Mechanical Stimulation of Skeletal Muscle Generates Lipid-Related Second Messengers by Phospholipase Activation[1]

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Running Title: Stretch-activated Phospholipid Breakdown

Text Figures: 7

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[1]Supported by NIH AR39998 and NASA NAG2-414.

Abstract

Repetitive mechanical stimulation of cultured avian skeletal muscle increases the synthesis of prostaglandins E_2 and $F_{2\alpha}$ which regulate protein turnover rates and muscle cell growth. Mechanical stimulation significantly increases the breakdown rate of ^{3}H-arachidonic acid labelled phospholipids, releasing free ^{3}H-arachidonic acid, the rate-limiting precursor of prostaglandin synthesis. Mechanical stimulation also significantly increases ^{3}H-arachidonic acid labelled diacylglycerol formation and intracellular levels of inositol phosphates from myo-[2-^{3}H]inositol labelled phospholipids. Phospholipase A_2, phosphatidylinositol-specific phospholipase C (PLC), and phospholipase D (PLD) are activated by stretch. The lipase inhibitors bromophenacylbromide and RHC80267 together reduce stretch-induced prostaglandin production by 73-83%. The stretch-induced increases in prostaglandin production, ^{3}H-arachidonic acid labelled phospholipid breakdown, and ^{3}H-arachidonic acid labelled diacylglycerol formation occur independently of cellular electrical activity (tetrodotoxin insensitive) whereas the formation of inositol phosphates from myo-[2-^{3}H]inositol labelled phospholipids are dependent on cellular electrical activity. These results indicate that mechanical stimulation increases the lipid-related second messengers arachidonic acid, diacylglycerol, and prostaglandins through activation of specific phospholipases such as PLA_2 and PLD, but not by activation of phosphatidylinositol-specific PLC.

Index Terms: Phospholipids; prostaglandins; skeletal muscle growth; diacylglycerol; inositol phosphates; phospholipase A_2; phospholipase C; phospholipase D.

Introduction

Skeletal muscle growth is regulated by many diverse factors including steroids, polypeptide growth factors, innervation, nutritional status, electrical activity, and tension development. Many second messengers are involved in transducing these varied growth regulators into alterations in muscle protein turnover rates. Prostaglandins are included in this group of second messengers and regulate muscle growth responses to insulin and tension/mechanical force (reviewed in Palmer, 1990). In tissue culture, repetitive passive stretch/relaxation of differentiated avian myofibers grown on an elastic substratum stimulates myofiber hypertrophy (Vandenburgh et al.1989) and this growth is coupled to stretch-induced increases in the production of prostaglandins (PG) E_2 and $F_{2\alpha}$ (Vandenburgh et al.1990). Similar mechanically-induced changes in prostaglandin production occur in adult organ cultured skeletal muscle (Smith et al.1983; Palmer et al.1983) and in exercising animals (Young and Sparks, 1979). Prostaglandin production is also increased by mechanical forces in a number of other cell types, including bone (Somjen et al.1980; Klein and Raisz, 1970) and endothelial cells (Frangos et al.1985). The mechanisms by which stretch alters prostaglandin production and by which prostaglandins regulate stretch/tension induced skeletal muscle growth are not known.

There are two main regulatory steps in cellular prostaglandin production. The first is the availability of arachidonic acid, the polyunsaturated fatty acid precursor of prostaglandin synthesis

(Irvine, 1982). Arachidonic acid is liberated from the 2-acyl position of phospholipids by the action of phospholipase A_2 (PLA_2) or phospholipase C and D (PLC, PLD) activity coupled to diglycerol lipase activity (Irvine, 1982). The second regulatory step is the modulation of the activity of cyclooxygenase which converts free arachidonic acid into the prostaglandin pathway (Raz et al.1988). As a first step in determining the mechanism by which mechanical force stimulates prostaglandin production in skeletal muscle, we have analyzed the effects of repetitive stretch/relaxation of cultured avian myofibers on the breakdown rate of cellular phospholipids, the major storage reservoir of arachidonic acid (Irvine, 1982). The results indicate that repetitive mechanical stimulation of the cells increases the breakdown rate of arachidonic acid containing phospholipids by specific activation of several phospholipases and that the stretch-induced increases in PGE_2 and $PGF_{2\alpha}$ are sensitive to lipase inhibitors. One mechanism for stretch-induced increases in prostaglandin production is thus by providing greater levels of free arachidonic acid for subsequent prostaglandin synthesis. In addition, stretch significantly increases diacylglycerol formation in the cultured cells by a mechanism which is independent of phosphatidylinositol specific PLC. This lipid-related second messenger may play a role in stretch regulation of skeletal muscle growth, possibly by activating protein kinase C (Komuro et al.1991).

Materials and Methods

Cell Cultures

Avian myoblasts are isolated from 11 to 12 days *in ovo* pectoralis muscle by standard dissection techniques (Vandenburgh et al.1988). Fertilized chicken eggs are obtained from Beaver River Farm (West Kingston, RI). The cells are plated at a very high density of 5,700 cells/mm^2 in 1 ml of growth medium and maintained in a humidified 5.0% CO_2 incubator at 37°C. Growth medium is Eagle's basal medium containing 50 U/ml penicillin (Sigma Chemical, St. Louis, MO), 10% horse serum (Hyclone Laboratories, Logan, UT), and 5% chicken embryo extract (85/10/5 medium). Cells are plated and grown in collagen-coated 15 mm diameter culture wells of a mechanical cell stimulator device. At 48-54 h postplating, the cells are embedded in a collagen gel matrix (Vandenburgh et al.1988). Briefly, the culture medium is removed and 0.5 ml of ice-cold collagen solution is added. The collagen solution is prepared immediately before use by mixing 85/10/5 medium with rat tail collagen (Type I, Collaborative Research, Bedford, MA) to a final concentration of 200-400 μg/0.5 ml. Sterile sodium hydroxide (0.1N) is added to the chilled solution to maintain a pH of 7.0. Stainless steel wire cloth (mesh no. 50, Newark Wire Cloth, Newark, NJ) is placed inside the perimeter of each well immediately after the collagen gel solution is added but before its polymerization. The screen acts as an "artificial tendon" by providing a surface to which the differentiating cells and collagen gel attach and are

