on projects directly related to Space Biology in laboratories that provide the necessary facilities and a relevant research environment. It is anticipated that Research Associates will develop careers in the evolving discipline of Gravitational Biology, a focused area of Space Biology. The field of Gravitational Biology is rapidly growing and its future will reflect the quality and training of its scientific personnel.

Accomplishments

The program began on June 1, 1980, with funding to support several Research Associates each year. As of May 1, 1993, awards have been granted to 59 individuals, of whom 33 have received a second year of funding. Table I shows the variety of projects submitted and institutions to which the 59 Research Associates have been assigned. These scientists represent different disciplines including: animal physiology, botany, cellular and molecular biology, developmental biology, immunology, plant physiology, and zoology.

Significance of Accomplishments

Many of the Research Associates have been asked to participate in NASA panels, national workshops, and national meetings. Each year, in the fall, the Research Associates attend the annual meeting of the American Society for Gravitational and Space Biology (ASGSB), where they are active participants, presenting papers and posters along with their senior colleagues. The Research Associates are also encouraged to participate in other national meetings in their own disciplines. The scientists who have completed this program have accepted positions in colleges and universities, with private industry, and with NASA. Table II shows the location of the Research Associates today.

A complete list is provided of the 59 Research Associates, the title of their research projects, the host laboratory and director, and their current location.

Table I. SPACE BIOLOGY RESEARCH ASSOCIATES

ANIMAL PROJECTS

NAME	INSTITUTION	RESEARCH AREA
G. ADAMS	U. Cal., Irvine	Muscle plasticity/Signal pathways
S. BAIN	SUNY, Stony Brook	Skeletal bone remodeling
W. BERRY	U. Louisville	Role of vitamin D/Immune function
M. BINDER	Dartmouth Col.	Cardiac pathology
S. BLACK	U. Cal., Berkeley	Amphibian developmental orientation
H. BLAIR	Washington U.	Cellular/Bone atrophy
J. BUCKEY	U. Texas, Dallas	Cardiovascular Responses
G. BURROWS	National Institutes of Health	Synaptogenesis/Neuropathology
D. CLOHISY	Washington U.	Osteoclastogenesis/Bone atrophy
J. CLOHISY	St. Louis U.	Bone/space flight and gene expression
M. COOPER	U. Cal., Berkeley	Osteoporosis/Bone atrophy
D. DICKMAN	U. Texas, Galveston	Semicircular muscle canals
S. GLOTZBACH	Stanford U.	Neurophysiology/Circadian rhythms
E. GOOLISH	U. Michigan	Weightlessness/Swim bladder function
C. GOULD	U. of Louisville	Immunology/Interferon functions
M. GRAY	Tufts U.	Mechanical environment in bone structure
E.GREENFIELD	Washington U.	Osteoblast role/Osteoclast activity
T. JONES	U. Cal., Davis	Neurophysiology/Brainstem potentials
T. KERR	Wayne State U.	Mammalian vestibular system
C. KIRBY	U. Texas, Houston	Weightlessness/Swim bladder function
D. KLIGMAN	National Institute of Mental Health	Neurite extension factor responses
A. LYSAKOWSKI	U. Chicago	Vestibular hair cells/Synaptic relations
K. MCLEOD	SUNY, Stony Brook	Electrical fields in bone remodeling
D. MEYERS	U. Pennsylvania	Gravity perception - Microcrustacean (FW)
E. MILLER	U. Louisville	Immunological memory/Infection
L. MINOR	U. Chicago	Responses of secondary vestibular neurons
D. MURAKAMI	U. Cal., Davis	Hyperdynamia/Visual systems
S. PERKINS	Washington U.	Vitamin D/Osteoclast differentiation
K. POTE	U. Virginia	Otoconia Ca binding protein
G. RADICE	Indiana U.	Gravity-sensors/Amphibian embryology
R. REITSTETTER	U. Arizona	Microgravity/Cell interaction
F. ROBINSON	U. Pittsburgh	Sensory motor properties in uvula
J. STEFFEN	U. Louisville	Glucocorticoid receptors/Muscle responses
J. SZILAGYI	Cleveland Clinic	Hypodynamic responses/Animal model
D. THOMASON	U. Texas, Houston	Decreased actin synthesis/Muscle atrophy
J. THOMPSON	Med. Col., WI.	Muscle fibers following atrophy
L. THOMPSON	Marquette U.	Muscle fatigability/cross-bridge
Y. TORIGOE	U. Cal., Irvine	Neurophysiology of gut

TABLE I. SPACE BIOLOGY RESEARCH ASSOCIATES (CONTINUED)

PLANT PROJECTS

NAME	INSTITUTION	RESEARCH AREA
S. BARSEL	Michigan St. U.	Plant cell physiology
T. BJORKMAN	U. Washington	Electrical responses/Gravity sensing
T. BROCK	U. Michigan	Auxin & protein synthesis in gravitropism
M. DESROSIERS	Michigan St. U.	Electrical potential in hormone transport
J. GARAVELLI	Texas A&M U.	Plant/Algae cell chemistry
J. GAYNOR	Yale U.	Amyloplast/Gravitational sensitivity
M. HARRISON	Washington U.	Environmental ethylene/Gravitropism
G. JAHNS	U. Houston	Lignin biosynthesis in plant development
J. KISS	U. Colorado	*Chara* rhizoids gravitropic response
W. KROEN	N.C. State U.	CO_2 enrichment and root/shoot ratios
K. KUZMANOFF	Stanford U.	Enzyme regulators in plant cell wall
V. LING	Harvard U.	Gravity signal transduction/Ca-calmodulin
M. MATILSKY	Princeton U.	Gravity perception/Coenocyte
M. MUSGRAVE	Duke U.	Plant respiratory metabolism/Space flight
D. REINECKE	Michigan St. U.	IAA distribution/Plant geosensing
M. RICE	Oregon St. U.	Gravicurvature/Auxins
B. SERLIN	U. Texas, Austin	Cell wall growth/Corn roots
R. SLOCUM	Yale U.	Role of calcium/Gravistimulation
J. SLONE	Washington U.	Auxin transport/Gravitropism
L. TALBOTT	Washington U.	Stem gravicurvature/Specific polymers
L. WIMMERS	U. CA., Davis	Gravitropism/Ca-ATPase

TABLE II.

LOCATION OF RESEARCH ASSOCIATES TODAY

59	=	Total Number
6	=	Current Recipients of this Award
33	=	Faculty Members in Universities
1	=	Located in NASA Facilities
3	=	Other Government & Private Research Foundation Facilities
11	=	Other Research Capacities
4	=	Industry
1	=	Deceased
1	=	One of the Faculty Members was the Alternate Payload Specialist Candidate for the SLS-2 Flight

RESEARCH ASSOCIATE AWARDEES

The 59 awardees are listed alphabetically, including their award terms (in parenthesis after their name), host laboratory, and current location. Current professional appointments are indicated in parenthesis as: Current Research Associate (C); Faculty (F); NASA facility (N); Other Government & Private Research Foundation facilities (G); Other Research Capacities (R); Industry (I); and Deceased (D).

GREGORY ADAMS, Ph.D. (11/1/92 - 10/30/93) is working on "Metabolic Modulation of Skeletal Muscle Phenotype During Unweighting Induced Atrophy and Compensatory Hypertrophy" in Dr. Kenneth Baldwin's laboratory at the University of California, Irvine, CA. (C)

STEVEN BAIN, Ph.D. (6/1/88 - 5/30/90) worked on "The Interaction of Skeletal Remodeling with Systemic Disorders: An Obstacle to Extended Space Flight?" in Dr. Clinton Rubin's laboratory at SUNY, Stony Brook, NY. He is now Senior Scientist of Biomedical Research at ZymoGenetics, Seattle, WA. (I)

SARA-ELLEN BARSEL, Ph.D. (6/1/87 - 5/30/88) worked on "Molecular and Genetic and Phototropism in *Arabidopsis thaliana*" in Dr. Kenneth Poff's laboratory at Michigan State University, East Lansing, MI. She is working for Chemical Abstracts in Columbus, OH. (I)

WALLACE BERRY, Ph.D. (7/1/88 - 6/30/90) worked on "Lymphokine Producing Capacity of Antiorthostatically Suspended Rats: Relationship to 1,25-dihydroxy-vitamin D3" in Dr. Gerald Sonnenfeld's laboratory at the University of Louisville, Louisville, KY. He is now an Assistant Professor in the Boyd Research Center, University of Georgia, Athens, GA. (F)

MICHAEL BINDER, M.D. (1/1/83 - 12/30/83) worked on "Congenital Heart Malformations and Situs Inversus" in Dr. W. M. Layton's laboratory at Dartmouth Medical School. He is now an Assistant Professor in the Department of Anatomy, Dartmouth College, Hanover, NH. (F)

THOMAS BJORKMAN, Ph.D. (10/1/86 - 9/30/88) worked on "The Mechanism of Gravity Sensing in Plants" in Dr. Robert Cleland's laboratory at the University of Washington, Seattle, WA. He is now an Assistant Professor in the Department of Horticultural Sciences at Cornell University, Geneva, NY. (F)

STEVEN BLACK, Ph.D. (7/1/82 - 6/30/84) worked on "Determination by gravitational and centrifugal force of the amphibian dorsal-ventral axis" in Dr. Raymond Keller's laboratory at the University of California, Berkeley. He is now an Associate Professor in the Department of Biology at Reed College, Portland, OR. (F)

HARRY BLAIR, M.D. (7/1/84 - 6/30/86) worked on "Cellular Mechanisms of Bone Degradation" in Dr. Steven Teitelbaum's laboratory at The Jewish Hospital/Washington University Medical Center, St. Louis, MO. He is now an Assistant Professor in the Department of Pathology at the University of Alabama, Birmingham, AL. (F)

THOMAS BROCK, Ph.D. (8/1/86 - 7/30/88) worked on "Comparison of Changes in Protein Synthesis Induced by Gravity and Auxin Treatment in Pulvini and Coleoptiles of Oat (*Avena sativa* L.)" in Dr. Peter Kaufman's laboratory at the University of Michigan. He is now a Research Associate in the Department of Pediatrics at the University of Michigan Medical School, Ann Arbor, MI. (R)

JAY BUCKEY, JR., M.D. (7/1/82 - 6/30/84) worked on "Three-Dimensional Echocardiography For Measuring Ventricular Volume" in Dr. C. Gunnar Blomqvist's laboratory at the University of Texas Health Sciences Center, Dallas. He an Assistant Professor of Medicine at the University of Texas Health Sciences Center, Dallas, TX. He was also the Alternate Payload Specialist for the SLS-2 flight. (F,N)

GEORGE H. BURROWS, Ph.D. (7/1/81 - 6/30/83) worked on "Studies of Synaptogenesis" in Dr. Marshall Nirenberg's laboratory at National Institute Health, Bethesda, MD. His last known position was as a staff member of the National Heart, Lung, and Blood Institute, Bethesda, MD. (G)

DENIS CLOHISY, M.D. (7/1/86 - 6/30/87) worked on "Mechanisms of Osteoclast Precursor Differentiation" in Dr. Steven Teitelbaum's laboratory at Washington University Medical Center, St. Louis, MO. He is now an Assistant Professor in the Department of Orthopaedic Surgery, University of Minnesota, St. Paul, MN. (F)

JOHN CLOHISY (7/1/91 - 6/30/92) worked on "Regulation of Gene Expression in Bone Remodeling" in Dr. Nicola Partridge's laboratory at St. Louis University Hospitals, St. Louis, MO. He is completing his clinical residency at St. Louis University Hospital, St. Louis, MO. (G)

MARK COOPER, Ph.D. (1/1/85 - 12/30/86) worked on "Osteoporosis of Weightlessness and the Electrophysiology of Bone" in Dr. John Miller's laboratory at The University of California at Berkeley, CA. He is now an Assistant Professor in the Department of Zoology, University of Washington, Seattle, WA. (F)

MARK DESROSIERS, Ph.D. (7/1/86 - 6/30/88) worked on "A Search for Voltage-gating of Plant Hormone Transport Channels" in Dr. Robert Bandurski's laboratory at Michigan State University. He is continuing to work with Dr. Bandurski at Michigan State University, East Lansing, MI. (R)

J. DAVID DICKMAN, Ph.D. (6/1/87 - 5/30/89) worked on "High Frequency Response Properties of Semicircular Canal Fibers" in Dr. Manning Correia's laboratory at the University of Texas, Galveston, TX. He is now an Assistant Professor in the Department of Surgery, Division of Otolaryngology at the University of Mississippi, Jackson, MS. (F)

