Modulation of Bone Remodeling via Mechanically Activated Ion Channels

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Description of Research

A critical factor in the maintenance of bone mass is the physical forces imposed upon the skeleton. Removal of these forces, such as in a weightless environment, results in a rapid loss of bone, whereas application of exogenous mechanical strain has been shown to increase bone formation. Numerous flight and ground-based experiments indicate that the osteoblast is the key bone cell influenced by mechanical stimulation. Aside from early transient fluctuations in response to unloading, osteoclast number and activity seem unaffected by removal of strain. However, bone formation is drastically reduced in weightlessness and osteoblasts respond to mechanical strain with an increase in the activity of a number of second messenger pathways resulting in increased anabolic activity. Unfortunately, the mechanism by which the osteoblast converts physical stimuli into a biochemical message, a process we have termed biochemical coupling, remains elusive.

Prior to the application of this grant, we had characterized a mechanosensitive, cation nonselective channel (SA-cat) in osteoblast-like osteosarcoma cells that we proposed is the initial signalling mechanism for mechanotransduction. During the execution of this grant, we have made considerable progress to further characterize this channel as well as to determine its role in the osteoblastic response to mechanical strain. To achieve these goals, we combined electrophysiologic techniques with cellular and molecular biology methods to examine the role of these channels in the normal function of the osteoblast in vitro.

Accomplishments

1. We demonstrated that parathyroid hormone (PTH) modulates the SA-cat channel in two ways. First, PTH produces a two- to four-fold increase in the average number of open channels \(N_{P_o}\) during stretch. Secondly, PTH increased the single channel conductance \(g_s\) of the SA-cat channel. Both of these effects were nongenomic, arising within two minutes of application of PTH. Interestingly PTH mediated these effects through two distinct pathways. Application of the membrane permeant form of cyclic AMP, 8-bromo cyclic AMP mimicked the PTH induced increase in single channel conductance but did not alter \(N_{P_o}\). We have yet to identify the second messenger involved in the PTH induced activation of \(N_{P_o}\).

2. We have also demonstrated that the SA-cat channel is dependent on attachment to the cytoskeleton of the cell. Application of cytochalasin D, a molecule which cleaves f-actin filaments to promote short chained actin filaments, increased SA-cat channel \(N_{P_o}\) ten-fold over a time course similar to PTH stimulation. However cytochalasin D had no effect on \(g_s\). These data would indicate that the SA-cat channel is closely associated with the cytoskeleton and that PTH modulates the osteoblastic response through rearrangement of the actin cytoskeleton. These data reinforce previous observations demonstrating that PTH produces a morphologic change in the cell through alteration of the cytoskeleton.
3. When osteoblast-like osteosarcoma cells were subjected to a chronic, intermittent mechanical strain, we found that application of additional strain produced a significant increase in whole cell conductance when compared to nonstrained control cells. The mechanosensitive channel blocker, gadolinium, added to the bathing media of the patch chamber effectively blocked this increase in whole cell conductance. Single channel studies indicated that the increase in whole cell conductance was the result of a three- to five-fold increase in SA-cat channel activity. Chronic intermittent mechanical strain also induced spontaneous channel activity heretofore unseen in other systems containing mechanosensitive channels. The sensitivity of the channels to stretch was also affected by chronic strain causing channels to become much more responsive at lower magnitudes of strain.

4. Using the same mechanical strain regimen that we used to examine the effects of chronic mechanical strain on SA-cat channel activity, we analyzed the expression and production of bone matrix proteins in human osteoblast-like osteosarcoma cells. We found that type-I collagen expression and production were increased within 24 hours of strain application. Additionally significant increases in other types of bone matrix proteins such as osteopontin expression and osteocalcin production were observed three to four days following chronic mechanical strain. Surprisingly, the increases in osteopontin and osteocalcin were independent, and synergistic to, 1,25-dihydroxyvitamin D stimulation.

5. In collaboration with Peter Friedmans' laboratory at Dartmouth University, we initiated studies to determine the molecular makeup of the mechanosensitive channel. Previously Friedmans' laboratory had demonstrated that there are three isoforms to the voltage sensitive L-type calcium channel in UMR-106.01 cells. Antisense oligodeoxynucleotides (ODN's) derived from nonconserved sequences in the S6 region of the IV membrane spanning domain of the \( \alpha_1 \) subunit of these voltage sensitive calcium channels were used to examine the physiologic role of these channels in UMR-106.01 cells. We found that the antisense ODN to the cardiac isoform of the L-type \( \alpha_1 \) subunit \( (\alpha_{ic}) \) completely abolish the mechanically induced increase in whole cell conductance of the UMR cells following chronic mechanical strain. Antisense ODN's to the \( \alpha_{1S} \) (skeletal) and \( \alpha_{1D} \) (neuroendocrine) as well as sense ODN's for the \( \alpha_{ic} \) subunit had no effect on the strain induced increase in whole cell conductance. Cell attached single channel studies demonstrated that the antisense ODN to the \( \alpha_{ic} \) subunit completely blocked the SA-cat single channel functional expression in UMR cells, whereas \( \alpha_{ic} \) antisense ODN did not effect SA-cat channel expression or activity.

6. Two technical problems are associated with the mechanical strain apparatus (Flexercell®) utilized in obtaining the data described in Accomplishments #3-5. The first problem is the lack of uniformity of mechanical strain applied across the growth well of the Flexercell apparatus. Strains range from 120,000 \( \mu \)E to zero at the center of the well. Thus, determination of the exact magnitude of strain applied to the cells is difficult. A second technical problem associated with the Flexercell is that physiologic levels of the magnitude of strain that the osteoblasts perceive in vivo are difficult to achieve. To answer these problems, we have devised a mechanical loading device based on the in vivo four-point bending model which produces uniform strain across the entire growth plate. We are now able to apply strains from 500 \( \mu \)E to 5,000 \( \mu \)E to MC3T3-E1 osteoblast-like cells. These studies have determined that mechanical stretch of the osteoblast at these levels has no influence on the expression or production of bone matrix proteins that we observed in Accomplishment #4. However by altering the degree of movement of the growth plate through the media, we have found that fluid shear plays a much more important role on the expression and production of these matrix proteins. We have demonstrated that while osteopontin expression is not increased at levels of mechanical stretch from 1,500 \( \mu \)E to 4,500 \( \mu \)E, increasing the movement of the growth plate through the media at all of these strain levels significantly increases expression of osteopontin. These data would...
indicate that fluid shear and not mechanical stretch is the critical mechanical factor in the osteogenic response of bone to mechanical strain.

**Significance of Accomplishments**

PTH is a calcitropic hormone released by the parathyroid gland in response to low serum calcium yet has paradoxical effects on bone. Acutely PTH increases bone resorption by stimulating the osteoblasts to signal an increase in osteoclastic activity. However, following this acute effect of PTH on bone resorption, PTH also stimulates bone formation. Several studies have indicated this anabolic effect of PTH on bone may be mediated by low concentrations of the hormone or intermittent application. The cellular mechanisms by which PTH exerts its effect on the osteoblast are still inconclusive. However since we propose that the SA-cat channel is an important signaling mechanisms for mechanically induced osteogenesis, the stimulation of these channels in a similar fashion by PTH would suggest that the SA-cat channel may be a site of convergence of two distinct osteoblast activator stimuli. PTH, like mechanical strain, modulates the channel in two ways; increasing channel activity (NP) and increasing single channel conductance (g.). PTH elevates intracellular cyclic AMP and modulates channel conductance through this second messenger, possibly by altering configuration of the channel. By increasing single channel conductance, the channel can increase the amount of ions which transverse the channel at a given channel opening. The close association of the SA-cat channel with the actin cytoskeleton would indicate that both mechanical strain and PTH may affect the NP of the channel by reorganizing the actin cytoskeleton. PTH has been shown to effect the shape of the osteoblast causing a shrinkage of the osteoblast into a stellate shape. Cytochalasin D, an f-actin severing molecule, produces similar morphologic changes. By increasing the length of channel opening, PTH and mechanical strain can effectively increase the number of ions that pass through the channel. The net result of the two effects of PTH and mechanical strain on SA-cat channel kinetics is to greatly increase the amount of ions passing through the channel at a given time, resulting in a much larger intracellular signal. Since we hypothesize that these channels are responsible for the signal transduction of mechanical strain to produce an anabolic effect, these data would suggest that these channels may also be responsible for the osteogenic activity of PTH.

While the mechanisms by which the osteoblast sense mechanical perturbations have yet to be identified, we have demonstrated that when osteoblasts are subjected to cyclic chronic mechanical strain, SA-cat channel kinetics are significantly altered. To date, this is the only membrane protein which has been shown alter its configuration in response to mechanical strain. This change in channel activity relates to large increases in whole cell conductance when the osteoblast is further stimulated. Previous studies have shown that intracellular calcium increases within milliseconds of the onset of mechanical strain suggesting that a mechanosensitive calcium conductive channel may be the primary response element for mechanotransduction. Interestingly Lanyon and associates had demonstrated the dynamic but not the static mechanical strain produces osteogenic effects in *in vivo* preparations. Our studies have shown that when osteoblasts are strained in the patch configuration without prior conditioning strain little change is observed in whole cell conductance. These data would suggest, that as in *in vivo* observations, multiple stimulation of events are required to modulate the SA-cat channel and that chronic intermittent mechanical strain primes the channel to respond to additional strain and promote osteogenic activity. To determine if the strain regimen which induced mechanosensitive channel kinetic changes were sufficient to induce an osteogenic response in osteoblasts, bone matrix protein expression and production were evaluated using the same strain regimen. We found that chronic cyclic mechanical strain increased expression and/or production of all of the matrix proteins we examined. These observations strengthened but did not confirm our hypothesis that the SA-cat channel is an integral part of the signalling mechanism for the osteogenic response to mechanical strain. One interesting observation made during these studies was that mechanical strain increased osteopontin and osteocalcin expression and production independent of 1,25-dihydroxyvitamin D stimulation.
Previously these matrix proteins were thought to be only increased with vitamin D stimulation. However vitamin D and mechanical strain produced a synergistic response in both osteopontin and osteocalcin. These data would indicate that mechanical strain induces an anabolic response in the osteoblast through a separate mechanism from vitamin D.

Barry and Friedman have demonstrated three $\alpha_4$ subunits to L-type calcium channels in UMR-106.01 osteoblast-like osteosarcoma cells using rTPCR. They found unique conservation of alternative splicing across each $\alpha_4$ subunit genes in this cell line. Previously they had demonstrated that antisense oligodeoxynucleotides to $\alpha_{1c}$ subunit blocked the volume regulatory response to hypotonic swelling in renal distal tubule cells. To determine if similar inhibition would occur in the UMR-106.01 cell, we employed a similar antisense oligodeoxynucleotide strategy.

We demonstrated that the $\alpha_{1c}$ subunit was integral to the osteoblast response to chronic mechanical strain. We found that antisense oligodeoxynucleotides to the $\alpha_{1c}$, but not the $\alpha_{1s}$ or $\alpha_{1P}$, subunits completely blocked the whole cell conductance increase in chronically strained cells. Additionally antisense to the $\alpha_{1c}$ subunit completely blocked the functional expression of SA-cat channels in the osteoblast. These studies have indicated that the SA-cat channel may be an alternatively spliced molecule, similar in molecular sequence to the voltage operated calcium channels found in other tissues. To date, isolation of mechanosensitive channels using molecular techniques has been difficult. The reason for this may be that the mechanosensitive channel is actually an isoform of the L-type voltage sensitive calcium channel. We are currently cloning the channel protein as well as introducing a promoter region onto the antisense oligodeoxynucleotide to knock out this channel over long periods of time and therefore examine the results in osteogenic activity in both control and transfected osteoblasts.

The objective of Specific Aim #1 of this grant was to examine the optimal magnitudes of mechanical strain to induce osteogenic activity in osteoblasts. Unfortunately the mechanical loading apparatus used to obtain the data outlined in Specific Aims #3-5 was insufficient to determine these optimal magnitudes. Therefore we have devised a new strain loading apparatus which produces uniform strain across the growth plate and can apply mechanical loads well within the physiologic range perceived by the osteoblast, in vivo. Using this device we have demonstrated that mechanical stretch applied to the osteoblast in these physiologic ranges is insufficient to produce an anabolic response in the osteoblast. However by altering the movement of the growth plate through the medium we have found that fluid shear plays a critical role in the response of the osteoblast and can induce a three- to five-fold increase in the bone matrix protein, osteopontin. These findings are significant in that they demonstrate that fluid shear and not stretch produces the osteogenic response in bone and that the signaling mechanisms may differ between the two stimuli.

**Publications**


**Invited Publications:**


Parathyroid hormone activation of stretch-activated cation channels in osteosarcoma cells (UMR-106.01)

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Cell-attached patches of membrane of osteoblast-like cells UMR-106.01 respond to bath application of parathyroid hormone (PTH) with an increase in the average activity, as well as the single channel conductance, of a stretch-activated non-selective cation channel. Correlations with whole cell membrane potential and conductance changes are considered.

Osteoblastic cell line; Stretch-activated channel; Parathyroid hormone; Cyclic AMP

1. INTRODUCTION

Parathyroid hormone (PTH) and mechanical strain are critical factors in regulating bone modelling, in part through their actions on osteoblasts [1–3]. Osteoblasts, in turn, synthesize bone matrix proteins and prime bone matrix for targeting by osteoclasts. It is not known whether these chemical and physical factors work through entirely separate pathways or interact at some critical juncture. However, it is known that osteoblasts have mechanosensitive or stretch-activated cation (C'(SA)) channels [4,5], which, in other cells, appear to be tied to cytoskeletal elements [6]. Osteoblasts exposed to PTH respond in various ways including membrane depolarization [7,8] and cytosolic retraction [9,10]. Additionally, in other cells, C'(SA) channels appear to be a target of action of growth-promoting hormones: platelet derived growth factor, for example, activates a C'(SA) channel in fibroblasts [11]. Against this background, we investigated whether PTH might affect C'(SA) channel gating in a clonal osteoblastic cell line (UMR-106.01), thereby making the C'(SA) channel a site of convergence of two distinct osteoblast activator pathways.

2. MATERIALS AND METHODS

Subcultures of PTH-responsive UMR-106.01 cells (passages 9–16), originally derived from rat osteosarcoma, were grown to 40–80% confluence on glass coverslips, which were transferred to a recording chamber (1 ml volume) (Biophysics Technologies, Baltimore, MD) which permitted rapid bath solution change with minimal perturbation to the cells. Cells were bathed in either a mammalian Na' Ringer's (NR) consisting of (in mM): 140 NaCl; 5.5 KCl; 1 MgCl2; 1 CaCl2; 3 glucose, and 20 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer, titrated to pH 7.3 with NaOH or a K+ Ringer's (KR) consisting of (in mM): 144 KCl; 1 MgCl2; 1 CaCl2; 20 HEPES; 3 glucose; titrated to pH 7.3 with KOH. Rat PTH (1–34 fragment), 8 br-cAMP or gadolinium (Sigma Chemical, St. Louis) were introduced to the chamber by perfusing the chamber with 10 ml of either NR or KR containing the final concentrations of these agents.

Standard electrophysiological techniques were used as previously adapted in our laboratory [4,12]. Single channel recordings were made with pipette solutions consisting of either KR or a Ca2+-free K+ Ringer's (KROCa) consisting of (in mM): 144 KCl; 1.3 EGTA; 20 HEPES; titrated to a pH of 7.3 with KOH. Single channel currents were filtered at 1 KHz and recorded at a sampling frequency of 3 KHz. The clamping potential $V_c$ is defined as $V_{pp}$, where $V_{pp}$ is the potential imposed on the pipette interior with the bath taken as ground. Membrane potentials were most reliably recorded under current clamp conditions using 'perforated patch' techniques. For these experiments, the pipette solution consisted of (in mM): 12 NaCl; 64 KCl; 28 K2SO4; 47 sucrose; 1 MgCl2; 0.5 EGTA; 20 HEPES, titrated to pH 7.35 with KOH. Nystatin was added at a concentration of 100 μg/ml to permeabilize the patch. Access resistances of <60 MΩ were sought. An interactive graphics program, which uses level crossings to determine in a segment of record the fraction of time when zero, one, or more channels are open, was used to measure the average number of channels open in a patch during a specified period of time ($NP_t$). Using a similar program, open channel amplitudes were determined and grouped into bins of 0.025 pA to produce amplitude histograms. Due to biological variability, comparisons were made between co-cultures of the same passage number.

3. RESULTS

Figure 1A illustrates the effects of PTH on C'(SA) channel activity in cell attached patches of UMR-106.01 membranes during application of suction to the interior of the pipette. PTH (50 nM), which has been shown to increase adenylyl cyclase activity [13] and intracellular Ca2+ concentration [14] as well as depolarize the cell membrane [8], increased the mean activity ($NP_t$) of a channel which was open during application of suction.
to the pipette. In the presence of PTH, note that on repeated application of suction, the single stretch-activated channel present in the patch opens every time suction is applied, spends much of the duration of the suction pulse in the open state and then closes down promptly with cessation of suction. This channel has previously been shown to be a non-selective cation channel in that it selects cations over anions but does not select between Na\(^+\) and K\(^+\) and passes Ba\(^{2+}\) [4]. It also shows little consistent voltage dependence over a

100 mV range around the resting membrane potential of the cell. In Fig. 1B, the same experimental protocol was repeated, but following application of 100 \(\mu\)M 8-br-cAMP, a membrane permeant cAMP analogue. Note that after addition of up to 1 mM 8-br-cAMP there is no obvious or computed change in mean channel activity evoked by stretch.

Figure 2A and 2B compares the stretch sensitivity and current/voltage (i-V) characteristic of the C\(^\prime\)(SA) channel prior to and after addition of PTH. These experiments and the remaining single channel studies were conducted with very low Ca\(^{2+}\) in the pipette. Low Ca\(^{2+}\) increases the peak conductance of the C\(^\prime\)(SA) channel from 19.7 \(\pm\) 1.9 pS (n=12) to 41.2 \(\pm\) 3.8 pS (n=6) (P<0.001) thereby amplifying changes in channel amplitudes and conductance. Figure 2A demonstrates that addition of PTH reduces the threshold level of suction required to activate the C\(^\prime\)(SA) channel. However, even though PTH enhanced the mean activity of the channel by 70\%, the amount of suction required to obtain maximal levels of activity was not different from control. Figure 2B demonstrates that PTH increased the single channel current amplitude seen at patch potentials near or hyperpolarized to the resting potential of the patch.

Fig. 2. Effects of PTH (50 nM) on the stretch sensitivity and single channel conductance of C\(^\prime\)(SA) channel. (A) Plot of average activity vs. applied pipette suction demonstrating that PTH reduced the threshold level of suction required to activate the C\(^\prime\)(SA) channels without altering the amount of suction required to obtain maximal levels of activity. (Experiment typical of a series of four.) (B) Single channel current vs. voltage curves demonstrating increase in single channel conductance after bath application of PTH. Small shift in zero current potential was not statistically significant.
(see also Fig. 1A). With Ca\(^{2+}\), present in the pipette, channel amplitudes were also increased with PTH, suggesting that Ca\(^{2+}\) was not a factor in the PTH-induced change in conductance. As multiple forms of mechanosensitive channels have previously been seen in osteoblast-like cells [5], we tested the possibility that the increase in single C\(^{+}(SA)\) channel conductance resulted from the condition that the C\(^{+}(SA)\) channel could occupy several conductance states and that the larger states were preferred in the presence of PTH. To do this we examined single channel current amplitudes (i) in the presence of both very low Ca\(^{2+}\) in the pipette and high K\(^{+}\) Ringer's in the bath. High K\(^{+}\) Ringer's in the bath prevents small shifts in \(i\) due to cell depolarization (see sample trace Fig. 3A). Figure 3A displays channel currents before and after PTH, while 3B shows histograms of open channel current amplitudes. In this cell, typical of 8 out of 12 cells, single channel current amplitudes were well fit to Gaussian distributions, suggesting one predominant conductance level for the channel in the presence or absence of PTH. Note however, that the peak amplitude for the open state is shifted from 1.25 pA in control conditions to 1.63 pA during PTH exposure. This increase in channel conductance was consistent in all experiments (n=12). Small fluctuations in current were occasionally visible at the leading or trailing edge of a burst of channel activity suggesting that the channel may open or close through a transient subconductance state(s). However, given the long channel open times, brief sojourns at these subconductance levels, highlighted by arrows in Fig. 3A, would be expected to contribute little area to the amplitude histograms.

Fig. 3. Effects of PTH on single channel amplitudes of the C\(^{+}(SA)\) channel. (A) Traces of single channel currents recorded before and after addition of PTH to the high K\(^{+}\), low Ca\(^{2+}\) bath to null out the cell resting potential. Arrows indicate that the channel may open or close through a transient subconductance state which contributes little to the amplitude histogram. (B) Amplitude histograms for single C\(^{+}(SA)\) channel currents recorded before and after addition of PTH. Currents were recorded during suction pulses of 15 mmHg with the patch held \(V_c = -40\) mV. Note the increase in peak channel amplitude from 1.25 pA to 1.63 pA after treatment with PTH. (C) Single channel tracings from a representative experiment demonstrating intermediate conductance states (arrows). (D) Amplitude histogram for single channel currents. Note the non-Gaussian distribution of channel openings.
Fig. 4. Correlation of single C'(SA) channel activity with whole cell membrane potential. (Panel A, upper traces): PTH-induced depolarization occurring within 1–2 min after addition, followed by slow repolarization with washout in Ringer’s. Reapplication of PTH resulted in a second depolarization. This was atypical, being seen in only 2 out of 8 PTH responsive cells. (A, lower trace): lack of effect of 0.5 mM 8-bromo-cAMP on the membrane potential (typical of an n=6), while subsequent application of PTH produced a substantial depolarization. (B, upper panel): effects of gadolinium on single C'(SA) channels in the cell attached patch. Channel activity was compared between patches in the same cell. Addition of 5 µM Gd³⁺ to the pipette solution significantly decreased NP from average maximum levels of 1.707 ± 0.071 to 0.429 ± 0.039 (n=7; P<0.001) without affecting the stretch sensitivity. (B, lower trace): block of PTH-induced membrane depolarization by pre-treatment with 20 µM gadolinium. Trace is typical of 6 similar experiments.

However, in 4 experiments, (e.g. Fig. 3C) frequent sojourns into a subconductance state were noticeable and open channel histograms were not well fit to a Gaussian distribution. Even in these experiments, PTH shifted the majority of channel openings to higher amplitudes. Interestingly, addition to the bath of 100 µM 8-bromo-cAMP, which failed to increase channel activity, none-the-less mimicked the PTH-induced increase in channel conductance (data not shown).

If the effects of PTH on C'(SA) channel activity in the cell-attached patch of membrane were representative of that of C'(SA) channels in the remainder of the cell and C'(SA) channels contributed substantially to the whole cell membrane conductance, then we might predict that addition of PTH would depolarize the UMR-106.01 cell membrane and increase its conductance. Using this line of reasoning, an agent which blocked the C'(SA) channel might be expected to substantially reduce the effect of PTH on membrane potential and conductance. Fig. 4 provides tests of both of these predictions.

In Fig. 4A, it is apparent that addition of PTH (50 nM) to the Ringer’s bath resulted in rapid membrane depolarization. In the perforated patch configuration, 8 out of 12 cells responded within 1–2 min to 50 nM PTH with an average 11.3 ± 2.5 mV depolarization (range 6.0–23.2 mV) which slowly reversed after washout. Similar results were also seen in conventional whole cell current clamp recording. The membrane potential was not measurably altered by bath application of 0.5 mM 8-bromo-cAMP (lower trace, panel A). Fig. 4B demonstrates that trivalent cation gadolinium (Gd³⁺), which when added to the patch pipette at 5 µM more than halves C'(SA) channel activity in the cell-attached patch (top panel), prevents PTH-induced depolarization at 20 µM. Gadolinium has been shown to block stretch-activated channels with similar characteristics to the C'(SA) channels in *Xenopus* oocytes [15].

4. DISCUSSION

We have obtained evidence that bath application of parathyroid hormone, in concentrations which effect other cell functions, results in an increase in activity of a stretch activated non-selective cation (C'(SA)) channel seen in cell-attached patches of membrane from a PTH-responsive osteoblast-like cell line UMR-106.01. This effect is accompanied by a small increase in single channel conductance. These observations suggest that the C'(SA) channel might be a locus at which the actions of membrane deformation and PTH converge. This interpretation is supported by evidence that PTH often depolarizes the cell membrane while pretreatment with Gd³⁺, which reduces the activity of C'(SA) channels in the cell-attached patch, prevents PTH-induced depolarization. However, our attempts to measure changes in membrane conductance underlying the de-
polarization, have produced scattered results which are not highly consistent with the Ca\(^{2+}\) (SA) channel being the sole or major electrophysiological target of PTH action. It is possible that PTH also effects other ion channels in the membrane. For example, by simultaneously closing a channel with a reversal potential negative to the cell's measured resting potential of \(-30\) to \(-40\) mV, while opening the Ca\(^{2+}\) (SA) channel, PTH could induce depolarization with very variable effects on membrane conductance. Recently, the physiological activity of stretch-activated channels has been questioned in other cells due to the inability to see macroscopic conductances activated by cell deformation which parallel the activity of stretch-activated channels recorded in the cell-attached patch [16]. More rigorous correlation of macroscopic and single channel currents shall be critical in clarifying these points. Analysis of results of microelectrode studies in the UMR-106.06 cell line, using specific channel antagonists, are consistent with PTH producing the membrane depolarization by promoting the closure of Ca\(^{2+}\)-activated K\(^+\) channels [7,8]. Other studies with whole cell recordings suggest that PTH activates chloride currents in osteoblasts [17]. Additionally, PTH has been shown to inhibit L-type Ca\(^{2+}\) channels in the neuroblastoma cell [18]. However, in pilot experiments done in conjunction with the current studies, neither nitrendipine, an L-type Ca\(^{2+}\) channel blocker, nor Ba\(^{2+}\), a K\(^+\) channel blocker, prevented PTH-induced depolarization.

The mechanism of action of PTH on Ca\(^{2+}\) (SA) channels in UMR-106.01 cells, as well as the identity of possible second messengers involved, is currently unknown. PTH regulation of osteoblast function is generally thought to be mediated through the adenylate cyclase pathway. However, in our experiments, the effect of PTH on mean channel activity is not duplicated by bath application of a membrane permeant analog of cAMP, although the smaller effect of PTH on single channel conductance is modulated by 8 br-cAMP. These data suggest that PTH stimulation of the Ca\(^{2+}\) (SA) channel occurs through a different second messenger pathway. PTH has been shown to elevate inositol polyphosphates and diacylglycerides and stimulate the phospholipase C pathway [14,19] in cells displaying osteoblast phenotypes. Interestingly, when cultured osteoblasts are subjected to mechanical stress, intracellular concentrations of inositol phosphates, as well as cAMP, are elevated [20].

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REFERENCES
An Arg-Gly-Asp peptide stimulates Ca\(^{2+}\) efflux from osteoclast precursors through a novel mechanism

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Yamakawa, Kensuke, Randall Duncan, and Keith A. Hruska. An Arg-Gly-Asp peptide stimulates Ca\(^{2+}\) efflux from osteoclast precursors through a novel mechanism. Am. J. Physiol. 266 (Renal Fluid Electrolyte Physiol. 35): F561–F567, 1994.—We examined the effect of a peptide containing the Arg-Gly-Asp (RGD) sequence on Ca\(^{2+}\) efflux from osteoclast precursors. 4Ca\(^{2+}\)-loaded osteoclast precursors were treated with GRGDSP (170 \(\mu\)M) for 10 min after 30 min of basal perfusion with a bicarbonate-containing buffer. GRGDSP significantly increased fractional efflux of Ca\(^{2+}\) from treated cells compared with vehicle-treated cells (\(P < 0.01\)) or cells treated with up to 200 \(\mu\)g/ml of a control peptide containing GRGESP. The effect of RGD was sustained for 15 min after the peptide was removed from the perfusate, but control levels of Ca\(^{2+}\) efflux returned by 1 h. The Ca\(^{2+}\) efflux effect of GRGDSP was most likely due to activation of the plasma membrane Ca\(^{2+}\)-adenosinetriphosphatase (Ca\(^{2+}\)-ATPase) pump, as indicated by its inhibition with vanadate and a calmodulin antagonist, N-(4-aminobuty1)-5-chloro-2-naphthalenesulfonamide, and the absence of an effect of Na\(^{+}\)-Ca\(^{2+}\) exchange inhibition. An inhibitor of cyclic nucleotide-dependent protein kinases, N-[2-(methylamino)ethyl]-5-isouquinolinesulfonamide (0.1 mM), failed to inhibit GRGDSP-stimulated Ca\(^{2+}\) efflux. However, genistein and herbimycin A, inhibitors of protein-tyrosine kinases, blocked Ca\(^{2+}\) efflux stimulated by GRGDSP. The results indicate that RGD sequences of protein-tyrosine kinases may stimulate Ca\(^{2+}\) efflux from osteoclasts through activation of protein-tyrosine kinases and suggest that GRGDSP-stimulated Ca\(^{2+}\) efflux is mediated via the plasma membrane Ca\(^{2+}\)-ATPase.