held under tension during long-term growth. Culture medium (85/10/5) is added to the top of the hardened collagen gel 24 h after solidifying and changed every 24 h thereafter. After several days, the collagen gel dehydrates, collapsing on top of the muscle cells as a thin 12-50 μm layer (Vandenburgh et al.1988) in the same manner as described for other cell types (Bell et al.1979). Under these growth conditions, the myogenic cells proliferate and fuse during the first 24 to 72 h in vitro, become striated and highly contractile by 96 to 120 h, and remain so for the duration of the experiments. Day 6-12 postplating cultures contain 40-50% of their nuclei in mononucleated cells, the majority of which are proliferating fibroblasts. Eighty-five to ninety percent of the noncollagenous protein in the cultures is located in the much larger multinucleated skeletal myofibers based on morphometric analysis of cell volumes (Vandenburgh et al.1989). Total noncollagenous protein per well is measured as described in reference (Vandenburgh et al.1989). In the experiments where avian fibroblasts are cultured, they are isolated from fused muscle cultures (7 days postplating) by trypsinization (0.2 % trypsin in calcium magnesium free saline), centrifugation, and filtration through 20 μm^2 nylon mesh. The fibroblasts are subcultured once before culturing in the mechanical cell stimulator device.

Mechanical Stimulation

The mechanical cell stimulator (Model 1) used to repetitively stretch and relax the culture substratum and the attached muscle

cells has been described previously (Vandenburgh, 1988). The cell growth chamber of the device contains 24 15-mm-diameter wells. Twelve of the wells are static controls whereas 12 wells are mechanically stimulated by circular prongs positioned under each well. There is a linear relationship between stretch of the substratum and stretch of the myofibers in this device (Vandenburgh, 1988). Mechanical activity program TRIAL51.PGM is used in all experiments and consists of five 20% substratum stretches and relaxations during a 20 s period followed by a 10 s rest; this activity is repeated twice more and is followed by a rest period of 15 min. Cells are thus mechanically active for 6.7% of the experimental time. The rate of stretch and relaxation is 2.5 mm/s. This frequency of mechanical activity is optimal for increasing prostaglandin production in the cultured cells (Vandenburgh et al.1990). Unstimulated controls are maintained in the same set-up and on the same elastic substratum as the cells that are mechanically stimulated. The mechanical cell stimulator is kept on a rotary shaker (20-30 rpm) in a water-jacketed 5% CO_2 incubator at 37°C during the period of mechanical activity. All experiments are repeated with at least two different preparations of primary cells.

Prostaglandin Assays

PGE_2 and $PGF_{2\alpha}$ production are assayed in the culture medium using commercial radioimmunoassay kits (Advanced Magnetics, Cambridge, MA) as outlined previously (Vandenburgh et al.1990). The

cross-reactivities of the antibodies at 50% binding (B)/initial binding (B_0) ratio are PGE_2 antibody: 1.3% for $PGF_{2\alpha}$, < 1% for 6-keto-$PGF_{2\alpha}$; $PGF_{2\alpha}$ antibody: 0.3% for PGE_2, 1.1% for 6-keto-$PGF_{2\alpha}$.

Phospholipid Labelling and Analysis

Day 5 to Day 7 postplating muscle cultures are prelabelled overnight (16-20 h) with 0.5 - 2 μCi/ml of ^{3}H-arachidonic acid, [methyl-^{14}C] choline, or myo-[2-^{3}H]inositol (Amersham, Chicago) in complete 85/10/5 medium. The cultures are rinsed for 1-3 h at 37° on a rotary shaker (40-60 rpm) and the rinse medium changed every 20 to 30 min to remove unincorporated radioactivity. Fresh 85/10/5 medium or unsupplemented Eagle's basal medium (500 μl) is added to each well and mechanical activity initiated for up to 24 h. Results on phospholipid breakdown are similar whether mechanical stimulation is performed in 85/10/5 medium or basal medium. For experiments performed in calcium-free medium, the rinses and experimental medium are calcium-free unsupplemented Eagle's basal medium. For cells labelled with myo-[2-^{3}H]inositol, the culture medium during mechanical stimulation also contains 10 mM LiCl to inhibit inositol phosphate breakdown. In the experiments in which phospholipase D activity is measured, the culture medium during mechanical stimulation contains 0.5 - 1.0% (v/v) ethanol.

The ^{3}H-arachidonic acid labelled total phospholipids are extracted following the method of Dixon and Hokin (Dixon and Hokin, 1984). Briefly, the cells are rinsed with phosphate buffered saline and homogenized first in 750 μl/well 0.2 M Tris-HCl, pH 7.4

followed by a further homogenization in 3 ml of methanol: chloroform: hydrochloric acid 2:1:0.032, v/v/v. One ml of chloroform followed by 1 ml Eagle's basal medium is added to each sample while vortexing. Phases are separated by centrifugation at 3000 x g for 5 min. The lower organic phase is removed and the upper aqueous phase washed with 2 ml chloroform. The organic phase wash and original extract are combined and dried under nitrogen. Phospholipids are separated from free arachidonic acid by thin layer chromatography. The lipid residue is first dissolved in a small volume of chloroform: methanol (1:1, v/v), and an aliquot counted for radioactivity. Phospholipid and arachidonic acid standards are applied with each sample for chromatography on Chromagram sheets (Kodak, Rochester, NY) using benzene: dioxane: acetic acid (20:20:1) as solvent (Betteridge, 1980). Lipids are visualized by charring with iodine vapors, the spots are cut from the plates and the radioactivity in each spot determined by liquid scintillation counting.