JOHN S. GARAVELLI, Ph.D. (1/1/82 - 12/30/82) worked on "Chemical Characterization of Volatile Products of Algal Cell Cultures" in Dr. Franklin Fong's laboratory at Texas A&M University. He is now Data Base Coordinator for Protein Information Resource at the National Biomedical Research Foundation, Washington, DC. (G)

JOHN GAYNOR, Ph.D. (1/1/81 - 12/30/82) worked on "Purification and Characterization of Amyloplasts from *Pisum sativum*" in Dr. Arthur Galston's laboratory at Yale University. He is now an Associate Professor in the Department of Biology at Montclair State College, Upper Montclair, NJ. (F)

STEVEN GLOTZBACH, Ph.D. (1/1/84 - 12/30/84) worked on "Neurophysiological Studies of Circadian Rhythm Control Mechanisms" with Dr. H. Craig Heller at Stanford University. He is a Senior Research Scientist in the Department of Pediatrics; Co-Director, Pediatric SIDS Research Program and Program Coordinator of the Perinatal Emphasis Research Center at Stanford University, Stanford, CA. (R)

EDWARD GOOLISH, Ph.D. (8/1/90 - 7/30/91) worked on "The Effects of Simulated Weightlessness on Swimbladder Function and Buoyancy Regulation in Fish" in Dr. Paul Webber's laboratory at the University of Michigan, Ann Arbor, MI. He is a Research Associate at Scripps/NOAA in LaJolla, CA. (R)

CHERYL GOULD, Ph.D. (7/1/84 - 8/30/85) worked on "Effect of Weightlessness on Various Immunological Functions using a Murine Simulated Space Flight Model" in Dr. Gerald Sonnenfeld's laboratory at the University of Louisville, Louisville, KY. She is now an Assistant Professor in Medical Technology at the University of Dayton, Dayton, OH. (F)

MARTHA GRAY, Ph.D. (7/1/86 - 6/30/87) worked on "The Correlation of Applied Strain Distributions to the Location of New Bone Formation: A Rigorous Mechanical Analysis of an in-vivo Bone Preparation" in Dr. Clinton Rubin's laboratory at Tufts University School of Veterinary Medicine, North Grafton, MA. She is an Associate Professor in the Department of Electrical Engineering and Computer Science at M.I.T. and in the Harvard-M.I.T. Division of Health Sciences and Technology in Cambridge, MA. (F)

EDWARD GREENFIELD, Ph.D. (7/1/88 - 6/30/90) worked on "Regulations of Osteoclastic Bone Resorption by Osteoblasts" in Dr. Steven Teitelbaum's laboratory at Washington University, St. Louis, MO. He is now an Assistant Professor in the Department of Orthopaedics, Case Western Reserve University, Cleveland, OH. (F)

MARCIA HARRISON, Ph.D. (7/1/83 - 8/30/85) worked on "Participation of Ethylene in Two Modes of Gravitropism of Shoots" with Dr. Barbara Pickard at Washington University, St. Louis. She is now an Assistant Professor in the Biology Department at Marshall University in Huntington, WV. (F)

GARY JAHNS, Ph.D. (1/1/83 - 4/30/84) worked on "Interactions of Light and Gravity on the Growth, Orientation, and Lignin Biosynthesis in Mung Beans" in Dr. Joe Cowles' laboratory at the University of Houston. He is working at NASA-Ames, Moffett Field, CA. (N)

TIMOTHY JONES, Ph.D. (1/1/81 - 12/30/82) worked on "The Effects of Hypergravic Fields on Brainstem Auditory-evoked Potentials" in Dr. John Horowitz's laboratory at the University of California, Davis. He is now an Assistant Professor at the University of Nebraska, Lincoln, NE. (F)

THOMAS KERR, Ph.D. (1/1/83 - 12/30/84) worked on "Cellular Localization of Na+, K+-ATPase in the Mammalian Vestibular System"; the first year in Dr. Muriel Ross' laboratory at the University of Michigan and the second year in Dr. Dennis Drescher's laboratory at Wayne State University. He was an Assistant Professor at Wayne State University, Detroit, MI. (D)

CHRISTOPHER KIRBY, Ph.D. (11/1/90 - 10/30/92) is working on "Eccentric Exercise as a Countermeasure to Unweighting Atrophy" in Dr. Frank Booth's laboratory at The University of Texas Medical School, Houston, TX. He is continuing to work with Dr. Booth at The University of Texas Medical School, Houston, TX. (R)

JOHN KISS, Ph.D. (9/1/90 - 8/30/91) worked on "Gravitropism and Golgi apparatus function in *Chara* rhizoids" in Dr. L. Andrew Straelin's laboratory at the University of Colorado, Boulder, CO. He is now an Assistant Professor in the Department of Biology at Hofstra University, Hempstead, NY. (F)

DOUGLAS KLIGMAN, Ph.D. (7/1/82 - 6/30/84) worked on "The Role of Neurite Extension Factor Nerve and Muscle Tissue Response to Stress or Injury" in Dr. David Jacobowitz's laboratory at the National Institute of Mental Health (NIMH), Bethesda, MD. The last known position was as a staff member at NIMH, Bethesda, MD. (G)

WILLIAM KROEN, Ph.D. (10/1/90 - 8/30/91) worked on "Balancing Reproductive and Root Demands for Carbohydrates and Nitrogen in Atmospheres with High CO_2 Concentrations" in Dr. Mary Peet's laboratory at North Carolina State University, Raleigh, NC. He is now an Assistant Professor in the Department of Natural Sciences at Wesley College, Dover, DE. (F)

KONRAD KUZMANOFF, Ph.D. (7/1/83 - 7/30/85) worked on "Isolation and Identification of B-glucan Synthetase: A Potential Biochemical Regulator of Gravistimulated Differential Cell Wall Loosening" in Dr. Peter Ray's laboratory at Stanford University. He is now a Consultant with Zoeticon, Berwyn, IL. (I)

VINCENT LING, Ph.D. (11/01/92 - 10/30/93) is working on "Calcium and Calmodulin Mediation of the Gravitropic Signal" in Dr. Sarah Assmann's laboratory at Harvard University, Cambridge, MA. (C)

ANNA LYSAKOWSKI, Ph.D. (7/1/89 - 6/30/91) worked on "Synaptic Relations of Type I and Type II Vestibular Hair Cells" in Dr. Jay Goldberg's laboratory at the University of Chicago, Chicago, IL. She is a Research Assistant Professor in the Department of Neurobiology at the University of Chicago, Chicago, IL. (F)

MICHAEL MATILSKY, Ph.D. (1/1/81 - 12/30/82) worked on "Gravity Perception in the Algal Coenocyte *Caulerpa prolifera*" in Dr. William Jacobs' laboratory at Princeton University. He is the Director of the Laboratory and Sperm Bank Fertility Unit in the Department of Obstetrics and Gynecology at Central Emek Hospital, Afula, Israel. (I)

KENNETH MCLEOD, Ph.D. (11/1/87 - 10/30/89) worked on "Regulation of Bone Remodeling Activity through the Control of Stress Generated Electric Fields" in Dr. Clinton Rubin's laboratory, SUNY, Stony Brook, NY. He now has a joint appointment as an Associate Professor in the Department of Orthopaedics and in the Department of Biophysics and Physiology at SUNY, Stony Brook, NY. (F)

DEWEY MEYERS, Ph.D. (7/1/81 - 6/30/83) worked on "Response, Adaptation and Gravitational Perception in a Parthenogenic Freshwater Microcrustacean, *Daphnia galeata mendotae*" in Dr. Allan Brown's laboratory at the University of Pennsylvania. He is now a medical student in the Medical School, Marshall University, Huntington, WV. (R)

EDWIN MILLER, Ph.D. (7/1/92-6/30/93) is working on "Influence of Antiorthostatic Suspension on the Generation and Expression of Immunological Memory" in Dr. Gerald Sonnenfeld's laboratory at the University of Louisville, Louisville, KY. (C)

LLOYD MINOR, Ph.D. (7/1/87 - 6/30/88) worked on "Primary Vestibular Afferent Inputs to Central Pathways Mediating the Vestibulo-ocular Reflex" in Dr. Jay Goldberg's laboratory at the University of Chicago. He is now a Clinical Instructor in the Department of Otolaryngology at Vanderbilt University in Nashville, TN. (F)

DEAN MURAKAMI, Ph.D. (1/1/85 - 12/30/86) worked on "Influences of the Hyperdynamic Environment on the Development of the Visual System in the Rat" in Dr. Charles Fuller's laboratory at the University Of California at Davis. He is now an Assistant Researcher in the Department of Animal Physiology at the University of California, Davis, CA. (R)

MARY MUSGRAVE, Ph.D. (6/1/86 - 5/30/88) worked on "Studies of Respiratory Metabolism" in Dr. Boyd Strain's laboratory at Duke University, Durham, NC. She is now an Associate Professor at Louisiana State University, Baton Rouge, LA. (F)

SHERRIE LYNN PERKINS, M.D. (7/1/88 -6/30/89) worked on "Vitamin D Effect on Osteoclast Precursor Differentiation" in Dr. Steven Teitelbaum's laboratory at Washington University, St. Louis, MO. She is now an Assistant Professor in the Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT. (F)

KENNETH POTE, Ph.D. (6/1/88 - 5/30/90) worked on "An Otoconial Calcium Binding Protein; Its Temporal Expression and Tissue Distribution" in Dr. Robert Kretsinger's laboratory at the University of Virginia, Charlottesville, VA. He is now an Assistant Professor in the Department of Otolaryngology, Harvard Medical School, Boston, MA. (F)

GARY RADICE, Ph.D. (7/1/81 - 6/30/83) worked on "Control of Gravity-Sensing Mechanism in Amphibian Eggs" in Dr. George Malacinski's laboratory at Indiana University. He is now an Assistant Professor in the Department of Biology, University of Richmond, Richmond, VA. (F)

DENNIS REINECKE, Ph.D. (10/1/88 - 9/30/89) worked on "Does Indole-3-Acetic Acid Turnover Correlate with Topically-induced Asymmetric Growth?" in Dr. Robert Bandurski's laboratory at Michigan State University, East Lansing, MI. He is now working as a Research Associate in the Department of Plant Biology at the University of Minnesota, St. Paul, MN. (R)

RAVEN REITSTETTER, Ph.D. (10/1/91 - 9/30/92) is working on "Effects of Altered Gravity on Single Channel Acetylcholine Receptor Kinetics" in Dr. Raphael Gruener's laboratory at the University of Arizona, Tucson, AZ. He is continuing to work with Dr. Gruener at the University of Arizona, Tucson, AZ. (R)

MARGARET S. RICE, Ph.D. (1/1/92 - 12/30/92) is working on "Immunolocalization and Molecular Characterization of an IAA Binding Protein" in Dr. Terri Lomax's laboratory at Oregon State University, Corvallis, OR. (C)

FARREL R. ROBINSON, JR., Ph.D. (7/1/84 - 6/30/86) worked on "Sensory Motor Properties of the Uvula and Nodulus" in Dr. David Tomko's laboratory at The University of Pittsburgh School of Medicine, Pittsburgh, PA. He is now working as an Assistant Professor in the Department of Physiology & Biophysics at the University of Washington School of Medicine, Seattle, WA. (F)

BRUCE SERLIN, Ph.D. (7/1/84 - 6/30/85) worked on "Differential Wall Growth in Gravistimulated Corn Roots: Its Timing and Regulation" in Dr. Stanley Roux's laboratory at The University of Texas at Austin. He is now an Assistant Professor in the Department of Biological Sciences at DePauw University, Greencastle, IN. (F)

ROBERT SLOCUM, Ph.D. (1/1/81 - 12/30/82) worked on "Studies on the Localization and Functional Role of Calcium in Gravistimulated Plant Organs"; the first year in Dr. Stanley Roux's laboratory at The University of Texas at Austin and the second year in Dr. Arthur Galston's laboratory at Yale University. He is now an Assistant Professor in the Department of Biological Sciences at Goucher College, Baltimore, MD. (F)

J. HENRY SLONE, Ph.D. (7/1/85 - 6/30/87) worked on "Characterization of the protein responsible for the lateral transport of auxin during gravitropism of pea shoots and determination whether phosphorylation participates in gravitropic activation" in Dr. Barbara Pickard's laboratory at Washington University in St. Louis, Missouri. He is now an Assistant Professor in the Department of Biology at Francis Marion University, Florence, SC. (F)

JOSEPH STEFFEN, Ph.D. (7/1/81 - 6/30/83) worked on "Glucocorticoid Receptor Levels in Hindlimb Skeletal Muscles and Diaphragm During Prolonged (2 Week) Antiorthostatic Hypokinesia and Recovery" in Dr. X. J. Musacchia's laboratory at the University of Louisville. He is now an Associate Professor in the Department of Biology at the University of Louisville, Louisville, KY. (F)