RGD proteins; synthetic peptides; protein kinases; N-[2-(methylamino)ethyl]-5-isouquinolinesulfonamide; genistein; herbimycin A

OSTEOCLAST PROGENITORS are recruited from hemopoietic tissue (32). They proliferate and differentiate into mononuclear osteoclasts and fuse to form multinucleated osteoclasts (32). When osteoclasts attach to bone matrix, they polarize their cell membrane into discrete domains consisting of the ruffled border, clear zone, and basolateral membrane. The clear zone surrounds the ruffled border membrane and seals the resorption space under the ruffled border membrane from the external environment, maintaining a microenvironment favorable for bone resorption. Protons and osteolytic enzymes are secreted into the resorption lacunae degenerating the bone matrix (32).

The clear zone contains numerous protrusions of the plasma membrane called podosomes, which contain a \(\beta_3\)-integrin (40). The osteoclast \(\beta_3\)-integrin, \(\alpha_\beta_3\), is critical to osteoclast function (7) and has recently been shown to bind to several Arg-Gly-Asp (RGD)-containing matrix proteins including osteopontin (30). Osteopontin is found concentrated in bone matrix underneath the clear zone of osteoclasts (28). In addition, osteoclasts adhere to the RGD-containing proteins, bone sialoprotein II, fibrinogen, fibronectin, von Willebrand factor, and vitronectin through the occupancy of \(\alpha_\beta_3\)-integrin (10). Although the mechanism of osteoclast attachment to bone matrix has not been completely elucidated, the recognition of RGD by \(\alpha_\beta_3\)-integrin is thought to play a key role (7, 10, 13, 40).

Recently, several investigators have demonstrated generation of immediate cell signals when RGD sequence-containing peptides (25, 29) bind to osteoclast \(\alpha_\beta_3\)-integrin. The signal-generating complex activated by occupancy of the \(\alpha_\beta_3\)-integrin by an RGD-containing peptide has several SH2-domain-type enzymes associated with pp60 \(c\)-, a nonreceptor protein-tyrosine kinase. These include phosphatidylinositol 3-kinase and phospholipase C\(\gamma\) (29). In avian osteoclast precursors, we have found that the RGD sequence-containing peptides, osteopontin and vitronectin, decreased intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)](22). The effect of osteopontin on [Ca\(^{2+}\)], was inhibited by vanadate [a potent inhibitor of plasma membrane Ca\(^{2+}\)-adenosinetriphosphatase (Ca\(^{2+}\)-ATPase)] but was not affected by numerous agents that control intracellular Ca\(^{2+}\) stores (22). These results suggested the possibility that RGD-containing peptides might stimulate Ca\(^{2+}\) efflux from osteoclast precursors (22). Therefore the purpose of this study was to directly measure the effects of an RGD-containing peptide on Ca\(^{2+}\) efflux from osteoclast precursors and to analyze the mechanism of activation of the Ca\(^{2+}\)-ATPase.

MATERIALS AND METHODS

Materials. Synthetic peptides, GRGDSP and GRGESP, were obtained from Telios Pharmaceuticals (San Diego, CA). \(4^{25}\)Ca\(^{40}\) was purchased from Amersham International (Amer- sham, UK). Herbimycin A and genistein were from GIBCO BRL (Gaithersburg, MD). N-(4-aminobuty1)-5-chloro-2-naphthalenesulfonamide (W-13) and N-[2-(methylamino)ethyl]-5-isouquinolinesulfonamide (H-8) were from Calbiochem (San Diego, CA).

Isolation and culture of osteoclast precursors. The method of Alvarez et al. (2) as modified by Medhora et al. (20) was used to isolate and culture avian osteoclast precursors. The bone marrow was isolated from the tibia and femora of laying chickens fed a calcium-deficient diet. Marrow suspensions were centrifuged at 350 g for 5 min. Pellets were resuspended in phosphate-buffered saline (PBS) containing antibodies and centrifuged in Ficoll-Hypaque gradient. The cells at the interface were collected, washed with PBS, and resuspended in minimum essential medium Eagle alpha modification (alpha-MEM) containing 5% of both fetal calf serum and chicken serum. The cells were plated onto 150-mm plastic dishes at 2 \(\times\) 10\(^4\) cells/dish and incubated in 5% CO\(_2\) in humidified air at
39.5°C. After 24 h, cells that did not adhere to plastic plates were obtained and pelleted by centrifugation. Those cells were resuspended in αMEM containing 5% of both fetal calf serum and chicken serum and 5 μg/ml cytoxin B-D-arabinofuranoside and replated onto 25-mm cover slips. At 4 or 5 days of culture the pure preparations of multinucleated cells (osteoclast precursors) that were obtained resorbed bone in "pit assays," were uniformly tartrate-resistant acid phosphatase (TRAP)-positive. and expressed several osteoclast antigens (2).

**Ca²⁺ efflux from perfused osteoclast precursors.** Osteoclast precursors on the cover slips were washed three times with Krebs-Ringer-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) bicarbonate with glucose (KRHBG). KRHBG contained (in mM) 100 NaCl, 5 KCl, 1.2 MgCl₂, 2 CaCl₂, 24 NaHCO₃, 6 glucose, and 25 HEPES (pH 7.4) and was preequilibrated with 95% O₂-5% CO₂ at 37°C or room temperature before experiments. Two cover slips on which osteoclast precursors were cultured were assembled into a Sykes-Moore chamber. Osteoclast precursors that adhered to the cover slips faced the inside of the chamber. Ca²⁺ efflux was analyzed by the method of Borle et al. (5). Osteoclast precursors were preloaded with KRHBG containing 40 μCi/ml ⁴⁵CaCl₂ at 37°C for 1 h before washing three times with KRHBG containing no labeled CaCl₂. Osteoclast precursors were perfused with KRHBG, which was equilibrated with 95% O₂-5% CO₂ at 37°C (pH 7.4), using a peristaltic pump at 0.5 ml/min. After 30-min of basal perfusion, either test substances or vehicle were added to the perfusate.

**Measurement of radioactivities in effluent and cell lysates.** The effluent from each chamber was collected directly into scintillation vials or test tubes at intervals of 2.5, 5, or 10 min. At the end of each experiment, osteoclast precursors on the cover slips were lysed in 1.5 ml of distilled water and residual ⁴⁵Ca²⁺ was determined for the calculation of fractional efflux of Ca²⁺ efflux. Ca²⁺ efflux was expressed as fractional efflux and fractional efflux ratios of Ca²⁺, calculated according to Borle et al. (5). The fractional efflux of Ca²⁺ is the percentage of the total ⁴⁵Ca²⁺ leaving the cells during the time interval of each collection. The fractional efflux ratio is the ratio obtained from dividing fractional efflux of Ca²⁺ of stimulated or inhibited cells by that of control cells. All experiments were performed in pairs with one test chamber and one chamber receiving vehicle as the treatment. All data were represented as means ± SE of experiments performed at least in triplicate. For statistical analyses, the Student's t-test was used, and P < 0.01 was recognized as statistically significant.

**RESULTS**

First, we examined the effect of GRGDSP (RGD sequence-containing peptide) on Ca²⁺ efflux from osteoclast precursors. The results are summarized in Fig. 1A and B. Fractional efflux of ⁴⁵Ca²⁺ approached near steady state after 30 min of perfusion (Figs. 1-7A), and all experimental maneuvers were conducted after this point. Then 100 μg/ml (170 μM) of GRGDSP were added to osteoclast precursors for 10 min. As shown in Fig. 1A, GRGDSP significantly and rapidly increased fractional efflux of Ca²⁺ efflux ~50% in GRGDSP-treated cells compared with those treated with vehicle. Ca²⁺ efflux continued to increase from 50 to 130% above baseline. After GRGDSP peptide was removed with perfusion, Ca²⁺ efflux eventually returned to basal levels by 40 min after stimulation. These results indicate that a peptide containing the RGD sequence stimulated sustained Ca²⁺ efflux from osteoclast precursors.

![Image](image-url)

**Fig. 1. Effect of a peptide containing Asp-Gly-Asp (RGD) sequence on Ca²⁺ efflux from osteoclast precursors.** Adherent osteoclast precursors on cover slips were perfused for 55 min. Cultures were treated with either 100 μg/ml GRGDSP (●) or vehicle (○) from 30 to 40 min. Results are expressed as fractional efflux of Ca²⁺, using a logarithmic scale (A) or as fractional efflux ratio of Ca²⁺ (B). Method for calculation was described in MATERIALS AND METHODS. Numerator is fractional Ca²⁺ efflux in cells treated with GRGDSP, and denominator is fractional Ca²⁺ efflux in cells treated with vehicle. Results were expressed as means ± SE; n = 6. *P < 0.01.

Next, to confirm whether the RGD sequence was necessary to stimulate Ca²⁺ efflux from osteoclast precursors, they were perfused with KRHBG containing a RGE-sequence peptide. GRGESP (200 μg/ml) did not increase efflux of Ca²⁺ from osteoclast precursors (Fig. 2), indicating the requirement for the RGD sequence in peptides stimulating Ca²⁺ efflux.

![Image](image-url)

**Fig. 2. Effect of a peptide containing RGE sequence on Ca²⁺ efflux from osteoclast precursors.** Osteoclast precursors were treated with either 200 μg/ml GRGESP (●) or vehicle (○) from 30 to 40 min. Results are expressed as fractional efflux of Ca²⁺. Numerator is fractional Ca²⁺ efflux in cells treated with GRGESP, and denominator is fractional Ca²⁺ efflux in cells treated with vehicle. Results were expressed as means ± SE; n = 3.
We have previously reported that the reduction of [Ca\(^{2+}\)]
stimulated by RGD-containing peptide was inhibited by vanadate (22).
That result suggested that the reduction of Ca\(^{2+}\) might be mediated by a Ca\(^{2+}\)-ATPase of the osteoclast precursor plasma membrane. We therefore tested this possibility. Preincubation and perfusion of osteoclast precursors with 10 \(\mu\)M vanadate blocked the effect of GRGDSP on Ca\(^{2+}\) efflux from osteoclast precursors (Fig. 3, A and B). These results indicate that GRGDSP-stimulated Ca\(^{2+}\) efflux is a vanadate-sensitive process, in which Ca\(^{2+}\) efflux is mediated by the plasma membrane Ca\(^{2+}\)-ATPase. Lower doses of vanadate (1 \(\mu\)M) were also effective in reducing the stimulation of Ca\(^{2+}\) efflux from osteoclast precursors, but the differences were smaller, making statistical assessment difficult. Thus we elected to utilize the 10 \(\mu\)M dose reported here. The basal rate of Ca\(^{2+}\) efflux in vanadate-treated precursors was similar to control despite the expected reduction of Ca\(^{2+}\) might be mediated by a Ca\(^{2+}\)-ATPase probably provided the basis for similar rates of basal efflux in the vanadate-treated and control cells. The plasma membrane Ca\(^{2+}\)-ATPases are calmodulin-dependent enzymes (27). We therefore examined the effect of preincubating osteoclast precursors with an antagonist of calmodulin, W-13 (0.1 mM) (12), for 5 min before addition of GRGDSP (Fig. 4, A and B). W-13 decreased GRGDSP-stimulated Ca\(^{2+}\) efflux, indicating that it was a calmodulin-sensitive process.

Plasma membrane Ca\(^{2+}\)-ATPases are regulated by adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase (26, 37). H-8 is a known antagonist of cyclic nucleotide-dependent protein kinases (11). The action of GRGDSP on Ca\(^{2+}\) efflux from osteoclast precursors was not inhibited by 0.1 mM H-8 (Fig. 5, A and B). Fractional efflux ratio of Ca\(^{2+}\) in the culture treated with both H-8 and GRGDSP increased 50% above baseline during addition of GRGDSP (Fig. 5B). GRGDSP continued to stimulate Ca\(^{2+}\) efflux after this peptide was

![Fig. 3. Effect of vanadate on Ca\(^{2+}\) efflux stimulated by GRGDSP. Osteoclast precursors were preincubated with 4\(^{2+}\)Ca and 10 \(\mu\)M vanadate for 1 h and perfused with Krebs-Ringer-HEPES bicarbonate buffer with glucose (KRBH) containing a similar dose of vanadate. Cells were treated with either 100 \(\mu\)g/ml GRGDSP (\(\bullet\)) or vehicle (C) from 30 to 40 min. Results are expressed as fractional efflux of Ca\(^{2+}\) (A) or as fractional efflux ratio of Ca\(^{2+}\) (B). Numerator is fractional Ca\(^{2+}\) efflux in cells treated with GRGDSP, and denominator is fractional Ca\(^{2+}\) efflux in cells treated with vehicle. Results were expressed as means \(\pm\) SE; \(n = 3\).

![Fig. 4. Effect of a calmodulin antagonist on Ca\(^{2+}\) efflux stimulated by GRGDSP. Osteoclast precursors to be perfused with GRGDSP were pretreated with KRHBG containing 0.1 mM W-13 for 25 to 40 min. Cells were treated with either 100 \(\mu\)g/ml GRGDSP (\(\bullet\)) or vehicle (C) from 30 to 40 min. Results are expressed as fractional efflux of Ca\(^{2+}\) (A) or as fractional efflux ratio of Ca\(^{2+}\) (B). Numerator is fractional Ca\(^{2+}\) efflux in cells treated with GRGDSP, and denominator is fractional Ca\(^{2+}\) efflux in cells treated with vehicle. Results were expressed as means \(\pm\) SE; \(n = 3\).

![Fig. 5. Effect of a cyclic nucleotide-dependent protein kinase antagonist on Ca\(^{2+}\) efflux stimulated by GRGDSP. Osteoclast precursors were perfused with KRHBG containing either 0.1 mM H-8 and 100 \(\mu\)g/ml GRGDSP (\(\bullet\)) or 0.1 mM H-8 (\(\bullet\)) from 30 to 40 min. A: Results are expressed as fractional efflux of Ca\(^{2+}\). B: Results are expressed as fractional efflux ratio of Ca\(^{2+}\). Numerator is fractional Ca\(^{2+}\) efflux in cells treated with GRGDSP and H-8, and denominator is fractional Ca\(^{2+}\) efflux in cells treated with H-8 alone. Results were expressed as means \(\pm\) SE; \(n = 3\).
removed, similar to results shown in Fig. 1. Pretreatment of osteoclast precursors with H-8 also failed to inhibit RGD-stimulated Ca\(^{2+}\) efflux (not shown), and other inhibitors of protein kinase A were also ineffective (not shown). Therefore these results suggest that Ca\(^{2+}\) efflux stimulated by GRGDSP was not mediated by a process of activation of cyclic nucleotide-dependent protein kinases, although the latter activity was not directly measured.

The occupancy of the \(\alpha\beta_3\)-integrin by RGD peptides activates a signal-generating complex consisting of several proteins expressing src homology 2 domains, which are tyrosine kinase substrates in chicken osteoclast precursors (29). We therefore examined whether activation of protein-tyrosine kinases are involved in GRGDSP-stimulated Ca\(^{2+}\) efflux. First, genistein, a known inhibitor of tyrosine kinases, was used (1). Genistein (10 \(\mu\)g/ml) was added to osteoclast precursors treated with GRGDSP or vehicle. GRGDSP-stimulated Ca\(^{2+}\) efflux was absent compared with efflux observed in control cells treated with genistein alone. However, there was a possibility that increases in [Ca\(^{2+}\)]\(_i\) might have affected Ca\(^{2+}\) efflux, since genistein increases [Ca\(^{2+}\)]\(_i\) (35). This would have increased efflux from the control and impaired ability to detect stimulated efflux. Thus the effect of GRGDSP on Ca\(^{2+}\) efflux from osteoclast precursors treated with genistein was compared with GRGDSP treatment alone (Fig. 6, C and D). Fractional Ca\(^{2+}\) efflux was greater in GRGDSP-treated osteoclast precursors compared with those treated with both GRGDSP and genistein (Fig. 6C). Fractional efflux of Ca\(^{2+}\) from GRGDSP-treated cells increased 60% compared with that of cells treated with GRGDSP and genistein (Fig. 6D). The effects of genistein or GRGDSP-stimulated Ca\(^{2+}\) efflux indicate that GRGDSP stimulation may depend on activation of protein-tyrosine kinases.

Furthermore, to confirm that activation of protein-tyrosine kinases is involved in GRGDSP-stimulated Ca\(^{2+}\) efflux, the effect of another protein-tyrosine kinase antagonist on GRGDSP-stimulated Ca\(^{2+}\) efflux was tested. Herbimycin A is an inhibitor of tyrosine kinase of pp60\(^-\)src (36). Preincubation of herbimycin A with osteoclast precursors inhibited GRGDSP-stimulated Ca\(^{2+}\) efflux (Fig. 7, A and B). These results indicate again that GRGDSP-stimulated Ca\(^{2+}\) efflux may be mediated via activation of protein-tyrosine kinases.

**DISCUSSION**

The studies reported here demonstrate that an RGD sequence-containing peptide, GRGDSP, stimulated Ca\(^{2+}\) efflux from osteoclast precursors. These data indicate that the reductions in [Ca\(^{2+}\)]\(_i\), observed previously (22) during osteoclast precursor treatment with RGD-containing proteins were, in fact, due to stimulation of Ca\(^{2+}\) efflux. The reduction in [Ca\(^{2+}\)]\(_i\) demonstrated previously by several RGD-containing proteins, which bind to osteoclast \(\alpha\beta_3\)-integrin, was blocked by an anti-\(\alpha\beta_3\) antibody (22). We did not have sufficient reagent to perform similar blocking experiments with the perfusion setup required for the experiments in this study. Thus we cannot claim \(\alpha\beta_3\) specificity for the GRGDSP stimulation of efflux observed in the experiments reported here, although it is likely because collagen and fibronectin ligands for \(\alpha\beta_3\) and \(\alpha\beta_1\), the other osteoclast integrins to which GRGDSP could potentially have bound, do not signal to changes in [Ca\(^{2+}\)]\(_i\) (22). Ca\(^{2+}\) efflux has been studied from multiple cells (4–6, 8,
alkalosis increases \([\text{Ca}^{2+}]\), in agreement with data from renal tubular cells, indicating that extracellular alkalosis decreases fractional \([\text{Ca}^{2+}]\) efflux (5). Thus our data from previous publications (34) and those reported herein suggest that regulation of the osteoclast \([\text{Ca}^{2+}]\)-ATPase stimulates \([\text{Ca}^{2+}]\) efflux and is sufficient to lower \([\text{Ca}^{2+}]\), an effect that may be functionally important to stimulation of bone resorption.

Cyclic nucleotides stimulate \([\text{Ca}^{2+}]\) influx through regulation of voltage-operated \([\text{Ca}^{2+}]\) channels, resulting in an increase in \([\text{Ca}^{2+}]\), and activation of \([\text{Ca}^{2+}]\) efflux in multiple cell types including renal tubular cells and osteoclast precursors (23, 24). In osteoclast precursors, cAMP increases \([\text{Ca}^{2+}]\), (19), and inhibition of protein kinase A activity fails to affect the reduction in \([\text{Ca}^{2+}]\), stimulated by matrix proteins, RGD-containing peptides (22), or extracellular acidosis (34). In the present studies, inhibition of protein kinase A by H-8 also failed to affect RGD-stimulated \([\text{Ca}^{2+}]\) efflux.

Finally, regulatory substances that stimulate inositol trisphosphate production from phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis increase \([\text{Ca}^{2+}]\), and also stimulate \([\text{Ca}^{2+}]\) efflux (4). Tepikin and associates (33) demonstrated that guanosine 3',5'-cyclic monophosphate cGMP stimulates \([\text{Ca}^{2+}]\) efflux by the plasma membrane \([\text{Ca}^{2+}]\)-ATPase and not by \([\text{Na}^{+}]/[\text{Ca}^{2+}]\) exchange. Therefore agents that increase \([\text{Ca}^{2+}]\) stimulate \([\text{Ca}^{2+}]\) extrusion from cells usually mediated by activation of \([\text{Ca}^{2+}]\)-ATPase through calmodulin. In osteoclast precursors, since \([\text{Ca}^{2+}]\), is reduced, calmodulin activation of the \([\text{Ca}^{2+}]\)-ATPase cannot be the mechanism of activation. An exception to stimulation of \([\text{Ca}^{2+}]\) efflux by activation of \([\text{Ca}^{2+}]\)-ATPase is the study by Furukawa and associates (9) demonstrating that guanosine 3',5'-cyclic monophosphate cGMP stimulates \([\text{Ca}^{2+}]\) efflux by \([\text{Na}^{+}]/[\text{Ca}^{2+}]\) exchange in vascular smooth muscle cells (9). In the osteoclast, cGMP does not affect \([\text{Ca}^{2+}]\), (18). In addition, our previous and current studies demonstrating activation of \([\text{Ca}^{2+}]\) efflux by activation or inhibition by extracellular acidosis or alkalosis and matrix proteins indicated that removal of extracellular \([\text{Na}^{+}]\) had no effect on the stimulation of reduction in \([\text{Ca}^{2+}]\).

Plasma membrane \([\text{Ca}^{2+}]\)-ATPase activity is known to be activated by cyclic nucleotide-dependent kinases (26, 37), protein kinase C (31), and acidic phospholipids in vitro (21). In the studies presented herein utilizing osteoclast precursors, protein kinases A and C appear not to be implicated. Therefore, in agreement with the protein-tyrosine kinase inhibitor studies, it seems likely that recognition of RGD peptides by osteoclasts may involve tyrosine kinase activation. One effect of tyrosine kinase was be activation of phosphatidylinositol 3-hydroxyl kinase (25, 29) and the resultant production of polyphosphoinositides, which may have stimulated the plasma membrane \([\text{Ca}^{2+}]\)-ATPase of osteoclast precursors. Phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), PIP2, phosphatidic acid (PA), and phosphatidylerine (PS) increased the affinity of the
smooth muscle and erythrocyte Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) in the following order of potency: PIP\(_2\) > PIP > PI = PS = PA (21). Because RGD peptides activate a signal-generating complex associated with the αβ\(_3\)-integrin (29), one possible explanation for the mechanism of stimulation of plasma membrane Ca\(^{2+}\)-ATPase by RGD peptides is production of polyphosphoinositides through activation of phosphatidylinositol 3-hydroxy kinase. We have shown stimulation of polyphosphoinositides, including phosphatidylinositol trisphosphate (PIP\(_3\)), by RGD-containing matrix protein occupancy of αβ\(_3\) (14). PIP\(_3\) production has also been reported to be stimulated by platelet-derived growth factor (PDGF) (3) in platelets and by α-thrombin, U-46619 (thromboxane A\(_2\)-like substance), and guanosine 5’-O-(3-thiotriphosphate) in smooth muscle cells (16). These studies would suggest the possibility that agents which stimulate polyphosphoinositide production may stimulate extrusion of Ca\(^{2+}\) through activation of plasma membrane Ca\(^{2+}\)-ATPase. Of interest is the fact that PDGF stimulates osteoclasts to increase [Ca\(^{2+}\)]\(_i\) and almost certainly Ca\(^{2+}\) efflux (38). A final point should be made regarding our previous studies (22) to subsequent studies demonstrating increases in [Ca\(^{2+}\)]\(_i\), produced by RGD-containing peptides using osteoclast-like cells from other species. Ca\(^{2+}\) efflux would be activated in the situation of transient elevations in [Ca\(^{2+}\)]\(_i\), and our data address signal transduction from RGD to Ca\(^{2+}\) efflux. What appears to be missing from the avian osteoclast precursor in our hands are the elements of signal transduction to the transient increase in [Ca\(^{2+}\)]\(_i\). Recent studies using transgenic mice with disrupted c-src gene, indicate that RGD-stimulated increases in [Ca\(^{2+}\)]\(_i\), are independent of the c-src tyrosine kinase pathway implicated by αβ\(_3\) activation of c-src in our studies. Further experiments are required to elucidate the mechanism of stimulation of plasma membrane Ca\(^{2+}\)-ATPase produced by RGD peptides to analyze the potential role of PIP\(_3\) or other polyphosphoinositides in mediating this effect.

In summary, the findings reported here are consistent with previous observations on the decrease in intracellular Ca\(^{2+}\) after exposure of chicken osteoclasts to RGD peptides (22). The involvement of Ca\(^{2+}\)-ATPase as the mechanism of Ca\(^{2+}\) efflux leading to reduced [Ca\(^{2+}\)]\(_i\) remains a valid hypothesis. The signal transduction pathway between integrin stimulation (presumably αβ\(_3\)) and activation of the pump requires further elucidation.

NOTE ADDED IN PROOF

It has been reported that osteoclast exposure to αβ\(_3\)-ligands increases intracellular Ca\(^{2+}\) (Shankar, G., I. Davison, M. H. Helfrich, W. T. Mason, and M. A. Horton. Integrin receptor-mediated mobilization of intranuclear calcium in rat osteoclasts. J. Cell Sci. 105: 61–68, 1993). This in itself could secondarily stimulate Ca\(^{2+}\) efflux via the Ca\(^{2+}\) pump. αβ\(_3\)-Ligands increase intracellular Ca\(^{2+}\) in other cells as well (Schwartz, M. A. Spreading of human endothelial cells on fibronectin or vitronectin triggers elevation of intracellular free calcium. J. Cell Biol. 120: 1003–1010, 1993). Stimulation of an increase in [Ca\(^{2+}\)]\(_i\), is independent of the tyrosine kinase pathway, since it was observed in mice with disrupted c-src (Baron, R., L. Neff, G. Yeh, J. Stadel, P. Soriano, and J. Levy. RGD-induced tyrosine phosphorylation in osteoclasts requires c-src expression. J. Bone Miner. Res. 8: S127, 1993).

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REFERENCES


Chronic, intermittent loading alters mechanosensitive channel characteristics in osteoblast-like cells

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Duncan, Randall L., and Keith A. Hruska. Chronic, intermittent loading alters mechanosensitive channel characteristics in osteoblast-like cells. Am. J. Physiol. 267 (Renal Fluid Electrolyte Physiol. 36): F909–F916, 1994.—The effects of chronic, intermittent strain on the mechanosensitive cation (SA-cat) channels in UMR-106.01 osteoblast-like osteosarcoma cells were studied using patch-clamp techniques. Chronically strained cells demonstrated significantly larger increases in whole cell conductance when subjected to additional mechanical strain than nonstrained controls (69.0 ± 15.1 vs. 14.1 ± 3.1%; P < 0.001). This increase could be blocked by the SA-cat channel inhibitor, gadolinium, and corresponded to a three- to fivefold increase in SA-cat channel activity. Chronic strain increased the number of open channels in response to stretch and induced spontaneous SA-cat channel activity in 33% of the patches of strained cells. Graded increases in negative patch pressure demonstrated that SA-cat channels in chronically strained cells were activated at significantly lower levels of mechanical perturbation than nonstrained controls. These data suggest that chronic, cyclic strain reduces the activation threshold of the SA-cat channel and further strengthen our hypothesis that this channel may act as a mechanotransducer for the activation of bone remodeling by physical strain.

scale-activated channel; mechanotransduction; osteoblasts; bone remodeling

THE EFFECTS OF BIOPHYSICAL force on bone remodeling have become increasingly evident in recent years. Clinical observations and in vivo studies have suggested that exercise or applied mechanical load has a positive effect on bone remodeling to increase bone mass. Compared with sedentary subjects, physically active people have a significantly greater bone mass (23). Physical exercise also appears to retard bone loss caused by postmenopausal osteoporosis (22). Conversely, prolonged immobilization can result in a decrease in bone mineral content (13) and an increase in calcium excretion, leading to a reduction in total body calcium (5). Extended periods of weightlessness can also mimic the effects of immobilization. In both human and animal studies, subjects exposed to weightlessness have shown diminished or arrested bone formation (14, 26), reduced collagen production (18), increased osteoclast number (25), and a decrease in mechanical properties of bone (25).