^{3}H-arachidonic acid labelled neutral lipids are extracted following the same procedure as for total phospholipids. Dried lipid extracts are dissolved in a small volume of chloroform, an aliquot is counted for radioactivity, and the remainder of each sample is mixed with 15 μg each of monolein, diolein (1,2 isomer), triolein, and arachidonic acid as carriers for chromatography. Neutral lipids are separated on Chromagram Plates (Kodak) using hexane: diethylether: glacial acetic acid (65/35/4, v/v), and lipid spots are visualized by iodine charring. The radioactivity is

determined in each spot by liquid scintillation counting.

The extraction of water soluble myo-[2-^{3}H]inositol labelled compounds is identical to the phospholipid extraction method of Dixon and Hokin (Dixon and Hokin, 1984) through the first centrifugation step. Three ml of the upper aqueous phase is removed and applied to 1 ml Dowex 1-X8 columns (Formate form). Free ^{3}H-inositol is eluted from the column with distilled water and glycerophosphoinositols are eluted with 5 mM disodium tetraborate/60 mM sodium formate before stepwise elution of the inositol phosphates with a formic acid/ammonium formate step gradient (Berridge et al.1983). The radioactivity in the inositol phosphate fractions is measured by liquid scintillation counting.

Phospholipase A_2 activation is measured as an increase in arachidonic acid free lysophosphatidylcholine (Bar-Sagi and Feramisco, 1986). The cells are labelled overnight with both ^{3}H-arachidonic acid and [methyl-^{14}C] choline. They are rinsed and mechanically stimulated as described previously for total phospholipids. The phospholipids are extracted by the method of Ben-Sagi and Feramisco (Bar-Sagi and Feramisco, 1986). Lysophosphatidylcholine is separated from phosphatidylcholine on LK6D thin layer chromatography plates (Whatman) using chloroform:methanol:acetic acid:water (75:45:12:3) as the solvent (Bar-Sagi and Feramisco, 1986). Standards are applied with the samples and phospholipids are visualized by charring with iodine vapor. Spots are scraped from the plates and radioactivity determined by liquid scintillation spectrometry.

Phospholipase D activity is assayed by the method of Yang, Freer, and Benson (Yang et al.1967). Phospholipase D catalyzes not only the hydrolysis of ^{3}H-arachidonic acid labelled phospholipids to generate phosphatidic acid, but also catalyzes a transphosphatidylation reaction in the presence of ethanol with the resulting production of ^{3}H-arachidonic acid labelled phosphatidylethanol. To assay for phospholipase D activity mechanical stimulation is performed in the presence of 0.5 - 1.0% (v/v) ethanol, the ^{3}H arachidonic acid labelled phospholipids are extracted by the method of Dixon and Hokin (Dixon and Hokin, 1984), and ^{3}H-arachidonic acid labelled phosphatidylethanol isolated by thin layer chromatography with chloroform: methanol: acetic acid (65:15:2, v/v/v) as the solvent system (Pai et al.1988). Static controls are incubated with an identical volume of ethanol. Phosphatidylethanol standard for thin layer chromatography is prepared from the reaction of phosphatidylcholine with purified phospholipase D (Sigma Chem. Co.) in the presence of ethanol and purified by silicic acid column chromatography (Liscovitch, 1989).

In the experiments where the phospholipase A_2 inhibitor bromophenacylbromide (Sigma Chemical Co.) and diglycerol lipase inhibitor RHC80267 (gift of Dr. Mabel Hokin, Univ. of Wisconsin) are used, the cells are preincubated for 30 - 60 min with the inhibitors before initiating mechanical activity.

Statistical analysis of all data is performed by t-tests for paired or unpaired values using statistical software (PC Statistician, Human Systems Dynamics, Northridge, CA). Data are

presented as mean $\pm$ S.E.

Results

Prostaglandin Production by Myofibers and Fibroblasts

Repetitive stretch/relaxation of cultured avian skeletal muscle stimulates PGE_2 and $PGF_{2\alpha}$ production which modulate protein turnover rates, leading to cell growth (Vandenburgh et al.1990). The primary muscle cultures used in these studies contained two main cell types - multinucleated, electrically active neonatal-like myofibers and mononucleated interstitial fibroblasts. To determine which cell type produces the stretch-sensitive prostaglandins, we compared PGE_2 and $PGF_{2\alpha}$ production in confluent fibroblast only cultures and cultures containing both myofibers and fibroblasts by measuring the accumulation of prostaglandins in the culture medium. Myofiber only cultures are not analyzed because of the difficulty in mechanically stimulating them. Without the extracellular matrix and basement membrane produced by the fibroblasts around each muscle fiber (Sanderson et al.1986), the myofibers are too fragile to be repetitively stimulated without rupturing (Hatfaludy et al.1989). Under static nonstretch culture conditions, fibroblast only cultures grown as described in Materials and Methods produce significantly greater amounts of PGE_2 and $PGF_{2\alpha}$ per/mg noncollagenous protein than mixed myofiber/fibroblast cultures in which 85% to 90% of total protein is in the myofibers (PGE_2: 1.40 ± 0.54 vs 0.60 ± 0.25 pg/μg protein; $PGF_{2\alpha}$: 2.29 ± 0.21 vs 0.88 ± 0.29 pg/mg protein; n=5-6; $p < .001$). As found previously (Vandenburgh et al.1990), stretch-induced PGE_2 and $PGF_{2\alpha}$ production increases in these cultures vary substantially between different

cell preparations. Stretch-induced alterations can therefore only be compared to static controls in the same experiment.