JULIANNA SZILAGYI, Ph.D. (7/1/81 - 12/30/81) worked on Progressive Hemodynamic Changes in Simulated Weightlessness" in Dr. C. Ferrario's laboratory at the Cleveland Clinic. She is now an Assistant Professor in the Department of Pharmacology at the University of Houston, Houston, TX. (F)

LAWRENCE TALBOTT, Ph.D. (7/1/89 - 12/30/89) worked on "Actuation of gravicurvature in pea stems by alterations of specific wall polymers" in Dr. Barbara Pickard's laboratory at Washington University, St. Louis, MO. He is now a Research Associate at the University of California, Los Angeles. (R)

DONALD THOMASON, Ph.D. (11/1/89 - 8/30/90) worked on "Mechanisms of decreased actin synthesis during rodent hindlimb unweighting" in Dr. Frank Booth's laboratory at the University of Texas Medical School, Department of Physiology and Cell Biology, Houston, TX. He is now an Assistant Professor in The Department of Physiology & Biophysics at the University of Tennessee, Memphis, TN. (F)

JOYCE THOMPSON, Ph.D. (7/1/90 - 6/30/92) worked on "Targetoid and Type IIC Fibers in Hypodynamic and Recovering Rat Muscle - Histochemistry, Ultrastructure and Myosin Isozyme Composition" in Dr. Danny Riley's laboratory at the Medical College of Wisconsin, Milwaukee, WI. She is a Research Assistant Professor in the Department of Cellular Biology and Anatomy in the Medical College of Wisconsin, Milwaukee, WI. (F)

LADORA THOMPSON, Ph.D. (7/1/92 - 6/30/93) is working on "The Role of H^+ and Inorganic Phosphate in the Fatigability of the Soleus Following Hindlimb Suspension" in Dr. Robert Fitts' laboratory at Marquette University, Milwaukee, WI. (C)

YASUHIRO TORIGOE, Ph.D. (1/1/84 - 12/30/85) worked on "Anatomical correlated underlying vestibulo-autonomic outflow to the gut" with Dr. Robert H.I. Blanks at the University of California, Irvine. He is now an Assistant Adjunct Professor in the Department of Anatomy and Neurobiology at the University of California, Irvine, CA. (F)

LARRY WIMMERS, Ph.D. (11/1/92 - 10/30/93) is working on "Role of the E.R.-Localized Ca^{2+}-ATPase in Higher Plant Gravity Perception" in Dr. Alan Bennett's laboratory at the University of California, Davis, CA. (C)

ECCENTRIC EXERCISE TRAINING AS A COUNTERMEASURE TO NON-WEIGHT BEARING MUSCLE ATROPHY

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Description of Research

The goal of this research is to examine the control of muscle gene expression during states of altered contractile activity. The specific aims of this research are: 1) to determine if eccentric resistance (lengthening contractions) exercise training can maintain normal protein content in non-weight bearing soleus, and 2) to examine whether the duration of an acute bout of aerobic exercise is associated with elevated skeletal muscle cAMP content post-exercise.

Muscle atrophy resulting from non-weight bearing is one of the principle alterations associated with space flight conditioning. Normal locomotion, posture, and activity incorporate a significant degree of eccentric contraction, which is absent during non-weight bearing. This provides a convincing rationale for testing eccentric resistance exercise as a countermeasure to non-weight bearing (e.g., space flight) muscle atrophy.

Increased aerobic work capacity in skeletal muscle undergoing chronic endurance training results, in part, from increases of mitochondrial density. Although, the mechanochemical link(s) between chronic endurance exercise and the increase in mitochondrial density is unknown, cAMP is one possible signal under consideration. cAMP concentration in skeletal muscle was doubled in the first couple of minutes following an acute bout of running at all exercise durations ranging from 5 to 30 minutes. Since 5 to 10 minute bouts of aerobic exercise, repeated daily, are insufficient to stimulate increased mitochondrial density, a dilemma exists: "If cAMP is a mechano-chemical link between endurance exercise and increased mitochondrial density, why is cAMP elevated after 5 or 10 minute runs, which when performed daily do not increase skeletal muscle mitochondrial density?"

Accomplishments

(1) Eccentric exercise training during non-weight bearing attenuated, but did not prevent, the loss of soleus muscle wet weight and noncollagenous protein by 77 percent and 44 percent, respectively.

(2) No increases in skeletal muscle cAMP were noted for the first 4 hours after a single 10 or 60 minute bout of running by untrained rats.

Significance of the Accomplishments

Because the soleus muscle exhibits the greatest degree of atrophy during non-weight bearing, it has been the focus of most exercise countermeasure studies. Virtually all exercise modalities attenuate soleus muscle atrophy. However, compared with other forms of exercise, resistance training is at least equal in its effectiveness and superior in its efficiency. This is supported by the finding that eccentric exercise during only 0.035 percent of the total non-weight bearing period attenuated 44 percent of soleus muscle noncollagenous protein loss.

The absence of change in skeletal muscle cAMP in the 4 hour time period after running for either 10 or 60 minutes in the present study could suggest the following: 1) cAMP values by themselves, are not a function of run duration, 2) elevated muscle cAMP levels could occur at higher exercise intensities, 3) increases in cAMP may require multiple bouts of exercise, or 4) increases in muscle cAMP may be transient, initiating more prolonged increases in factors distal to cAMP in the adenylate cyclase cascade. Future studies should investigate the correlative relations between run duration and protein kinase A, or some protein phosphorylated by protein kinase A, rather than cAMP.

COMPETITION BETWEEN FRUIT DEVELOPMENT AND ROOT GROWTH IN TOMATOES GROWN AT HIGH CO_2

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Description of Research

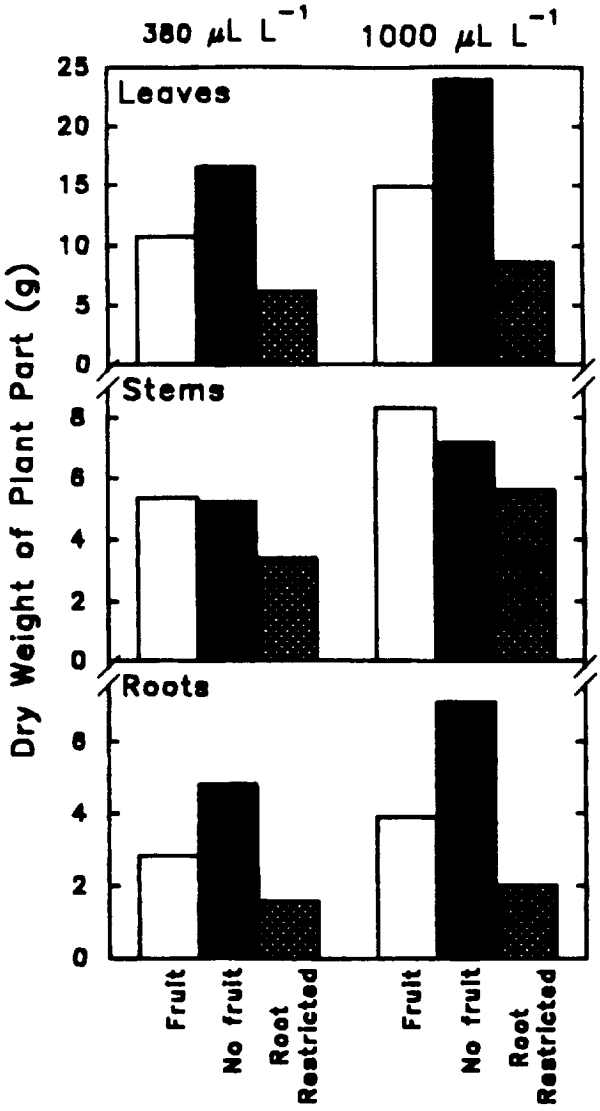
Long-term growth of plants at elevated CO_2 (approximately 1000 $\mu l\ l^{-1}$) generally leads to increased growth despite only transient increases in photosynthetic carbon uptake. We have previously shown increased growth of leaves, stems, and roots during the first 2 weeks of enrichment in two varieties of tomato; this growth was accompanied by slight increases in carbon exchange rate. Leaves and stems remained larger in plants grown at high CO_2, though growth rate of plants in the two CO_2 treatments was similar after the first 2 weeks. Root growth slowed or stopped at the time of fruit set in plants growing at elevated CO_2, resulting in similar or reduced total root biomass relative to ambient-grown plants. This decreased rooting ability was hypothesized to account for nutrient deficiency-like symptoms exhibited by plants grown at high CO_2. Tomato fruit weight, but not necessarily number, significantly increased at increased CO_2. Decreased root biomass and increased fruit weights would be beneficial in a CELSS system, since less non-edible material would be produced in systems anticipated to operate at high CO_2 concentrations.

A second set of experiments, conducted during the summer of 1991, examined plant biomass, fruit production, photosynthetic carbon uptake, and nutrient and carbohydrate partitioning during growth of the small cherry tomato variety "Florida Petite" at normal (380 $\mu l\ l^{-1}$) and elevated (1000 $\mu l\ l^{-1}$) CO_2 concentrations when the plants were subjected to root restriction or fruit removal. We were interested in testing the hypothesis that there was internal competition for carbon and nutrients between fruit and root systems.

Accomplishments

(1) Plants grown at high CO_2 had significantly higher biomass than those grown at ambient levels of CO_2. When growth was reduced by tight spacing and root restriction, biomass of all plant parts was significantly reduced at both CO_2 levels. However, leaves and stems of root-restricted plants growing at high CO_2 were larger than those at ambient CO_2, though root biomass remained the same. (See accompanying figure of plant biomass after 6 weeks of growth.) There was no significant reduction in growth of unripe fruit due to root restriction at either CO_2 level. Space and funding constraints precluded running the experiment until fruit had ripened.

(2) Removal of fruit significantly increased the remaining vegetative biomass of leaves and roots in plants grown at both ambient and elevated CO_2, though the plants exhibited severe leaf deformations. Photosynthetic carbon uptake declined in defruited plants under both CO_2 regimes.



(3) Plants grown at high CO_2 had significantly higher levels of leaf starch and soluble sugars than did leaves of plants grown at ambient levels of CO_2. There were no changes in carbohydrate concentrations of stems and roots. We have previously described both of these results. No significant changes in nutrient concentrations were found in any part of plants growing at high CO_2 at the time of fruit ripening.

(4) Fruit removal at each CO_2 level resulted in significant reductions in leaf starch and hexose concentrations (relative to plants bearing fruit) particularly at high CO_2, whereas there were significant accumulations of hexose and sucrose in roots. Fruit removal caused an increase in phosphorus concentration and a decrease in calcium concentration in leaves. Stems accumulated significant amounts of nitrogen, phosphorus, and magnesium, while roots also accumulated magnesium.

Significance of Accomplishments

(1) Root restriction and tight spacing at elevated CO_2 may not severely reduce fruit production while bringing about less vegetative growth in bushy tomatoes. This line of research needs to be explored further in reference to potential nutrient delivery systems and their effect on root growth and viability, particularly since there is evidence in the literature suggesting that root-restricted plants show symptoms of water deficiency.

(2) Fruits are such potent sinks for the products of photosynthesis that root growth appears to be limited by internal competition with fruit. Removal of this carbon sink allows for increased root growth, even when photosynthetic carbon uptake decreases to compensate for the reduced demand for carbon.

(3) The reduced root-to-shoot ratio during fruit development in these plants grown at elevated CO_2 does not appear to result in deficiencies for carbohydrates or nutrients within leaves. Leaves of high CO_2 -grown plants still accumulate massive amounts of carbohydrates. Whether growth of plants at elevated CO_2 has any effect on the nutritional value of tomato fruit is still unknown, though we found no difference in carbohydrate or nutrient concentrations of ripening fruit due to elevated CO_2.

(4) Fruit removal, despite the apparent sink-limited reduction in carbon uptake, allows for increased levels of carbohydrates to be moved to the roots under either CO_2 regime. Nutrients also accumulate in the stem when not required by ripening fruit. All three results bear out the hypothesis that root growth in tomatoes is restricted by fruit load.

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CHARACTERIZATION OF A CALMODULIN-BINDING PROTEIN FROM PLANT ROOTS

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Description of Research

The long-term objective of this research is to dissect the biochemical components comprising the calcium-mediated signal transduction pathways within plant cells. The current focus is on the characterization and purification of a calcium-dependent calmodulin-binding protein preferentially localized in plant roots.