The response of osteoblasts and osteoblast-like osteosarcoma cells in culture to mechanical strain appears to be dependent on the magnitude of the strain applied. In a recent review, Burger and Veldhuijzen (4) observed that high levels of strain (>10,000 microstrain (με)) produced increases in cell proliferation, prostaglandin E₂ (PGE₂) synthesis, and adenosine 3′,5′-cyclic monophosphate (cAMP) production and decreased alkaline phosphatase activity and collagen synthesis. In contrast, at lower levels of strain, alkaline phosphatase activity and protein synthesis were stimulated while cell proliferation was reduced. These observations suggest that the osteoblast can differentiate between different levels of strain and modify its response accordingly. Therefore, osteoblasts must possess some signaling mechanism by which this mechanical stimuli is converted into chemical message. We have previously characterized a mechanosensitive, cation-selective (SA-cat) channel in the UMR-106.01 cell line, which is modulated by parathyroid hormone (PTH) (8). We postulate that this channel may act as a signal transducer for mechanically induced osteogenesis. We demonstrate that, following 2–24 h of chronic, cyclic mechanical strain, whole cell conductance is markedly increased in response to cell stretch via modulation of the mechanosensitive channel.

METHODS

UMR-106.01, a well-characterized osteoblast-like osteosarcoma cell line (10), was a gift from Nicola Partridge (St. Louis Univ.). UMR-106.01 cells (passage 10–20) were plated from muder cultures onto either rigid or flexible, collagen-coated Flexercell silicone-bottomed 6-well culture plates (Flexcell, McKeeport, PA) and were grown in minimal essential medium with Eagle's modification, nonessential amino acids, and Earle's salts (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Cells were fed twice weekly and maintained in a humidified atmosphere of 95% air-5% CO₂ at 37°C. When the cells were visually estimated to be 50–90% confluent, the culture plates were placed on the Flexercell apparatus, which uses vacuum to stretch the silicone bottoms. Cyclic stretch was applied at 3 cycles/min. The maximal stretch produced was 12% displacement at the edge of the wells. After the cells had been stretched for 2–24 h, the silicone bottom of the cluster was then removed and transferred to a recording chamber (1 ml total volume) (Biophysics Technologies, Baltimore, MD), which was modified to permit rapid exchange of the bathing solution with minimal perturbation to the cells. Cells were bathed in a mammalian Na⁺ Ringer solution consisting of (in mM) 136 NaCl, 5.5 KCl, 1 MgCl₂, 1 CaCl₂, and 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, titrated to pH 7.3 with NaOH.

The strain pattern associated with the Flexcell apparatus is nonuniform (1). The profiles of strain range from 120,000 με at the edge of the well (12% maximal displacement) to 0 με at the center. The cells used in this study were subjected to similar magnitudes of strain, since patches were always performed in an area 7 mm from the edge of the well. Strain applied to this area was estimated at 20,000–35,000 με using the strain curve described by Banes et al. (1). Control patches were made in the same area of the plate in nonstretched silicone-bottomed wells. To impose membrane strain on the UMR cells during the patch-clamp studies, 10 ml of 100 mM NaCl (247 mosM), 80 mM NaCl (220 mosM), or 65 mM NaCl (182 mosM) hypotonic Ringer solutions were perfused into the chamber. The UMR-106.01 cell line is not homogeneous, with 2–3 morphologically different cell types present. We only
patched cells with a rounded, polygonal morphology in the center of colonies of ≥ 40 cells. We have previously shown that cells in this region respond to PTH stimulation with activation of the SA-cat channel (8). Cells on the periphery of colonies do not demonstrate this response to PTH and were excluded from this study. Confluent UMR-106.01 cells can produce multiple layers of cells; therefore, subconfluent plates were used in this study. However, no differences were observed in channel characteristics or activity between cultures of different levels of confluence provided the rounded, polygonal cells were patched. We used cultures from 18 different passages for these experiments. There was no variability in channel activity or characteristics between different passages of cells. However, comparisons between chronically stretched and control cells were made between cocultures of the same passage number and level of confluence.

Standard patch-clamp techniques were used to study channel activity in the UMR-106.01 cells. Single-channel recordings were made using 3–5 MΩ, fire-polished, wax-coated, borosilicate pipettes. The pipettes were filled with a Ca2+-free solution consisting of (in mM) 40 KCl, 107 K4-acetate, 6.7 HEPES, 1.3 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), titrated to 7.5 with KOH. This pipette solution was used, since we have previously demonstrated that SA-cat single-channel conductance is significantly increased when Ca2+ is removed from the pipette solution (8). Pipette-to-membrane seals of 10–40 GΩ were attained by gentle application of suction to the tip of the pipette holder. Single-channel currents were amplified with a List EPC-7 patch-clamp amplifier (Adams-List, Westbury, NY), filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) and displayed on a Tectronix oscilloscope. The filtered signal was digitized with a DT2821 analog and digital input/output board (Data Translation, Marlboro, MA) and recorded on the hard drive of a 386DX computer (Dell Computers, Austin, TX) at a sampling frequency of 3 kHz. This data acquisition system was controlled by a software program written by L. C. Falke (Obtech, Boston, MA). The clamping potential is defined as \(-V_{\text{pipette}}\), where \(V_{\text{pipette}}\) is the potential imposed on the pipette interior with the bath as ground. During single-channel recordings, membrane stretch was induced in the patch membrane using pulses of suction applied to the sideport of the pipette via a micropipette-controlled on/off valve.

Analyses of single-channel properties were achieved using a graphics-based analysis program (L. C. Falke, Obtech), which uses level crossings of the digitized data to determine from a segment of record the fraction of time when zero, one more channels were open. This analysis yields the average number of channels open in the patch during a given period of time (\(N_P\)). Open-channel amplitudes were determined utilizing another data analysis program by grouping the currents into bins of 0.025 pA and generating amplitude histograms. These histograms were then fit to a Gaussian distribution curve.

To measure membrane potentials and whole cell conductances, the nystatin perforated-patch technique was used under current clamp conditions. The pipette solution in these experiments consisted of (in mM) 12 NaCl, 64 KCl, 28 K2SO4, 47 sucrose, 1 MgCl2, 0.5 EGTA, 20 HEPES, titrated to 7.35 with KOH. Nystatin was added at a concentration of 300 µg/ml to permeabilize the patch. Access resistances of < 40 MΩ were consistently achieved with this concentration of nystatin. Whole cell conductance measurements were made by pulsing ±50 pA across the membrane.

Data are expressed as means ± SE. In studies where a comparison was made between treatment and control, the Student’s t-test was used to determine significance. If multiple comparisons were made against the control, Dunnett’s method of multiple comparisons was employed.

**RESULTS**

Application of 2–24 h of cyclic stretch to UMR-106.01 cells prior to patch-clamp studies significantly increased the resting membrane potential (\(V_m\)) from −32.1 ± 1.96 mV in control nonstretched cells \((n = 32)\) to −42.4 ± 1.88 mV in stretched cells \((n = 43); P < 0.001\). However, the resting whole cell conductance was not significantly altered. The resting whole cell conductance of control cells averaged 15.8 ± 1.2 nS compared with stretched whole cell conductance values of 12.2 ± 1.3 nS. To determine conductance changes in response to stretch during the patch studies, 182 mosM Na+ Ringer was perfused into the chamber. UMR-106.01 cells responded with a transient hyperpolarization followed by a rapid depolarization, yet control cells only exhibited a small, slow rise in whole cell conductance (Fig. 1A), averaging a 14.1 ± 3.1% increase after 2 min of hypotonic stretch. Chronically stretched cells responded with a much larger and more rapid increase in whole cell conductance (69.0 ± 15.1%; \(P < 0.001\)) usually peaking within 1 min after hypotonic challenge (Fig. 1B). These data suggest that chronic stretch primes certain channels in the cell to respond to additional mechanical stimulation. When isotonic Na+ Ringer was perfused into the chamber following hypotonic stretch, an additional transient increase in whole cell conductance was observed in both control and prestretched cells. Cell \(V_m\) and whole cell conductance slowly returned to resting levels following this transient, usually within 5–10 min. No differences in resting cell \(V_m\), whole cell conductance, or response to hypotonic stretch were observed in the UMR-106.01 cells during the time interval of applied chronic, mechanical strain. To determine whether the SA-cat channel was responsible for this increase in whole cell conductance, the SA-cat channel blocker, gadolinium, was added to the bath prior to hypotonic stretch. A typical response to gadolinium block is illustrated in Fig. 2. Prior to addition of 10 µM GdCl3, the 8-h chronically stretched cell responded to hypotonic stimulation with a 31.9% increase in whole cell conductance (Fig. 2A). Following an isotonic wash, \(V_m\) and whole cell conductance returned to resting levels within 9 min. Addition of GdCl3 (Fig. 2B) produced a pronounced, sustained hyperpolarization (−25.6 to −36.9 mV) with a nonsignificant decrease in whole cell conductance (18.8–13.9 nS). Hypotonic challenge in the presence of Gd3+ produced a typical \(V_m\) response. However, after 2 min, whole cell conductance had only increased 11.1%. A similar response was seen in control nonstretched cells. The transient rise in whole cell conductance following return to isotonic Ringer was also reduced by Gd3+. When Gd3+ was washed out and the cell allowed to equilibrate in isotonic Ringer for 15 min, \(V_m\) returned to resting levels observed prior to Gd3+ block. Hypotonic challenge following Gd3+ washout produced a rapid and sustained increase in whole cell conductance (60.5%) within 1 min of hypotonic Ringer perfusion. After isotonic Ringer wash, the transient increase in whole cell conductance
was again present. In six experiments, Gd$^{3+}$ blocked the response to hypotonic swelling by reducing the increase in whole cell conductance from $65.3 \pm 15.2$ to $14.2 \pm 5.3\%$ ($P < 0.05$). Previously, lanthanides, including Gd$^{3+}$, have been shown to block calcium channels in skeletal (12) and neural (24) tissue. To determine whether the voltage-dependent, dihydropyridine-sensitive calcium channels found in UMR cells were involved in the whole cell conductance response to hypotonic challenge, nitrendipine was added to the chronically stretched UMR cells prior to hypotonic swelling. No significant deviation from the time course or magnitude of changes in whole cell conductance was observed with nitrendipine (data not shown).

To determine whether hypotonic swelling induced single SA-cat channel activity, single-channel analyses were made in the cell-attached configuration ($n = 6$). Figure 3 illustrates a representative experiment that demonstrates that SA-cat channel activity increases with a decrease in the osmolarity of the bathing Ringer solution. When an SA-cat channel was found to be present in a patch via application of suction to the backside of the pipette, suction was removed, and various hyposmotic solutions were perfused through the chamber. Figure 3A demonstrates that, during application of negative pressure to chronically stretched cells in isotonic Ringer, 1–2 SA-cat channels opened, but no spontaneous activity was observed. Spontaneous SA-cat channel activity was observed when 267 mosM Ringer was perfused into the chamber. Measurement of channel activity after 2 min of hypotonic challenge found that $NP_o$ was significantly higher ($0.13 \pm 0.09; P < 0.05$). When the cell was further challenged with 220 and 182 mosM Ringer, SA-cat channel activity increased to $0.34 \pm 0.12$ and $3.18 \pm 1.03$, respectively, with up to 5 channels opening simultaneously in the patch in 182 mosM Ringer. SA-cat channel activity remained high immediately following return to an isotonic bathing solution ($3.52 \pm 1.27$), but spontaneous activity disappeared 5–10 min after isotonic Ringer perfusion. Hypotonic challenge did not produce spontaneous activity in nonstretched control cells; however, return to isotonic Ringer did induce SA-cat channel spontaneous activity ($NP_o = 0.92 \pm 0.50$; data not shown). The activation of SA-cat channel activity by hypotonic challenge was completely blocked in cells pretreated with 10 $\mu$M GdCl$_3$ ($n = 6$; data not shown).

To determine whether chronic stretch altered the stretch sensitivity of the SA-cat channel, graded increases in negative patch pressure were applied to the patch membrane of both control and 2- to 6-h chronically stretched cells from the same passage. In 3 of 9
chronically stretched cells tested, spontaneous SA-cat channel activity was observed (Fig. 4A, arrows). Spontaneous activity was not seen in nonstretched controls. Chronic stretch produced a significant shift of stretch sensitivity to lower levels of suction (Fig. 4B) with maximal channel activity observed at −20 mmHg compared with control cells where maximal channel activity was observed at −40 mmHg. Significant differences in activity in response to increasing negative pressure were achieved at −15 mmHg. Chronic stretch also produced a twofold increase in maximal channel NP, over control values (P < 0.001).

Examination of single-channel records from chronically stretched UMR cells in response to negative patch pressure indicated that channel openings were different from nonstretched control recordings. Figure 5 exemplifies these changes in SA-cat open-channel amplitude following 2 h of chronic stretch. In Fig. 5A, current traces from control and 2 h stretched cells demonstrate larger amplitudes with smaller openings and closings (Fig. 5A, arrows) occurring during the applied stretch. At a clamp voltage of 40 mV, peak open-channel amplitude shifted from 1.22 ± 0.09 pA for nonstrained UMR cells to 2.20 ± 0.12 pA (n = 10; P < 0.001) for stretched cells (Fig. 5B). Although the peak amplitude is not equal to the calculated sum of two channel open amplitudes, the observed values were not significantly different from the calculated value. The distribution of the amplitude histogram for chronically strained channel openings is non-Gaussian for two SA-cat channel openings; however, the small side peak in the strained histogram in Fig. 5B corresponds to the control open amplitude. We have previously observed a similar shift in SA-cat open-channel amplitudes during PTH stimulation of UMR cells, which we hypothesized was the result of activation of an additional conductance state of the SA-cat channel. Occasional simultaneous openings in these records would support this hypothesis. However, these observations suggest three possible explanations for the shift in open-channel amplitude following 2 h of chronic mechanical strain: 1) increased sensitivity to stretch following strain causes two SA-cat channels to open simultaneously, 2) an additional mechanosensitive channel with a slightly smaller amplitude becomes active in chronically stretched cells that we have not previously observed in nonstretched UMR cells, or 3) chronic
MECHANICAL LOAD MODULATES CHANNELS IN OSTEOLASTS

A 30mmHg 30mmHg

B
Isotonic
267mosm
220mosm
182mosm
Isotonic (20 sec post)
Isotonic (10 min post)

Fig. 3. SA-cat channel activity in response to increasing levels of hypotonicity. A: mechanosensitive cation (SA-cat) channel activity in isotonic Ringer solution when negative pressure is applied to back of patch pipette. Suction is then removed, and channel activity is measured without patch stretch (B). In isotonic Ringer solution, no spontaneous activity was observed in this patch, but, with increasing hypotonicity, channel activity increases. Finally, at 182 mosM Ringer, up to 5 channels are opening. Channel amplitude is reduced as hypotonicity increases, since membrane potential is depolarizing and resting membrane potential ($V_m$) is nearing reversal potential for SA-cat channel. Return to isotonic Ringer solution produces further SA-cat channel activity immediately after washout, but, 10-min postwashout, no channel activity is observed.

stretch activates an additional conductance state of the SA-cat channel. Further studies are required to determine which of these possibilities occur with chronically applied mechanical strain.

DISCUSSION

In the studies reported herein, we have demonstrated that chronic, intermittent mechanical stretch modulates the SA-cat channel by increasing the sensitivity of the channel to stretch and the average number of open channels. Chronic stretch also increased whole cell conductance which was due to the changes in SA-cat channel activity, since blocking the channel reversed the whole cell conductance change. These results are important, since they demonstrate a change in whole cell conductance related to alterations in SA-cat channel activity.

Mechanically sensitive channels have been characterized in a wide variety of cells from diverse tissue (15), including the osteoblast-like osteosarcoma cell lines, UMR-106.01 (9) and MG-63 (6). Recently, however, Morris and Horn (16) have questioned the physiological relevance of these channels. At levels of mechanical perturbation which should have stimulated mechanosensitive channels in the growth cones of snail neurons, they could find no evidence for activation of whole cell currents. The lack of whole cell current activation suggested that the mechanosensitive channels may be an artifact of the patch gigaohm seal. However, mechanically induced whole cell current activation consistent with mechanosensitive channel activity has been observed in yeast spheroplasts (11), fungal cells (28), and mammalian smooth muscle (7). In addition, calcium-
permeable mechanosensitive channels have been implicated in the intracellular calcium increases observed during hypotonic challenge in hepatocytes (2) and mechanical stimulation of lung airway epithelia (20).

In this study, we demonstrated that UMR cells subjected to chronic strain exhibited a significant increase in whole cell conductance following hypotonic challenge, which could be attributed to activation of SA-cat channels. We have shown that PTH depolarizes only those UMR cells in contact with other cells (8), suggesting that normal osteoblastic function requires cell-to-cell contact in this cell type. Therefore, whole cell currents were difficult to measure due to the extensive electrical coupling between cells, and only whole cell conductance was used as a measurement of whole cell channel activity. Previously, we have been unable to demonstrate whole cell conductance changes with PTH stimulation or hypotonic challenge alone, although PTH stimulates SA-cat single-channel activity and conductance. Although it is puzzling that whole cell conductance is not significantly altered by PTH and hypotonic challenge alone, chronic mechanical strain prior to the patch could either activate previously inactive SA-cat channels in the membrane or incorporate more channels into the membrane. This, coupled with the increased sensitivity of the channel to stretch, could cause the large, significant increases in whole cell conductance and "prime" the cell for additional encounters with mechanical strain. The studies reported here significantly clarify the physiological potential of SA-cat channel activity. Induction of mechanical loads produces spontaneous channel activity and whole cell conductance attributed to the opening
of this channel. Thus, in this osteoblast-like cell line, the SA-cat channel is an integral component of the electrical environment and ion flux of the cell.

We have also demonstrated that 10 μM GdCl₃ blocks the membrane depolarization associated with PTH stimulation (8) and the whole cell conductance increase with hypertonic challenge. Gadolinium has been shown to block stretch-activated, cation-nonselective channels in Xenopus oocytes (27), vascular smooth muscle (7), cardiac myocytes (19), hepatocytes (2), and rat capillary endothelial cells (17). However, gadolinium was also found to block voltage-activated calcium currents in cardiac and skeletal muscle (3) at similar concentrations to the gadolinium inhibition of the SA-cat channel. In the UMR-106.01 cells, we have previously identified a dihydropyridine-sensitive, voltage-dependent calcium channel resembling the L-type calcium channel in neural and cardiac tissue (9). We attempted to block any changes in the whole cell conductance resulting from the dihydropyridine-sensitive calcium channel response to hypertonic challenge with nitrendipine. The lack of a significant block of the whole cell conductance with nitrendipine would indicate that this increase is due solely to the increase in SA-cat channel activation.

The effects of skeletal unloading during weightlessness or immobilization have been well described in the literature. The balance between bone formation and bone resorption is rapidly lost during these conditions, and the body begins to lose large amounts of calcium and bone mineral density. The effects of skeletal reloading on bone restoration are not as clear, with the restoration dependent on species, age, bone type, method of skeletal unloading, duration of unloading, and the time of testing after unloading (13, 21). The response of osteoblasts in culture to mechanical loading would indicate that the osteoblast responds differently to different magnitudes of strain (4). High levels of strain produce proliferative responses in the cell, stimulating the cell cycle, PGE₂ synthesis, and cAMP production. Lower magnitudes of strain promote anabolic responses with increases in matrix protein production and alkaline phosphatase activity while reducing cell proliferation. These data would imply that a sensing mechanism in the osteoblast is capable of discerning different magnitudes of strain and altering the cellular function accordingly. Burger and Veldhuijzen (4) offer a theory to explain the different responses to variable strain. An osteoblast aligned in the direction of mechanical strain would experience little deformation, and matrix production would proceed in a normal fashion. However, if the osteoblast were to obliquely align with the lines of force, the cell would incur high levels of strain, and matrix production would decrease. The result would be increased bone formation and strength along the force vectors in bone. The levels of mechanical strain used in this study are quite high compared with strains that would be normally incurred by bone in vivo; however they are similar to the strains used in previous in vitro studies, which demonstrated proliferative responses in the osteoblast. One effect of stimulation of the SA-cat channel may be to initiate these processes; however, channel parameters must be studied when the osteoblast is subjected to reduced magnitudes of strain before more conclusive statements can be made.

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Human Osteoblast-Like Cells Respond to Mechanical Strain with Increased Bone Matrix Protein Production Independent of Hormonal Regulation*

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ABSTRACT
Exposure of osteosarcoma cell lines to chronic intermittent strain increases the activity of mechanosensitive channel (SA-cat) channels. The impact of mechano-transduction on osteoblast function has not been well studied. We analyzed the expression and production of bone matrix proteins in human osteoblast-like osteosarcoma cells, OHS-4, in response to chronic intermittent mechanical strain. The OHS-4 cells exhibit type I collagen production, 1,25-Dihydroxyvitamin D-inducible osteocalcin, and mineralization of the extracellular matrix. The matrix protein message level was determined from total RNA isolated from cells exposed to 1–4 days of chronic intermittent strain. Northern analysis for type I collagen indicated that strain increased collagen message after 48 h. Immunofluorescent labeling of type I collagen demonstrated that secretion was also enhanced with mechanical strain. Osteopontin message levels were increased several-fold by the application of mechanical load in the absence of vitamin D, and the two stimuli together produced an additive effect. Osteocalcin secretion was also increased with cyclic strain. Osteocalcin levels were not detectable in vitamin D-untreated control cells. However, after 4 days of induced load, significant levels of osteocalcin were observed in the medium. With vitamin D present, osteocalcin levels were 4 times higher in the medium of strained cells compared to nonstrained controls. We conclude that mechanical strain of osteoblast-like cells is sufficient to increase the transcription and secretion of matrix proteins via mechano-transduction without hormonal induction. (Endocrinology 136: 528–535, 1995)

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duration of strain varied from 1–3 days during this set of experiments, 10 nM 1,25-(OH)₂D₃ was added 24 h before harvesting the cells. Figure 4 illustrates that nonstretched, vitamin D-treated control cells and strained cells exhibited basal levels of osteopontin message. After 72 h of mechanical strain, OHS-4 cells exhibited a 4-fold increase ($P < 0.02$) in message levels of osteopontin in vitamin D-treated strained cells compared to vitamin D-treated controls. These studies would indicate that both vitamin D and mechanical strain regulate osteopontin message independently, and the two stimuli may be synergistic.

Osteocalcin is one of the most abundant noncollagenous proteins secreted by osteoblasts. Like osteopontin, the osteocalcin message in osteoblasts is up-regulated by 1,25-(OH)₂D₃ (22, 23). Osteocalcin secretion is also increased in OHS-4 osteoblast-like cells by vitamin D (17). We analyzed the effects of vitamin D and mechanical strain on osteocalcin secretion to determine whether the osteoblast also up-regulates the production of this protein in response to the two stimuli. As in the osteopontin study, we applied chronic mechanical load to OHS-4 cells for 1–4 days. Twenty-four hours before harvest, we treated the cells with serum-free medium containing 10 nM 1,25-(OH)₂D₃. We demonstrated that cells exposed to 4 days of strain and 24 h of vitamin D exhibited a 3-fold increase in osteocalcin secretion compared to cells exposed to vitamin D alone (Fig. 5). When cells were not treated with vitamin D, detectable levels of osteocalcin secretion were only observed after 4 days of mechanical load; however, these levels were lower than the levels of osteocalcin secreted in response to vitamin D stimulation alone. These data would suggest that, like osteopontin message, both mechanical load and vitamin D increase osteocalcin secretion, and the two together produce a synergistic response.

**Discussion**

The human osteosarcoma cell line OHS-4 has been shown to have many of the characteristics of normal differentiated osteoblasts (17). These cells produce alkaline phosphatase, express message for type I, but not type III, collagen, and secrete osteocalcin in response to 1,25-(OH)₂D₃ treatment. When OHS-4 cells were injected into mice, they formed mineralized nodules that had a calcium to phosphate ratio of 1.6, similar to human hydroxyapatite. We have shown that
OHS-4 cells exhibit SA-cat channels exactly like the UMR-106.01 cells that we have studied (data not shown). In this study, we demonstrate that OHS-4 cells, like normal osteoblasts in primary culture, also respond to mechanical strain. When subjected to chronic intermittent strain, these cells increased the production of type I collagen and osteopontin and the secretion of type I collagen and osteocalcin. Also, mechanical stimulation of the expression and production of the noncollagenous matrix proteins was independent of stimulation of 1,25-(OH)$_2$D$_3$, and the two stimuli together indicated synergism, suggesting that the stimulation of these proteins by mechanical strain is mediated through a separate pathway from that of vitamin D.

Multiple in vitro techniques have been used to stimulate mechanical loading at the cellular level, including hypotonic swelling, fluid shear, atmospheric pressure, and mechanical stretch. However, the response of the osteoblast as well as chondrocytes to mechanical strain is variable. Several studies have reported increases in cell proliferation (24, 25), total protein production (25, 26), and DNA synthesis (27) in response to mechanical strain, whereas others have observed a decrease in these proliferative responses during strain (28, 29). These apparent conflicting observations also hold true for the production of proteins and enzymes associated with osteogenesis. Alkaline phosphatase activity (28, 30) and collagen synthesis (30, 31) have been shown to increase or decrease with mechanical strain, although these responses are inversely related to proliferation (28). Although the lack of a consistent response to mechanical strain could be explained by the different cell types used, a recent review by Burger and Veldhuijen (16) offers an interesting hypothesis. By compiling the existing data and correlating the magnitudes of strain, they concluded that at high levels of strain, the osteoblast responds with proliferative activity and a decrease in the production of the osteoblast phenotypic markers, such as alkaline phosphatase and bone matrix proteins. At lower levels of strain, the response of the osteoblast indicates a more differentiated state, with an increase in alkaline phosphatase activity and matrix protein production and a decrease in proliferation. These observations led to the hypoth-
OSTEOGENIC EFFECTS OF MECHANICAL LOADING

Fig. 5. Effects of mechanical strain on osteocalcin secretion in OHS-4 osteosarcoma cells. With 1,25-(OH)₂D₃ present 24 h before osteocalcin determination, both control and mechanically strained cells had basal levels of osteocalcin secretion into the medium (A). After 4 days of strain, OHS-4 cells treated with vitamin D had significantly higher levels of secretion (3.41 ± 0.594 ng/mg protein) than vitamin D-treated nonstrained controls (0.79 ± 0.251 ng/mg protein; P < 0.001). Without prior hormonal treatment, no basal secretion of osteocalcin was observed (B). However, after 4 days of mechanical strain, a significant level of osteocalcin secretion was observed. This figure represents one of three experiments.

Another possible mechanism could be the influence of mechanical strain on matrix protein production. Mechano-sensitive channels have been characterized in osteoblast-like osteosarcoma cells (44, 45), which respond to mechanical perturbation and are up-regulated by chronic intermittent strain (15). These channels are capable of conducting calcium, which could trigger a number of second messenger responses in the osteoblast. Another possible mechanism could be the influence of mechanical strain through the actin cytoskeleton and the induction of transcription factors. Rearrangement of the actin cytoskeleton with cytochalasin-B has been reported to reestablish the chondrocyte phenotype and collagen message after dedifferentiation with retinoic acid (46). Another mechanism that could be influenced by mechanical strain is the prostaglandin pathway. High levels of mechanical strain in-
crease PGE₂ secretion in osteoblasts and osteoblast-like osteosarcoma cell lines. Although these levels of strain have been associated with accelerated proliferation and reduced differentiation (16), recent studies have shown effects on mineralization. A metabolite of PGE₂ has been shown to stimulate type I collagen synthesis (47), and a PG₄ metabo-
lite increases osteocalcin production (48). Therefore, me-
chanical strain could exert its influence through this path-
way. However, further study is required to elucidate the pathway for conversion of biophysical stimuli into gen-
eral expression.

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Antisense oligodeoxynucleotide inhibition of a swelling-activated cation channel in osteoblast-like osteosarcoma cells

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ABSTRACT By patch-clamp analysis, we have shown that chronic, intermittent mechanical strain (CMS) increases the activity of stretch-activated cation channels of osteoblast-like UMR-106.01 cells. CMS also produces a swelling-activated whole-cell conductance ($G_m$) regulated by varying strain levels. We questioned whether the swelling-activated conductance was produced by stretch-activated cation channel activity. We have identified a gene involved in the increase in conductance by using antisense oligodeoxynucleotides (ODN) derived from the $\alpha_1$-subunit genes of calcium channels found in UMR-106.01 cells ($\alpha_{1G}$, $\alpha_{1C}$, and $\alpha_{1P}$). We demonstrate that $\alpha_1C$ antisense ODNs abolish the increase in $G_m$ in response to hypotonic swelling following CMS. Antisense ODNs to $\alpha_{1G}$, and $\alpha_{1P}$, sense ODNs to $\alpha_{1C}$, and sham permeabilization had no effect on the conductance increase. In addition, during cell-attached patch-clamp studies, antisense ODNs to $\alpha_{1C}$ completely blocked the swelling-activated and stretch-activated nonselective cation channel response to strain. Antisense ODNs to $\alpha_{1S}$ treatment produced no effect on either swelling-activated or stretch-activated cation channel activity. There were differences in the stretch-activated and swelling-activated cation channel activity, but whether they represent different channels could not be determined from our data. Our data indicate that the $\alpha_{1C}$ gene product is involved in the $G_m$ and the activation of the swelling-activated cation channels induced by CMS. The possibility that swelling-activated cation channel genes are members of the calcium channel superfamily exists, but if $\alpha_{1C}$ is not the swelling-activated channel itself, then its expression is required for induction of swelling-activated cation channel activity by CMS.