Mechanical stimulation of myofiber/fibroblast cultures for 24 to 48 h increases PGE_2 production 0.4 to 2.8 fold and $PGF_{2\alpha}$ production 1.3 to 1.8 fold (Figure 1). Mechanical stimulation of fibroblast only cultures has no significant effect on PGE_2 production compared to static controls (Figure 1A) and increases $PGF_{2\alpha}$ production by only 48-54% (Figure 1B). Similar results were obtained with a second preparation of primary cells. Based on the fact that myofiber/fibroblast cultures contain 85% to 90% of their total noncollagenous protein in the much larger myofibers (Vandenburgh et al.1989), it can be calculated that all of the stretch-induced PGE_2 production and 90% of stretch-induced $PGF_{2\alpha}$ production occurs in the myofibers present in mixed myofiber/fibroblast cultures. These results indicate that PGE_2 and $PGF_{2\alpha}$ production in skeletal myofibers are more sensitive to mechanical loading and unloading than interstitial fibroblasts. It is possible that co-culturing fibroblasts with myofibers increases the mechanical sensitivity of fibroblast prostaglandin production, but this seems unlikely.

Electrical Activity Requirements for Prostaglandin Production

Repetitive mechanical stimulation of the cultured myofibers might alter prostaglandin production and cell growth by altering their pattern of spontaneous electrical activity since electrical stimulation of the cultured myofibers is known to stimulate their growth rate (Brevet et al.1976). We therefore measured

prostaglandin production in myofibers made electrically quiescent with tetrodotoxin, a voltage-sensitive sodium channel blocker which inhibits all electrical activity in the cultured cells (Crisona and Strohman, 1983). Mechanical stimulation of the muscle cell cultures for 24 h in the presence of tetrodotoxin increases the production of both PGE_2 and $PGF_{2\alpha}$ to the same level as in its absence (Table 1). Prostaglandin production is therefore a function of mechanical rather than electrical activity. Stretch-induced increases in the muscle cells' metabolic and growth rates are also independent of electrical activity in this model system (Hatfaludy et al.1989; Vandenburgh et al.1989).

Phospholipid Breakdown and Prostaglandin Production

The breakdown rate of phospholipids to release free arachidonic acid is one of the major controlling steps in prostaglandin synthesis. To determine whether mechanical stimulation of the cultured cells might regulate prostaglandin production by stimulating this pathway, we first measured the calcium sensitivity of stretch-induced prostaglandin production since phospholipases are calcium dependent enzymes (Irvine, 1982). Rinsing of the cells for several hours in calcium-free medium before initiation of mechanical stimulation for 24 h in calcium-free medium significantly decreases the stretch-induced production of both PGE_2 and $PGF_{2\alpha}$ (Figure 2). Stretch-induced $PGF_{2\alpha}$ production is more sensitive to reduced calcium than PGE_2 production; $PGF_{2\alpha}$ production is decreased 65% ($P<.001$) whereas PGE_2 is reduced by only 23% ($P<.03$). Both PGE_2 and $PGF_{2\alpha}$ production are still

significantly increased by mechanical stimulation in the absence of extracellular calcium - PGE_2 by 41% ($P<.05$) and $PGF_{2\alpha}$ by 58% ($P<.01$). These results indicate that the stretch-induced increases in prostaglandin production are partially calcium sensitive and that stretch activation of phospholipases may be involved.

Stretch-induced prostaglandin production occurs in the absence of serum or other medium arachidonic acid sources (Vandenburgh et al.1990). It is therefore likely that the arachidonic acid for stretch-induced prostaglandin production is derived from the action of phospholipases A_2, C, and/or D on cellular phospholipids. To confirm this experimentally, the effect of phospholipase A_2 inhibitor bromophenacylbromide and diglycerol lipase inhibitor RHC80267 on stretch-induced prostaglandin production was measured. Mechanical stimulation of the muscle cells in the presence of either inhibitor alone had no effect on stretch-induced prostaglandin production (data not shown) but in combination inhibited stretch-induced PGE_2 production by 83% and $PGF_{2\alpha}$ production by 73% (Table 2). These results support the conclusion of the calcium-free medium experiments and indicate that stretch-stimulated phospholipase activation is a major regulator of stretch-induced PG production. Because neither inhibitor alone was effective, it appears that several different phospholipase pathways can produce the arachidonic acid needed for stretch-induced prostaglandin production.

<u>PLA_2 Activity</u>

To directly assay phospholipid breakdown in the mechanically

stimulated cultures, we measured the rate of release of free ^{3}H-arachidonic acid from prelabelled phospholipids. Within 30 min of initiating mechanical activity, there is a significant 34% increase in free intracellular ^{3}H-arachidonic acid (Figure 3A) and subsequent significant loss of ^{3}H-arachidonic acid from the cellular phospholipid fraction (Figure 3B). After 2 to 4 h, there is a significant reduction in free ^{3}H-arachidonic acid in the mechanically stimulated cultures (Figure 3A). Similar results were obtained in three separate experiments with different cell preparations. These data indicate that mechanical activity accelerates the breakdown of arachidonic acid containing phospholipids early after initiating mechanical activity.