Although the process of development in plants is greatly affected by a variety of environmental conditions, relatively little is known about the mechanisms which link perception of external signals to alterations in growth. Stimuli such as light and gravity, when applied to plants, result in transient and localized increase of subcellular calcium ion concentration prior to growth response. This system appears to be analogous to the transient calcium fluxes that occur during the stimulation of mammalian cells, especially those of neural origin. In addition, pivotal proteins involved in neural signal transduction pathways have also been found in plant cells, most notably calmodulin.

The calmodulins are a small family of highly conserved calcium-binding proteins that function as activators of disparate regulatory pathways. Calmodulin has no intrinsic enzymatic property. Through calmodulin, transient calcium increases result in the temporal and indirect activation of many calcium-calmodulin dependent enzymes (including protein kinases and phosphatases)[Figure 1]. In this nascent field of plant signal transduction, only 4 calmodulin-dependent enzymes have been described, as opposed to the dozens documented in animal systems. Due to this paucity of information, a detailed model of the calcium signaling network in plants has yet to emerge. Clearly, further elucidation of calmodulin-dependent enzymes in plant systems is necessary.

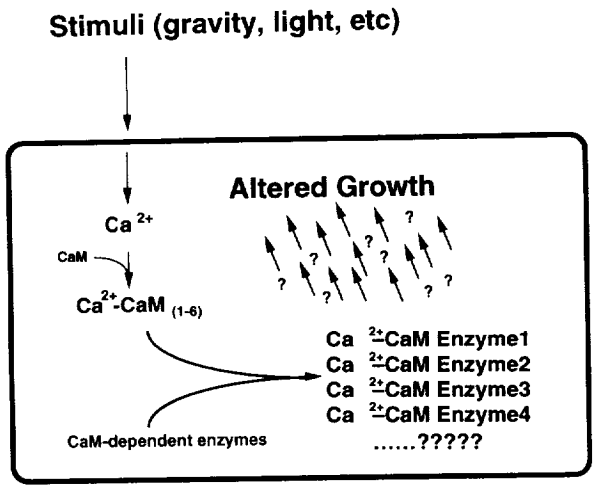

Figure 1.

Protein extracts of various plant cell protoplasts, tissues, and organs were analyzed for the presence of these target calmodulin-binding proteins by electrophoretic separation, immobilization onto PVDF membranes, and exposure to radiolabeled calmodulin. In this way, proteins bound to the tagged calmodulin were detected. With this assay, these calmodulin-binding proteins could then be subjected to biochemical purification and subsequent identification. The characterization of these proteins would thus lead to a better understanding of the biochemical signaling networks that link sensory perception and physiological response within plants.

Accomplishments

(1) Calmodulin levels and calmodulin-binding proteins are not distributed uniformly between protoplast types, tissues, or organs in *Vicia faba* (fava bean).

(2) Two categories of calmodulin-binding proteins are present in plants. One set is ubiquitously distributed throughout the plant, and the others appear to be unique and found in root, stem, or guard cell protoplasts.

(3) One prominent calmodulin-binding protein with a molecular weight of approximately 62 kilodalton was detected in both the cortex and stele of *Vicia* roots. This protein was detected along the axis of the root but not at the root tip.

(4) Other major calmodulin-binding proteins were detected in stems and nodules. These proteins were similar in mass but not identical to the root protein.

(5) Development of a purification protocol for the root 62 kilodalton calmodulin-binding protein resulted in a method that offered an overall 200-fold purification of this protein from crude extracts. This protein was characterized as a calmodulin-stimulated enzyme, glutamate decarboxylase.

Significance of Accomplishments

Finding 1: Differential distribution of calmodulin and calmodulin-binding proteins suggests that calcium signaling pathways are not identical in all parts of the plant. This finding is consistent with the evidence that stimuli-induced calcium increases lead to disparate physiological responses in plants, depending on tissue type.

Finding 2: The presence of two categories of calmodulin-binding proteins suggests two classes of calmodulin-regulated activity: The first class being proteins involved with "housekeeping" functions and the second class involved with responses unique to that tissue.

Finding 3: The preferential accumulation of the 62 kilodalton calmodulin-binding protein in the mature regions of the root suggest a developmental regulation of this protein.

Finding 4: Discovery of other similar, but not identical, calmodulin-binding proteins in stems and nodules suggests post-translational modification(s) of proteins or presence of different protein isoforms may be involved in specialized signaling activity in specific tissues.

Finding 5: Identification of this calmodulin-stimulated protein as glutamate decarboxylase within the framework of calcium signal transduction offers insight into the mechanism(s) by which the root responds to gravity and other signals via the production of gamma-aminobutyrate (GABA).

Publication

Ling, V. and Assmann, S.M. 1992. Cellular distribution of calmodulin and calmodulin-binding proteins in *Vicia faba* L. *Plant Physiology* 100: 970-978.

SYNAPTIC RELATIONS OF TYPE I AND TYPE II HAIR CELLS IN THE MAMMALIAN CRISTA AMPULLARIS

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Description of Research

The long-range goal of this research is to understand the structural and functional organization of the sensory epithelium in the vestibular end organs. These end-organs include the crista ampullaris, which detects three-dimensional motion, and the utriculus, which detects gravity. Our studies to date have concentrated primarily on the crista ampullaris, but preliminary data from the utriculus indicate similar regional synaptic organization.

Previous work at the light microscopic level has characterized three types of afferent nerve fibers innervating two types of receptor cells (type I and type II hair cells). The three types of nerve fibers are: (1) calyx (or chalice) fibers, which germinate in a cup-like ending around type I hair cells; (2) bouton fibers, which terminate as synaptic boutons at the base of type II hair cells; and (3) dimorphic fibers, which terminate in both calyx and bouton endings.

In the adult pigmented chinchilla, type I and type II hair cells are present in equal proportion throughout the sensory epithelium. In contrast, the three types of nerve fibers show an unequal distribution. Calyx fibers (10 percent of the total) are found primarily in the central zone. Bouton fibers (20 percent of the total) are found primarily in the peripheral zone. Dimorphic fibers (70 percent of the total) are found throughout the epithelium. These three types of fiber also have different physiological properties. Simply stated: Calyx fibers have irregular firing patterns, bouton fibers have regular firing patterns, and dimorphic fibers have a broad range of firing patterns. Dimorphic fibers are irregular in the central zone and regular in the peripheral zone. In addition to these differences in discharge regularity, there are differences among the three fiber types in galvanic sensitivity and in response dynamics. From these earlier studies, it was concluded that the physiology of an afferent fiber is more closely related to its location in the sensory epithelium than to its branching pattern or to the types and number of hair cells it contacts.

Building upon these studies, we have been making progress on three projects: (1) ultrastructural analysis of physiologically characterized afferent fibers; (2) further analysis of the regional variations in synaptic organization; and (3) comparative studies on a primate model, the squirrel monkey.

In the first project, we have observed some morphological features that may serve to distinguish hair cells contained in the two different types of afferents in unlabeled material. Previously, it has not been possible to distinguish type I hair cells belonging to pure calyx units from those belonging to dimorphic units without some form of dye labeling of the afferent fiber. Based upon serial section analysis of 10 samples from cristae in four animals, there appear to be at least two classes of type I hair cells. We have confirmed this difference in several horseradish peroxidase (HRP)-labelled calyx and dimorphic units.

Figure 1. An HRP-injected physiologically characterized calyx unit. This unit had a cv of 0.63 and a low gain to head rotation at 2 Hz. Note the large subcuticular mitochondria and thick stereocilia on the type I hair cell enclosed with the unlabeled calyx.

In our previous studies of the chinchilla cristae, we found it convenient to divide the neuroepithelium into central, intermediate, and peripheral zones of equal areas. The synaptic ultrastructure of these three zones differed in several ways. We have now found that the central zone can, itself, be divided longitudinally into two subzones. Conclusions are based on a serial ultrastructural examination of 10 samples, each 2 μm thick. Four of the samples were cut in the longitudinal plane of the cristae and the others were sectioned transversely. Stereological methods were used to estimate the number of synaptic features per hair cell.

Having completed our studies of the ultrastructural synaptic organization of the chinchilla crista, we initiated a parallel study in the monkey. We had previously shown that the ratio of type I to type II hair cells was higher in the monkey (3:1), than in the chinchilla (1:1). We were interested in how the difference in hair-cell ratios would be reflected in the synaptic organization of the crista in the two species. Our results are based on five samples from two cristae in two squirrel monkeys.

Accomplishments

(1) There appear to be two classes of type I hair cells in mammalian cristae. These two classes are distinguished by certain ultrastructural features. The first class has large mitochondria subjacent to the cuticular plate and thicker stereocilia. These mitochondria were twice as large as those found elsewhere in the same cell. Cells containing these larger mitochondria were found mainly in the central zone at the apex of the crista and occasionally in the intermediate zone. So far they have not been observed in the peripheral zone.

The second class of type hair cells has smaller subcuticular mitochondria and thinner stereocilia. This second class was found in units identified as dimorphic units by the presence of calyceal collaterals. We have confirmed this difference in several units unlabeled by extracellular injections of HRP and in a small number of HRP-unlabeled and physiologically characterized calyx and dimorphic units. Type II hair cells had subcuticular mitochondria similar in size to the second class, but their stereocilia were thinner than those of type I hair cells in any region of the crista. Preliminary studies of the utricular macula indicate that the same two classes of type I hair cells are also present in the striolar region.

(2) In the chinchilla, the central zone can be divided longitudinally into two subzones: one (CA) occupying the apex of the crista, and the other (CS) located along its slopes. The CA region differs from the CS region in having (1) larger, more widely space hair cells; (2) thicker calyx endings; (3) most of the complex calyx endings present in the neuroepithelium; (4) larger numbers of calyceal invaginations per type I hair cell; (5) a higher concentration of ribbon synapses between type II hair cells and calyx outer faces; and (6) fewer afferent boutons per type II hair cell. Within the CA zone, type I hair cells are innervated by complex, as compared to simple, calyx endings that have more calyceal invaginations and fewer outer-face ribbon synapses.

(3) From our ultrastructural studies in the monkey we found that there are 6–10 type ribbons per type I hair cell and some indication that central type I hair cells have more ribbons that peripheral type I hair cells. In addition, each central type I hair cell had ≅ 20 calyceal invaginations, whereas each peripheral type I hair cell had ≅ 2 invaginations. Although qualitatively similar gradients were observed in the chinchilla, the numbers of ribbon synapses and calyceal invaginations were smaller in the monkey.

Type II hair cells in the monkey had similar numbers of ribbons and afferent boutons; there were fewer boutons and ribbons centrally, than peripherally (≅ 13 vs. ≅ 40 per type II hair cell). Very few ribbons from type II hair cells onto the outer surface of calyces were seen in the monkey as compared to the chinchilla. As in the chinchilla, the majority of these were found in the central

zone. Such ribbons constituted only 1 percent (12/952) of the total number of synaptic ribbons in the monkey; whereas in the chinchilla, they constituted 11 percent (456/4007) of the central outer-face ribbons. In the chinchilla, outer-face synapses are so common that most calyx afferents could be functionally dimorphic; this is not the case in the monkey.

In the monkey, the number of efferent boutons per hair cell differs between the central and peripheral zones with ≅ 2 efferent boutons per type I hair cell in the central zone, and ≅ 0.5 per hair cell in the periphery. Type II hair cells had ≅ 4 in the central zone and ≅ 10 in the periphery. In contrast, the efferent innervation of type I and type II hair cells was more uniform in the chinchilla.

Significance of the Accomplishments

Finding 1: The use of purely morphological features to distinguish subclasses of type I hair cells belonging to different afferent classes would provide a significant contribution to studies of the vestibular periphery. Such criteria would obviate the necessity of physiological characterization, such that space flight or human material could be analyzed. The functional significance of the features used to determine the two different classes of type I hair cells is yet to be determined; but, e.g., it is conceivable that the thicker stereocilia found on type I hair cells in calyx afferents are responsible for the lower gain of these calyx afferents due to their greater viscous drag and mechanical loading on the hair cell.

Finding 2: It may be of functional importance that 75–80 percent of the calyx fibers found in the central zone in the chinchilla are located in CA, the subdivision along the apex of the crista. We have yet to determine whether the synaptic differences are a reflection of the specific innervation of calyx fibers. In addition, we are currently attempting to determine whether this subdivision of the central zone also holds for the squirrel monkey.

Finding 3: The general trends in regional synaptic innervation that we observed in the crista of the chinchilla, a rodent model, seem to be valid, as well, in the monkey, a primate model. This has implications for studies of human synaptic innervation of the vestibular periphery.