Mechanical strain increases bone formation and remodeling activity resulting in a net increase in bone mass (1-3). However, the mechanisms by which the osteoblasts and other bone-forming cells sense mechanical stimuli and transduce biochemical signals have yet to be identified. We previously characterized a mechanosensitive, cation nonselective channel in osteoblast-like cells (4) that is modulated by parathyroid hormone (5). These channels are not voltage regulated; they are dihydropyridine insensitive, and they are inhibited by the trivalent gadolinium (Gd$^{3+}$). We hypothesize that these channels act as signal transducers for the anabolic effects of mechanical strain and parathyroid hormone. Application of chronic, intermittent mechanical strain (CMS) to osteoblasts increases stretch-activated cation channel open probability and sensitivity of the channel to mechanical strain as well as eliciting spontaneous channel activity (6). Spontaneous channel activity and identification of a component of cell conductance due to stretch-activated cation channel activity had not been previously demonstrated for these channels (7). Application of CMS to osteoblasts also produces an increase in whole-cell conductance ($G_m$) when the osteoblast is challenged by hypotonic swelling (6). Our observations suggest that, during physical loading of the osteoblast, stretch-activated or swelling-activated cation channels are an integral component of the electrical environment and mediate ion flux into the cell.

Osteoblasts not only respond to CMS by modulating the stretch-activated cation channel, but gene transcription for bone-matrix proteins is altered as well (8). Application of CMS to human osteoblast-like osteosarcoma cells for 24-72 hr increases type I procollagen message and type I collagen secretion. CMS also up-regulates osteopontin message and osteocalcin secretion independent of 1,25-dihydroxyvitamin D$_3$. Whether the effects of CMS on bone matrix protein expression and production are modulated through the stretch-activated cation channel has not been determined.

Using homology-based reverse transcriptase PCR, Barry et al. (9) isolated partial cDNA clones of three $\alpha_1$ subunits of calcium channels in UMR-106.01 osteoblast-like osteosarcoma cells. They found a unique conservation of alternative splicing across each of the $\alpha_1$-subunit genes in UMR-106.01 cells, $\alpha_{1G}$, $\alpha_{1C}$, and $\alpha_{1P}$ (see ref. 10 for nomenclature). Alternative splicing of $\alpha_1$ subunit was described by Perez-Reyes et al. (25) who found two variants of $\alpha_{1S}$ and four of $\alpha_{1C}$ and $\alpha_{1D}$. Barry et al. (9) found that rodent osteoblast-like cells express only the b variant (Perez-Reyes designation) of all three $\alpha_1$ subunits. Employing an antisense strategy in the studies reported here, we demonstrate that antisense to the $\alpha_{1C}$ channel subunit is capable of blocking the $G_m$ increase in UMR-106.01 osteoblast-like osteosarcoma cells in response to hypotonic swelling following CMS. Furthermore, the $\alpha_{1C}$ antisense oligodeoxynucleotide (ODN) blocks the single cation channel response to hypotonic strain and stretch activation in cells exposed to CMS.

MATERIALS AND METHODS

Cell Culture. UMR-106.01 cells (passages 12-18) were grown in minimal essential medium with Eagle's modification, nonessential amino acids, and Earle's salts (Sigma) supplemented with 10% fetal bovine serum (GIBCO). Cells were plated onto flexible, type I collagen-coated, silicone-bottomed six-well culture plates (Flexercell, McKeenPs, PA), fed twice weekly, and maintained in a humidified atmosphere of 95% air/5% CO$_2$ at 37°C. When the cells were ~75% confluent, sense or antisense ODNs were introduced into the cells as described below. To induce CMS, culture plates were placed on an apparatus that uses vacuum to stretch the silicone-bottomed plates. Cyclic stretch was applied for 12-30 hr at

Abbreviations: CMS, chronic, intermittent mechanical strain; $G_m$, whole-cell conductance; ODN, oligodeoxynucleotide; NP$_o$, open channel activity.

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three cycles per min. The strain pattern associated with the Flexercell apparatus is nonuniform (12). Strain \( E \) was measured as a fraction of deformation: \( E = \Delta l/l \), where \( l \) is length of a cell; \( \mu E = E \times 10^{6} \). The profiles of strain range from 120,000 \( \mu \)E at the edge of the well (12\% maximal displacement) to 0 \( \mu \)E at the center. The cells used in this study were subjected to similar magnitudes of strain since patches were always performed in an area 7 mm from the edge of the well. Strain applied to this area was estimated at 80,000–100,000 \( \mu \)E using the strain curve described by Banes et al. (12). Comparisons were made between chronically strained control and ODN-treated cells from the same passage number and at the same level of confluence.

Introduction of ODNs. A pair of antisense/sense ODNs (24-mer) and a 20-mer antisense ODN were developed from the sequence of a \( a_{12}c \) cDNA of the L-type calcium channel genes isolated from the UMR-106.01 cell line (9) by reverse transcriptase PCR.\(^{3} \) The sequence of the antisense ODN (24-mer) was 5'-CTCATTGCGTTTGCATGTGC-3' and that of the sense ODN was 5'-TGAGCAGCAACAGCACCGGAAGG-3'. The sequence of the antisense 20-mer was ACTCTGGAGCACACTTCTTG. ODNs were synthesized by MacroMolecular Resources (Fort Collins, CO) and introduced into UMR-106.01 cells using streptolysin O (Sigma) permeabilization (13). After the UMR-106.01 cells had been plated onto flexible silicone-bottomed culture plates and grown to 75% confluence, the medium was removed and the cells were washed with a permeabilization buffer consisting of 137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 2.7 mM EGTA, 1 mM NaATP, 100 mM Pipes, 0.1% bovine serum albumin (pH 7.4). The permeabilization buffer containing 0.5 unit of streptolysin O per ml and the appropriate ODN at 100 \( \mu \)M was then placed on the cells for 5 min at room temperature. This solution was then removed and the normal medium with 10% fetal bovine serum was added to the cells. To test the effects of streptolysin alone, UMR-106.01 cells were permeabilized with the same concentration of streptolysin O with no ODN present. These experiments are referred to in the figures and text as "sham permeabilized." Similar procedures were used for application of antisense ODNs to the \( a_{12} \) and \( a_{12} \) subunits of the L-type calcium channels. Cell membrane permeabilization by the above technique was uniform, nonenthal, and completely reversible as assessed by complete recovery of membrane potential \( (V_m) \) and cell resistances following removal of the streptolysin O. The initial \( V_m \) of patched untreated cells was \(-33.8 \pm 4.7 \) mV (range: -21.9 to -69; \( n = 23 \)), while that of streptolysin O-treated cells was \(-32.6 \pm 3.3 \) mV (range: -18.7 to -70 mV; \( n = 51 \)).

Patch-Clamp Studies. Following application of CMS, the silicone bottom of the cluster was removed and transferred to a recording chamber (1 ml total vol) (Biophysics Technologies, Baltimore), which was modified to permit rapid exchange of the bathing solution with minimal perturbation to the cells. Cells were bathed in a "normal" mammalian Na+ Ringer's solution consisting of 138 mM NaCl, 5.5 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 20 mM HEPES buffer titrated to pH 7.3 with NaOH. To impose membrane strain on the UMR cells during the patch-clamp studies, 10 ml of 65 mM NaCl (182 mOsm) hypotonic Ringer's solution was perfused into the chamber. The circumferential strain of a spherical cell can be approximated as \( E = (d_2^2 - d_1^2)/d_1 \), which simplifies to \( E = d_2/d_1 \), where \( d_1 \) is diameter of a spherical cell prior to swelling and \( d_2 \) is the diameter after swelling. This computation assumes even distribution of force across the plasma membrane and no addition of new membrane after swelling. Although many laboratories use hypotonic swelling as a means of imposing mechanical strain on cells, we realize that stretch and swelling are very different and the distinction is maintained throughout this report, which is focused on swelling-induced channel activity in order to analyze the increase in swelling-induced whole-cell conductance \( (G_m) \) produced by CMS.

To measure \( V_m \) and \( G_m \), the nystatin perforated-patch technique was used under current-clamp conditions (14). The pipette solution in these experiments consisted of 12 mM NaCl, 56 mM KCl, 10 mM HEPES, 20 mM HEPES buffer, 1 mM MgCl\(_2\), 0.5 mM EGTA, 20 mM Hepes, titrated to 7.35 with KOH. Nystatin was added at a concentration of 300 \( \mu \)g/ml to permeabilize the patch. Access resistances of \(<40 \) M\( \Omega \) were consistently achieved with this concentration of nystatin. Whole-cell conductance \( (G_m) \) measurements were made by pulsing \(<-50\) pA across the membrane. Acceptable patches for analysis were determined by maintenance of negative cell polarization after returning to isosmotic conditions, the persistence of the large cell membrane capacitive current spike in response to a 20-mV voltage pulse at the beginning and end of the experiment, and the ability to reseal the patch in the outside out configuration. These criteria were used to confirm that the perforated-patch configuration remained intact throughout the experimental period.

Patch-pipettes for single channel recordings contained either 144.0 mM KCl, 10.0 mM KHepes, 1.0 mM MgCl\(_2\) or 138.0 mM NaCl, 5.5 mM KCl, 1.0 mM MgCl\(_2\), 8.0 mM NaHepes in the pipette. To determine the channels' monovalent ion selectivity ratios, bath solutions were exchanged between the normal bathing solution (see above), the high potassium pipette solution, and an isotonic low NaCl solution containing 65.0 mM NaCl, 5.5 mM KCl, 1.0 mM MgCl\(_2\), 8.0 mM NaHepes and 154.0 mM mannitol. The channel's selectivity to calcium was determined by changing bath solutions between the normal NaCl bathing solution and a high calcium solution containing 75.0 mM CaCl\(_2\), 5.5 mM KCl, 1.0 mM MgCl\(_2\), 8.0 mM NaHepes and 60.0 mM mannitol. All bath solutions were maintained at pH 7.3.

Voltage-pulse protocols for construction of current–voltage (\( I-V \)) plots consisted of holding inside-out patches at \(-70\) mV referenced to the pipette for 5 sec between 4-sec pulses to holding potentials, which were stepped from \(-100\) or \(-150\) mV to \(+100\) or \(+150\) mV in 10-mV increments. Electrophysiological signals were amplified with a List EPC-7 patch-clamp amplifier (Adams–List, Westbury, NY), filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), and displayed on a Tektronix oscilloscope. A Digitada 1200 interface (Axon Instruments) was used to digitize the filtered signal, which was stored on the hard drive of a 386DX computer (Dell Computers, Austin, TX). Data were acquired and analyzed using the PCLAMP 6.02 software (Axon Instruments). Cursor measurements were made to determine the magnitude of discrete single-channel openings, and these current magnitudes were plotted against the holding voltage. \( I-V \) plots were fitted to the GHK current equation (15) using the nonlinear fitting algorithm in the SIGMAPLOT software program (Jandel Scientific, San Rafael, CA).

Statistical Analysis. The significance of differences between means for the experimental groups was assessed by using the Bonferroni correction (16).

RESULTS

We have previously shown that hypotonic challenge induces a large increase in \( G_m \) in UMR-106.01 cells only after the cells have been subjected to CMS (Table 1). This increase in conductance correlates with increased stretch-activated cation channel activity (figure 3 in ref. 6). Both swelling-activated and stretch-activated cation channel activity and the

\(^{3}\) A patent application has been filed for the discovery described in this publication.

\(^{\text{14}}\) The publication has been filed for patent application.
FIG. 1. Effect of an antisense 24-mer ODN from the \( \alpha_{1C} \) subunit of the UMR-106.01 calcium channel 2 gene (\( \alpha_{1C} \)) on the response of \( V_m \) and \( G_m \) to hypotonic stretch. (A) UMR-106.01 cell \( V_m \) and \( G_m \) response to hypotonic stretch (top) and reversal in isotonic media (iso). The cells had been exposed to chronic mechanical cyclic strain for 18 hr. (8) As in A, except the cells had been treated with the \( \alpha_{1C} \) antisense ODN during the 19 hr of mechanical strain. Data are representative of several similar experiments (see Table 1). (C) Cells were loaded with the sense ODN in parallel with the antisense ODN used 18 hr prior to study, during which time the cells were exposed to chronic cyclic strain as described. (D) As in A, except the cells were exposed to anti-streptolysin O but no ODN. Data are representative of several similar experiments (see text).

Hypotonic challenge-induced conductance are completely inhibited by the stretch-activated cation channel blocker, Gd\(^{3+}\) (6). In this study, we examined the effects of antisense ODNs derived from nucleotide sequences of the \( \alpha_{1S} \), \( \alpha_{1C} \), and \( \alpha_{1D} \) on the \( G_m \) increase observed after hypotonic challenge of cells exposed to CMS. Antisense ODNs produced a time-dependent inhibition of the chlorothiazide-induced increase in intracellular calcium in mouse distal convoluted tubule cells (17). This inhibition started 6 hr after introduction of the antisense ODN and was maximal at 18 hr. The delay in inhibition was attributed to turnover of existing proteins. We observed a similar time course of inhibition of CMS-induced increase in swelling-activated cell conductance by the \( \alpha_{1C} \) antisense ODNs. Inhibition of the increase in conductance began at 12 hr, and it was maximal at 18–22 hr. \( G_m \) measurements of untreated cells, mechanically strained for 18–19 hr, were increased 71.2% \( \pm \) 2.7% \( (n = 21) \) after hypotonic swelling. Representative experiments are illustrated in (Figs. 1A and 2A).\(^1\) There was complete inhibition of the CMS-induced increase in swelling-activated conductance in cells of companion wells treated with \( \alpha_{1C} \) antisense ODN (24-mer) (Fig. 1B). Blockage of CMS-induced increase in conductance was observed in 18 separate cultures of UMR-106.01 cells. Comparable observations were made using the \( \alpha_{1C} \) 20-mer antisense ODN derived from a sequence upstream of the 24-mer ODN. The \( \alpha_{1C} \) antisense (20-mer) \( (n = 7) \) (Fig. 2B) abolished the increase in conductance produced by CMS in the stretched control cells (Fig. 2A). UMR-106.01 cells permeabilized with streptolysin O in the absence of ODN, the sham permeabilized, exhibited increased conductance in response to swelling (67.2% \( \pm \) 5.9%; \( n = 6 \) (Fig 1D) with no differences compared to cells not treated with streptolysin O. Introduction of the 24-mer \( \alpha_{1C} \) sense ODN had no significant effects on the increase in \( G_m \) resulting from hypotonic swelling (\( n = 4 \)) (Fig. 1C). In cells exposed to the \( \alpha_{1C} \) antisense ODNs, return of the swelling-activated \( G_m \) response was observed after 26 hr. Interestingly, this response was usually greater than the control response (R.L.D. and K.A.H., unpublished data), suggesting a feedback mechanism resulting in increased expression of the channel following recovery from antisense inhibition. Antisense ODNs (24-mers) to the \( \alpha_1 \) subunit of two other calcium channel genes (\( \alpha_{1S} \) and \( \alpha_{1D} \)) of the L-type found in UMR-106.01 cells were also tested. Neither \( \alpha_{1D} \) (Fig. 3A) nor \( \alpha_{1S} \) antisense ODNs (Fig. 3B) from the same IVS6 region of the \( \alpha_{1S} \) subunit altered the conductance response to hypotonic swelling in UMR-106.01 cells \( (n = 6 \) for both \( \alpha_{1S} \) and \( \alpha_{1D} \)).

The \( G_m \) increase in response to hypotonic swelling following CMS correlates with an increase in stretch-activated cation single channel activity (6) and is blocked by Gd\(^ {3+} \) (figure 2 in

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Table 1. Effects of ODNs on increase in cell conductances produced by hypotonic swelling of UMR-106.01 cells

<table>
<thead>
<tr>
<th>No ODN, no CMS</th>
<th>Peak change in conductance, nS</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ODN, CMS 18 hr</td>
<td>15.3 ( \pm ) 1.8*</td>
<td>21</td>
</tr>
<tr>
<td>Sham ODN, CMS 18 hr</td>
<td>11.0 ( \pm ) 1.7</td>
<td>6</td>
</tr>
<tr>
<td>( \alpha_{1C} ) 24-mer antisense, CMS 18 hr</td>
<td>2.8 ( \pm ) 0.7†</td>
<td>18</td>
</tr>
<tr>
<td>( \alpha_{1C} ) 24-mer sense, CMS 18 hr</td>
<td>11.6 ( \pm ) 0.6</td>
<td>4</td>
</tr>
<tr>
<td>( \alpha_{1C} ) 20-mer antisense, CMS 18 hr</td>
<td>3.7 ( \pm ) 0.9†</td>
<td>7</td>
</tr>
<tr>
<td>( \alpha_{1C} ) 20-mer sense, CMS 18 hr</td>
<td>12.3 ( \pm ) 2.8</td>
<td>6</td>
</tr>
<tr>
<td>( \alpha_{1D} ) 24-mer antisense, CMS 18 hr</td>
<td>11.7 ( \pm ) 1.1</td>
<td>6</td>
</tr>
</tbody>
</table>

Data for peak change in conductance are means \( \pm \) SEM.  
*\( P < 0.001 \) compared to no ODN, no CMS.  
†\( P < 0.001 \) compared to no ODN, CMS 18 hr. Sham ODN CMS 18 hr cells were exposed to streptolysin O but no ODN.

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\(^1\)The recently described consensus nomenclature for calcium channel subunits is used in this report (10). According to this convention, \( \alpha_{1S} \) is the same as CaCh1, \( \alpha_{1C} \) is CaCh2, and \( \alpha_{1D} \) is CaCh3 (18).
have reported (6) (first trace, isotonic). Two minutes following perfusion of hypotonic Ringer's solution (182 mOsm) into the patch chamber, swelling-activated cation channel \( N_{P_o} \) increased to a mean of 1.93 ± 0.32 (\( n = 12 \)). Following washout of the hypotonic bath with normal Na Ringer's, \( N_{P_o} \) remained high (isotonic, 2 min post). However, 10 min after washout of hypotonic Ringer's, channel activity was not different from baseline before hypotonic challenge, although a small percentage of cells still exhibited spontaneous channel activity (isotonic, 10 min post). When UMR-106.01 cells were treated with \( \alpha_{1C} \) antisense ODN, \( N_{P_o} \) of swelling-activated cation channels in response to hypotonic challenge was not significantly different (2.21 ± 0.71; \( n = 6 \)) from control cells (Fig. 4B, 182 mOsm). Washout of the hypotonic Ringer's resulted in the return of volume-activated cation channel activity to baseline over 10 min (Fig. 4B, isotonic 2 min post and 10 min post). However, Fig. 4C demonstrates that the \( \alpha_{1C} \) antisense ODNs (24-mer) eliminated the swelling-activated cation channel activity in response to hypotonicity (\( n = 6 \)) (182 mOsm). The \( \alpha_{1C} \) antisense ODN also eliminated stretch-activated cation channel activity. The results were exactly similar to Fig. 4C showing no channel activity induced by back pressure in the patch pipette (\( n = 20 \)) and are not further illustrated. \( \alpha_{1C} \) or \( \alpha_{1D} \) antisense ODNs had no effect on stretch-activated cation channel activity, and the increased stretch-activated channel activity following CMS was as reported (6).

Gadolinium also inhibited the increase in swelling-activated cation activity in response to perfusion with 182 mOsm bath similar to its inhibition of the increase in stretch-activated cation channel activity following CMS (figure 4 in ref. 6). These data suggest that the inhibition of the swelling-induced increase in \( G_m \) following CMS may have been mediated through inhibition of increased swelling-activated cation channel activity.

Characterization of the swelling-activated cation channel activity by single channel recordings revealed that these channels were insensitive to changes in \( V_m \), insensitive to nifedipine and nitrendipine and blockable by Gd\(^{3+}\). Thus, in these characteristics, they were similar to stretch-activated cation channels of UMR-106.01 cells, which have been previously characterized (4). To determine whether the swelling-induced conductance in cells subjected to CMS was due to activation of stretch-activated cation channels, we measured the conductance and ion selectivity of single channels in inside-out patches activated by hypotonic swelling. When bathed in symmetrical or asymmetrical NaCl/KCl solutions (see Materials and Methods), the swelling-activated cation channels had a conductance of 28 pS, significantly greater than the 18 pS reported for stretch-activated cation channels (4). To further clarify the similarities and differences of the swelling-activated and stretch-activated ion channels, we determined the ion selectivity ratios for Na\(^+\), K\(^+\), Cl\(^-\), and Ca\(^{2+}\). When patches were bathed in asymmetrical NaCl/KCl solutions, the reversal potential of the \( I-V \) relation was not significantly different from 0 mV, indicating that the channel was equally conductive to both Na\(^+\) and K\(^+\) (Fig. 5A). These data indicate that the swelling-activated cation channel is nonselective for Na\(^+\) and K\(^+\) as is the stretch-activated cation channel described earlier (4). When bath solutions were changed from 138 mM NaCl to 65 mM NaCl/mannitol solutions, the reversal potential of the \( I-V \) relation was shifted to the right by 9.0 ± 1.0 mV (Fig. 5A). These data indicate that the swelling-activated cation channel has a \( P_{Na}/P_{Cl} \) of 5.5:1 and has, therefore, a relatively low selectivity for cations over anions. This is in contrast to the high cation selectivity of the stretch-activated cation channel which, under similar conditions, gives a rightward shift of the \( I-V \) relation of ∼17 mV, very close to the theoretical limit for a perfectly cation-selective channel (4). To determine the permeability of the channel to Ca\(^{2+}\), we switched the bath solution to one containing 75 mM Ca\(^{2+}\) and fitted the resulting \( I-V \)

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**Fig. 2.** Effect of an antisense 20-mer ODN from the UMR-106.01 \( \alpha_{1C} \) gene on the response to hypotonic stretch. (A) UMR-106.01 cells exposed to 18 hr of chronic cyclic strain prior to hypotonic stretch. (B) As in A, except the cells were loaded with the 20-mer antisense ODN. Data are representative of several similar experiments.

**Fig. 3.** Effect of antisense ODNs derived from the I\( V \) S5-S6 regions of the \( \alpha_{1D} \) and \( \alpha_{1S} \) genes on the response of \( V_m \) to hypotonic strain. (A) UMR-106.01 cell \( V_m \) and \( G_m \) response to hypotonic stretch and reversal by return to isotonic media. Cells were loaded with the ODN to the \( \alpha_{1D} \) subunit gene and exposed to chronic cyclic strain for 18 hr. (B) As in A, except the cells had been loaded with an antisense ODN to the \( \alpha_{1S} \) subunit. Data are representative of several similar experiments.
A 18 HR STRAINED CONTROL

VC = 30mV

182 mOsm

CLOSED

- 1

- 2

- 3

ISOTONIC

2 min post

CLOSED

- 1

- 2

ISOTONIC

6 min post


B α15 TREATED 18 HR STRAINED CELLS

VC = 20mV

182 mOsm

CLOSED

- 1

- 2

- 3

ISOTONIC

2 min post

CLOSED

- 1

- 2

ISOTONIC

6 min post


C α1C TREATED 18 HR STRAINED CELLS

VC = 30mV

182 mOsm


Fig. 4. Single channel recordings of swelling-activated cation channel activity in cells exposed to chronic, cyclic strain. (A) Swelling-activated cation channel activity after 18 hr of cyclic strain. Spontaneous swelling-activated cation channel activity and a high level of channel activity upon exposure to hypotonic media (182 mOsm) was observed. Upon return to isotonic media, there was decreased channel activity by 2 min and restoration to basal levels by 10 min. (B) Cells treated with antisense to α15 and 18 hr of cyclic strain. Loading UMR-106.01 cells with the antisense ODN to the α15 subunit gene failed to affect swelling-activated cation channel activity. Exposure to hypotonic media produced a major increase in channel activity, which returned to baseline following 10 min in isotonic media. (C) α1C antisense-treated 18 hr strained cells. Failure to detect swelling-activated cation channel activity in UMR-106.01 cells loaded with the antisense ODN to the α1C subunit in response to hypotonic stimulus.

Fig. 5. Single swelling-activated cation channel current-voltage plots of excised patches bathed in asymmetrical solutions to determine channel ion selectivity. Lines represent fits of the data with the GHK current equation. (A) Data obtained with 144 mM KCl in the pipette and 138 mM NaCl in the bath solution. Reversal potential of near zero indicates a 1:1 selectivity ratio for Na⁺ and K⁺. (B) Data obtained with 144 mM KCl in the pipette and 65 mM NaCl in the bath solution. Shift of reversal potential toward positive values indicates a 2.8:1 selectivity ratio of Na⁺ to Cl⁻.

DISCUSSION

In the studies reported here, we demonstrate that the response of chronically strained cells to hypotonic challenge is blocked by antisense ODNs to the α1C subunit of the high-voltage, L-type calcium channel family (19). The α1 subunit of this family of channels contains the channel pore, the voltage sensor, and the dihydropyridine receptor (11, 20–22). These data suggest that the swelling-activated cation channel, which is voltage insensitive and dihydropyridine insensitive and Gd³⁺ blockable, similar to the stretch-activated cation channel (4–6), may either contain homology to an α1 subunit of the high-voltage L-type calcium channel family, or α1C expression
that cell-attached patches of hypotonically swollen cells also exhibit differences in conductance and cation/anion selectivity characteristics could have been produced by the two different activated cation channels. The insensitivity of the swelling-activated channel to stretch-activated and swelling-activated cation channels. There is not a uniform observation due to additional mechanisms of action besides RNAse H activity (18, 24). The amount of ODNs needed to perform Northern blot analysis for αIC in the presence of antisense was too great to be feasible because of the large amount of RNA required for Northern analysis (9), the amount of cells required for application to the strain apparatus, and the concentrations of unmodified phosphodiester ODN that we used. Therefore, we were unable to assess the effects of antisense ODN on αIC message levels. However, the controls, based on failure of sense and αIS and αID antisense to affect single channel activity and Gm, adequately demonstrate the specificity of the αIC antisense ODN in eliminating swelling-activated cation channel activity and its adaptation to CMS. The ODNs used in the above studies were all unmodified phosphodiester ODNs. Thus, the streptolysin O permeabilization may have avoided expected degradation in culture media (18, 20, 24) and facilitated the superior sequence-specific effects of phosphodiester ODNs.

The antisense ODNs used in these studies were against the region 5' of the S6 in domain IV. This region is upstream of the dihydropyridine receptor (23). Since the stretch-activated cation channel, the swelling-activated cation channel, and the strain-induced Gm increase are not inhibited by 10 μM nifedipine (6), the dihydropyridine receptor must not be expressed or is very insensitive to dihydropyridines in the isoform of the αIC, which is being acted on by the antisense ODN. Furthermore, this isoform could have key divergences from other dihydropyridine-sensitive channels since the cation channel inactivated by antisense αIC ODN is voltage independent and sensitive to Gd³⁺. These trivalent cation-sensitive and cation nonselective characteristics suggest significant differences in the gene splicing between this isoform and others of the dihydropyridine-sensitive L-type calcium channels. Clarification of these issues requires reconstitution of swelling-activated cation channel activity with expression of the full-length αIC protein. This will allow sequence structure-function analysis.

Finally, our data do not allow us to distinguish between stretch-activated and swelling-activated cation channels. There were significant similarities in the channel properties, but there were also differences in conductance and cation/anion selectivity. One possibility is that the effect of swelling on the cell (i.e., the cytoskeleton) differs from that of back pressure on the patch pipette. This is assuredly the case and, since the swelling-activated cation channels are sensitive to cytoskeletal disruption (R.L.D., unpublished observations), changes in channel characteristics could have been produced by the two different forces. The insensitivity of the swelling-activated channel to stretch may also be attributable to swelling-induced changes in the channel's relationship to the cytoskeleton. We have noted that cell-attached patches of hypotonically swollen UMR-106-01 cells subjected to CMS typically require ~60 mmHg or greater pipette suction to measurably increase NPm. This is in contrast to the stretch-activated cation channel, which shows maximal increase in NPm at ~30 mmHg (4-6).

In summary, we have shown that antisense ODNs directed against the αIC subunit of the L-type calcium channel family specifically inhibit a swelling-activated, Gd³⁺-sensitive cell conductance and both swelling-activated and stretch-activated, nonselective cation channels in cells subjected to CMS. While we cannot determine from our data that the swelling-activated and stretch-activated cation channels are subunits of the L-type calcium channel family, it is clear that the αIC gene product is critical to the transduction of mechanical strain into a process that regulates transmembrane cation permeability.