Several pathways exist for the mechanically-induced release of ^{3}H-arachidonic acid from cellular phospholipids. These include: 1) activation of phospholipase A_2 (PLA_2), releasing arachidonic acid directly from the 2-acyl position of the phospholipid moiety; 2) sequential action of phospholipase C (PLC) and diglycerol lipase; or 3) sequential activation of phospholipase D (PLD), phosphatidate phosphohydrolase, and diglycerol lipase. Stretch-induced change in PLA_2 activity is measured by prelabelling the cells with ^{3}H-arachidonic acid and [methyl-^{14}C] choline overnight and measuring the formation of lysophosphatidylcholine after initiating mechanical stimulation. A decrease in the ^{3}H/^{14}C ratio of the lysophosphatidylcholine fraction isolated by thin layer chromatography indicates PLA_2 activation. Within 18 min of initiating mechanical stimulation of the cultured cells there is a

significant 26% increase in the formation of ^{14}C-lysophosphatidylcholine and its $^{3}H/^{14}C$ ratio is significantly reduced by 12% compared to static control cultures (Figure 4). Similar results were obtained in a second experiment. Mechanical stimulation for a shorter time of 1.5 min has no significant effect on either ^{14}C-lysophosphatidylcholine formation or its $^{3}H/^{14}C$ ratio (data not shown). These results indicate that there is a time dependent increase in PLA_2 activity when the muscle cultures are mechanically stimulated and this PLA_2 activation could act as one source of free arachidonic acid at 30 min of stimulation for subsequent prostaglandin production.

Phospholipases C and D Activities

If mechanical stimulation activates either the phospholipase C or D phospholipid breakdown pathway, there should be a stretch-induced increase in ^{3}H-arachidonic acid labelled diacylglycerol. Stretch-induced activation of PLA_2 would not produce radioactive diacylglycerol. We therefore measured diacylglycerol formation at varying times after mechanical stimulation. Thirty minutes after initiating mechanical stimulation, there is no significant increase in ^{3}H-arachidonic acid labelled diacylglycerol in the cells but diacylglycerol formation does increase by 60 min, peaks at 120 min, and returns to control levels by 240 min. (Figure 5A). These results indicate that stretch stimulates phospholipase C and/or D activity. The fact that the stretch stimulated peak of free intracellular ^{3}H-arachidonic acid occurs earlier (30 min) than the peak increases in ^{3}H-diacylglycerol formation (120 min) suggests

that the early increase in arachidonic acid results from PLA_2 activation rather than PLC and/or PLD activation. Similar results were obtained with two separate cell preparations. ^{3}H-Arachidonic acid labelled monoacylglycerol is also significantly increased by mechanical stimulation 30 to 60 min after initiating mechanical activity (Figure 5B); this increase could result from increased activity in the phospholipase A_1 and/or the PLC/PLD pathways. By 4 h ^{3}H-arachidonic acid labelled monoacylglycerol formation is significantly less than in static control cultures (Figure 5B).

Stretch-induced increases in ^{3}H-arachidonic acid labelled diacylglycerol may result from stretch-induced increases in phosphatidylinositol-specific phospholipase C activity. Cellular phospholipids are therefore labelled overnight with myo-[2-^{3}H]inositol, rinsed, and mechanically stimulated. Two hours of stretch/relaxation activity significantly increases the release of both total ^{3}H-inositol phosphates and free myo-^{3}H-inositol by 32% and 27%, respectively (Figure 6A). This indicates that mechanical stimulation activates phosphatidylinositol-specific phospholipase C activity at a similar time period when ^{3}H-arachidonic acid labelled diacylglycerol levels are increased (Figure 5A). Similar results were obtained in a second experiment. To determine whether stretch also increases diacylglycerol formation by activating phospholipase D (PLD), we measured the stretch-induced alterations in PLD activity as outlined in Materials and Methods by measuring phosphatidylethanol formation in the presence of 1% ethanol. PLD activity is increased within 30 min of initiating mechanical

activity by 65% and returns to control levels by 2 h (Figure 6B). Similar results were obtained in a second experiment where phosphatidylethanol formation was measured in control and stretched cells exposed to 0.5% ethanol (data not shown). Stretch-induced activation of PLD activity is thus a second mechanism by which diacylglycerol formation increases in the mechanically stimulated cultures. Nonphosphatidylinositol-specific PLC may also be partially responsible for stretch-induced diacylglycerol formation, but its activity was not measured in the present study.

Electrical Activity and Phospholipase Activity

Stretch-induced prostaglandin efflux and muscle cell growth are both independent of electrical activity in the cells since they occur in cultures pretreated with the voltage-sensitive sodium channel inhibitor tetrodotoxin [Table 1 and (Vandenburgh et al.1989), respectively]. We measured ^{3}H-arachidonic acid labelled phospholipid breakdown in tetrodoxin containing medium to separate the mechanically activated phospholipases from those which might be related to mechanically-induced alterations in the electrical activity of the cells. Pretreatment of the muscle cultures with tetrodotoxin has no effect on stretch-induced increases in ^{3}H-arachidonic acid labelled phospholipid breakdown or diacylglycerol formation but completely inhibited stretch-induced increases in myo-[2-^{3}H]inositol labelled inositol phosphates (Figure 7). Stretch-induced activation of phosphatidylinositol-specific PLC activity thus appears to be related to mechanically-induced

electrical activity rather than pure mechanical activity. Stretch-induced activation of the other phospholipases which increase phospholipid breakdown and stimulate arachidonic acid release and diacylglycerol formation are mechanically regulated, independently of electrical activity in the cells.

Discussion

Prostaglandins are mechanogenic second messengers which regulate skeletal muscle growth rates in vivo (Palmer, 1990; Strelkov et al.1989) and in tissue culture (Vandenburgh et al.1990). The main conclusion of the present study is that mechanical stimulation of skeletal muscle activates cellular phospholipases, which generate a number of lipid-related second messengers including arachidonic acid (Kim et al.1989) and diacylglycerol (Nishizuka, 1986). Increased free arachidonic acid by mechanically-induced phospholipase activation means that the first of the two main regulatory steps in prostaglandin synthesis is stimulated. It is not known whether the second regulatory step, cyclooxygenase activation, is also altered by mechanical activity.