However, two features were different in the monkey: (1) *The numbers of outer-face synapses.* In the chinchilla, outer-face synapses are so common that most calyx afferents could be functionally dimorphic; this is not the case in the monkey. This means that most likely the only innervation that a calyx unit in the monkey receives is from the hair cells enclosed within it. (2) *Regional variations in efferent innervation.* The most significant data that we have on efferent effects on afferent discharge rates are from the squirrel monkey. These data show that efferents exert a greater effect upon irregular afferents. Subsequent studies have shown that irregular afferents are found predominantly in the central zone. The present findings suggest that the greater numbers of efferent boutons per central type I hair cells may be responsible for the greater effects of efferent innervation on irregular afferents.

An understanding of the synaptic relations of hair cells with their afferent and efferent endings is fundamental to understanding alterations during space flight, as well as during development of the vertebrate vestibular apparatus in extended space flights. These changes will probably be subtle, at best; and, so, quantitative baseline data from normal adult tissue, including non-human primates, such as that shown in the present study, are needed for comparison to space flight experiments.

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INFLUENCE OF ANTIORTHOSTATIC SUSPENSION ON RESISTANCE TO INFECTION

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Description of Research

This research project was designed to evaluate how antiorthostatic suspension, a ground-based modeling system that simulates certain aspects of weightlessness that occur during space flight, influences the capacity of mice to resist microbial infection.

When humans are exposed to pathogenic microorganisms, either through childhood vaccination programs or through natural contact with environmental microorganisms, the immune system generates protective immunity to that pathogen. Sometimes this immunity lasts for the life span of the host (e.g., polio vaccine), and in other instances the immunity lasts only for several years (e.g., tetanus vaccine). Long-term immunological memory plays an important role in the health of adult humans.

Since space flight has been shown to alter many of the effector mechanisms of the adult immune system, some of which include those responsible for mediating resistance to infection and immunological memory, this research project has addressed the question of whether space flight, which is a stressful event, could alter the ability of the immune system to resist microbial infection and generate and express immunological memory. If space flight inhibits the capacity of the immune system to resist infection and generate and express immunological memory, this could predispose subjects exposed to space flight to an increased risk of microbial infections.

The antiorthostatic suspension model has become the most widely accepted ground-based modeling system employed to simulate certain changes in immune cell effector function that occur as a result of space flight, and has been utilized in this project. This model entails suspending rodents with a head-down tilt of ~20°, such that their hind limbs no longer bear weight. This induces a forward shift in body fluids similar to that which occurs as a result of weightlessness during space flight, and also induces some changes in immunological function that have been reported during and following space flight.

Mice were suspended in the antiorthostatic suspension system or in the control orthostatic position, in which there is no head-down tilt, to distinguish the immunological effects induced by the stress of the head-down tilt from the effects induced by the stress of attaching the suspension apparatus, and were infected with pathogenic microorganisms to study their ability to resist infection and generate and express protective immunological memory.

Accomplishments

It is well documented that stressful events can depress certain facets of immunological function. However, the relevance of these alterations in the context of resistance to infection have yet to be studied in any detail. The predominant number of reports in the literature contend that since stress can depress some functional capacities of the immune system, a correlated decrease in resistance to infection should accompany it.

The data generated in this project have, in fact, shown the exact opposite. Mice have been suspended in the antiorthostatic position and infected at various timepoints throughout the suspension. Using the pathogenic bacterium *Listeria monocytogenes*, a microorganism commonly used to evaluate cell-mediated resistance to infection, we found that resistance to the challenge infection was not altered if mice were infected concurrently with the onset of the suspension.

However, if the infection was started on day 2 or 4 of the suspension, which represents the most stressful period of the suspension experiments, mice were completely resistant to the challenge infection. In fact, mice infected on day 2 or 4 of the suspension were completely resistant to a lethal dose of *L. monocytogenes*. Control mice infected with *L. monocytogenes* typically require 7 to 10 days to clear the challenge infection. Mice infected on day 2 or 4 of the suspension were able to clear the infection within 1 day, which represents a remarkable enhancement in resistance to infection, even though the subjects were being subjected to the stressful event of antiorthostatic suspension. As the mice adapted to being in the antiorthostatic position, an attenuation of the enhancing effect was observed. Mice infected on day 7 of the suspension no longer displayed the same magnitude of enhanced resistance to *L. monocytogenes*, and the immune system required 7 days to clear the infection.

Unfortunately, the enhancement of resistance was not always viewed as advantageous, in that mice infected on day 2 or 4 of the infection failed to generate a long-lived state of protective immunological memory to the listeria infection. This has been attributed to the host being able to clear the challenge infection so rapidly that stimulation of effector T-lymphocytes, which mediate memory immunity to *L. monocytogenes*, did not occur. Mice that were infected concurrently with the onset of infection, or after adapting to the stress of antiorthostatic suspension, generated normal levels of protective immunological memory.

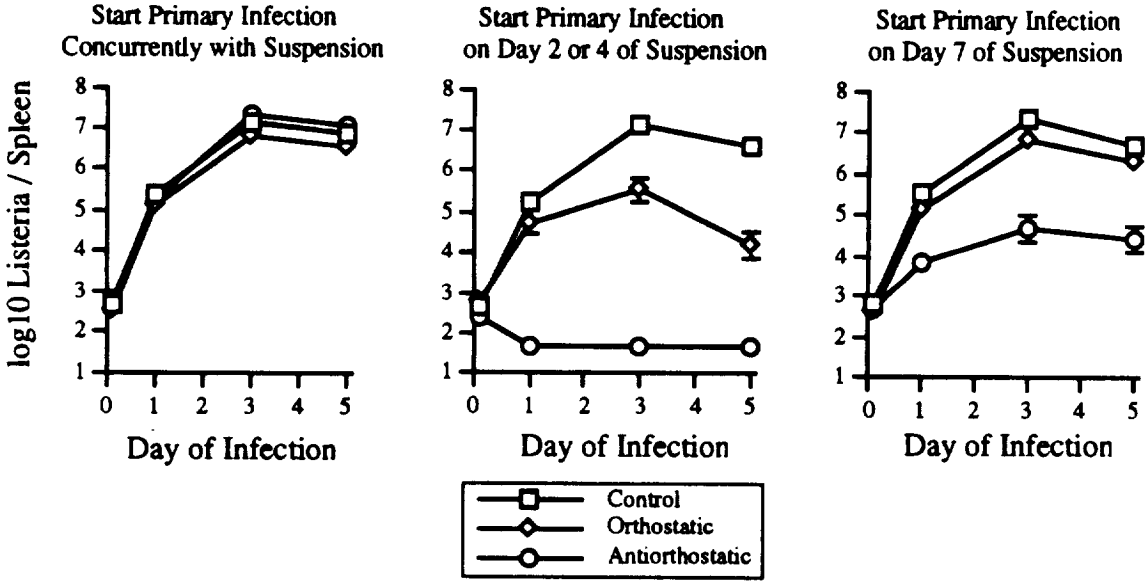

Figure 1. Growth of a primary *L. monocytogenes* infection in mice suspended in the orthostatic or antiorthostatic positions. Suspending mice for 2–4 days prior to the onset of infection enhanced the capacity of the immune system to resist infection.

Since the macrophage is the cell lineage responsible for the killing of *L. monocytogenes*, we studied marcrophage functional activities. We were able to correlate the enhancement of resistance during suspension with increased levels of marcrophage activation.

Significance of the Accomplishments

The novel findings of this project are not consistent with the predominant reports in the literature, which suggest that suspension, like many other forms of stress, depress immune cell function and therefore are expected to decrease host resistance to disease. The mechanisms underlying the capacity of antiorthostatic suspension to enhance resistance to *L. monocytogenes* were not apparent, but may have involved direct macrophage activation, macrophage activation via other cells of the immune system (such as natural killer [NK] cells), and by other tissues and organs, most notably the neurological and endocrine systems, which are affected by stress and can exert immunomodulatory capacities during stressful events. While it is possible that certain forms of stress may depress some facets of the immune system and in fact render the host more susceptible to disease, some forms of stress may actually enhance certain immune cell effector mechanisms, which may endow the host with increased resistance to infection by particular classes of pathogenic microorganisms during stressful periods.

The number of animal models available for evaluating how stressful events influence immunological function is small. Of these models, only a small percentage is utilized for studies of how stress can influence resistance to infection. These results are among the first which conclusively demonstrate that a physiological stressor can, in fact, enhance resistance to infection.

Identifying the mechanisms by which the enhancement to infection was mediated can contribute to our knowledge of how other physiological systems interact with the immune system. The immune system is not an isolated entity that works in an independent fashion; but instead, bidirectionally communicates with other tissues and organs by mechanisms which are largely unknown. Studies such as these can help to elucidate how the immune system participates in mediating systemic physiological homeostasis, and how stressful events, be they on Earth or as a result of manned space missions, can affect these normal modes of communication. By understanding what the normal modes of communication are and how they are altered as a result of stress, it may be possible to design and test therapeutic regimens that help the host better adapt to certain stressful events.

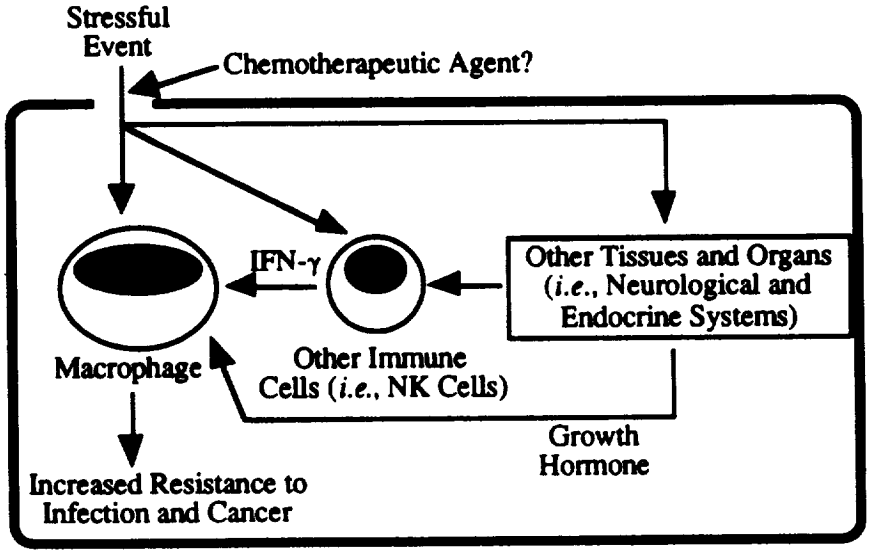

Figure 2. Possible mechanisms by which antiorthostatic suspension, and other potential physiological stressors, may activate macrophages to enhance resistance to infection. If the mechanisms by which enhanced macrophage activation occurs can be elucidated, it may be possible to develop chemotherapeutic agents that stimulate these mechanisms, which could be of benefit to NASA and to basic biomedical sciences.

Moreover, identifying how the enhanced resistance to infection was mediated may lead to advances in preventing and treating microbial infections. By developing chemotherapeutic agents that can stimulate the underlying mechanisms which enhance resistance to infection, it may be possible to activate the immune system in persons at increased risk of infection, including astronauts, trauma patients, and burn victims. In addition, it may be possible to stimulate macrophage activation in patients with dysfunctional immune systems, such as transplant recipients that are receiving immunosuppressive therapy, or AIDS patients, who have macrophages but lack the T-cells necessary for stimulating macrophage activation.

Such therapies may not be restricted to effectiveness against microbial infection, but also may include resistance to neoplastic diseases, since the macrophage can exert potent effects against transformed cells. By altering normal events that are occurring within the host, it may be possible to alleviate many of the toxicities associated with administering exogenous materials, such as high levels of cytokines, that are encountered with many of the therapies used today. Indeed, there is enthusiasm and potential for this NASA project in contributing not only to the manned space program but also to basic biomedical sciences.

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DEVELOPMENTAL STUDY OF RESPONSES TO AUXIN IN THE *DIAGEOTROPICA* TOMATO MUTANT

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Description of Research

My research is aimed at understanding the cellular events in plants that take place after the perception of a gravity stimulus. This signal transduction chain may involve calcium ions, growth inhibitors, and other factors. However, it is generally believed that during the response to gravity in plant shoots, indole-3-acetic acid (IAA) plays an important role. According to the prevailing Cholodny-Went hypothesis, IAA is transported across the stem in the direction of the gravity vector, which causes asymmetric growth (elongation of cells on the lower side), leading to upward curvature of the plant.