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CELLULAR AND MOLECULAR BIOLOGY OF BONE

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SIGNAL TRANSDUCTION IN
OSTEOBLASTS AND
OSTEOCLASTS
KEITH A. HRUSKA, FELICE ROLNICK,
RANDALL L. DUNCAN, MEETHA MEDHORA,
and KENSUKE YAMAKAWA

I. Introduction
II. Substances with Effects in Bone Cells
A. Hormones
B. Cytokines
C. Growth Factors
D. Paracrine/Autocrine Factors
E. Cell Adhesion Molecules

III. Mechanisms of Signal Generation
A. Receptors
B. Signal-Generating Complexes
C. Effector Elements
D. Signals

IV. Specific Examples of Signal Transduction in Osteoblasts: Parathyroid
Hormone/Parathyroid Hormone-Related Peptides
A. The Parathyroid Hormone/Parathyroid Hormone-Related
Peptide Receptor
B. Parathyroid Hormone/Parathyroid Hormone-Related Peptide Signal-
Generating Complexes
C. Effectors
D. Signals
E. Biological Effects

Cellular and Molecular Biology of Bone
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V. Specific Examples of Signal Transduction in Osteoclasts
   A. Role of Specific Paracrine Substances
   B. Hydrogen Ion
   C. Calcium
   D. Osteopontin/α,β Integrin Signaling
   E. Calcitonin

References

I. INTRODUCTION
Signal transduction in the major bone cells, osteoblasts and osteoclasts, is a broad topic. Osteoblasts are somewhat mysterious cells in that their ontogeny and differentiation are still being described. For purposes of this chapter, an osteoblast will refer to cells that can be isolated from tissue sources that express a specific phenotype. This phenotype is the early expression of type 1 collagen production and alkaline phosphatase followed by secretion of specific noncollagenous bone matrix proteins such as osteocalcin, osteonectin, osteopontin, and others. Responsiveness to parathyroid hormone and the ability of the cells to calcify the extracellular matrix, which may be stimulated by ascorbic acid and β-glycerol phosphate, are additional properties of the osteoblast phenotype. Surprisingly, the identification of these cells in vivo, and their relationship to such cells as the lining cells, the osteocytes, and precursor cells off of the bone surface, has not been clearly established. Osteoblasts have proven to be extremely pleiotropic cells possibly related to their ontogeny. Because they share common ancestry with adipose tissue, muscle cells, and fibroblasts, it is not be surprising that they exhibit many responses of these cells to specific substances. As a result, the topic of signal transduction in the osteoblast is a vast one. The list of substances that activate the cells and the mechanisms by which activation produces specific biological effects are exceedingly complex and incompletely described. In this chapter, the general mechanisms of signal transduction are discussed along with the general list of specific substances and their mechanisms of action. A specific example of cell activation and the pathways of signal transduction from parathyroid hormone are discussed for osteoblasts.

Osteoclasts are also enigmatic cells. Their ontogeny has recently been somewhat elucidated. One of the fascinating features of osteoclast development is the loss of many receptors that are expressed in progenitor cells and that are present in osteoblasts. Several substances that regulate bone resorption do so despite the absence of receptors for these substances on the differentiated osteoclast. Thus, in the osteoclast, signal transduction by paracrine substances and cell-to-cell communication are important mechanisms of regulating cell function. In addition, the osteoclast has several unique mechanisms of signal transduction, which are described in this chapter. Also, a novel mechanism of signal trans-
13 Signal Transduction in Osteoblasts and Osteoclasts

For purposes of isolation, these cells are in that their phenotype is the alkaline phosphatase matrix proteins other. Responsive cells to calcify the y ascorbic acid and ilacitropic hormones and their effects on bone remodeling play a central role in skeletal homeostasis. Although this basic tenant still has substance, our current understanding of bone physiology is much more complex. Nevertheless, systemic hormones play key roles in skeletal remodeling.

II. SUBSTANCES WITH EFFECTS IN BONE CELLS

Because of the diverse nature of bone cell ontogeny, the list of substances that affects osteoblasts and osteoclasts is prodigious. The list may be too large to enumerate usefully in a chapter such as this. The list in Table I is by no means inclusive, but it sets the stage for description of the mechanisms of signal generation used by several classes of substances. Classical bone physiology has considered the actions of calcitropic hormones and their effects on bone remodeling to play a central role in skeletal homeostasis. Although this basic tenant still has substance, our current understanding of bone physiology is much more complex. Nevertheless, systemic hormones play key roles in skeletal remodeling.

A. Hormones

The hormones that affect skeletal remodeling can be divided into two general groups: the peptide hormone class and the steroid hormone class.

1. Peptide Hormones

The major peptide hormones that effect osteoblast skeletal remodeling are parathyroid hormone (PTH), calcitonin, calcitonin gene-related peptide, and growth hormone. Other circulating peptides such as thrombin that are not considered to be calcitropic hormones also have dramatic effects on osteoblast function in vitro. Peptide hormones generally activate their target cells through binding to surface receptor protein with membrane-spanning domains. The receptors, in turn, couple to intracellular effectors through their cytoplasmic domains, which generally interact through guanosine triphosphate (GTP)-binding proteins. The effectors include adenylate cyclase, phospholipases, and ion channels.

2. Steroid Hormones

The list of steroid hormones with major actions on skeletal remodeling is large. Generally, the mechanism of steroid hormone action is thought to occur through receptors that are transiently in the cytoplasm but that have the capability of translocating the steroid–receptor complex into the nucleus. Here, the receptor complex binds to DNA along with accessory proteins serving as regulatory factors in gene transcription. Recent
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state with effector molecules that, when activated by the ligand-
receptor complex, are capable of generating cell signals.

A. Receptors

The receptors for the substances listed in Table I fall into several types. There are three known classes of cell surface receptor proteins: G protein-linked, catalytic, and channel-linked (Berridge, 1985; Kahn, 1976; Levitski, 1984; Rees et al., 1982; Snyder, 1985). Cell surface receptor proteins are defined by the signal transduction mechanism used. The GTP-
bounding protein-linked receptors indirectly activate or inactivate a separate plasma membrane-bound enzyme or ion channel. The interaction between the receptor and the enzyme or ion channel is mediated by a third protein, a GTP-binding regulatory protein (or G protein). The G protein-linked receptors usually activate a chain of events that alters the concentration of one or more small intracellular signaling molecules, often referred to as intracellular messengers. These intracellular messengers act, in turn, to alter the behavior of yet other target proteins in the cell. The G protein-linked receptors fit into several classes. They are generally proteins with seven membrane-spanning domains with large cytoplasmic loops and carboxyl-terminal tails. The β-adrenergic receptor is perhaps the best described (Benovic et al., 1990). Recently, two receptors for calcitropic hormones have been cloned that appear to form a new subfamily of the class of seven membrane-spanning domain receptors. These receptors are the PTH receptor and the calcitonin receptor (Juppner et al., 1991; Lin et al., 1991).

B. Signal-Generating Complexes

Association of ligands with their receptors generates complexes capable of producing cell signals. The signal-generating complex may consist only of the receptor and the bound ligand in the case of steroid hormones. These ligand–receptor complexes are then capable of translocation into the nucleus, where they bind to DNA along with an accessory protein forming dimers that activate DNA transcription. The translocation of the receptor–ligand complex is affected by phosphorylation, which in some instances results in activation of the hormone receptor complex (Orti et al., 1992). For other ligands that bind to receptors with seven transmembrane-spanning domains, which classically associate with G proteins, the signal-generating complex is much more complicated. In this instance, a hormone, receptor, G protein, and effector element together form the signal-generating complex (Fig. 1). Changes in the receptor associated with ligand binding increases the association of the receptor with the trimeric forms of G protein. The α subunit of the
FIGURE 1 Ligand (L) binding to receptors (R) leads to generation of multi-unit complexes referred to here as signal generating complexes. Several examples are diagrammatically described. (A) Hormones that are ligands for receptors with seven membrane spanning domains (1) classically associate with trimeric GTP-binding proteins, (2) associated with numerous effector elements, and (3) forming a signal generating complex producing multiple second messengers (as shown in the figure). Second messengers activate a series of enzymes and release of calcium from intracellular stores as diagrammed (4). The kinases and calcium produce direct biological effects and/or participate in phosphorylation cascades leading to biological effects. (B) Growth factor receptors (R) are usually single transmembrane spanning proteins with intrinsic protein tyrosine kinase activity. The receptors contain src-2 (SH2) and src-3 (SH3) domains, which produce association with other signal generating enzymes including the src family of tyrosine kinases (E), PI-3 kinase, PLCγ, and ras-GAP. These enzymes associate with the tyrosine phosphorylated receptor through the
G proteins then is stimulated to bind GTP and associate and activate various effectors. In some instances, the effector may represent an ion channel that directly couples to α subunits of trimeric G proteins (Fig. 1). A third general type of signal-generating complex is represented by a class of growth factor receptors that are tyrosine kinases. Upon ligand association, these receptors activate the receptor tyrosine kinase and become autophosphorylated on src homology 2 (SH2) domains. The phosphorylation of the SH2 domains then produces association of multiple effectors with the activated receptor (Fig. 1).

C. Effector Elements

Several effector elements with prominent actions in bone cells are listed in Table II. There are two general categories for discussion. The first is the G protein-linked effectors, regulated by a heterotrimeric class of GTP-binding proteins; the second are the effectors, regulated through tyrosine phosphorylation.

1. G Protein-Linked Effectors

a. Adenylate cyclase

The plasma membrane-bound enzyme, adenylate cyclase, when activated produces the ubiquitous intracellular messenger cyclic adenosine monophosphate (cAMP). Cyclic AMP is rapidly and continuously synthesized and destroyed. Destruction occurs by one or more cAMP phosphodiesterases, which hydrolyze the natriuretic peptide (ANP) to AMP. Receptor proteins, which activate adenylate cyclase, usually do so by a stimulatory G protein (G_s) (Gautier et al., 1989). Individuals who are genetically deficient in G_s have decreased responses to many hormones, and this includes the action of PTH. Reconstitution of cAMP production by insertion of epinephrine receptors, G_s, and adenylate cyclase molecules into phospholipid vesicles indicates that no other proteins are required for activation of the adenylate cyclase effector. The adenylate cyclase molecule is activated by the receptor hormone complex through binding of GTP to G_s. G_s keeps the adenylate cyclase active as long as...
TABLE II  Effectors

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<td>Guanylate cyclase</td>
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GTP is intact. Hydrolysis of GTP to guanosine diphosphate by the $G_\alpha$ protein terminates activation of the cyclase. The adenylate cyclase effector protein can also be inhibited by coupling to ligand-occupied inhibitory receptors and inhibitory G proteins. The $G_\alpha$-adrenergic receptors coupled to the inhibitory G protein ($G_i$) inhibit adenylate cyclase activity. $G_i$ $\alpha$ and the $\beta\gamma$ subunits are believed to contribute to the inhibition of adenylate cyclase activity.

Bacterial toxins have been shown to have specific actions on G proteins that have assisted greatly in the elucidation of their biological roles. Cholera toxin is an enzyme that catalyzes the transfer of adenosine diphosphate ribose from intracellular nicotinamide adenine dinucleotide to the $G_\alpha$ subunit $G_\alpha$. The ribosylation alters $G_\alpha$ so that it can no longer hydrolyze bound GTP. This produces an indefinitely active state of the G protein and results in prolonged elevations in cAMP levels. Pertussis toxin, made by the bacterium that causes whooping cough, produces the same effect by ADP ribosylating $G_i$. In this case, however, the $G_i$ complex is prevented from interacting with receptors and therefore fails to inhibit adenylate cyclase in response to receptor activation.

b. Guanylate cyclase

The discovery that atrial natriuretic peptide (ANP) activates particulate guanylate cyclase leading to the production of the second-messenger cyclic guanosine monophosphate (cGMP) has renewed interest in guanylate cyclase in recent years. Besides atrial natriuretic peptide, brain natriuretic peptide, *Escherichia coli* toxin, and nitrous oxide serve to increase cGMP levels in target tissues. In bone cells, nitrous oxide has been shown to have a major inhibitory action on osteoclast function (MacIntyre et al., 1991). This action was reported to be independent of guanylate cyclase activity, although confirmation of the latter point is required.

In target tissues of peptide substances capable of activating guanylate cyclase, the functions correlate with the distribution of particulate
guanylate cyclase rather than that of the soluble form of the enzyme. Recently, the isolation sequencing and expression of a complete complementary DNA (cDNA) clone coding for the membrane guanylate cyclase of rat brain clearly showed that an ANP receptor domain is present in the enzyme. The ANP receptor–guanylate cyclase molecule is a transmembrane protein that contains an extracellular ANP-binding domain and an intracellular guanylate cyclase catalytic domain (Chnkers et al., 1989). Another type of guanylate cyclase receptor cloned recently appears to be more specific for brain natriuretic peptide than ANP. However, because of the high concentrations needed to stimulate guanylate cyclase activity, it is possible that other natural endogenous ligands of this receptor exist. Radiation inactivation studies have indicated that particulate guanylyl cyclase is a multidomain protein with separate domains for ANP binding and cGMP synthesizing activity. There is an additional functional domain on particulate guanylate cyclase with high homology to protein kinases. This domain appears to function as regulatory element of the enzyme (Potier et al., 1991).

The soluble form of guanylate cyclase has been reported to exist as a heterodimer; it appears to contain heme as a prosthetic group, and it is activated by nitroprusside, nitric oxide, and reactive free radicals. Soluble guanylate cyclase is a heterodimer of 82- and 70-kDa proteins (Waldman et al., 1991), and its activation by endothelium-derived vaso-dilators suggest that it may have a role in the angiogenesis associated with bone modeling and remodeling.

c. Phospholipases

Phospholipases are a family of enzymes responsible for phospholipid hydrolysis. They are designated by letter, depending wherein the phospholipid molecule hydrolytic cleavage is stimulated by the enzyme. Phospholipase A₂ is responsible for removing the fatty acid from the second position of the glycerol backbone of the target phospholipid. This phospholipid is usually arachidonic acid. Phospholipase A₂ is a major source of arachidonate release leading to eicosanoid production. In contrast, phospholipase C acts to cleave at the phosphoric acid residue coupling the glycerol backbone to the polar head group of phospholipids. A specific group of phospholipase C enzymes, phosphatidylinositol-specific phospholipase C, is responsible for hydrolysis of phosphoinositides into diacylglycerol and inositol phosphates. Phosphatidylinositol-specific phospholipase C are enzymes that have been identified to couple with transmembrane-spanning receptors and G proteins. Thus, they are activated by a host of signal-transducing molecules active in bone cells. Phospholipase D is an enzyme that cleaves at the head group of phospholipids producing phosphatidic acid.
and the free polar head group. Phosphatidic acid is an important signal molecule. In addition, glycosyl phosphatidylinositol (GPI)-specific phospholipase D degrades the GPI anchor of alkaline phosphatase. This anchor-degrading activity is abundant in mammalian plasma and serum. Although the physiologic function of this enzyme remains to be determined, it is proposed to play a role in the regulation of cell surface expression of GPI-anchor proteins.

d. Ion channels

Because certain ion channels couple directly to receptor proteins, they must be considered as effector elements of activated membrane receptors. In addition, G proteins may directly activate ion channels, indicating that the latter are G-protein effectors (Brown 1991). The first pathway for which a membrane-delimited G-protein activation was deduced was that involving the muscarinic $M_2$ atrial receptor, the G protein called $G_{M_2}$, and the specific atrial potassium channel gated by this protein (Brown and Birbaumer, 1990).

2. Tyrosine Kinase-Linked Effectors

In recent years, several steps involved in signal transduction pathways mediated by receptors with intrinsic tyrosine kinase activity have been elucidated. Early responses to ligand occupancy of these receptors include the clustering and internalization of the receptors, activation of the intrinsic tyrosine kinase activity, autophosphorylation of the cytoplasmic domain of the receptor, phosphorylation of exogenous substrates on tyrosine residues, generation of ion fluxes, stimulation of phosphoinositide turnover, and induction of the protooncogenes c-myc and c-fos (Bjorge et al., 1990). The induction of phosphatidylinositol turnover is produced by the association of an isoform of phospholipase C, phospholipase $C_{M_2}$, to the receptor through its homology two domains, SH2. Likewise, phosphatidylinositol is phosphorylated in the 3 position by the association of phosphatidylinositol 3 (OH) kinases with receptors through its SH2 domain. Also stimulated to associate to the receptor through its SH2 domain is a GTP-activating protein, which binds to the ras oncogene product forming the ras–gap complex. In this setting, the small molecular weight G-protein ras is provided with guanosine triphosphate hydrolytic capabilities and is thus activated.

There exists an additional mechanism activating enzymes with SH2 domains when the receptor protein is not an intrinsic tyrosine kinase. This is the association of a large family of cytosolic tyrosine kinases, which are myristilated and associated with the inner leaflet of the plasma membrane, with ligand–receptor complexes. This family of tyrosine
an important signal (GPI)-specific phosphatase. This receptor proteins, activated membrane ion plasma and se- nzyme remains to be the tivation of cell surface iation of receptor proteins, niemembrane active ion channels, awn 1991). The first activation was de-receptor, the G pro-annel gated by this

kinesases, the src family, possesses SH2 domains and is capable of forming signal generating complexes similar to the epidermal growth factor (EGF) and plate-derived growth factor (PDGF) receptors, which are intrinsic tyrosine kinases. This mechanism of activation also appears to operate for the family of tyrosine phosphatases, the prototype of which is CD45, the leukocyte common antigen.

a. Phospholipase Ca

Cloning of the various isoforms of phospholipase C has revealed that three members of the family have two conserved domains considered to be catalytic domains for phospholipase C activity in common. Phospholipase Ca has a totally different amino acid sequence showing similarity to the dioxin of E. coli. Phospholipase Cγ contains homologous regions related to the NH2 terminal regulatory domains of oncogenes of the src family. Two isoforms of phospholipase Cγ, PLCγ1 and PLCγ2, have been cloned (Ryu et al., 1987; Takenawa and Nagai, 1981). The distribution of phospholipase C isoforms in bone are unknown, but both forms of phospholipase Cγ are ubiquitously expressed.

b. Phosphatidylinositol 3-OH-kinase

A new category of phosphoinositides phosphorylated at the 3 position of the inositol ring have recently stimulated significant interest. Phosphoinositol 3-OH-kinase (type 1) is associated with ligand occupied PDGF and EGF receptors (Zhang et al., 1992; Auger et al., 1989; Bjorge et al., 1990; Whitman et al., 1988; Stephens et al., 1989). Mutant PDGF receptors competent to activate PLCγ, but unable to bind and activate phosphatidylinositol 3-OH-kinase, do not exert mitogenic effects in fibroblasts (Coughlin et al., 1989). This implies an important signaling function for 3-phosphorylated phosphoinositides. Phosphoinositol 3-OH-kinase from several organs has an apparent size of 190 kDa, determined by gel filtration, and is a heterodimer consisting of 85- and 110-kDa subunits (Carpenter et al., 1990; Shibasaki et al., 1991; Morgan et al., 1990). The β85 subunit is phosphorylated on serine, threonine, and tyrosine after stimulation (Kaplan et al., 1987; Escobedo et al., 1991; Courtneidge and Heber, 1987). It contains one SH3 and two SH2 regions homologous to the nonkinase regions of PP60 c-src (Otsu et al., 1991) which appear to mediate the specific association of the phosphoinositol 3-OH-kinase with tyrosine protein kinases of both receptor and nonreceptor classes. In contrast, the P110 protein is considered to be the catalytic component of the 3-kinase (Otsu et al., 1991). We have recently demonstrated activation of P13 kinase by matrix proteins, and this enzyme appears to be an important regulator of osteoclast function.
c. ras-guanosine triphosphatase-activating protein

The ras protein is a GTP-binding protein that acts as a transducer mediating the signals of growth or differentiation in many types of cells (Barbacid, 1987; Kaziro et al., 1991). In fibroblasts, accumulation of active ras-GTP complexes was observed in response to EGF or PDGF (Satoh et al., 1990a,b). ras also accumulates in response to other ligands that bind to tyrosine kinase receptors. The oncogene products of the src family, which are tyrosine kinases, also induce the increase of ras-GTP (Satoh et al., 1990b; Gibbs et al., 1990). GTPase-activating protein (GAP) is rapidly phosphorylated on tyrosine residues when cells are stimulated by EGF, PDGF, or oncogenes encoding tyrosine kinases (Morla et al., 1988; Ellis et al., 1990). Tyrosine phosphorylation of GAP reduces GTPase-stimulating activity and causes the accumulation of active ras-GTP. GAP forms complexes in a ligand-dependent manner with EGF and PDGF receptors, which include phosphatidylinositol-OH-3 kinase, phospholipase Cy, and src family tyrosine kinases. This complex triggers signal-transducing events (Ullrich and Schlessinger, 1990). In the case of interleukin 3 and GM-CSF, two receptors that are important in bone and activate ras-GAP complexes and ras-GTP levels, the receptors are not tyrosine kinases, and the tyrosine kinase associated with these receptors has not been described. (Satoh et al., 1992).

d. src family of tyrosine kinases

The src family of non-receptor cytosolic protein tyrosine kinases includes eight closely related representatives whose proteins, when tested, are localized to the inner face of the cell membrane by amino-terminal myristylation (Cooper, 1990). One isoform of p59-src, found primarily in lymphocytes, has been shown to regulate T-cell receptor signaling (Cooke et al., 1991). The src family of tyrosine kinases is extremely important in bone cell physiology. Recent knock-out experiments using an anti-sense strategy have demonstrated that transgenic mice devoid of src develop a metabolic bone disease similar to osteopetrosis (Soriano et al., 1991). The nonreceptor protein tyrosine kinases are capable of associating with multiple receptors following ligand occupancy (Eiseman and Bolten, 1992). This process thus enables multiple receptors that are not intrinsic tyrosine kinases to activate signaling complexes associated with activation of the src family and production of the effector complexes through src homology-binding domains (SH2 and SH3 domains).

e. Tyrosine phosphatases

Protein tyrosine phosphatases have an increasingly appreciated and important role in signal transduction. A prototype for a transmembrane
protein tyrosine phosphatase is CD45. CD45 is a structurally heterogeneous family of isoforms distributed in cells of the hematopoietic system. The structure of CD45 indicates that it has a single transmembrane-spanning protein with an extracellular NH2-terminal domain rich in O-linked sugars. It has a large, highly conserved, cytoplasmic domain that possesses protein tyrosine phosphatase activity. Thus, CD45 is a prototype of a novel class of receptors that play an active role in the regulation of cell growth. The ligand for CD45 has not been discovered yet. However, in lymphocytes, the adhesion molecule, CD22, interacts with T cells by binding to the smallest isoform of CD45. The protein phosphatase family is a large group of proteins that include transmembrane proteins of the single transmembrane-spanning type and cytosolic proteins with carboxyl-terminal regions that are important in determining their intracellular localization in regulation of their enzymic activity. The intracellular protein tyrosine phosphatases are associated with the particulate fraction of cell homogenates. They have hydrophobic carboxyl termini that may serve as membrane anchors. One intracellular protein tyrosine phosphatase, PTP1C, is characterized by the presence of SH2 domains. Another, MPH1, is characterized by a talin-related domain suggesting that it may play an important role in focal adhesions and regulation of the actin cytoskeleton. Tyrosine phosphatases are important in the regulation of the cell cycle and cell transformation. They do not block function simply by dephosphorylation of proteins. They can synergize with kinases to produce specific functions. CD45 specifically activates the src family of kinases through dephosphorylation of the tyrosine residue in their regulatory domain. The protein tyrosine phosphatase PTPH1 exhibits homology to erzin and is associated with the cytoskeleton.

D. Signals

A list of the substances produced by the process of signal-generating complexes activating effector molecules is provided in Table III. Many of these intracellular signal substances are known to have major effects in the process of bone remodeling. Others have been shown to play signifi-

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cant roles in isolated cells, especially osteosarcoma cells, but their action remains to be determined in vivo. Also, many of these signals participate in cell function through a complex array and cascade of events. This clouds our ability to interpret the biologic role of these signals. Thus, in this chapter an attempt will not be made to describe the biologic effects of each of these signals, because much is still required before such a task could be successfully performed. Rather, specific examples of signal transduction in bone cells will be provided, and the roles of individual signals will be discussed in this context.

IV. SPECIFIC EXAMPLES OF SIGNAL TRANSDUCTION IN OSTEOBLASTS: PARATHYROID HORMONE/PARATHYROID HORMONE-RELATED PEPTIDES

PTH regulates calcium and phosphorous homeostasis by binding to specific G protein-coupled receptors in bone and kidney (Rosenblatt et al., 1989). Parathyroid hormone-related peptide (PTHrP) which shares 8 of the 13 amino-terminal residues of PTH, binds to the same 80-kDa receptor glycoprotein (Orloff et al., 1989; Jüppner et al., 1988; Shigeno et al., 1988; Karpf et al., 1987, 1991). An important issue yet to be resolved is the mechanism by which non-homologous domains of PTH and PTHrP activate the same receptor or whether additional specific receptors will be discovered.

A. The Parathyroid Hormone/Parathyroid Hormone-Related Peptide Receptor

The receptor for PTH/PTHrP has recently been cloned (Jüppner et al., 1991) from a cDNA library prepared from opposum kidney cells using an expression cloning strategy. The cloned PTH receptor bound PTH 1-34 and PTHrP 1-36 equivalently. Nucleotide sequencing revealed an open reading frame encoding a 585-amino acid protein that showed no similar sequences in nucleic acid or protein databases. The receptor protein is predicted to have seven membrane-spanning domains similar to other G protein-coupled receptors (Fig. 1). The subsequent cloning of the calcitonin receptor (Lin et al., 1991) and the secretin receptor indicates conservation of glycosalation sites, and extracellular cysteines, suggesting that these receptors form a subfamily of receptors sharing functional features and distinguishing them from the other G protein-linked receptors.

B. Parathyroid Hormone/Parathyroid Hormone-Related Peptide Signal-Generating Complexes

The cloning of the PTH receptor should clarify the nature of the signal-generating complex associated with the PTH receptor. At the present
cells, but their action on these signals participate in a cascade of events. This is an example of signal integration. Thus, in the biologic effects of PTH and PTHrP, the roles of individual genes are of a composite nature.

**Introduction in Parathyroid**

Parathyroid hormone (PTH) is by binding to specific receptors (Rosenblatt et al., 1987) which share 8 of the same 80-kDa receptors (Shigeno et al., 1988; Shigeno et al., 1988; Sheff et al., 1990). Yet to be resolved is the question of PTH and PTHrP specific receptors with regard to their roles in PTH signal transduction.

**G-protein related**

The parathyroid hormone receptor couples through a G-protein, probably Gs, to phospholipase C. However, the nature of the G-protein responsible for parathyroid activation of phospholipase C activity remains to be determined. Studies also suggest that the small molecular weight GTP-binding protein rho may be associated with PTH function (Reshkin and Murer, 1992). Whether or not this protein associates with the signal-generating complex remains to be determined. Thus, as suggested in Fig. 1 (top), one possibility regarding the diversity of signals generated through a single receptor for both PTH and PTHrP is variable activation of multiple GTP binding proteins.

**C. Effectors**

The effector elements associated with the signal-generating complex of the PTH receptor include adenylate cyclase, phospholipase C, and ion channels. There exists additional data that suggests phospholipase A2 and D are also activated by PTH. PTH receptor coupling through the Gs and activation of adenylate cyclase is a classic pathway of PTH-based signal transduction. However, it is clear that in osteoblastic PTH also activates phospholipase C (Civitelli et al., 1988; Abou-Samra et al., 1989; Farnandale et al., 1988; Suzuki et al., 1989).

We have recently shown that PTH modulates the action of stretch-activated cation channels in the osteoblastic osteogenic sarcoma cell line UMR 106 (Duncan et al., 1992), an effect independent of cAMP generation. The mechanism of this ion channel activation remains to be determined. However, it is unlikely that this channel associates directly with the receptor, and, thus, it must be excluded as a potential effector component of the PTH signal-generating complex. Other ion channels have also been shown to be activated by PTH (Chesnoy-Marchais, 1989; Edelman et al., 1986; Ferrier and Ward, 1986; Ferrier et al., 1988). The PTH effects on these ion channels appear to be mediated through the actions of cAMP and calcium. However, neither cAMP nor calcium mimic the actions of PTH on the stretch-activated cation channel of the UMR 106 cell (Duncan et al., 1992).