Mechanical stimulation of the cultured cells stimulates the activity of several phospholipases based on the breakdown of ^{3}H-arachidonic acid, [methyl- ^{14}C] choline, and myo-[2-^{3}H] inositol labelled phospholipids. A number of pathways exist for the release of free arachidonic acid from cellular phospholipids (Irvine, 1982). These include direct release of arachidonic acid by PLA_2 activity, and indirect release by activation of the PLC/PLD and diglycerol lipase pathways. Both pathways are activated by mechanical stimulation of the muscle cells and, based on the lipase inhibitor results, regulate prostaglandin production in the cells. Repetitive stretch/relaxation of the muscle cultures stimulates PLA_2 activity by 18 min of mechanical activity, increasing the intracellular levels of free ^{3}H-arachidonic acid within 30 min. ^{3}H-

Arachidonic labelled diacylglycerol formation is increased by 60 to 120 min after mechanical stimulation is started, indicating activation of the PLC/PLD-diglycerol lipase pathways. These results support the hypothesis that PLA_2 activation early after stretch initiation provides free arachidonic acid for prostaglandin production. At later stimulation times when ^{3}H-arachidonic acid labelled diacylglycerol levels are elevated and prostaglandin production is increasing (2 to 4 h), free ^{3}H-arachidonic acid levels are significantly reduced compared to static control cultures (Figure 3). This could mean either that subsequent diacylglycerol breakdown by the diacylglyceride lipase/monoglyceride lipase pathway is not an important arachidonic acid source for prostaglandin production in the muscle cultures, or that the coupling of arachidonic acid release from the phospholipids to cyclooxygenase conversion into prostaglandins is enhanced by mechanical stimulation of the cells. The efficiency of conversion of released arachidonic acid to prostaglandin production is dependent on the localization of the cyclooxygenase enzyme relative to the site of release of arachidonic acid by activated phospholipases (Hsueh and Needleman, 1978), and mechanical stimulation could influence the relationship of these two processes.

Phosphatidylinositol-specific PLC is activated by mechanical stimulation of the muscle cells based on stretch-induced increases in inositol phosphate formation. But this activation is eliminated as a potential source of arachidonic acid for prostaglandin

production in the stretch stimulated, electrically quiescent cells since the PLC activation is prevented by the voltage sensitive channel blocker tetrodotoxin whereas prostaglandin production is not. Inositol phosphates are involved in excitation-contraction coupling in skeletal muscle (Vergara et al.1985) and therefore it is not surprising that stretch-induced inositol phosphate production is inhibited in electrically quiescent cells. The increased inositol phosphate formation by stretch in electrically active cells probably indicates that mechanical stimulation of the muscle cells alters their level and/or pattern of spontaneous electrical activity. But these alterations are unrelated to the mechanical effects on prostaglandin production or cell growth which are both tetrodotoxin insensitive. These results support the idea that stretch induced alterations in other phospholipase activities and prostaglandin production, which are both tetrodotoxin insensitive, are unrelated to the muscle cell's electrical activity. They do not mean that skeletal muscle electrical activity in itself is not an important regulator of muscle cell growth under certain conditions (Brevet et al.1976). The cultured muscle mechanical cell stimulator model system allows the separation of mechanical effects from electrical effects which are difficult to separate in vivo.

In contrast to its inhibitory effect on stretch-induced inositol phosphate formation, tetrodotoxin has no effect on stretch-induced increases in ^{3}H-arachidonic acid labelled diacylglycerol formation. Nonphosphatidylinositol specific PLC

and/or PLD must therefore be activated by mechanical stimulation and may be an important pathway for producing free arachidonic acid by subsequent breakdown of diacylglycerol by diglycerol lipase and/or monoacylglyceride lipase. Phospholipase D is directly activated within 30 min of initiating mechanical stimulation of the cultured cells by a mechanism which is not inhibited by tetrodotoxin.

The mechanism by which cell stretch activates phospholipases A_2 and D is not known. Several different models can be proposed. First, phospholipase activation and phospholipid metabolism link growth factor receptor binding to stimulation of cell growth by generating lipid-related second messengers in several cell types (Burch and Axelrod, 1987; Tsai et al.1990). But since stretch-induced increases in prostaglandin production, phospholipid breakdown, and arachidonic acid formation occur in medium devoid of exogenous growth factors, stretch appears to activate the phospholipases independently of growth factor-receptor interactions. Stretch may directly alter G protein regulation of phospholipase activities (Kim et al.1989), bypassing the growth factor-receptor binding step.

Secondly, since the majority of phospholipases are calcium dependent enzymes (Irvine, 1982) and stretch-induced increases in prostaglandin production are sensitive to calcium removal from the medium, mechanical activity may activate the phospholipases and increase prostaglandin production by stretch-induced alterations in calcium influx through the plasma membrane, possibly by stretch-

sensitive channels (Guharay and Sachs, 1984). Skeletal muscle protein turnover rates are sensitive to calcium influx by an as yet undetermined mechanism (Kameyama and Etlinger, 1979; Baracos et al.1986) and calcium regulates some steps in protein turnover in adult skeletal muscle by a prostaglandin dependent mechanism (Rodemann et al.1982). Although we have not measured stretch-induced calcium fluxes in this paper, we have previously shown that mechanical stimulation of the cultured muscle cells activates fluxes of other ions such as sodium and potassium and that muscle growth is coupled to these alterations (Vandenburgh and Kaufman, 1981). Since stretch-induced prostaglandin production is frequency-dependent (Vandenburgh et al.1990), the oscillation rate of calcium movements rather than total fluxes (Ambler et al.1988) may be important in stretch-induced prostaglandin regulation of skeletal muscle growth. These findings may indicate that intermittent mechanical stimulation increases prostaglandin production by altering calcium ion oscillations which are then coupled to phospholipase activation, elevated free arachidonic acid levels, and stimulated prostaglandin production.