The *diageotropica* (*dgt*) mutant of tomato offers insight into the role of IAA in gravicurvature. The *dgt* is insensitive to exogenous IAA in elongation assays, and has an impaired perception of gravity (the mature plant has a horizontal growth habit, for which it was named). Recently we have found that *dgt* seedlings can respond to gravity. Within the framework of the Cholodny-Went hypothesis, it is puzzling how *dgt* can respond to gravity if it is indeed insensitive to IAA. Therefore, we have undertaken a careful developmental study to determine whether IAA indeed plays a role in the *dgt* gravitropic response.

Accomplishments

(1) The gravitropic response of *dgt* has been measured over a developmental time course, and compared to that of the isogenic wild type tomato, VFN8. As shown in Figure 1, 10-day-old *dgt* seedlings respond more slowly than VFN8, but after 22 hours *dgt* achieves essentially the same upward curvature as wild type plants. This pattern continues for approximately 10 more days, with *dgt* always slower at early time points (e.g., 6 hours), but then eventually achieves the same degree of curvature as VFN8 (see Figure 2). However, 20 days after germination *dgt* can no longer curve upward as well as VFN8. This impaired graviresponse corresponds to development of the first internode of the tomato seedlings. Some developmental event apparently occurs during formation of the internode which makes *dgt* less responsive to gravity.

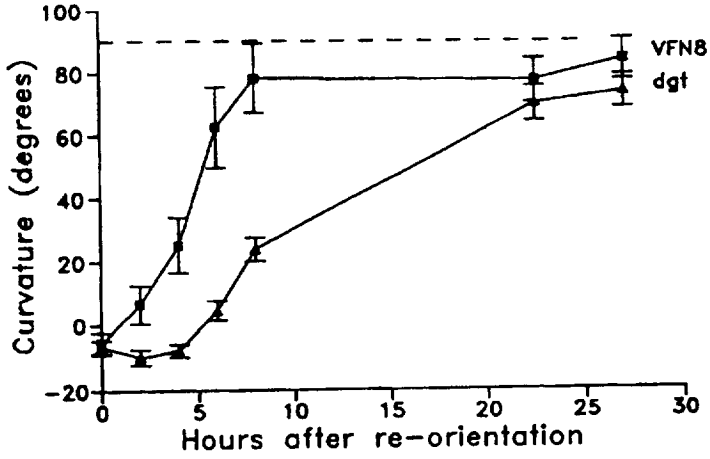

Figure 1.

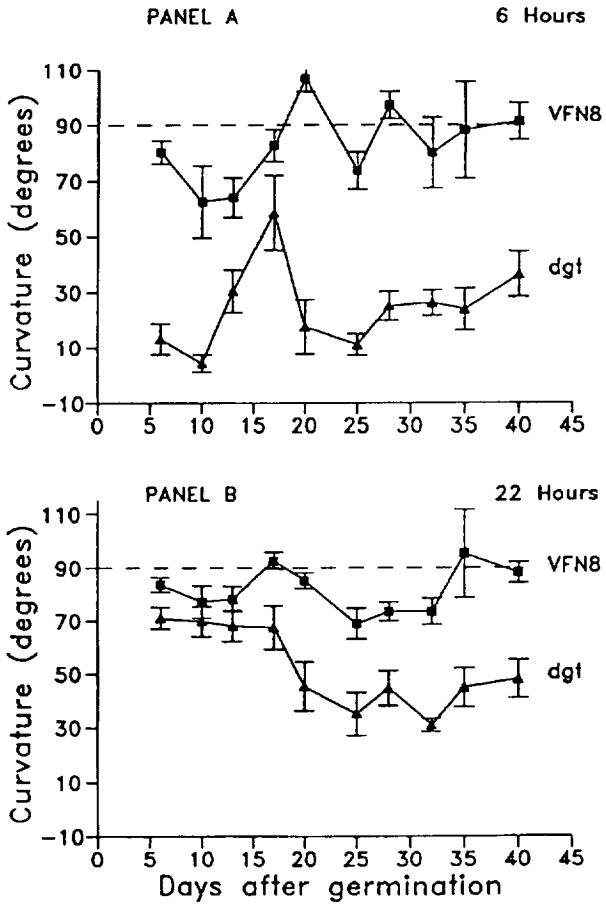

Figure 2.

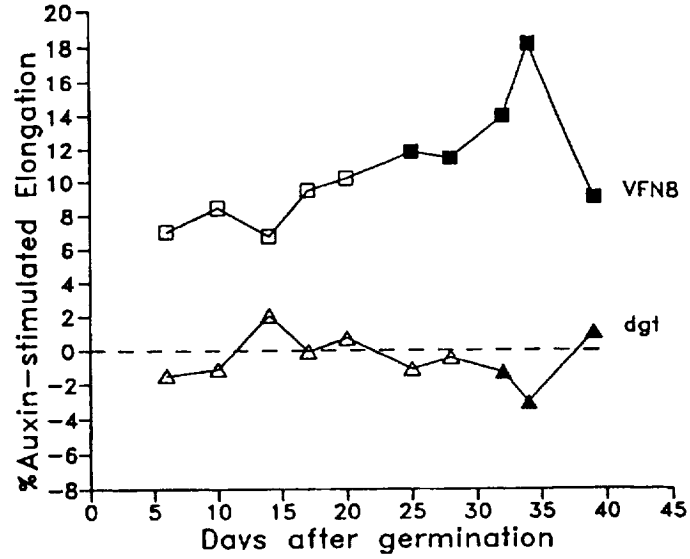

Figure 3.

(2) During our study, we tested *dgt* and VFN8 for stimulation of stem elongation by IAA solutions. Figure 3 shows that *dgt* never significantly responds to IAA, while VFN8 exhibits IAA-stimulated elongation of 8 to 15 percent as compared to stem segments in buffer only. Therefore, at no point in development do the *dgt* seedlings in our study elongate in response to exogenously applied IAA.

(3) The above findings led us to investigate further whether IAA is indeed involved in the response of young tomato seedlings to gravity. We immersed 7-day-old intact seedlings in the buffer with various IAA transport inhibitors and, 2 hours later, reoriented the seedlings horizontally and measured the upward curvature. Figure 4 shows that 10 and 100 µmol 2,3,5-tri-iodobenzoic acid (TIBA, which specifically blocks polar IAA transport at the cellular efflux carrier) greatly reduces the gravitropic response of both *dgt* and VFN8. N-1-naphthylphthalamic acid (another polar transport inhibitor) and IAA produce very similar responses. The effect of IAA transport inhibitors and high levels of IAA itself on the gravitropic response in *dgt* and the wild type tomato indicates that IAA is involved in the gravitropic response of both normal tomatoes and the *diageotropica* mutant.

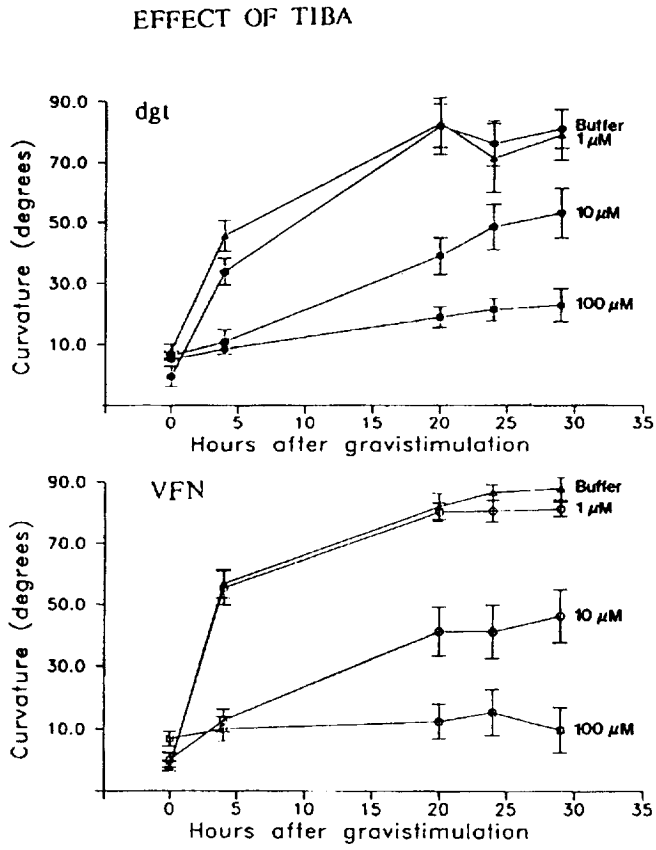

Figure 4.

Significance of the Accomplishments

We have shown that *dgt* does not elongate in response to exogenous IAA but does respond to gravity in an IAA-dependent manner. Taken together, these results suggest either that IAA perception or response is different or shifted between the elongation and curvature assays, or that *dgt* may respond to gravity by inhibition of growth of the upper side, rather than elongation of the lower side, of the hypocotyl. We are testing these hypotheses further, and confirmation of either hypothesis would lead to a better understanding of the mechanism of the gravitational response in plants.

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SARCOMERE LESIONS IN ATROPHIED RELOADED ADDUCTOR LONGUS

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Description of Research

The overall goal of the current research is to investigate alterations in muscle structure and function during and following exposure to microgravity (space) or simulated microgravity (hindlimb suspension unloading, HSU) which predisposes such muscle to injury on reloading. The higher tensions produced in eccentric work and the association of eccentric work with greater damage in normal muscle have led workers to postulate that the absence of the high-tension eccentric component of muscle contraction during unloading or microgravity renders such muscle more susceptible to the high-tension of eccentric contractions on reloading. Specifically, the primary goals of the present line of investigations are to test the hypotheses that HSU adductor longus (AL) is more susceptible to lengthening contraction damage than the normal AL, and that elevations in tension (P) and dP/dt during the eccentric protocol correlate with morphological damage. A secondary goal is to investigate the mechanism of any elevated sensitivity.

Accomplishments

(1) Using the 5-minute AL eccentric contraction model (Thompson, 1993) at a Fast (F), Medium (M), or Slow (S) lengthening rate, it has been determined that:

A. The muscle damage produced, as assessed by occurrence of myofiber phagocytic invasion and degree of sarcomeric disruption, increases with faster lengthening rates (rate=mm/ms) and higher rates of rise in tension (dP/dt=g/ms) over the first minute (Table 1).

Table 1

Muscle Damage Produced as Result of Faster Lengthening Rates and Higher Rates of Rise in Tension					
	Lengthening Rate	Rates of Rise in Tension	Damage	Tetanus Duration	Tetanus Frequency
F	2/200	0.5–0.8	Multiple fiber phagocytic invasion.	300(ms)	30x/m
M	2/500	0.4–0.5	Focal sarcomeric disruptions, no invasions.	600(ms)	30x/m
S	2/800	0.3–0.4	Sarcomeres indistinguishable from control.	900(ms)	15x/m

B. The distal tendon of the AL could be surgically severed, attached to the transducer for physiological data acquisition, then reattached to its origin in the surviving animal without causing the extensive damage to the AL that is seen following the Medium and Fast stretch rates.

C. Application of a standardized bout of eccentric contractions (Table 2) produced at least five distinct lesion types .

Table 2

Eccentric Contractions				
Lengthening Rate:	Duration	Repetitions	Bout Duration	Stimulation Parameters
2 mm/400 ms	The last 400 ms of a 500 ms tetanus.	20x/minute	5 minutes	3 v, 100 Hz

All five lesion types (Figure 1) were observed in AL muscles reloaded for 12–48 hours following a 14-day HSU treatment. The most prominent lesion seen in pilot studies that eccentrically stretched normal muscle was the hypercontraction type (lesion illustrated in Figure 1C; lesion frequency in parenthesis, Figure 2).