**D. Signals**

The signals associated with the PTH receptor include cAMP, calcium (Reid et al., 1987; van Leeuwen et al., 1988; Donahue et al., 1988;
Cyclic AMP is the most important signal generated by the PTH signal-generating complex. By stimulating protein kinase A-mediated protein phosphorylation, it directly regulates numerous protein functions in its target cells. However, although detection of cAMP by sensitive radioimmunoassay methods has been accomplished, a discrepancy remains between the physiologic levels of circulating PTH and the ability to determine cAMP production. PTH circulates at the $10^{-11}$-$10^{-12}$M levels, whereas stimulation of cAMP generation, at best, can be accomplished at $10^{-10}$M doses. Utilizing the effect of cAMP to dissociate the regulatory subunit from protein kinase A, one can measure the saturation of protein kinase a catalytic activity with the regulatory subunit. This indirect measure of cAMP generation is more sensitive than cAMP assays and affords greater correlation between protein kinase A activity and the biological effects of PTH. However, many of the biologic effects of PTHs have not been carefully correlated with activation of protein kinase A, and, thus, doubt remains regarding the role of cAMP in some of PTH biologic effects. Cyclic AMP besides stimulating protein kinase A also serves as a gene transcription factor through the cAMP response element and cAMP response element-binding proteins (Habener, 1990). Many of the long-term actions of the PTH are regulated through cAMP-dependent regulation of gene transcription.

Observations from the laboratory of Herrmann-Erlée et al. (1983) demonstrated a failure to correlate PTH-stimulated bone resorption and cAMP production. These studies were supported by the further observation that PTH fragments, shortened at the amino-terminus and unable to stimulate cAMP production, were still capable of stimulating bone resorption. Subsequently, studies from multiple laboratories, including our own, have indicated direct effects of the PTH signal-generating complex on increasing cytosolic calcium. The mechanisms by which PTH increases calcium fluxes in target cells have only been partially elucidated. First, the activation of phospholipase C produces a release of calcium from intracellular stores through the actions of inositol 1,4,5-trisphosphate, serving to increase open time of calcium channels in the endoplasmic reticulum or closely associated organelles (Hruska et al., 1987; Reid et al., 1988). In addition, PTH stimulates calcium entry through calcium channels of the plasma membrane. These calcium channels appear to be of two types: voltage-operated calcium channels of the L type and receptor-operated calcium channels (Bidwell et al., 1991; Yamaguchi et al., 1987; Reid et al., 1988). In PTH target cells which do not exhibit voltage-operated calcium channels, PTH stimulates a calcium entry through a putative receptor-operated calcium channel, which
hate (Civitelli et al., 1988; Aboul-Enein et al., 1988; Reil et al., 1988; Yamaguchi et al., 1987; Reid et al., 1988). The direct action of an increase in cytosolic calcium on osteoblast function remains to be clearly elucidated. Many of the effects of cAMP appear to be enhanced by the change in cytosolic calcium, possibly through an amplification of calcium calmodulin-dependent kinase activities.

PTH and PTHrP increase inositol trisphosphate production upon binding to the PTH receptor of osteoblasts (Civitelli et al., 1989; Farndale et al., 1988). The increase in inositol trisphosphate appears to occur through activation of phospholipase C. However, the isform of phospholipase C affected by PTH has not been determined, nor has the mechanism of phospholipase C activation clearly been determined. One possibility is that the PTH signal-generating complex includes association with G, and a phospholipase C isoform; however, this remains to be determined. Another possibility would be that the direct actions of PTH on ion channels could produce an activation of phospholipase C through changes in either sodium concentration or calcium concentration. This would explain recently described differences between thrombin, a substance known to activate a plasma membrane phospholipase C, and PTH in osteoblast-like cells. The biologic effects of inositol trisphosphate, besides contributing to the changes in cytosolic calcium stimulated by PTH, are unclear.

Associated with the stimulation of phospholipase C activity by PTH, diacylglycerol has also been shown to be produced, leading to protein kinase C translocation to the plasma membrane (Abou-Samra, 1989). The activation of protein kinase C activity by a PTH suggests a multitude of actions that have largely yet to be clearly demonstrated for the hormone. This continues to be a puzzling issue related to PTH-based signal transduction. One area of special concern is whether or not at some stage in osteoblast development PTH is a growth factor through its actions on phospholipase C.

E. Biological Effects

The biologic effects of PTH and PTHrP on the osteoblast are prodigious. They include many actions that have as yet to be described. This topic is beyond the scope of this chapter. One interesting aspect of the topic of biologic effects is the mechanism of action of the nonamino terminal regions to these molecules, which have recently been shown to stimulate placental Ca++ transport and inhibits osteoblast function (Fenton et al., 1991; Care et al., 1990).
The cellular basis of bone remodeling is not completely understood. The osteoclast, the multinucleated cell involved in bone resorption, is a complex unit that develops a specialized apparatus for dissolving the bone matrix (King and Holtrop, 1975; Holtrop and King, 1977). Using cell culture systems, several advances have recently been made indicating the molecular events involved in osteoclast bone resorbing activity. For bone resorption to be initiated, the osteoclast polarizes (Baron et al., 1985) and directly attaches to the bone surface by a specialized area termed the clear zone (Holtrop and King, 1977), in which the contact with the substrate is established by specific adhesion structures called podosomes (Marchisio et al., 1984, 1987; Zambonin-Zallone et al., 1988). Morphologically, podosomes appear as short membrane protrusions with a core of microfilaments linked to the plasma membrane by talin and vinculin (Marchisio et al., 1984, 1987). Recent data suggest that podosomes play a pivotal role in substrate recognition by osteoclasts because a specific β₃ integrin of the RGD superfamily of matrix receptors is expressed on their cell membrane surface (Davies et al., 1989; Zambonin-Zallone et al., 1989). Substrate recognition is a necessary early step in the initiation of bone resorption, and it may induce phenotypic differences in cellular responses as the osteoclast changes from a motile cell seeking bone substrate to an actively resorbing cell.

The organization of the podosome-containing clear zone allows tight sealing of the resorbing compartment between the osteoclast plasma membrane and the bone surface. The acidification of this extracellular microenvironment (Baron et al., 1985; Blair et al., 1989) produces hydroxyapatite solubilization. Lysosomal enzymes, secreted into this space by a mannose 6-phosphate receptor-driven mechanism (Baron et al., 1988; Blair et al., 1988) and activated by the acid pH, digest the organic components of the bone matrix (Blair et al., 1986). Tight sealing of the compartment is needed to maintain the pH of 5 and the Ca²⁺ concentrations of up to 40 mM (Silver et al., 1988).

While the mechanisms of osteoclast regulation are incompletely understood, it is clear that the osteoclast is a unique cell in that its plasma membrane is devoid of many receptors that activate osteoblast and regulate bone remodeling. For example, PTH, interleukin 1, prostaglandin E₂, 1,25(OH)₂D₃, and several other hormones known to stimulate bone resorption do so despite the absence of receptors in the osteoclast. Thus, it appears that paracrine factors will have a special importance on the regulation of osteoclast function.

A. Role of Specific Paracrine Substances
An example of a paracrine substance stimulated by a systemic hormone known to function in stimulation of bone resorption is the release of
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GM-CSF from murine osteoblasts by PTH (Horowitz et al., 1989). GM-
CSF does not directly induce bone resorption (Lorenzo et al., 1988) but, rather, induces the increased formation of osteoclasts from marrow precursors. This suggests that these cytokines augment resorption by increasing the number of osteoclasts available for activation. The exact role of this action of PTH to increase osteoblast GM-CSF production in the bone resorption stimulated by PTH remains to be determined, but this is an important example of potential mechanisms of indirect osteoclast regulation functioning in bone remodeling.

Another paracrine substance stimulated by PTH has recently been shown to directly activate osteoclasts function is interleukin-6 (Girasole et al., 1989). This important observation indicates that one factor released by osteoblast and other cells in the bone micro-environment, IL-6, can account for some of the paracrine stimulation of osteoclast function induced by PTH and other factors, especially interleukin-1. Whether the combined actions of GM-CSF and interleukin-6 account for the osteoclast stimulation induced by PTH and other factors remains to be determined.

B. Hydrogen Ion

Because metabolic acidosis is known to stimulate bone resorption in vitro and in vivo, we have analyzed the effects of extracellular protons on the regulation of osteoclast function. We have found that exposure of the osteoclast to metabolic acids produces a fall in intracellular pH and cytosolic calcium. The reductions in both of these ions participates in rearrangement of the microfilament cytoskeleton with a rapid increase in the expression of podosomes (Fig. 2). The increase in podosome formation is followed shortly by a very significant stimulation in bone resorption (Teti et al., 1989). The mechanism of the reduction in cytosolic calcium appeared to be an activation of the plasma membrane residing Ca$^{2+}$-ATPase. Because the Ca$^{2+}$-ATPase is electrogenic and functions as a Ca$^{2+}$/H$^+$ exchanger, it is possible that the effect of intracellular protons was directly on the Ca$^{2+}$-ATPase at an internal modifier site. The direct role of intracellular calcium in the regulation of podosome formation may represent the function of cytoskeletal-associated proteins such as gelsolin or profilin. These proteins regulate actin filament polymerization and severing through regulation of phosphatidylinositol bisphosphate levels bound to the proteins in a Ca$^{2+}$-dependent complex (Bryan and Coluccio, 1985; Chaponnier et al., 1986).

C. Calcium

Because of the role of Ca$^{2+}$ in the control of podosome formation, we have analyzed the mechanisms of Ca$^{2+}$ entry in the osteoclast. We have
demonstrated that increasing extracellular Ca\(^{2+}\) produces a remarkable increase in cytosolic Ca\(^{2+}\), which derives mainly from Ca\(^{2+}\) release from intracellular stores (Miyauchi et al., 1990; Malgaroli et al., 1989; Zaidi et al., 1988). Furthermore, the increase in intracellular Ca\(^{2+}\) produced by changes in extracellular Ca\(^{2+}\) are associated with rapid reorganization of the actin cytoskeleton and disruption of podosome expression. This is associated with a remarkable reduction in bone resorptive activity (Fig. 3) (Miyauchi et al., 1990).

Recently, we have shown that the increase in extracellular Ca\(^{2+}\) activates an osteoclast plasma membrane-associated phospholipase C, suggesting that the osteoclast possesses a Ca\(^{2+}\) sensor protein. Furthermore, this Ca\(^{2+}\) sensor appears to represent a G protein-linked receptor because the Ca\(^{2+}\)-induced activation of phospholipase C is markedly increased by AlF\(_4^{-}\) and fluoride. This is analogous to the function of a Ca\(^{2+}\) sensor protein on the parathyroid chief cell, where changes in
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extracellular calcium regulate PTH secretion (Brown, 1991). We have also shown that osteoclast precursors exhibit voltage-operated Ca2+ channels in their plasma membrane that rapidly disappear upon binding of the osteoclast to bone matrix.

These data suggest that the osteoclast regulates Ca2+ concentrations in the resorption space by changes in the adhesion of the cell to the bone and the sealing of the resorption space. As resorption space Ca2+ concentrations increase, the resorption products stimulate the osteoclast to decrease podosome expression and osteoclast bone adhesion. This results in incompetency of the resorption space and release of its contents to the interstitial bone fluid. This is an energy-conserving mechanism of returning the bone resorption products to the interstitial and eventually plasma fluid. It avoids the energy-expensive transcellular transport of ions and resorption products through the osteoclast cell.

D. Osteopontin/α,β3 Integrin Signaling

The mechanism of osteoclast attachment to bone has not been clearly determined, but the α,β3 integrin is thought to be a key mechanism of attachment to matrix proteins (Horron, 1988; Davies et al., 1989;
Zambonin-Zallone et al., 1989). The bone matrix proteins recognized by the vitronectin receptor (α,β) of the podosome have recently been identified (Fig. 4). Reinholdt et al., (1990) suggested that osteopontin, a protein with tight binding hydroxyapatite, is one protein recognized by the vitronectin receptor in bone. Osteopontin contains a functional RGD cell-binding sequence by cDNA cloning and sequencing (Olgberg et al., 1986, 1988). Osteopontin is an osteoblast product whose synthesis is genomically regulated by 1,25-dihydroxycholecalciferol (Yoon et al., 1987; Heath et al., 1989; Butler, 1989). We have recently shown that osteopontin plays a key role in anchoring the osteoclast to the bone surface, and that another candidate protein for a function similar to that proposed for osteopontin is bone sialoprotein, another RGD-containing bone matrix protein. (Miyauchi et al., 1991; Ross et al., 1993).

Recently, we demonstrated that recognition of osteopontin peptides from the osteopontin and bone sialoprotein sequence stimulate immediate reductions in osteoclast cytosolic Ca^{2+}. The changes in cytosolic Ca^{2+} required the RGD sequence and were blocked by a monoclonal antibody to the α,β integrin (Fig. 4). The decrease in cytosolic Ca^{2+} stimulated by osteopontin and related peptides appeared to be due to activation of a plasma membrane Ca^{2+}-ATPase. The mechanism of signal transduction from the occupied integrin to activation of the Ca^{2+}-ATPase is not clear. Recent studies demonstrating the critical role of cytosolic protein tyrosine kinases (c-src) in osteoclast function raises the possibility that this protein, through association with the occupied integrin, could serve to regulate osteoclast function. We have preliminary evidence indicating that the src protein is, in fact, associated with the α,β integrin and serves to regulate osteoclast function through effector elements with SH2 to domains, as already discussed.

E. Calcitonin

The recent cloning of the calcitonin receptor (Lin et al., 1991) has clarified the mechanisms of signal transduction related to the ability of this hormone to inhibit osteoclast function. The calcitonin receptor is closely related to the PTH receptor, discussed earlier. Although the PTH/PTHrP receptor is more than a 100 amino acids longer than the calcitonin receptor, overall there is 32% identity and 56% similarity between the sequences of the two receptors. Both receptors activate adenylate cyclase (Lin et al., 1991; Juppner et al., 1991). The calcitonin receptor is thought to couple to Gs and an additional signaling pathway has been reported through a pertussis toxin-sensitive Gi protein in isolated osteoclasts and in LLC-PK-1 cells (Zaidi et al., 1988; Chakerborty et al., 1991). Zaidi (1990) demonstrated that calcitonin, besides increasing cAMP production, also stimulates an increase in osteoclast cytosolic Ca^{2+}, release of inositol
teins recognized by recently been identified osteopontin, a protein recognized by the a functional RGD motif (Olgberg et al., whose synthesis is interfer (Yoon et al., recently shown that osteoclast to the bone interaction similar to that seen RDG-containing il., 1993).

Osteopontin peptides stimulate immediate changes in cytosolic Ca2+ by a monoclonal antibody in cytosolic Ca2+ seen to be due to a mechanism of signaling of the Ca2+-the critical role of function raises the possibility that the occupied integrin have preliminary studies associated with the on through effector d.

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FIGURE 4 Effect of the monoclonal antibody, LM609, on changes in cytosolic calcium induced by osteopontin and bone sialoprotein peptides. LM609 (antivitronectin receptor) recognizes the osteoclast a,3 integrin and completely inhibited the effects of intact osteopontin (A), OPN-A (B), and BSP-A (C). However, the monoclonal antibody, LM142 (D and F) had no effect on changes in cytosolic calcium stimulated by OPN-A or BSP-2C. A peptide, CD48, from an irrelevant IgG also failed to affect the changes in cytosolic calcium produced by osteopontin (not shown) and bone sialoprotein peptides (E). The representative tracings demonstrate results observed with each antibody at least five times. These studies demonstrate that bone matrix proteins are ligands for the a,3 integrin and that integrin occupancy generates immediate cell signals.
trisphosphate, and production of diacylglycerol. These mechanisms of signal transduction by the calcitonin receptor signal-generating complex appear to control the biologic effects of calcitonin in the osteoclast. Specifically, osteoclast retraction and inhibition of bone resorption appear to result from an increase in cAMP, [Ca^{2+}], and protein kinase C activity. Recent studies by Teti et al. (1990) confirmed that protein kinase C activity is inhibitory to osteoclast function. This raises a paradigm that mechanisms of signal transduction such as cAMP and phospholipase C activity are inhibitory in the osteoclast. It raises specific issues about the mechanisms of signal transduction related to osteoclast stimulation. A key role of the src tyrosine kinases in this are suggested by our studies on the stimulatory effects of matrix proteins and by recent preliminary studies from Kato et al. (1991); Boyce et al., (1992).

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Transduction of Mechanical Strain in Bone

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ABSTRACT

One physiologic consequence of extended periods of weightlessness is the rapid loss of bone mass associated with skeletal unloading. Conversely, mechanical loading has been shown to increase bone formation and stimulate osteoblastic function. The mechanisms underlying mechanotransduction, or how the osteoblast senses and converts biophysical stimuli into cellular responses has yet to be determined. For non-innervated mechanosensitive cells like the osteoblast, mechanotransduction can be divided into four distinct phases: 1) mechanocoupling, or the characteristics of the mechanical force applied to the osteoblast, 2) biochemical coupling, or the mechanism through which mechanical strain is transduced into a cellular biochemical signal, transmission of signal from sensor to effector cell and 4) the effector cell response. This review examines the characteristics of the mechanical strain encountered by osteoblasts, possible biochemical coupling mechanisms, and how the osteoblast responds to mechanical strain. Differences in osteoblastic responses to mechanical strain are discussed in relation to the types of strain encountered and the possible transduction pathways involved.

INTRODUCTION

A primary biomedical concern in the exploration and development of space is the rapid and continuous loss of bone mass during extended flights. Reduction in bone formation (Morey and Baylink, 1978; Wronski and Morey, 1983a; Shaw et al., 1988), mineral content (Vico et al., 1987; Rambaut and Johnston, 1979), bone matrix protein production (Simmons et al., 1986; Patterson-Buckendahl et al., 1985) and total body calcium (Rambaut and Johnston, 1979; Turner et al., 1985; Cann and Adachi, 1983) characterize the physiologic response to weightlessness. In ground based studies, similar negative effects on bone can be observed in humans and animals subjected to prolonged immobilization and disuse (Whedon and Heaney, 1993) or skeletal unloading (Globus et al., 1986; Wronski and Morey, 1983b). At the cellular level, weightlessness and skeletal unloading appear to modulate bone turnover through the osteoblast. While the number of osteoclasts increase during short-term flights (Vico et al., 1987; Wronski et al., 1987), this effect is transient with normal numbers of osteoclasts observed during longer flights (Jee et al., 1983). Also, calcium kinetic studies have found no change in bone resorption rate in rats during eighteen days of weightlessness (Cann and Adachi, 1983). However, tetracycline labeling studies have shown a marked reduction in periosteal bone formation in growing rats subjected to weightlessness (Wronski et al., 1987) suggesting an impairment of osteoblastic function and perhaps histogenesis. Osteoblast number, size, and covered surface area are all decreased during space flight (Wronski and Morey, 1983a; Jee et al., 1983; Vico et al., 1988). Osteocalcin and type I collagen are significantly reduced in flown rats after seven days (Patterson-Buckendahl et al., 1985). In addition, weightlessness appears to inhibit the differentiation of osteoblasts from osteoprogenitor cells to preosteoblasts (Roberts et al., 1981; Roberts et al., 1987).

Bone loss due to weightlessness, immobilization, or skeletal unloading can be reversed upon return to normal weight bearing although this restoration of bone is dependent on a number of factors such as species, age, bone type, method, duration, and time of testing after skeletal unloading (Abram et al., 1988; Donaldson et al., 1970; Sessions et al., 1989; Jaworski and Uhloff, 1986; Lindgren and Mattsson, 1977). In conjunction with these data are the numerous studies indicating that increased mechanical loading can stimulate bone formation. Increased mechanical loading through exercise has been shown to increase bone mass (Smith and Gilligan, 1990; Eisman et al., 1990) and retard bone loss caused by postmenopausal osteoporosis (Krolner et al., 1983; Simkin et al., 1987). Additionally, in vivo and in vitro experiments have demonstrated that exogenous mechanical strain can increase bone mass and stimulate osteoblastic function. In this review the direct effects of increased mechanical load on whole bone and osteoblasts will be examined. The possible mechanisms of transduction of mechanical stimuli to the osteoblast will also be discussed.

MECHANICAL STIMULATION OF BONE IN VIVO

While the effects of gravity on the determination of bone size and shape were first introduced by Galileo (1638), the effects of mechanical strain on bone architecture was first described as a mathematical law by Wolff over a century ago (Wolff, 1892). Since then, numerous studies have demonstrated that mechanical strain increases bone mass and that bone will alter its structure to accommodate unique loads. Many techniques have been devised to examine the effects of exogenous mechanical strain on bone homeostasis. One which has provided valuable evidence for the osteogenic response of bone to mechanical strain is the ulnar osteotomy model (Goodship et al., 1979; Lanyon et al., 1982; Burr et al., 1989). Initially,
OSTEOGENIC RESPONSE TO MECHANICAL STRAIN

This technique involved the removal of the ulnar diaphysis to subject the intact radius to increased compressive strain. These studies were limited to animals in which the ulna and the radius were approximately equal in size so that minimal alteration in the normal gait of the animal occurred. Goodship et al. (1979) found that the radius underwent rapid remodeling following the osteotomy. Three months after the osteotomy, the cross sectional area of bone of the radius approximately equaled that of the radius and ulna together in the contralateral limb and the magnitude of strain was not significantly different between the experimental and control limbs. This study and others indicated that bone will alter its morphology to accommodate increased or aberrant mechanical strain (Goodship et al., 1979; Burr et al., 1989; Lanyon et al., 1982). A variation of the ulnar osteotomy model isolates the ulna in situ via proximal and distal osteotomies. Exogenous mechanical strain is applied through stainless steel caps attached to the ends of the isolated ulna. When mechanical strain was applied intermittently to this preparation, significant increases in cross-sectional area of bone in the ulna was observed when compared to non-strained or statically loaded ulnae (Lanyon and Rubin, 1984). This increase exhibited a dose dependent response to the magnitude of strain applied and principally involved the periosteal surface (Rubin and Lanyon, 1984). Interestingly, active bone formation could be induced by a single period of intermittent loading (Rubin and Lanyon, 1984) and minimal loading was required to maintain bone homeostasis (Lanyon et al., 1986).

While these models have produced significant advances in the understanding of mechanically induced osteogenesis, the surgical invasion of the animal required by the preparation can induce anomalies in the data. The inflammatory response to the surgery can accelerate bone metabolism, possibly through the prostaglandin pathway, to produce alterations in bone homeostasis (Harris, et al., 1973; Ayers, et al., 1989). Two models which have recently been developed to circumvent this problem and still apply exogenous strain to bone are the four point tibia bending model (Turner et al., 1991) and the tail vertebral loading model (Chow et al., 1993). An added advantage to these preparations is that rats can be used as an experimental model which allows many of the rat antibody probes to be used. The four point bending model applies strain in the medial to lateral direction which compresses the lateral surface of the tibia. Turner et al. (1991) demonstrated increased bone formation after twelve days of daily strain with poorly organized bone resembling woven bone noted in areas of higher strain. Better organized bone similar to lamellar bone was found in areas of lower strain. Woven bone formation was found five days after initial mechanical challenge but after three to four weeks of daily strain, the new bone mineralized into lamellar type bone (Turner et al., 1992). Further examination found that woven bone was principally seen on the periosteal surface following four point bending, whereas lamellar type bone formation was observed on the endocortical surface. Lamellar bone formation exhibited an activation threshold for both magnitude and frequency of mechanical strain. When four point bending was applied to rat tibia for two weeks at 2 Hz for 3 cycles/day, formation increased six-fold when the magnitude of mechanical strain exceeded 1.050 µ strain (Turner et al., 1994b). A similar activation threshold was observed when the frequency of mechanical strain was increased above 0.5 Hz with constant strain magnitude (Turner et al., 1994a). Analogous responses were observed in the tail vertebrae model which subjects the eighth caudal vertebrae to compression. Chambers et al. (1993) have reported 30-fold increases in bone formation in animals subjected to daily loading cycles of mechanical strain for five weeks. Animals subjected to a single loading cycle exhibited a 4-fold increase in bone. Interestingly, bone formation was not only increased but the osteoclasts surface and the number of osteoclasts were significantly decreased following both the single loading regime and the daily loading. Lamellar bone formation correlated with the magnitude and duration of daily loading (Chow et al., 1993).

While these studies have defined the mechanically-induced osteogenic response of bone in vivo, the mechanism of how bone senses these changes in the physical environment remains elusive. In the next section of this review, the mechanisms of mechanotransduction will be discussed in relation to the responses observed in the osteoblast in vitro preparations.

MECHANOTRANSDUCTION

Mechanotransduction, or the conversion of a biophysical force into a biochemical response is an essential mechanism for a wide variety of physiologic functions which allow living organisms to respond to the mechanical environment. Balance, hearing, and the sense of touch are commonly known examples of mechanotransduction. However, almost every tissue in the body responds to mechanical stimuli. While some tissues have specialized response elements to transduce mechanical force (e.g., the cochlea for hearing and balance) many cells respond to mechanical strain independent of specialized structures. Examples of this type of mechanotransduction are the vasoregulation of blood pressure by endothelial cells and the osteogenic response of osteoblasts to mechanical load. While a vast amount of literature exists which detail the responses of tissues to mechanical strain, the mechanism by which a single cell transduces a mechanical signal into a response remains unclear. Mechanotransduction can be divided into four distinct phases: 1) mechanocoupling, or the characteristics of the mechanical force applied to the cell 2) biochemical coupling, or the mechanism by which mechanical strain is transduced into a cellular signal 3) transmission of signal from sensor to effector cell, and 4) the effector cell response. This review will focus on each
OSTEOGENIC RESPONSE TO MECHANICAL STRAIN

Figure 1. Cellular deformations induced by different methods of in vitro strain application. Due to the curvature of the appendicular skeleton, the predominant response perceived by the osteoblasts or osteocytes in vivo would be uniaxial stretch and fluid shear. However hydrostatic pressure may play a role in bone marrow cells. Copyright R.L. Duncan and C.H. Turner, 1995, used with permission.

of these phases individually in relation to the osteogenic response of osteoblasts and osteoblast-like cells.

MECHANOCOUPLING

Mechanocoupling in specialized mechanosensory tissues describes the physical connection between the site of mechanical stimulation and the responding cell (e.g., the bones of the middle ear connecting the tympanic membrane with the cochlea). In mechanosensory cells which have no specialized mechanosensory apparatus yet respond to mechanical stimuli, the concept of mechanocoupling is not well defined. Here, mechanocoupling will refer to the characteristics of mechanical strain which modulate the response of the osteoblast to mechanical perturbation.

To simulate in vivo mechanical strain on cultured cells, multiple in vitro techniques have been used. Choosing which type of strain to use in in vitro experiments is obvious in certain preparations. However, the type of mechanical strain experienced by osteoblasts or osteocytes in vivo is not readily discerned. While logic dictates that compression would be the principle type of strain incurred by the osteoblast in vivo, strain measurements made in animal models indicate that, due to the curvature of the appendicular skeleton, the predominant type of strain experienced by weight bearing bone during normal movement occurs via bending (Bertram and Biewener, 1988; Biewener and Taylor, 1986). These observations would suggest mechanical stretch may be the principal type of deformation perceived by the osteoblast in vivo. However, cultured osteoblasts have been subjected to a variety of strain deformations, including hypotonic swelling, hydrostatic pressure, fluid shear, and mechanical stretch. As illustrated in Figure 1, these different methods of in vitro application of strain produce different types of cellular deformation. Variations in cell deformation caused by these methods of strain application raise the question whether osteoblasts respond differently to dissimilar applications of strain. Mechanical loading of osteoblasts or osteoblast-like cells have produced variable, and seemingly contradictory, responses (Burger and Veldhuijzen, 1993). Additionally, modulation of osteoblast function, in vitro, by the magnitude and frequency of strain are not as evident in in vivo studies. These disparate responses may reflect these variations in cellular deformation, making correlation between in vitro studies and in vivo studies difficult.

Fluid shear, or the force generated by the flow of interstitial fluid through channels in bone as a result of mechanical bending, may also influence the response of the osteoblast to strain. Fluid shear, also termed shear stress, is instrumental in a number of physiologic functions, including the endothelial control of vascular tone (Pohl et al., 1986). In vitro studies have demonstrated that osteoblasts respond to fluid shear with increases in the cellular levels of inositol trisphosphate (IP3), cAMP and prostaglandin E2 (PGE2)(Reich and Frangos, 1991; Reich et al., 1990; Reich and Frangos, 1993). These in vitro data may explain observations made in simulated weightlessness studies in which cranial bone mass is increased during skeletal unloading using head down tilt or hindlimb tail suspension (Roer and Dillaman, 1990; Arnaud et al., 1992), suggesting that the fluid shift associated with these positions may influence osteoblast function. Dillaman, et al. (1991) support this hypothesis, suggesting that the decrease in hind limb bone mass during tail suspension studies may be associated with a decrease in fluid flow in these regions.