Finally, plasma membrane associated phospholipase activity may be increased by enhancing enzyme accessibility to phospholipid substrate. In lipid bilayer model systems, phospholipase activity can be increased by elevating surface pressure on the monolayer which expands the space between the membrane phospholipid moieties, and allows phospholipase accessibility to the hydrophobic fatty acid portions of the molecules (Verger et al.1973; Hirasawa et

al.1981). A similar process may occur during stretch of intact cells. The tissue culture mechanical cell stimulator provides a means for testing these different hypotheses to determine the molecular mechanisms by which lipid-related mechanogenic second messengers are generated in skeletal muscle and lead to muscle cell growth.

Acknowledgements

We thank Michelle Guillemette for manuscript preparation; Joseph Chromiak and Ira Mills for critical reading of the manuscript.

Literature Cited

Ambler, S.K., Poenie, M., Tsien, R.Y., and Taylor, P. (1988) Agonist-stimulated oscillations and cycling of intracellular free calcium in individual cultured muscle cells. J. of Biol. Chem., *263*:1952-1959.

Bar-Sagi, D. and Feramisco, J.R. (1986) Induction of membrane fuffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science, *233*:1061-1068.

Baracos, V., Greenberg, R.E., and Goldberg, A.L. (1986) Influence of calcium and other divalent cations on protein turnover in rat skeletal muscle. Am. J. Physiol., *250*:E702-E710.

Bell, E., Ivarsson, B., and Merrill, C. (1979) Production of tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc. Natl. Acad. Sci USA, *76*:1274-1278.

Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F. (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J., *212*:473-482.

Betteridge, A. (1980) Role of Ca2+ and cyclic nucleotides in the

control of prostaglandin E production in the rat anterior pituitary gland. Biochem. J., *186*:987-992.

Brevet, A., Pinto, E., Peacock, J., and Stockdale, F.E. (1976) Myosin synthesis increased by electrical stimulation of skeletal muscle cell cultures. Science, *193*:1152-1154.

Burch, R.M. and Axelrod, J. (1987) Dissociation of bradykinin-induced prostaglandin formation from phosphatidylinositol turnover in Swiss 3T3 fibroblasts: Evidence for G protein regulation of phospholipase A2. Proc. Natl. Acad. Sci. (U. S. A.), *84*:6374-6378.

Crisona, N.J. and Strohman, R.C. (1983) Inhibition of contraction of cultured muscle fibers result in increased turnover of myofibrillar proteins but not of intermediate -filament proteins. J. Cell Biol., *96*:684-692.

Dixon, J.F. and Hokin, L.E. (1984) Secretogogue-stimulated phosphatidylinositol breakdown in the exocrine pancreas liberates arachidonic acid, stearic acid, and glycerol by sequential actions of phospholipase C and diglyceride lipase. J. Biol. Chem., *259*:14418-14425.

Frangos, J.A., Eskin, S.G., McIntire, L.V., and Ives, C.L. (1985) Flow effects on prostacyclin production by cultured human

endothelial cells. Science, *227*:1477-1479.

Guharay, F. and Sachs, F. (1984) Stretch-activated single ion channel currents in tissue cultured embryonic chick skeletal muscle. J. Physiol., *352*:685-701.

Hatfaludy, S., Shansky, J., and Vandenburgh, H.H. (1989) Metabolic alterations induced in cultured skeletal muscle by stretch/relaxation activity. Am. J. Physiol., *256*:C175-181.

Hirasawa, K., Irvine, R.F., and Rex, M.C. (1981) The hydrolysis of phosphatidylinositol monolayers at an air/water interface by the calcium ion dependent phosphatidylinositol phosphodiesterase of pig brain. Biochem. J., *193*:607-614.

Hsueh, W. and Needleman, P. (1978) Sites of lipase activation and prostaglandin synthesis in isolated, perfused rabbit hearts and hydronephrotic kidneys. Prostaglandins, *16*:661-681.

Irvine, R.F. (1982) How is the level of free arachidonic acid controlled in mammalian cells? Biochem J., *204*:3-16.

Kameyama, T. and Etlinger, J. (1979) Calcium-dependent regulation of protein synthesis and degradation in muscle. Nature, *279*:344-346.

Kim, D., Lewis, D.L., Graziadei, L., Neer, E.J., Bar-Sagi, D., and Clapham, D.E. (1989) G-protein Beta gamma-subunits activate the cardiac muscarinic K+-channel via phospholipase A2. Nature, *337*:557-560.

Klein, D.C. and Raisz, L.G. (1970) Prostaglandins: Stimulation of bone resorption in tissue culture. Endocrin., *86*:1436-1440.

Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E., Takaku, F., and Yazaki, Y. (1991) Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. Possible role of protein kinase C activation. J. Biol. Chem., *266*:1265-1268.

Liscovitch, M. (1989) Phosphatidylethanol biosynthesis in ethanol-exposed NG108-15 neuroblastoma x glioma hybrid cells. Evidence for activation of a phospholipase D phosphatidyltransferase activity by protein kinase C. J. Biol. Chem., *264*:1450-1456.

Nishizuka, Y. (1986) Studies and perspectives of protein kinase c. Science, *233*:305-312.