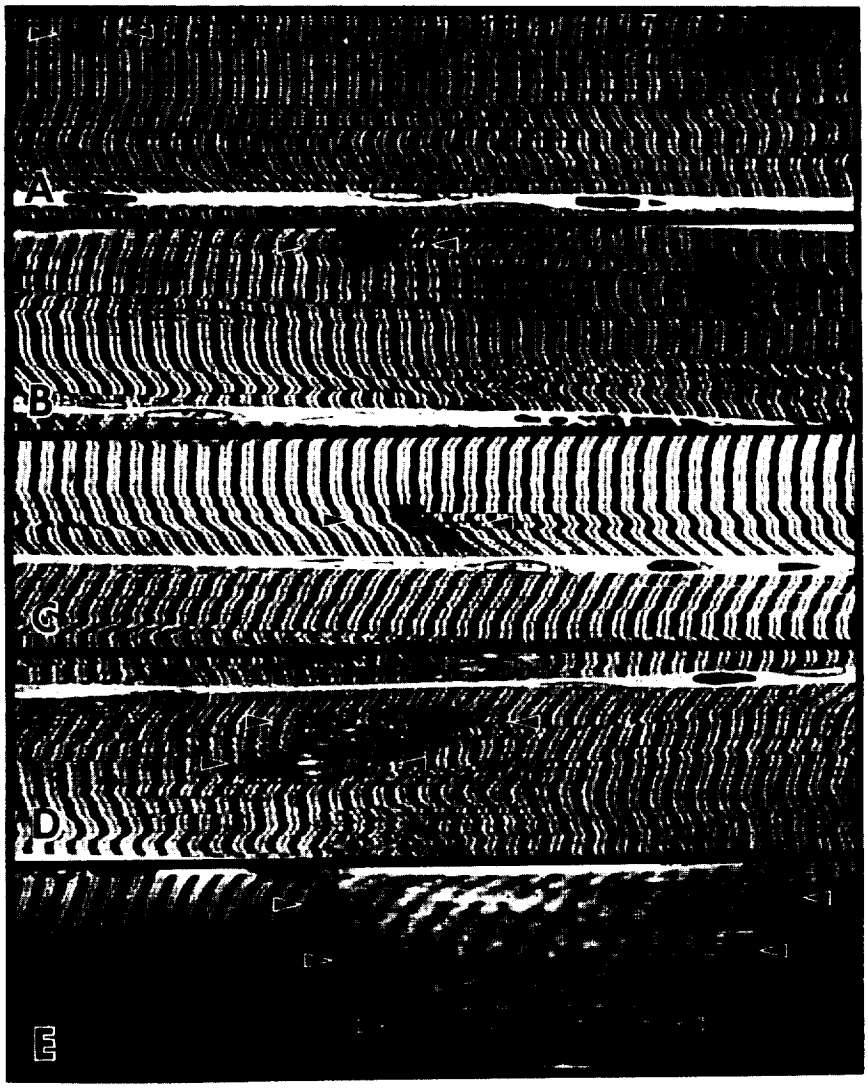

Figure 1. Photomicrographs of the five lesion types seen following the eccentric contraction protocol: (A) Wide-A: focal widening of A-band; (B) Opaq.: uniform opaque regions covering 3< sarcomeres; (C) H.C.: hypercontracted focal regions of 3< sarcomeres; (D) Miss-A: missing A-band focal regions of 3< sarcomeres; (E) H.S.: hyperstretched focal regions of 3< sarcomeres. The abrupt nature of these disruptions (between arrowheads), appositioning the seemingly normal sarcomeric pattern, strongly suggests mechanical failure of some element of the muscle fiber. Bar equals 10 microns.

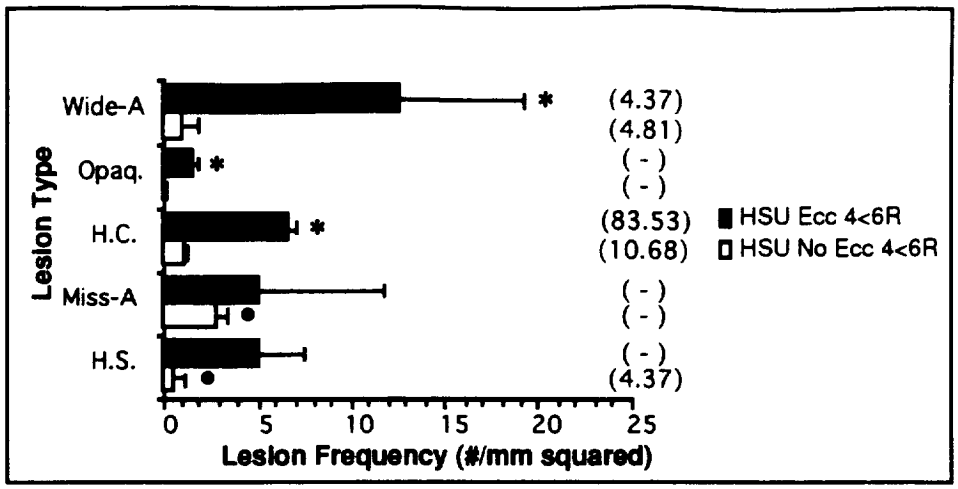

Figure 2. Bar graph depicting the lesion frequency for the five types of lesions observed following the AL eccentric contraction protocol. Wide-A, Opaq., H.C., Miss-A, H.S. are abbreviated as in Figure 1. HSU Ecc 4<6R: 14-day hindlimb suspension unloaded animals exposed to eccentric protocol and reloaded for 4–6 hours. HSU No Ecc 4<6R: 14-day hindlimb suspension unloaded animals exposed to no eccentric protocol and reloaded for 4–6 hours. Asterisk (*) indicates more elevated in HSU-Ecc-4<6R group over the HSU No Ecc 4<6R group (Wilcoxon paired sample test, $p \leq 0.05$; n=6/group, unless dotted "•" indicating n=5). Lesion frequency for a normal rat AL, exposed to the eccentric contraction protocol, is parenthetically indicated (in progress group, n=1); (-)≤0.5/mm squared.

D. The rank in total lesion frequency was very highly correlated with the rank of pre-lengthening contractile tension (Spearman's Rank Correlation Coefficient, 0.950) and directly and highly correlated to the rank of %-decrease in optimal tension, Po, 15 minutes following the eccentric protocol (Spearman's Rank Correlation Coefficient 0.829).

(2) Methacrylate embedded muscle tissue remains immunoactive to goat anti-rabbit (GAR) or goat anti-mouse (GAM) antibodies (anti-sarcomeric myosin, GAR IgG; anti-titin 9D10, GAM IgM), if mildly fixed prior to embedment (Fixation protocol: 1. Immersion fixed in 1–4 percent paraformaldehyde and 0.1–0.5 percent glutaraldehyde, or 2. Freeze substituted in 0.5 percent uranyl acetate in methanol or acetone at -80°C).

Significance of Accomplishments

Finding 1A: Since tension has been shown to increase in contracting muscle as the speed of eccentric lengthening increases, this implicates elevated tension in eccentric damage production. By controlling lengthening rate, while monitoring P and dP/dt, correlation of the effect of changes in P and dP/dt with the specific sarcomeric sites of damage may be executed.

Finding 1B: Demonstration of the ability to sever and reattach the distal tendon of the AL without producing artifactual damage to the AL allows the collection of physiological data (Po, dP/dt, Lo) on this muscle under controlled conditions and the ability to follow the progress over time of subsequent lesion development and repair in normal and atrophic antigravity muscles. Such muscles, predominately composed of slow-twitch oxidative fibers, are deep and adjacent to bone. To investigate the eccentric component on reloading of atrophic antigravity muscles, a surgical model is required in order to free the distal tendon and monitor physiological parameters

under controlled eccentric conditions for later correlation to actual damage. It has also been observed by the sponsoring laboratory (Dr. Danny A. Riley, personal communication) that reloading the soleus muscle in rats is a less reliable model because the conscious ambulatory rat often behaviorally unloads the soleus by walking on its heels to avoid using the soleus when reloaded. This is not the case with the AL; this muscle has been documented to consistently undergo atrophic modification on unloading and to consistently demonstrate early eccentric-like lesions that are not present in the simultaneously harvested soleus (at 6–12 hours reloading).

Finding 1C: The appearance of the different lesion types may represent differing stages in a sequel of damage (leading to segmental necrosis?), or may represent independent lesion types arising from differing and unrelated "weak points." The sudden appearance of these sarcomeric disruptions adjacent to seemingly normal sarcomeres, particularly the wide A-band, strongly suggest an initial mechanical failure of some components (myofibrillar, cytoskeletal, and/or membrane components: sarcolemma, t-tubule, or sarcoplasmic reticulum), which may later be acted upon by other factors; i.e., enzymatic digestion (calcium activated neutral protease) and compromised vascular supply (anoxia). Similar lesion morphology, previously seen in reloaded 14-day HSU muscle, indicates operation of a common mechanism in both eccentric HSU and reloaded HSU muscle; therefore, this eccentric model is suitable to study eccentrically induced lesion progression and repair in atrophic versus normal muscle. The pilot work on normal eccentrically stretched muscle suggests that the sarcolemma is more sensitive (most prominent lesion is hypercontracture, indicative of calcium leakage due to membrane damage), whereas, in the atrophic muscle the sarcomeric elements are more susceptible (most prominent lesion is the wide A-band). Atrophic muscle possesses more redundant sarcolemma than does normal muscle, thus, atrophic muscle may be under less sarcolemmal stress and more contractile element stress for a given level of tension than is normal muscle.

Finding 1D: The correlation between damage frequency and pre-lengthening tension level supports the premise that tension is a factor in lesion production. More damage is produced in muscle exposed to high initial tension and faster stretch rates than if the muscle is exposed to the same faster stretch rate under lower initial tension. Such high-tension, high-stretch rate conditions are produced in the "braking actions" of the antigravity muscles. And, finally, correlating the damage morphology with decreased post-lengthening Po confirms the use of 15-minute post-treatment Po decrease as an index of sarcomeric damage, as opposed to fatigue.

Finding 2: At the high-magnification light microscopy (LM) level and low-magnification electron microscopy (EM) level, the vulnerable sites of high-tension damage may be identified using both conventional LM and EM visualization techniques and the more recently developed immunohistochemical and immunoelectron microscopic techniques. This will assist in visualizing the immunoantigenicity and continuity of the normally invisible sarcolemmal and myofibrillar cytoskeletal elements (titin, desmin, dystrophin) within normal and lesioned muscle, and will assist in the detection of isozyme shifts in contractile and regulatory proteins (myosin and protein C isoforms) in lesioned and non-lesioned muscle.

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THE ROLE OF H^+ IN THE FATIGABILITY OF THE SOLEUS FOLLOWING HINDLIMB SUSPENSION

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Description of Research

The long-term goal of our space biology research program is to understand the effects of space travel (zero-g) on limb skeletal muscle function with particular emphasis on the cellular alterations in single slow- and fast-twitch muscle fibers. A second goal is to develop countermeasures to prevent the deleterious effects of zero-g travel on limb skeletal muscle. Weightlessness has been shown to cause considerable muscle atrophy in slow-twitch antigravity muscles such as the soleus (SOL) and to lead to an increased fatigability. The hindlimb suspension (HS) rat model has been extensively employed by us and others to investigate the functional changes (cellular and molecular) associated with zero-g. The model has been shown to induce muscle fiber atrophy, reduce peak force and power, increase maximal shortening speed (V_{max}), and increase fatigability of the SOL muscle. Additionally, HS has been shown to increase the percentage of fast type IIa fibers in the SOL. Since H^+ ion inhibits force to a greater extent in fast compared to slow-twitch fibers, I hypothesized that the increased fatigability of the SOL following HS could be in part attributed to an enhanced susceptibility of the actomyosin cross-bridge to the force inhibiting effects of H^+.

In order to test this hypothesis, the single-skinned fiber preparation was used and the H^+ concentration of the solution surrounding the contractile proteins varied such that the independent effect of H^+ on contractile function was determined. The effects of variations in pH of 7.0 and 6.2 on contractile properties were examined; these pH conditions were selected, as they mimic those observed before and following intense contractile activity in limb skeletal muscle.

Accomplishments

HS induced significant atrophy of the SOL muscle, and the SOL muscle to body weight ratio declined 35 percent. The change in SOL fiber diameter, peak force and tension (P_0) is presented in Table 1. Fiber diameter and peak force and tension decreased following HS. Peak tension (kN/m^2) was depressed by 15 percent at pH 6.2 for both groups.

Table 1. Diameter, Peak Force and Tension in Single Soleus Fibers.
Values are means ± SE. Number of observations is in parentheses.

Group	Diameter (μm)	Force (x 10^{-4} N)		P_0 ($kN \cdot m^{-2}$)	
		pH 7.0	pH 6.2	pH 7.0	pH 6.2
Control (43)	70±2	4.7±0.1	4.0±0.1**	129±6	109±5**
2 wk HS (43)	49±2*	2.0±0.1*	1.7±0.1	110±6*	93±5**

**Significantly different from pH 7.0, $p<0.05$. *Significantly different from control, $p<0.05$.

The maximum shortening velocity of single SOL fibers is summarized in Table 2. Fiber V_0 (maximum shortening velocity determined by the slack test technique) was significantly elevated by 64 percent following HS. The V_{max} (maximum shortening velocity calculated using the straight line form of the Hill equation) was not different from controls following HS. After 2 weeks, HS fiber stiffness (E_0 = elastic modulus) was depressed by 31 percent (Table 3). Since P_0 indicates the force per CSA, and E_0 represents the number of attached cross-bridges per CSA, the P_0/E_0 ratio was markedly increased following 2 week HS (62 percent), suggesting an increased force per cross-bridge (Table 3).

Table 2. Maximum Shortening Velocity.
Values are means ± SE. Number of observations is in parentheses.