A secondary effect of interstitial fluid flow in bone may be created by streaming or stress generated potentials. Streaming potentials are generated when ionized fluids pass across a charged surface. The surface of bone is negatively charged. thus cations in the interstitial fluid that is being forced through channels are attracted to the surface, producing a surplus of anions in the fluid. The voltage resulting from this imbalance of ions is positive in the direction of flow (Chakkalakal, 1989). Turner, et al. (1994a) have measured these potentials in rat tibia ex vivo. Streaming potentials were found to increase with increasing load frequencies. These increases correlated with increased bone formation rates in rats subjected to in vivo four point bending of the tibia (Turner et al., 1994a). The streaming potentials produced by the flow of interstitial fluid in bone could produce a number of responses in the osteoblast including activation of voltage operated channels in the cellular membrane.

BIOCHEMICAL COUPLING

While the mechanism for the initial detection and
OSTEOGENIC RESPONSE TO MECHANICAL STRAIN

Figure 2. Schematic illustration of the extracellular matrix-integrin-cytoskeletal axis (adapted from Pavalko et al., 1991). Integrins, membrane-spanning proteins composed of an α and β subunit, externally bind to specific extracellular matrix proteins. The β subunit is linked to actin associated proteins on the cytosolic side, depicted here as talin and α-actinin. Other actin associated proteins, such as tensin, have also been shown to be important in the linkage of actin with integrins.

conversion of mechanical force into a chemical signal in the osteoblast has yet to be determined. Several likely candidates have been proposed. One possible transduction pathway is the extracellular matrix-integrin-cytoskeletal axis (See Figure 2). Cells attach to the extracellular matrix through binding to membrane-spanning glycoproteins termed integrins. Integrins, in turn, are linked to the actin cytoskeleton through several actin associated proteins such as vinculin, talin, tensin and α-actinin (Pavalko et al., 1991). The cytoskeleton has been shown to form a network, connecting the extracellular matrix with the nucleus and the cytoplasmic constituents of the cell (Sims et al., 1992). Modeling and experimental evidence indicate that the cell generates an internal force through the cytoskeleton which exerts a tension on the extracellular matrix (Ingber et al., 1993; Ingber, 1993; Sims et al., 1992). This internal tension, similar in concept to the architectural system of tensegrity (Fuller, 1975), produces forces on the adhesion sites of the cell in excess of those forces produced by exogenous mechanical stimuli (Ingber et al., 1993). Without attachment, these internal forces would produce a spherical cell. The binding of integrins to matrix proteins on a rigid substrate must overcome the tensional forces of the cell, evoking changes in the cytoskeletal structure. Due to the tension of the cytoskeleton, physical stimulus could be rapidly transmitted to the nucleus, possibly altering gene expression. Indeed, alteration of the cytoskeletal organization has been shown to alter phenotypic expression in chondrocytes. When grown in culture, chondrocytes assume a flattened morphology and do not express differentiation markers. However, when treated with cytochalasin B, a mold metabolite which induces actin repolymerization, the cells assume a spherical shape and produce type IV collagen (Brown and Benya, 1988). Furthermore type IV collagen production is stimulated even when cytochalasin B is given at concentrations which induces actin repolymerization but does not alter cell shape (Benya et al., 1988). These observations suggest that the modulation of the cytoskeletal, and not cell shape changes, which mediate alterations in gene expression during cell adhesion and mechanical stimulation.

Mechanical strain also alters cell shape and cytoskeletal organization. When subjected to fluid shear stress, endothelial cells align parallel to the direction of flow (Dartsch and Betz, 1989). This response to mechanical stimulation is accompanied by an increase in filamentous actin (F-actin) stress fibers which also align in the direction of flow (Dartsch and Betz, 1989). Interestingly, when endothelial cells or osteoblasts are grown on flexible, silicone-bottomed culture plates and subjected to chronic cyclic deformation, the cells align perpendicular to the vector of strain (Buckley et al., 1988; Dartsch and Betz, 1989). This realignment is also accompanied by an increase in cytoskeletal stress fibers aligned in the same direction as the cell. These observations would suggest that different types of mechanical strain produce different cellular responses.

Integrins are composed of two subunits, denoted as α and β, both of which are required for cell adhesion (Hynes, 1992; Rouslahti, 1991). Numerous α and β subunits have been identified and sequenced. These subunits can interchange which permits different binding specificities for different extracellular matrix proteins (Hynes, 1992; Rouslahti, 1991). Ligand binding to specific integrins has been implicated in a number of bone cell functions, including attachment and differentiation (Dedhar, 1989; Grzesik and Gehron-Robey, 1995) and bone formation and resorption (Gronowicz and Derome, 1994). In addition, integrin stimulation has been
OSTEOGENIC RESPONSE TO MECHANICAL STRAIN

associated with increases in intracellular second messengers (Miyauchi et al., 1991; Zimomo et al., 1994; Schwartz, 1993; McNamee et al., 1992), tyrosine phosphorylation (Kornberg et al., 1991) and Na"/H" exchange (Ingber et al., 1990; Schwartz et al., 1991). Integrins have also been directly linked to the cellular response of mechanical strain (Schwartz and Ingber, 1994). When endothelial cells are subjected to shear stress, integrins rapidly realign with the direction of flow, indicating that cell adhesion is a dynamic process responding to mechanical strain (Davies et al., 1994). Furthermore, physical strain applied directly to integrins using a magnetic twisting device produces an increase in cytoskeletal stiffness in proportion to the magnitude of the strain applied (Wang et al., 1993). This increase in stiffness requires intact microfilaments, intermediate filaments and microtubules. These results suggest that the extracellular matrix-integrins-cytoskeletal axis plays an active role with the signal transduction of mechanical strain.

Another possible mechanotransduction pathway is the gating of ion channels through direct mechanical strain. Mechanosensitive channels are likely candidates for the primary transduction of mechanical strain since no second messenger is required for activation of these channels (Sachs, 1988). Mechanosensitive channels can be divided into two general categories: stretch activated channels, which open during increased membrane tension, and stretch inactivated channels which are normally open and close when mechanical strain is applied (Sachs, 1991). These channels can be further subdivided based on their activation properties, kinetic characteristics and ion selectivity (Morris, 1990). Stretch activated (SA-cat) channels have been identified in both rat (Duncan and Misler, 1989) and human (Davidson et al., 1990) osteoblast-like osteosarcoma cells. These channels open in response to low magnitudes of strain, conduct Na" and Ca"++ equally and are inhibited by gadolinium. The SA-cat channels in the osteoblast are similar to those found in a variety of tissues (Morris, 1990). We have recently demonstrated that osteoblast-like cells respond to chronic mechanical strain by altering SA-cat channel kinetics (Duncan and Hruska, 1994). Chronic stretch applied to osteoblasts for 2 to 24 hours prior to the patch study, changed SA-cat channel characteristics in two ways. First, SA-cat channel activity (NP) was increased 3 to 5 fold above non-stretched control cells (Figure 3A). This increase corresponded to an increase in whole cell conductance in response to additional mechanical perturbation during the patch. Secondly, single channel conductance was increased following chronic stretch (Figure 3B), suggesting that strain induces a configurational change in the channel which allows more ions to traverse the channel during opening. In addition, chronic intermittent mechanical stretch increased the stretch sensitivity of the SA-cat channels and induced spontaneous channel activity. These alterations in channel kinetics indicate that chronic mechanical strain "primes" the SA-cat channel to respond to additional perturbation.

Guharay and Sachs (1984) have demonstrated that stretch activated channels respond to changes in tension of the lipid membrane in response to mechanical strain, however they have proposed that changes in membrane tension are focused on the stretch channels by the cytoskeleton. Using cytoskeletal severing agents, they found that the stretch activated channels found in chick skeletal muscle were not linked to nublin or actin filaments. However, we have examined the interaction of the actin cytoskeleton on SA-cat channels in osteoblasts based on two observations. First, we have previously demonstrated that parathyroid hormone (PTH) modulates SA-cat channel kinetics in a manner similar to chronic mechanical strain (Duncan et al., 1992). PTH has been shown to induce a stellated morphology in osteoblasts in primary culture (Miller et al., 1976), which has been attributed to reorganization of the actin microfilaments (Egan et al., 1991; Lomri and Marie, 1983; Aubin et al., 1983). Secondly, channel studies in renal cells have demonstrated that depolymerization of actin using cytochalasins activates the epithelial sodium channels (Cantiello et al., 1991). We have demonstrated that when cytochalasin D alters polymerization of F-actin, SA-cat channel activity increases 10-fold within 4 minutes of application (Duncan et al., 1992). These data suggest a tight interaction between the cytoskeleton and the stretch activated channels in the osteoblast.

Upon application of stretch to the substrata, osteoblasts experience an almost instantaneous, large, transient increase in intracellular calcium (Jones et al., 1991). This increase in intracellular calcium appears to initially arise from the release of intracellular stores, followed by calcium entry through ion channels (Jones et al., 1991). One proposed mechanotransduction mechanism which could explain this rapid release of calcium is a direct link of the cytoskeleton with the phospholipase C pathway (Jones and Bingmann, 1991). Phospholipase C activates the protein kinase C pathway which in turn produces inositol triphosphate (IP,) and diacylglycerol. IP, stimulates the release of calcium from intracellular stores. Deformation of the cell due to mechanical strain would physically dislocate a proposed phospholipase C inhibitor attached to the cytoskeleton. This, in turn, would allow phospholipase C to activate. A similar mechanism has been proposed in a tumor suppressor gene for colo-rectal carcinoma (Kinzler et al., 1991).

While each of these candidates have been treated as a primary mechanotransduction mechanisms, it should be noted that each have a high degree of association. The tight interaction of each of these pathways would suggest that the entire cell is required to sense mechanical stimulation and that there is no single transduction pathway. An alternative explanation would be that these various pathways are expressed differently depending on cell type or stage of cell differentiation. The observations that endothelial cells align differently in response to fluid shear...
OSTEOGENIC RESPONSE TO MECHANICAL STRAIN

Figure 3. SA-cat channel kinetic changes in response to chronic intermittent mechanical stretch in UMR-106.01 osteoblast-like cells. SA-cat channel activity in chronically stretched cells was increased 3-5 fold over non-stretched controls. In this figure (A), note that channel activity was greater in strained cells than non-strained cells even though less suction was applied to the patch. Examination of channel amplitudes found that single channel amplitudes were significantly shifted (B), suggesting a conformational change in the channel to allow the passage of more ions during channel openings. Used with permission. American Journal of Physiology: Renal, Fluid and Electrolyte Physiology.

Two lines of reasoning exist to explain how the chemical signal in the osteoblast is propagated to the effector cell to increase osteogenic activity in response to mechanical strain. The first suggested explanation states that the osteoblast which senses mechanical strain is also the effector cell which responds to strain with increases in bone formation products and, ultimately, mineralization of the matrix. This hypothesis is supported by observations that human osteoblast-like osteosarcoma cells increase expression and production of matrix proteins in response to cyclic mechanical stretch (Harter et al., 1995). In this situation, the chemical signal generated by mechanical strain would be predominately transmitted by intracellular second messengers. Mechanosensitive cells have been shown to respond to mechanical strain with increased
OSTEOGENIC RESPONSE TO MECHANICAL STRAIN

levels of second messengers (Vandenburgh, 1992; Watson, 1991; Sandy and Farndale, 1991), including osteoblasts (Reich et al., 1990; Sandy et al., 1989; Rodan et al., 1975; Binderman et al., 1988; Brighton et al., 1992). Most mechanosensitive cells respond to mechanical strain with a rapid elevation of cAMP, which has been associated with growth and proliferation (Vandenburgh, 1992; Watson, 1991; Burger and Veldhuizen, 1993). In osteoblasts, cAMP is significantly increased after 5 minutes of mechanical stretch (Binderman et al., 1988; Sandy et al., 1989) and within seconds of application of fluid shear (Reich et al., 1990). While the exact mechanism of stimulation of adenylate cyclase is unknown, disruption of the cytoskeleton has been shown to elevate levels of cAMP (Kennedy and Insel, 1979; Insel and Koachman, 1982). IP3 levels have also been shown to increase with application of strain in osteoblasts (Reich and Frangos, 1991; Sandy et al., 1989; Brighton et al., 1992; Jones and Bingmann, 1991). IP3 significantly increases within seconds of strain application (Brighton et al., 1992; Jones and Bingmann, 1991) supporting the postulate of a phospholipase C activating mechanosensor associated with the cytoskeleton (Jones et al., 1991). However, Jones and Bingmann have timed the course of release of second messengers in the osteoblast in response to strain and have found that the increase in intracellular concentration of calcium precedes the rise in IP3 in osteoblasts (Jones and Bingmann, 1991). These data suggest calcium entry into the cell through activation of channels may be the initial cellular signal for the osteoblastic response to mechanical strain. This possibility is supported by the observation that cyclic loading increases calcium incorporation into osteoblasts and that this incorporation can be blocked by the calcium channel inhibitor verapamil (Vadiakas and Banes, 1992).

A second hypothesis for the transmission of chemical signal states that the mechanical strain is sensed by either osteoblasts or osteocytes which respond to mechanical strain by secreting paracrine factors. These paracrine factors can then either stimulate other osteoblasts to increase mineralization or recruit osteoblast precursor cells to differentiate into mineralizing osteoblasts. One paracrine released in the osteoblast in response to mechanical strain is PGE2 (Somjen et al., 1980; Ozawa et al., 1990; Yeh and Rodan, 1984; Murray and Rushton, 1990). Studies have shown that prostaglandins stimulates bone resorption in vivo and increases bone resorption by osteoclast in organ cultures (Klein and Raisz, 1970; Harvey, 1988a). This may explain the bone loss associated with chronic inflammation and wound healing (Harvey, 1988b; Ayers, et al., 1989). Recently several studies have indicated that PGE2 has an anabolic effect on bone as well (Nordin et al., 1990; Miller and Marks, 1993; Yang et al., 1993). PGE2 has been implicated in maintaining bone following ovariectomy (Ma et al., 1994; Jee et al., 1990; Mori et al., 1990; Ke et al., 1992; Ke et al., 1993) and during periods of weightlessness or immobilization (Li et al., 1993; Jee et al., 1992). Prostaglandin also regulate osteoblastic function in culture. PGE2 has been shown to stimulate proliferation (Hakeda et al., 1986; Yamaguchi et al., 1989; Nagai, 1989), alkaline phosphatase activity (Hakeda et al., 1985) and collagen synthesis (Hakeda et al., 1985; Nagai, 1989). Perhaps a more important function of PGE2 may be in the recruitment of osteoblast precursor cells into mineral forming osteoblasts. PGE2 has been shown to increase preosteoblast proliferation in rat calvarial organ cultures (Gronowicz et al., 1994) and promote attachment of these precursor osteoblasts (Scott and Bertram, 1995). This attachment would be important to the differentiation of precursors into osteoblasts. Therefore PGE2 would not only stimulate osteogenic function in existing osteoblasts but increase the production of osteoblasts through recruitment of precursor cells into mineralization osteoblasts.

In all likelihood, both of these pathways probably play a significant role in the transmission of signal to elicit an osteogenic response in bone. Histomorphometric measurements of bone would indicate that osteoblasts and collagen fibers are oriented in the direction of mechanical strain (Martin and Burr, 1989). These observations may indicate that the response of bone to mechanical strain is a localized phenomenon and suggest that the osteoblast acts as both sensor and effector of the osteogenic response to mechanical strain. However, prostaglandin effects on histogenesis would be extremely important as well. Studies using the rat maxillary molar periodontal ligament on COSMOS and Spacelab missions have indicated that there is an inhibition of differentiation from osteogenic cells to preosteoblasts during weightlessness (Roberts et al., 1981; Roberts et al., 1987). Recovery to normal differentiation patterns is quite rapid following return to normal gravity (Garetto et al., 1992; Garetto et al., 1990), indicating that mechanical load can quickly modify histogenesis. In light of recent reports indicating modulation of marrow stromal precursors by PGE2, stimulation of prostaglandin production would be a likely pathway to increase the number of osteoblasts available to affect bone formation.

EFFECCTOR RESPONSE

While the in vivo anabolic response of bone to mechanical strain is apparent, the response of cultured osteoblasts to strain is inconclusive. Proliferation and DNA synthesis has been shown to both increase (Hasegawa et al., 1985; Buckley et al., 1988) or decrease (Burger et al., 1992) upon application of in vitro mechanical strain. Similar discordant observations have been made in studies measuring differentiation markers such as alkaline phosphatase and type I collagen, in response to strain (Harter et al., 1995; Ozawa et al., 1990; Burger et al., 1992). In a recent review of these conflicting reports, Burger and Veldhuizen (1993) have suggested that these differences in experimental observations could be the
OSTEOGENIC RESPONSE TO MECHANICAL STRAIN

result of differences in the magnitude of strain applied. Examining existing data, they concluded that high magnitudes of strain stimulate proliferation. PGE₂ secretion and cAMP production while physiologic levels of strain induce a more differentiated response with increases in alkaline phosphatase activity, matrix protein production and a decrease in cell proliferation (Burger and Veldhuijzen, 1993).

While the magnitude of mechanical strain is likely to contribute to these observed differences in osteoblast response to mechanical strain, other possibilities exist which could explain these variable results. As previously mentioned, one of the most critical factors is the type of mechanical strain applied to osteoblasts. Stretch, hydrostatic compression, hypotonic swelling and fluid shear have been used to simulate in vivo mechanical strain. These different methods of strain application have profound differences on cellular deformation and, by extension, the proposed biochemical coupling mechanisms. It is the diverse stimulation of these biochemical coupling mechanisms which could produce dissimilar responses in the osteoblasts. Although each of the biochemical coupling mechanisms appear closely associated through the cytoskeleton, the physiologic relevance of multiple transduction pathways may be related to the different types of strain incurred by the osteoblast. For example, osteoblasts or osteocytes which do not perceive mechanical bending of the bone could be subjected to fluid shear in response to the mechanical strain. In this situation, the SA-cat channels may produce a response quite different from the response induced by mechanical strain applied to the cellular adhesion sites. These multiple transduction mechanisms may also be important in the response to varied magnitudes of strain. SA-cat channels have been shown to respond to superphysiologic levels of strain and are modulated by cAMP (Duncan and Hruska, 1994; Morris, 1990). Therefore, activation of these channels may be required for the proliferative response of osteoblasts to mechanical strain.

In summary, mechanical loading of bone is essential for maintenance of bone mass. The effects of loading on bone is mediated through alteration in osteoblastic function, although strain studies using cultured osteoblasts have produced conflicting results. Conversion of physical force into a cellular response could be transduced through integrins, the cytoskeleton, or mechanosensitive ion channels although these cellular components appear tightly coupled in the osteoblast. The varied responses of cultured osteoblasts to mechanical strain could be in response to subtle distinctions in the activation of the biochemical coupling mechanisms due to different perturbations of the cell by dissimilar mechanical strain applications.

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OSTEOGENIC RESPONSE TO MECHANICAL STRAIN


OSTEOGENIC RESPONSE TO MECHANICAL STRAIN


OSTEOGENIC RESPONSE TO MECHANICAL STRAIN


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OSTEOGENIC RESPONSE TO MECHANICAL STRAIN


Laboratory Investigations

Mechanotransduction and the Functional Response of Bone to Mechanical Strain

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Abstract. Mechanotransduction plays a crucial role in the physiology of many tissues including bone. Mechanical loading can inhibit bone resorption and increase bone formation in vivo. In bone, the process of mechanotransduction can be divided into four distinct steps: (1) mechanocoupling, (2) biochemical coupling, (3) transmission of signal, and (4) effector cell response. In mechanocoupling, mechanical loads in vivo cause deformations in bone that stretch bone cells within and lining the bone matrix and create fluid movement within the canaliculae of bone. Dynamic loading, which is associated with extracellular fluid flow and the creation of streaming potentials within bone, is most effective for stimulating new bone formation in vivo. Bone cells in vitro are stimulated to produce second messengers when exposed to fluid flow or mechanical stretch. In biochemical coupling, the possible mechanisms for the coupling of cell-level mechanical signals into intracellular biochemical signals include force transduction through the integrin-cytoskeleton-nuclear matrix structure, stretch-activated cation channels within the cell membrane, G protein-dependent pathways, and linkage between the cytoskeleton and the phospholipase C or phospholipase A pathways. The tight interaction of each of these pathways would suggest that the entire cell is a mechanosensor and there are many different pathways available for the transduction of a mechanical signal. In the transmission of signal, osteoblasts, osteocytes, and bone lining cells may act as sensors of mechanical signals and may communicate the signal through cell processes connected by gap junctions. These cells also produce paracrine factors that may signal osteogenic cells to differentiate into osteoblasts and attach to the bone surface. Insulin-like growth factors and prostanoids are possible candidates for intermediaries in signal transduction. In the effector cell response, the effects of mechanical loading are dependent upon the magnitude, duration, and rate of the applied load. Longer duration, lower amplitude loading has the same effect on bone formation as loads with short duration and high amplitude. Loading must be cyclic to stimulate new bone formation. Aging greatly reduces the osteogenic effects of mechanical loading in vivo. Also, some hormones may interact with local mechanical signals to change the sensitivity of the sensor or effector cells to mechanical load.

Key words: Bone density — Calcium channels — Integrins — Osteoporosis — Osteoblasts.

The mass and architecture in bones are governed, to some extent, by adaptive mechanisms sensitive to their mechanical environment. In the 19th century, the idea that mechanical forces shaped the architecture of the skeleton began to emerge in the ideas of Roux [1] and Meyer [2]. However, it was Julius Wolff [3] whose name became inseparable with the idea of bone adaptation. Based upon the observations made by Meyer concerning cancellous bone structure, Wolff proposed that mechanical stress was responsible for determining the architecture of bone. More importantly, he stipulated that the form of bone is related to mechanical stress by a mathematical law—Wolff's law of bone transformation [3]. Although we know today that many basic tenets of Wolff's law are incorrect [4], the idea that mechanical stresses affect the form of bone has garnered general acceptance [5, 6]. Experimental support is abundant. Reduction in bone formation [7–9], mineral content [10–14], and bone matrix protein production [15] result from the skeletal unweighting associated with spaceflight. Conversely, increased skeletal loading through exercise has been shown to increase bone mass [16–18] and retard bone loss caused by postmenopausal osteoporosis [19–22]. Recent interpretations of Wolff's law have proposed that changes in bone structure are brought about by a feedback system in which changes in local mechanical signals drive bone cells to change bone structure [6, 23–28]. The most biologically relevant of these theories is the "mechanostat" hypothesis put forth by Frost [24, 26, 29–30]. Frost's mechanostat theory is unique in its distinction between modeling and remodeling processes, thresholds for activating lamellar or woven bone formation, and its application to the etiology of osteopenia and osteoporosis. Frost's theory describes a window of mechanical usage that should be considered normal or physiological. When local mechanical signals in bone exceed the upper boundary of the physiological window, called the minimum effective strain (MES), bone will undergo modeling, or
Woven bone formation

Fig. 1. The four mechanical usage windows defined by mechanostat theory (adapted from Burr and Martin [25]). Each window is separated by a minimum effective strain (MES) for which approximate values are given in microstrain. In the disuse window, strains are low and bone is lost due to increased remodeling with negative bone balance. In the physiological window, bone is at a state of normal turnover and bone balance is maintained. In the overuse window, lamellar bone is gained on bone surfaces due to increased modeling. In the pathological overload window, woven bone is added to bone surfaces similar to a repair reaction. The strain values given for the MES levels were estimated by Frost based upon literature values. However, these levels tend to be variable and site-specific suggesting that strain magnitude alone does not predict bone adaptation.

sculpting, and change its structure to reduce the local strains to below the MES. If the mechanical loads on the skeleton are very large, the bone strains will be pushed into a pathological overload zone causing woven bone formation on bone surfaces (Fig. 1). This theory also incorporates a lower MES below which bone tissue will be resorbed until the local strains are increased. Frost further suggested that certain hormones and biochemical agents may fool the "mechano-" of bone to alter the boundaries of the physiological window, thus allowing normal mechanical usage to increase bone mass and bone strength significantly [26].

The mechanisms by which the mechanostat works are unknown, however, they require some form of cellular mechanotransduction. Mechanotransduction, or the conversion of a biophysical force into a cellular response, is an essential mechanism for a wide variety of physiological functions that allow living organisms to respond to the mechanical environment. Presumably, mechanotransduction in bone must include four distinct phases: (1) mechanocoupling, the transduction of mechanical force applied to the bone into a local mechanical signal perceived by a sensor cell; (2) biochemical coupling, the transduction of a local mechanical signal into a biochemical signal and, ultimately, gene expression; (3) transmission of signal from the sensor cell to the effector cell, i.e., the cell that will actually form or remove bone; and (4) the effector cell response, the final tissue-level response. This model for bone adaptation parallels the model for mechanotransduction in the vascular endothelium. In the endothelium, mechanocoupling occurs through fluid shear stresses caused by blood flow; endothelial cells are sensitive to fluid shear stresses [31, 32]. When blood flow rate is high, resulting in large fluid shear stresses, endothelial cells produce a paracrine factor which travels from the sensor cells in the endothelium to the effector cells in the vascular smooth muscle, relaxing the smooth muscle and reducing the flow rate [33, 34]. Unlike other mechanosensitive systems (e.g., cochlear hair cells [35] and muscle spindle fibers [36]), mechanotransduction in the vascular endothelium and bone does not require innervation. This review will focus on each of the four phases of mechanotransduction in relation to the osteogenic response of bone.

Mechanocoupling

Mechanocoupling in mechanosensory tissues refers to the physical transduction of mechanical energy to a form that can be detected by cells. For example, the bones of the middle ear transduce sound waves in air into stress waves within the fluid of the cochlea. In bone, the mechanical loads imposed by normal use cause local deformations in the tissue called strains (1 μstrain equals 1 μm of deformation per meter of length). The peak levels of these strains range from
Fig. 3. When a region of bone is compressed or stretched, it expands or contracts in the perpendicular direction. This phenomenon is called Poisson’s effect and causes a biaxial strain field on osteocytes embedded in the matrix. This strain field is different than the biaxial fields created by flexible membrane cell stretching systems used in vitro (in vitro loading systems typically cause cells to be stretched in two directions). The difference between physiological strain fields in vivo and nonphysiological strain fields created in vitro make it difficult to extrapolate cell culture findings to the tissue-level physiology.

Multiple in vitro techniques have been used to simulate in vivo bone strain on cultured osteoblasts including hypotonic swelling [56], hydrostatic pressure [57–61], mechanical stretch [62–67] and biaxial membrane stretch [68–71], and fluid shear [72–74]. These different methods of in vitro strain application produce different types of cellular deformation (Fig. 4). Variations in cell deformation induced by these methods of applying strain raise the question of whether bone cells respond differently to dissimilar types of strain. Numerous studies examining the effects of mechanical strain on bone cell function have reported a variety of different proliferative and/or differentiative responses. The cause for these disparate responses may reflect differences in the method of applying mechanical strain, making correlation between in vitro observations and in vivo studies difficult. Considering the way bone cells are probably loaded in vivo (i.e., by mechanical stretch and fluid shear, hypotonic swelling and biaxial stretch are the least physiological of the in vitro loading methods). Hypotonic swelling never occurs in bone under physiological conditions, but has been used, for technical reasons, to investigate mechanotransduction during patch-clamp analysis. Biaxial stretch does not accurately simulate the Poisson’s effect that occurs in mineralized tissue in vivo. Hydrostatic pressure almost never occurs in mineralized bone but can occur in bone marrow in the epiphyseal regions [75] and in the bone anlage during endochondral ossification [76, 77]. Therefore, in studies of mechanotransduction of marrow cells or mineralization of embryonic cartilage cultures [59, 60, 78], hydrostatic pressure may be an appropriate mechanical stimulus.

The response of bone cells to mechanical strain is modulated by the parameters of the strain applied. Application of static loads in vivo have no effect on bone formation [45, 79] yet dynamic loading of bone increases bone formation significantly when peak strains are greater than 1000 µstrain [80, 81]. This dichotomy of effects between static and dynamic loading is not as evident in cell culture experiments.
Similar responses have been observed for osteoblasts in culture whether exposed to static strains of 10,000 μstrain [63, 64] or dynamic strains of 20,000 μstrain [69]. It is the magnitude of these strains applied in the culture conditions that may produce these variable responses. Many of the studies of mechanical stretch in bone cell cultures have applied deformations ranging from 10,000 to 240,000 μstrain [62-64, 66, 68-70]. Since bone tissue only reaches strains of 1500-2500 μstrain during strenuous exercise and begins to fail when strains reach 7000 μstrain, the strains applied in the culture setting represent supraphysiological conditions. More recently, \textit{in vitro} experiments with osteoblast-like cells subjected to more physiological levels of strain (1700-3000 μstrain) have demonstrated both proliferative and synthetic responses [67, 71].

\textit{In vivo} loading studies in our laboratory have shown that mechanically induced bone formation is not increased if loading is applied at less than 0.5 Hz, but increases fourfold when loading frequency is increased to 2 Hz [79]. Loading frequency is proportional to strain rate within the bone tissue which, in turn, is approximately proportional to the degree of bone adaptation [46]. Frequency response to loading has not been established in the cultured osteoblast. Jones et al. [67] found no effect on cellular responses when load frequencies were varied from 0.5 Hz to 3.0 Hz \textit{in vitro}. However, Jones et al. did not use lower loading frequencies or static stretch as a comparison, so it is unclear whether dynamic loading is more effective for bone cells in culture. It is important to note that varying loading rates in culture causes bone cells to move through the culture medium at varying rates, thereby varying the amounts of fluid pressure or shear stress on the osteoblasts. These secondary fluid effects are confounding factors in the interpretation of results from culture experiments with different rates of applied stretch.

Several labs have demonstrated that the rate of stress-generated fluid flow within the bone matrix increases with increasing strain rate [49, 51, 53, 54, 79]. As a result, it is possible that fluid flow within bone \textit{in vivo} plays an important role in coupling mechanical loads into cellular signals. \textit{In vitro} studies have demonstrated that osteoblasts respond to fluid shear with increases in the cellular levels of inositol triphosphate (IP$_3$), cyclic AMP (cAMP), and prostaglandin E$_2$ (PGE$_2$) [72-74]. These data may explain the observations of increased bone mass in the skull and mandible made in simulated weightlessness studies in which humans are subjected to 6° head down tilt and rats were exposed to tail suspension [82, 83]. In these studies, a fluid shift occurred causing increased extracellular fluid pressures and perfusion in the head. Dillaman et al. [84] support this hypothesis, suggesting that the decrease in hindlimb bone mass during tail suspension studies is caused by a decrease in fluid flow in these regions.

A secondary effect of interstitial fluid flow in bone is the creation of electric potentials through the process of streaming. The surface of bone is negatively charged, thus cations in the interstitial fluid that is being forced through channels are attracted to the surface, producing a surplus of anions in the fluid. The voltage resulting from this imbalance of ions is positive in the direction of flow [52]. The streaming potentials produced by the flow of interstitial fluid in bone could produce a number of responses in osteoblasts including activation of voltage-operated channels in the cellular membrane, and could serve as mechanism of mechanotransduction. However, the studies of Reich et al. [73] suggested that the effects of fluid flow on osteoblasts was mediated by shear stresses on the cell rather than streaming potentials. More study of this important question is clearly warranted.

Another form of mechanical coupling involves microdamage that occurs in bone over time due to repetitive loading [85, 86]. Microdamage accumulates in bone very slowly under normal loading conditions, but when strains on the bone exceed 3000 μstrain, microdamage can accumulate rapidly [87]. This damage probably plays a role in initiating bone remodeling. Burr et al. [88, 89] have shown that microcracks within an osteon and along the cement line cause local bone resorption. However, it is unclear whether microdamage can cause modeling responses in bone that lead to increased bone mass.

Biochemical Coupling

Though the mechanism for the initial detection and conversion of mechanical force into a biochemical signal has yet to be determined, several likely candidates have been proposed. One possible transduction pathway is the extracellular matrix-integrin-cytoskeletal axis (Fig. 5). Cells attach to the extracellular matrix through binding to membrane span-
ning glycoproteins called integrins. Integrins attach to the actin cytoskeleton through several actin-associated proteins such as vinculin, talin, tensin, and α-actinin [90]. The cytoskeleton has been shown to form a network, connecting the extracellular matrix with the nucleus and the cytoplasmic constituents of the cell [91]. Modeling and experimental evidence indicate that the cell generates an internal force through the cytoskeleton which exerts a tension on the extracellular matrix [92–94]. This internal tension, similar in concept to the architectural system of tensegrity [95], produces forces on the adhesion sites of the cell in excess of those forces produced by exogenous mechanical stimuli [94]. Without attachment these internal forces would produce a spherical cell. The binding of integrins to the matrix proteins on a rigid substratum must therefore overcome the tensile forces of the cell, evoking changes in the cytoskeletal structure. Due to the tension of the cytoskeleton, physical stimulation would be rapidly transmitted to the nucleus, possibly altering gene expression. Indeed, cellular attachment to the extracellular matrix has been shown to play an important role in the regulation of cellular proliferation, differentiation, morphogenesis, and gene expression [94, 96–99]. Recruitment and/or differentiation of osteoblasts and osteoclasts similarly are modulated by cellular adhesion to the extracellular matrix [100, 101] and attachment of the osteoblasts to specific extracellular matrix proteins appears to be dependent on the differentiated state of the cell [102, 103]. Experimental evidence suggests that modulation of cellular function by attachment to the extracellular matrix occurs through changes in the cytoskeleton which, in turn, alters phenotypic expression. Chondrocytes assume a flattened morphology and do not express differentiation markers when grown in culture; however, when treated with cytochalasin B, a mold metabolite that induces repolymerization of filamentous actin, the cells assume a spherical shape and produce type IV collagen [104]. Furthermore, type IV collagen production is stimulated even when cytochalasin B is given at concentrations that induce actin repolymerization but do not alter cell shape [105]. These observations suggest that it is the modulation of the cytoskeleton, and not cell shape changes, that mediates alterations in gene expression during cell adhesion and mechanical stimulation.

Mechanical strain also has been shown to alter cell shape and cytoskeletal organization. When subjected to fluid shear stress, endothelial cells align parallel to the direction of flow [106]. This response to mechanical stimulation is accompanied by an increase in filamentous actin (F-actin) stress fibers which also align in the direction of flow [106]. Interestingly, when endothelial cells, fibroblasts, or osteoblasts are grown on flexible, silicone-bottomed culture plates and subjected to cyclic biaxial deformation, the cells align perpendicular to the major vector of strain [70, 106]. This realignment is also accompanied by an increase in cytoskeletal stress fibers aligned in the same direction as the cell. These observations suggest that different types of mechanical strain produce different cellular responses.

Integrins are composed of two subunits, denoted as α and β, both of which are required for cell adhesion [107, 108]. Numerous α and β subunits have been identified and sequenced. These subunits can be interchanged which permits different binding specificities for different extracellular matrix proteins [107, 108]. Ligand binding to specific integrins has been implicated in a number of bone cell functions, including attachment and differentiation [109, 110] and bone formation and resorption [111]. In addition, integrin stimulation has been associated with increases in intracellular second messengers [112–115], tyrosine phosphorylation [116], and Na+/H+ exchange [117, 118]. Integrins have been directly linked to the cellular response to mechanical strain as well [119]. When endothelial cells are subjected to shear stress, integrins rapidly realign with the direction of flow, indicating that cell adhesion is a dynamic process responding to mechanical strain [120]. Furthermore, physical strain applied directly to integrins using a magnetic twisting device is resisted by the cytoskeleton [121]. These results suggest that the extracellular matrix-integrins-cytoskeletal axis plays an active role with the signal transduction of mechanical strain.

Since the first observation that ion channels can be gated by mechanical strain [122], mechanotransduction has been proposed as a primary function of these channels. Moreover, mechanosensitive channels are likely candidates for the initial biochemical coupling mechanism of mechanical strain since no second messenger is required for channel activation [123]. Mechanosensitive channels make up a large family of channels which can be subdivided based on their activation properties, kinetic characteristics, and ion selectivity [124]. Stretch activated, cation nonselective (SA-cat) channels have been identified in both rat [125] and human [126] osteoblast-like cells. We have recently reported that chronic, intermittent mechanical stretch increases SA-cat channel ac-

**Fig. 5.** Diagram illustrating the cytoskeletal components at the point of attachment with the extracellular matrix in vitro (adapted from Pavalko et al. [90]). The integrin, made up of two heterodimers, α and β, spans the plasma membrane of the cell. The extracellular domain binds to extracellular matrix proteins. The intracellular domain interacts with either talin or α-actinin. Either of these proteins, in turn, attach to actin. Vinculin and paxillin also may have a role in local adhesions. Other proteins, e.g., tensin, have been identified in focal adhesions but are not shown here.
Mechanotransduction in Bone

R. L. Duncan, C. H. Turner: Mechanotransduction in Bone

Fig. 6. Effects of chronic, intermittent mechanical stretch on SA-cat single channel kinetics. Panel A illustrates that SA-cat channel activity was increased in chronically strained UMR-106.01 osteoblast-like cells, even though the negative pressure applied to the patch was smaller than in control cells. Examination of the single channel amplitudes showed that following 2 hours of chronic strain, single channel amplitude was significantly shifted (B). Noting the smaller openings and closings of the channel from the stretched cell (A, arrowheads), these data suggest that chronic strain modulates the SA-cat channel in two ways: (1) by increasing channel activity and (2) by increasing single channel conductance. This increase in single channel conductance could be due to the activation of an additional conductance state of the same channel. However, the gaussian distribution of the amplitude histogram does not rule out the possibility of a second channel being activated by chronic strain (adapted from Duncan and Hruska [56], used with permission).

Fig. 7. Effects of chronic, intermittent mechanical stretch on SA-cat single channel kinetics. Panel A illustrates that SA-cat channel activity was increased in chronically strained UMR-106.01 osteoblast-like cells, even though the negative pressure applied to the patch was smaller than in control cells. Examination of the single channel amplitudes showed that following 2 hours of chronic strain, single channel amplitude was significantly shifted (B). Noting the smaller openings and closings of the channel from the stretched cell (A, arrowheads), these data suggest that chronic strain modulates the SA-cat channel in two ways: (1) by increasing channel activity and (2) by increasing single channel conductance. This increase in single channel conductance could be due to the activation of an additional conductance state of the same channel. However, the gaussian distribution of the amplitude histogram does not rule out the possibility of a second channel being activated by chronic strain (adapted from Duncan and Hruska [56], used with permission).

Activity and single channel conductance in rat osteoblast-like cells [56] (Fig. 6). In addition, mechanical strain enhanced stretch sensitivity of these channels and induced spontaneous channel activity. These alterations in channel kinetics indicate that chronic, intermittent mechanical strain primes the SA-cat channel to respond to additional perturbation.

Guharay and Sachs [122] have proposed that, although stretch activated channels respond to changes in tension of the lipid membrane in response to mechanical strain, this tension is focused on the stretch channels by the cytoskeleton. However, using cytoskeletal severing agents, they were unable to link the tubulin or actin filaments with mechanosensitive channel activation in chick skeletal muscle. We also have examined the interaction of the actin cytoskeleton on SA-cat channels in the osteoblast based on three observations. First, we have demonstrated that parathyroid hormone (PTH) modulates SA-cat channel kinetics in a manner similar to chronic mechanical strain [127]. Second, PTH has been shown to induce a stellated morphology in osteoblasts in primary culture [128], which has been attributed to the reorganization of the actin microfilaments [129–131]. Third, channel studies in renal cells have demonstrated that depolymerization of actin using cytochalasins activates the epithelial sodium channels [132]. We have demonstrated that, when F-actin is repolymerized using cytochalasin D, SA-cat channel activity increases 10-fold within 4 minutes of application (Fig. 7) [133]. These data suggest a tight interaction between the cytoskeleton and the stretch-activated channels in the osteoblast.

Other channels besides the SA-cat channel could influence the osteoblastic response to mechanical strain. Olesen et al [134] have identified potassium-selective channels which respond to fluid shear in vascular endothelial cells. These channels are activated by flow rates below those which would activate stretch-activated channels in the endothelium [135]. This channel is rapidly activated by flow, remains active for the duration of flow, but immediately inactivates upon removal of fluid shear. The authors suggest that this potassium channel may be responsive to flow whereas the SA-cat channel in endothelial cells would be more sensitive to stretch. This would be physiologically advantageous to the control of vascular flow since there is a high degree of variability of the hemodynamic forces in arterial circulation [134, 136]. Potassium channels have been identified in osteoblasts [137], however, no determination of their mechanosensitivity has been made.

Upon application of stretch to the substrata, osteoblasts experience an almost instantaneous, large, transient increase in intracellular calcium [138]. This increase appears to initially arise from the release of intracellular stores, followed by calcium entry through ion channels [138]. One proposed mechanotransduction mechanism that could explain this...
rapid release of calcium is a direct link of the cytoskeleton with the phospholipase C pathway [138]. Phospholipase C activates the protein kinase C pathway, which in turn produces inositol triphosphate (IP$_3$) and diacylglycerol. IP$_3$ stimulates the release of calcium from intracellular stores. Deformation of the cell due to mechanical strain would physically dislocate a proposed phospholipase C inhibitor attached to the cytoskeleton (D. Jones, personal communication). The removal of this inhibitor would allow phospholipase C to activate. A similar mechanism has been proposed in a tumor suppressor gene for colorectal carcinoma [139].

Another possible mechanism for mechanotransduction at the cell membrane involves guanine nucleotide binding proteins (G proteins). In cultured endothelial cells exposed to fluid flow, nitric oxide is produced in an initial burst followed by sustained steady-state production. The G protein inhibitor guanosine 5'-O-(2-thiodiphosphate) blocks the flow-mediated burst in nitric oxide production [140]. This inhibition of nitric oxide was not sustained and was shown to be nonresponsive to pertussis toxin.

Though each of these candidates have been treated as a primary mechanotransduction mechanisms, it should be noted that they have a high degree of association with one another. The tight interaction of each of these pathways suggests that the entire cell is required to sense mechanical stimulation and that there is no single transduction pathway. The observations that endothelial cells align differently in response to fluid shear or biaxial mechanical strain suggests that different mechanical signals may influence the cellular response by stimulating these interconnected pathways differently. For example, fluid shear applied to the osteoblast may stimulate the stretch-activated cation channels or G proteins initially creating a different response than biaxial stretch which could be signaled through the integrin-cytoskeletal network.

Transmission of Biochemical Signal

There are two possible pathways by which a biochemical signal in the sensor cell is propagated to the effector cell to increase osteogenic activity after a mechanical stimulus. First, active osteoblasts on the bone surface can sense mechanical strain and also act as the effector cell that increases bone formation products. This scenario is supported by observations that osteoblast-like cells increase expression and production of matrix proteins in response to cyclic mechanical stretch [141, 142]. However, active osteoblasts make up only 5% of the bone surface in the adult human; 94% of the bone surface is covered by bone lining cells (the other 1% is covered by osteoclasts) [143]. Therefore, stimulation of active osteoblasts alone is not sufficient to create a modeling response that substantially increases bone mass. Osteocytes and bone lining cells make up over 95% of all cells of osteoblastic lineage that are attached to the bone [143-145]. These cells are responsive to mechanical loading in vivo [146-148] and have the ability to communicate with other bone cells through an extensive network of cellular processes connected at gap junctions [149-153]. However, osteocytes and bone lining cells cannot proliferate or produce substantial amounts of new bone matrix. Therefore, it is probable that a second means of communication of the strain stimulus exists which involves communication of a biochemical signal from nonproliferative, strain sensing cells (osteocytes and bone lining cells) to osteoprogenitor cells and osteoblasts through paracrine factors (Fig. 8).

Mechanosensitive cells, including osteoblasts [63-66, 69, 71-74], have been shown to respond to mechanical strain with increased levels of second messengers [154-156]. Most mechanosensitive cells respond to mechanical strain with a rapid elevation of cAMP which has been associated with growth and proliferation [142, 154, 155]. In osteoblasts, cAMP is significantly increased after 5 minutes of mechanical stretch [69, 157] and within seconds of application of fluid shear [73]. The exact mechanism of stimulation of adenylate cyclase is unknown, but disruption of the cytoskeleton has been shown to increase levels of cAMP [158, 159]. Inositol phosphates also increase with application of strain in osteoblasts [67, 69, 71, 72, 138]. Inositol triphosphate levels significantly increase within seconds of strain application [67, 71, 138] supporting the postulate of a phospholipase C-activating mechanosensor associated with the cytoskeleton [138]. However, Jones and Bingman [138] have examined the time course of release of second messengers in the osteoblast and have found that the increase in intracellular
concentration of calcium precedes the rise in inositol triphosphates in osteoblasts. These data suggest that calcium entry into the cell through activation of channels may be the initial cellular signal for the osteoblastic response to mechanical strain. This postulate is supported by the observation that cyclic loading increases calcium incorporation into osteoblasts and that this incorporation can be blocked by the calcium channel inhibitor verapamil [160].

The strongest evidence for paracrine communication of a mechanical signal from osteocytes to osteoblasts is provided by the experiments of Lean et al. [161]. They have shown that insulin-like growth factor I (IGF-I) expression increases in osteocytes 6 hours after loading followed by increased expression of type-I collagen and osteocalcin on the bone surface after 48 hours. These data can be interpreted to mean osteocytes are producing anabolic growth factors that are transported to the bone surface and recruit osteoprogenitor cells. IGF-I stimulates bone matrix formation and bone cell proliferation in rat calvaria cultures [162] and bone formation in vivo [163]. IGFs are thought to act as an intermediary for the anabolic effects of PTH [164, 165]. IGF-II production by osteoblasts in culture can be stimulated by low-frequency electric fields [166]. Therefore, it is possible that electric fields caused by stress-generated streaming currents in bone might cause anabolic effects through production of IGF-II by cells of osteoblastic lineage.

Other paracrines released by osteoblasts in response to mechanical strain are prostaglandin E (PGE$_2$) [61, 63, 65, 66] and prostacyclin [167]. Both of these prostaglandins are released by bone in organ culture after a mechanical loading stimulus [167] and, when prostaglandin production is blocked by indomethacin, the anabolic effect of mechanical load in vivo is greatly depressed [168]. Furthermore, prostacyclin infusion causes early metabolic changes in osteocytes and bone lining cells similar to mechanical loading [169]. Prostacyclin also increases IGF-II release in bone culture [169]. PGE$_2$ has anabolic effects on bone [170-179] and has been shown to stimulate proliferation [180-182], alkaline phosphatase activity [183], and collagen synthesis [182, 184] in cultured bone cells. Perhaps a more important function of PGE$_2$ is the recruitment of osteoblast precursor cells. PGE$_2$ has been shown to increase preosteoblast proliferation in rat calvarial organ cultures [185] and promote attachment of these precursor osteoblasts [186]. Therefore, PGE$_2$ would not only stimulate osteogenic function in existing osteoblasts but would increase the production of osteoblasts through recruitment and differentiation of precursor cells into active osteoblasts.

The paracrine communication resulting from a mechanical stimulus may be similar to the anabolic effects of PTH. Intermittent administration of PTH causes a modeling response in bone that increases bone mass [187-191]. The anabolic effects of PTH may be mediated by the same intermediary as that proposed for mechanical loading effects, namely, IGF-I or IGF binding proteins [164, 165, 192]. IGF-I antibodies prevented the PTH stimulation of collagen synthesis in culture [193], and IGF binding protein 3 levels increased after infusion of PTH in human subjects [192]. The anabolic effects of PTH on trabecular and endocortical surfaces of bone are thought to result from a stimulation of differentiation of osteoprogenitor cells in the bone marrow [194-195]. PTH also stimulates metabolic activity in osteocytes and lining cells that mimics the effects of mechanical loading [196]. It is unknown whether osteocytes and lining cells produce paracrine factors after PTH stimulation, and that question is worthy of more study.

**Effectors Response**

Over the years, numerous techniques have been developed to examine the effects of exogenous mechanical strain on bone homeostasis including overload of the radius by ulnar osteotomy [197-199], external force application through implanted pins [41, 80, 200-203], unilateral hind-limb immobilization [204-209], compression of rat caudal vertebrae [210-211], in vivo axial loading of rat ulnae [212], and external force application in rat tibiae using 4-point bending [79, 81, 213-215]. These studies have demonstrated several important characteristics of the in vivo effects of mechanical loading on bone.

Only dynamic loading causes anabolic effects on bone tissue in vivo: the greater the rate of change of applied strain in bone the more bone formation is increased. This important observation has been replicated several times [45, 79, 216] and demonstrates that static stretch of cells in situ is not sufficient to activate a bone modeling response.

The absence of mechanical loading leads to increased bone turnover and negative bone balance [41, 80, 204-206, 209]. Application of dynamic mechanical loads that cause 1000 µstrain in the bone tissue for 100 load cycles per day inhibits bone resorption and maintains bone mass [41]. Application of mechanical strains greater than 1000 µstrain causes increases in bone modeling that leads to increased bone mass [41, 81]. The effect of applied strains on bone is dictated by the magnitude and duration of the applied load. Lower magnitude loads applied for longer durations can cause the same anabolic effects as larger loads. For instance,
Rubin and Lanyon [41, 80] demonstrated that an applied strain of 2050 μstrain applied for four cycles per day produced the same maintaining effect on bone mass in immobilized limbs as an applied strain of 1000 μstrain applied for 100 cycles per day.

Anabolic responses occur in bone only if the mechanical loads surpass a threshold [81, 211]. We have shown that lamellar bone formation on the endocortical surface of rat tibiae increases linearly with increasing load for applied dynamic bending loads above 40 N [Fig. 9]. The bone formation rate on the endocortical surface of the tibia was six fold higher than control levels for an applied load of 64 N [81]. However, for applied bending loads less than 40 N, no increase in bone formation was observed. Chow et al. [211] showed no increase in lamellar bone formation in the rat caudal vertebra for applied dynamic loads of 15 and 50 N, but a load of 150 N increased the bone formation rate significantly. These findings are consistent with Frost's minimum effective strain (MES) concept that is illustrated in Figure 1 [24]. It is important to note that these findings are also consistent with a positive relationship between bone formation and strain rate [46]. This is because the maximum strain rate was proportional to the peak strain magnitude in the foregoing study.

Despite methodological differences, the timing of events leading to new bone formation is similar among different models of mechanical loading. After a single period of mechanical loading in vivo, there is a delay of 3–5 days before increased collagen and mineral apposition are observed on the bone surface [146, 148, 197]. The events that precede bone formation include immediate release of prostacyclin from osteocytes and lining cells, and a loading-related increase in glucose-6-phosphate dehydrogenase (G6PD) 5 minutes later, followed 6–24 hours later by increases in RNA synthesis and IGF-1 message in osteocytes [161, 196]. We recently demonstrated that 4-point loading in the rat tibia did not increase bone formation rate (BFR) over the first 5 days following loading, but BFR was significantly increased in the period between day 5 and 12, largely due to increases in bone-forming surface [214]. These results suggest that each loading bout activates a packet of osteoprogenitor cells that differentiate and start forming osteoid about 4 days after activation. In vivo modeling responses in bone occurring after a mechanical loading stimulus may involve activating packets (quanta) of cells with each new loading bout. Thus, a "quantum concept" similar to that applied to bone remodeling [6, 217], may be equally applicable to bone modeling during adaptation.

The ability of the skeleton to respond to mechanical stimuli is greatly reduced in older animals. Rubin et al. [218] demonstrated that the bone modeling response in 3-year old turkeys was almost nonexistent after a mechanical loading stimulus that activated exuberant bone formation in 1-year old turkeys. Likewise, recent data from our laboratory demonstrate that bone formation rate on the endocortical surface of the tibia was 16-fold less in 19-month old rats after a mechanical loading stimulus than it was in 9-month old rats [219]. It is unclear what age-related changes are responsible for this reduction in the effectiveness of mechanotransduction in older animals. For instance, it is not known what step or steps of mechanotransduction are rendered less effective with age. This seems a fruitful area for future research.

The interaction between mechanotransduction and hormones, although postulated by Frost [26] in the mechanostat hypothesis, has not been demonstrated definitively in experimental systems. However, the results from many studies suggest an effect of hormones on mechanical adaptation. For example, Burkhart and Jossey [220] demonstrated that intact parathyroid and thyroid glands were necessary for the development of disuse osteopenia in dogs. Their results suggest that a local change in the region of disuse acts by increasing the sensitivity of the bone to stimulation of resorption by thyroid or parathyroid hormones. PTH treatment also has been shown to stimulate the same metabolic changes in osteocytes and bone lining cells in tissue culture that are observed after a mechanical stimulus [196]. This suggests that the anabolic effects of PTH result from a similar biochemical pathway as mechanical loading effects. Indeed, both PTH and mechanical strain produce similar changes in the activation and kinetics of the mechanosensitive channel of the osteoblast [56, 127]. Another hormone that is indirectly linked with mechanical loading effects is estrogen. The rapid bone loss observed following loss of estrogen due to ovariectomy in adult animals, or menopause in women, is morphologically and temporally identical to that observed following disuse [221]. These results have been interpreted by Frost as a shift in the remodeling MES caused by the loss of estrogen such that estrogen deprivation mimics disuse [26]. Furthermore, estrogen receptors in bone are predominantly located in the osteocytes [222] which are often considered sensors for mechanical loads, suggesting that estrogen could modulate the sensitivity of osteocytes to mechanical stimuli. None of these results by themselves prove that hormones modulate the effects of mechanical stimuli but, when taken together, become provocative. The interaction between local mechanical effects and system hormones, if proven, could play a critical role in normal bone biology and, when aniss, may contribute to osteoporosis.

Conclusions

Mechanotransduction plays a crucial role in the physiology of many tissues including bone. The response and adaptation to local physical stimuli allows organisms to be better adapted to life in a gravitational field. The importance of gravity in determining bone size and shape was first discussed by Galileo in 1638 [223]. Since then, the influence of
mechanical forces on the ontogeny and phylogeny of the skeleton has been examined by countless investigators [e.g., 1-3, 5, 6, 224, 225]. Today, our knowledge of mechanotransduction in bone is still fairly primitive, but recent experiments have begun to outline some of its mechanisms. What is needed is a conceptual framework to tie the experimental findings together. We propose that mechanotransduction in bone can be divided into four distinct steps: (1) mechanotransduction, (2) biochemical coupling, (3) transmission of signal, and (4) effector cell response.

Mechanical loads in vivo cause deformations in bone that stretch bone cells within and lining the bone matrix. Normal loading is dynamic and creates fluid movement within the canalicles of bone. Bone cells in culture are stimulated to produce second messengers when exposed to mechanical stretch or fluid flow. However, the levels of stretch (strain) used in most of these studies were 5-100 times the normal strain levels that are found in living bone. Experimental studies in vivo have shown that static deformation of bone tissue does not cause an increase bone formation, but cyclic loading can increase it significantly. Thus, dynamic loading, which is associated with extracellular fluid flow and the creation of streaming potentials within bone, is most effective for stimulating new bone formation in vivo.

Several mechanisms have been described for the coupling of an external mechanical signal into an intracellular biochemical signal. These include force transduction from the extracellular matrix to the cytoskeleton and nuclear matrix through the integrins, stretch-activated cation channels within the cell membrane, G protein-dependent pathways in the cell membrane, and linkage between the cytoskeleton and the phospholipase C or phospholipase A pathways. These signal pathways are not independent as they have a high degree of association with one another. The tight interaction of each of these pathways would suggest that the entire cell is a mechanosensor and there are many different pathways available for the transduction of a mechanical signal. The variety of mechanical stimulus, i.e., fluid shear or mechanical stretch, perceived by the cell probably determines which pathway for mechanotransduction will be activated.

In culture, mechanical stimulation of osteoblasts leads to increased matrix formation, so the signal pathway from stimulus to response is straightforward. The mechanism for increasing bone formation in vivo is probably more complicated. In adult bone, only 5% of the surface is lined with active osteoblasts; the rest of the surface is either resorbing or quiescent. Even if all of these osteoblasts were activated by a mechanical stimulus, the resulting increase in bone formation would not be sufficient to create a bone modeling response that significantly increases bone formation. More osteoblasts must be recruited to the bone surface to facilitate an adaptive modeling response. These osteoblasts probably come from osteoprogenitor cells in the bone marrow or periosteum. The osteoprogenitors may be signaled to differentiate into osteoblasts and attach to the bone surface by paracrine factors produced by osteocytes or bone lining cells. IGF and prostaglandin production following a mechanical stimulus have been observed in osteoblasts and lining cells. Prostaglandin infusion creates the same metabolic changes in osteocytes as is seen after a mechanical stimulus, and inhibition of prostaglandins using indomethacin inhibits the mechanically induced bone formation response in vivo. Also, IGFs and some prostaglandins have anabolic effects on bone in vivo and stimulate matrix production by bone cells in vitro.

Mechanical loading can inhibit bone resorption and increase bone formation in vivo. The effects of mechanical loading are dependent upon the magnitude, duration, and rate of the applied load. Longer duration, lower amplitude loading has the same effect on bone formation as loads with short duration and high amplitude. Loading must be cyclic to stimulate new bone formation. Bone formation is roughly proportional to the rate of change of the applied load. There is a delay of 3–5 days after a mechanical stimulus before bone formation is observed on bone surfaces. Presumably this delay reflects the time required for communication of the mechanical signal to osteoprogenitor cells in the bone marrow or periosteum and proliferation and differentiation of osteoprogenitor cells into active osteoblasts. Aging greatly reduces the osteogenic effects of mechanical loading in vivo. Also, some hormones may interact with local mechanical signals to change the sensitivity of the sensor or effector cells to mechanical load.

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