Group	V_0 (fl•sec^{-1})			V_{max} (fl•sec^{-1})	
	pH 7.0	pH 6.2		pH 7.0	pH 6.2
Control (43)	1.55±0.07	1.58±0.05	(39)	0.91±0.05	1.01±0.05
2 wk HS (43)	2.54±0.19*	2.15±0.14**	(43)	0.96±0.08	1.01±0.06

**Significantly different from pH 7.0, $p<0.05$. *Significantly different from control, $p<0.05$.

Table 3. Elastic Modulus and Tension/Elastic Modulus Ratio.
Values are means ± SE. Number of observations is in parentheses.

Group	E_0 (x 10^7 N•m^{-2})		P_0/E_0	
	pH 7.0	pH 6.2	pH 7.0	pH 6.2
Control (19)	1.71±0.15	1.76±0.14	6.9±0.4	5.7±0.2**
2 wk HS (19)	1.19±0.21*	1.30±0.21	11.1±1.1*	8.1±0.8**

**Significantly different from pH 7.0, $p<0.05$. *Significantly different from control, $p<0.05$.

In control single SOL fibers, as pH was lowered, V_0, V_{max} and E_0 were unaltered, whereas the P_0/E_0 ratio was significantly depressed by 18 percent (Tables 2 and 3). In contrast, altered pH had a pronounced effect upon V_0 following HS, with V_0 decreasing 16 percent (Tables 2 and 3). Additionally, P_0/E_0 ratio was reduced (Table 3). This finding suggests that the fibers from HS animals are less resistant to H^+.

As pH was lowered, the force-pCa relationship shifted to the right (i.e., to higher Ca^{2+} concentrations) and was depressed in amplitude, indicating a reduction in the Ca^{2+} sensitivity of force development. The Ca^{2+} necessary to achieve half-maximal tension in fibers from both groups increased when pH was lowered from 7.0 to 6.2 (pCa_{50} 5.8 to 5.1). Furthermore, the Ca^{2+} threshold for force development was greater at pH 6.2 than at pH 7.0 in both groups by 1.0 pH unit. As pH was lowered, the steepness of the force-pCa curve increased in SOL control fibers, with pCa_{50} values of 5.79 at pH 7.0 and 5.06 at pH 6.2. Similarly, pCa_{50} values of 5.87 at pH 7.0 and 5.14 were observed in SOL fibers following HS. This suggests that the apparent co-operativity of force development had increased in both groups. Presently, the fibers are being typed on SDS gels; therefore, these preliminary results have not been subgrouped into fast- and slow-twitch fibers. It is anticipated that following fiber typing, HS and pH effects will be more apparent.

Significance of the Accomplishments

The effects of HS on muscle function (fiber atrophy, decreased peak tension, and increased V_0 of the slow type I fibers of the SOL) are confirmed in this study. The results of this study suggest that an increased H^+ ion concentration contributes to depressed contractile function, since both force and velocity decreased significantly as pH changed from 7.0 to 6.2. The relationship between V_0 and peak force ratio (pH 6.2/7.0) is presented in Figure 1. HS induces a shift from type I fibers with slower velocities to fibers with faster velocities. Importantly, the fibers exhibiting the fastest velocities showed the greatest reduction in force. This result supports the hypothesis that the increased fatigability following HS (and presumably zero-g travel) is in part caused by an increased susceptibility of the contractile apparatus to H^+ ions. Future efforts should be directed at the development of exercise countermeasures that prevent the myosin isozyme change (slow to fast) responsible for the increased sensitivity to H^+.

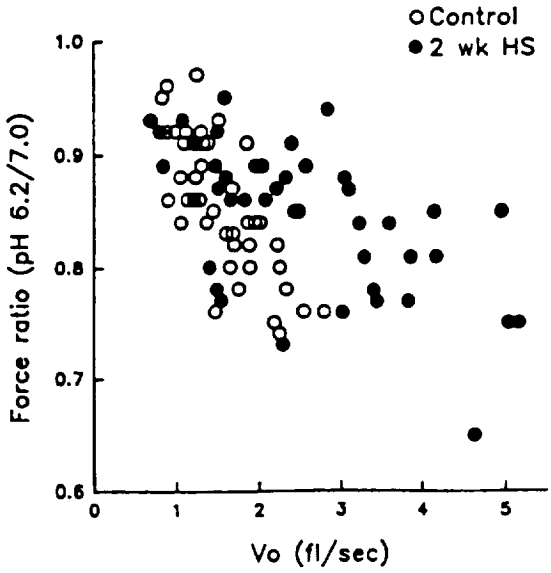

Figure 1. Relationship between V_0 and peak force ratio.

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MOLECULAR CHARACTERIZATION OF A PLANT Ca^{2+} ATPase

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Description of Research

The goal of our research is to elucidate the role of Ca^{2+} as a second messenger in gravity perception. We are approaching this goal using molecular, genetic, and biochemical techniques to investigate the role of higher plant Ca^{2+} translocating ATPases (Ca^{2+}-ATPases) which are critical to the regulation of cytosolic calcium levels.

Gravitropism, the ability of plants to grow directionally relative to the gravitational field is universal among higher plants. Despite years of research, the mechanisms by which plants detect and respond to gravity have not been exhaustively explored. One commonly cited model proposes that release of endoplasmic reticulum-localized Ca^{2+} to the cytoplasm acts as a second messenger in gravity perception. According to this model, a mass moving within the gravitational field causes Ca^{2+} channels to open, resulting in an increase in cytosolic Ca^{2+}, which might activate (directly, via calmodulin, or calmodulin sensitive protein kinases) Ca^{2+}-ATPases, H^{+}-ATPases, and auxin carriers.

The participation of Ca^{2+} in plant gravity perception is supported by three lines of recent evidence. First, oat coleoptiles exposed to chlorpromazine, a calmodulin inhibitor, showed reduced gravitropism. Second, in maize seedling roots, there is a strong correlation between the induction of graviresponsiveness by light, and the level of calmodulin in the apical millimeter where graviperception occurs. Third, using the fluorescent Ca^{2+} dye fluo-3 and scanning laser confocal microscopy, researchers were able to measure rapid increases in the level of cytosolic Ca^{2+} in the cells of maize coleoptile tips when horizontally oriented. Taken together, these results support a role for changes in the cytosolic Ca^{2+} concentration in graviperception.

Implicit in this model is the maintenance of a very low resting level of cytosolic Ca^{2+}, the maintenance of Ca^{2+} stores in the endoplasmic reticulum, and the ability to reestablish this condition after gravity induced Ca^{2+} fluxes. The regulation of cytosolic Ca^{2+} levels is widely recognized as a central element of regulatory processes in plants and is essential to the proposed role for Ca^{2+} as a second messenger in gravitropism. Active Ca^{2+} transport is responsible for maintaining low levels of cytosolic Ca^{2+} and in this way interacts with other mechanisms of Ca^{2+} release (channels) to effect regulated modulation of cytosolic Ca^{2+} that is important in several signal transduction pathways, including graviperception. Although active Ca^{2+} transport is catalyzed by both Ca^{2+}-ATPases and Ca^{2+}/H^{+} antiports in plant cells, the high affinity of Ca^{2+}-ATPase-driven transport is proposed to play the most significant role in modulating cytosolic Ca^{2+} levels in the physiological range. The goal of our research is to use molecular probes to test the hypothesis that the E.R.-localized Ca^{2+}-ATPase is involved in the signal transduction pathway leading to gravitropic growth of higher plants.

Ca^{2+}-ATPases have been difficult to study biochemically in plants because of the high levels of H^{+}-ATPase associated with membrane preparations that also possess Ca^{2+}-ATPase activity. To overcome this biochemical limitation and achieve the proposed research goal, we have identified cDNA and genomic clones encoding a Ca^{2+}-ATPase (identified as LCA) from tomato, which may be localized in the endoplasmic reticulum. The Ca^{2+}-ATPase cDNA can be distinguished from H^{+}-ATPase cDNA clones and has provided the basis for developing both Ca^{2+}-ATPase-specific nucleic acid and antibody probes. The specific objectives of our research have been to:

Objective 1: Identify Ca^{2+}-ATPase(s) localized in the endoplasmic reticulum (ER).
Objective 2: Determine the tissue localization of ER Ca^{2+}-ATPase in relation to the graviperceptive regions of the organs.
Objective 3: Determine whether the expression of the gene encoding ER Ca^{2+}-ATPase is regulated in a tissue or cell specific manner.
Objective 4: Critically assess the role of the ER-localized Ca^{2+}-ATPase in gravity perception by the expression of an antisense Ca^{2+}-ATPase gene in transgenic plants and determine whether reduced Ca^{2+}-ATPase levels interfere with gravitropic growth.

Accomplishments

(1) Genomic Sequencing: In order to finalize analysis of the primary sequence of the tomato ER-localized Ca^{2+}-ATPase and to identify its regulatory regions that may control tissue-specific expression, it was necessary to complete sequencing its genomic clone. We have completed nucleotide sequencing of the entire 10.3 kb LCA1 genomic clone (32, GenBank-accession number M96324) which includes 3 kb upstream of the coding region.

(2) Reporter Gene Fusion: We originally proposed to analyze LCA1 gene expression at the level of mRNA abundance in the gravity sensing region of the root tip by *in situ* RNA blot hybridizations. We have discovered that the very low abundance of LCA1 transcript makes this approach impractical. As an alternative, we have constructed a fusion gene comprising 3.0 kb of the sequences upstream of the LCA1 coding region linked to the ß-glucuronidase (GUS) coding sequence as a reporter gene. This construct has been transformed into tomato, and plants expressing GUS are now being analyzed.

(3) Identification of a Second Ca^{2+}-ATPase: We have recently identified and cloned a second gene encoding a putative Ca^{2+}-ATPase. The deduced sequence of this protein is dramatically different from LCA but shares very high amino acid homology with a yeast Ca^{2+}-ATPase which is localized in the endomembrane system. The subcellular localization and functional analysis of this protein will now be carried out in tandem with LCA.

(4) Analysis of Transgenic LCA1 Antisense Plants: In order to directly test the role of the ER Ca^{2+}-ATPase in gravity perception, we proposed analyzing the gravitropic response of roots in transgenic tomato and tobacco plants expressing an antisense LCA1 construct. We have successfully regenerated tobacco plants expressing an antisense gene. The T1 plants have reached maturity and have produced T2 seed. However, no reduction in LCA1 sense message levels was detected by RNA blot hybridization analysis. It is possible that the tomato and tobacco ER-localized Ca^{2+}-ATPase gene sequences are dissimilar enough to make antisense inhibition ineffective.

Regeneration of transgenic antisense tomato plants has been particularly difficult. Most antisense constructs were apparently lethal. No antisense plants could be regenerated while numerous plants were regenerated from vector only transformations. Five antisense plants have been regenerated, but their growth has been very slow, requiring nearly 1 year to reach maturity. We are now collecting T2 seed, and phenotypic analysis will be carried out in the T2 plants.

The difficulty in regenerating transgenic antisense tomato plants could be due to a direct effect of the antisense gene. In order to overcome this potential difficulty, we are now employing an inducible expression system based upon the tetracycline binding protein. In this system, exposure of plants to low levels of tetracycline activates transcription of the antisense gene.

Significance of the Accomplishments

Each of our accomplishments has contributed to our understanding of the role of Ca^{2+} in plant cell signal transduction, or set the stage for immediate progress in that area. Specifically, the LCA reporter gene fusions will tell us if the expression of a gene involved in Ca^{2+} homeostasis is correlated to sensory perception. Similarly, the antisense transgenic plants will help elucidate the importance of cytosolic Ca^{2+} in gravity perception. Lastly, the identification of a second Ca^{2+}-ATPase may reform our models of plant cell Ca^{2+} control and, thus, the role of Ca^{2+} in signal transduction.

Publications

Wimmers, L.E., Ewing, N.N. and Bennett, A.B. 1992. Higher plant Ca^{2+}-ATPases: primary structure and regulation of mRNA abundance by salt. *Proceedings of the National Academy of Sciences of the United States of America* 89: 9205-9209.

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This report consists of individual technical summaries of research projects of NASA's Space Biology Program, for research conducted during the calendar years of 1992 and 1993. This program includes both plant and animal research, and is dedicated to understanding the role of gravity and the effects of microgravity on biological processes; determining the effects of the interaction of gravity and other environmental factors on biological systems; and using the microgravity of the space environment as a tool to advance fundamental scientific knowledge in the biological sciences to improve the quality of life on Earth and contribute to NASA's goal of manned exploration of space. The summaries for each project include a description of the research, a list of the accomplishments, an explanation of the significance of the accomplishments, and a list of publications.

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