Pai, J.-K., Siegel, M.I., Egan, R.W., and Billah, M.M. (1988) Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. J. Biol. Chem.,

263:12472-12477.

Palmer, R.M., Reeds, P.J., Atkinson, T., and Smith, R.H. (1983) The influence of changes in tension on protein synthesis and prostaglandin release in isolated rabbit muscles. Biochem. J., *214*:1011-1023.

Palmer, R.M. (1990) Prostaglandins and the control of muscle protein synthesis and degradation. Prostaglandins Leukotrienes Essen. Fatty Acids, *39*:95-104.

Raz, A., Wyche, A., Siegel, N., and Needleman, P. (1988) Regulation of fibroblast cyclooxygenase synthesis by interleukin-1. J. Biol. Chem., *263*:3022-3028.

Rodemann, H.P., Waxman, L., and Goldberg, A.L. (1982) The stimulation of protein degradation in muscle by Ca2+ is mediated by prostaglandin E2 and does not require the calcium activated protease. J. of Biol. Chem., *257*:8716-8723.

Sanderson, R.D., Fitch, J.M., Linsenmayer, T.R., and Mayne, R. (1986) Fibroblasts promote the formation of a continuous basal lamina during myogenesis in vitro. J. Cell Biol., *102*:740-747.

Smith, R.H., Palmer, R.M., and Reeds, P.J. (1983) Protein synthesis in isolated rabbit forelimb muscles. The possible role of

metabolites of arachidonic acid in the response to intermittent stretching. Biochem. J., *214*:153-161.

Somjen, D., Binderman, I., Berger, E., and Harell, A. (1980) Bone remodelling induced by physical stress is prostaglandin E2 mediated. Biochim. Biophys. Acta, *627*:91-100.

Strelkov, A.B., Fields, A.L.A., and Baracos, V. (1989) Effects of systemic inhibition of prostaglandin production on protein metabolism in tumor-bearing rats. Am J Physiol, *257*:C261-C269.

Tsai, M-H., Yu, C-L., and Stacey, D.W. (1990) A cytoplasmic protein inhibits the GTPase activity of H-ras in a phospholipid-dependent manner. Science, *250*:982-985.

Vandenburgh, H.H. (1988) A computerized mechanical cell stimulator for tissue culture: Effects on skeletal muscle organogenesis. In Vitro, *24*:609-619.

Vandenburgh, H.H., Karlisch, P., and Farr, L. (1988) Maintenance of highly contractile skeletal myotubes in collagen gels. In Vitro, *24*:166-174.

Vandenburgh, H.H., Hatfaludy, S., and Shansky, J. (1989) Skeletal muscle growth is stimulated by intermittent stretch/relaxation in tissue culture. Am. J. Physiol., *256(Cell Physiol.25)*:C674-C682.

Vandenburgh, H.H., Hatfaludy, S., Sohar, I., and Shansky, J. (1990) Stretch-induced prostaglandins and protein turnover in cultured skeletal muscle. Am. J. Physiol., *259(Cell Physiol.28)*:C232-C240.

Vandenburgh, H.H. and Kaufman, S. (1981) Stretch-induced growth of skeletal myotubes correlates with activation of the sodium pump. J. Cell Physiol., *109*:205-214.

Vergara, J., Tsien, R.Y., and Delay, M. (1985) Inositol 1,4,5-trisphosphate: a possible chemical link in excitation-contraction coupling in muscle. Proc. Natl. Acad. Sci. (U. S. A.), *82*:6352-6356.

Verger, R., Mieras, M.C.E., and deHaas, G.H. (1973) Action of phospholipase A at interfaces. J. Biol. Chem., *248*:4023-4034.

Yang, S.F., Freer, S., and Benson, A.A. (1967) Transphosphatidylation by phospholipase D. J. Biol. Chem., *242*:477-484.

Young, E.W. and Sparks, H.V. (1979) Prostaglandin E release from dog skeletal muscle during restricted flow exercise. Am. J. Physiol., *236*:H596-H599.

Figure Legends

Figure 1. **Mechanical activity stimulates prostaglandin production from myofibers to a greater extent than fibroblasts.** Day 6 cultures of myofibers/fibroblasts or fibroblasts only are rinsed and mechanically stimulated as outlined in Materials and Methods. Medium is collected at 4, 24, and 48 h after initiating mechanical activity and assayed for PGE_2 and $PGF_{2\alpha}$ by radioimmunoassay. Results are expressed as mean ± SE percent increase in prostaglandin efflux from mechanically stimulated cultures compared to time matched static cultures. Each point represents 3-5 values and statistical analyses are by t-tests for unpaired values. Standard error bars are smaller than symbols where not shown.

Figure 2. **Mechanical stimulation of prostaglandin production is partially dependent on extracellular calcium.** Day 6 myofiber/fibroblast cultures are rinsed and mechanically stimulated in control 1.8 mM calcium medium or in calcium-free medium for 24 h as outlined in Materials and Methods. Prostaglandin efflux into the culture medium is measured and compared to time matched static cultures. Each point represents 3-5 values and statistical analyses are by t-tests for unpaired values.

Figure 3. **Mechanical stimulation increases the intracellular level of free arachidonic acid by increasing the breakdown of cellular phospholipids.** Day 5-6 muscle cultures are labelled overnight with

^{3}H-arachidonic acid (AA), extensively rinsed for 3 h and mechanical stimulation initiated at time 0 as outlined in Materials and Methods. The intracellular levels of (A) free ^{3}H-arachidonic acid and (B) the ^{3}H-arachidonic acid radioactivity remaining in cellular phospholipids (PL) are determined by thin layer chromatography at 2 to 24 h after initiating mechanical activity and compared to time matched static control cultures. Data are calculated as the mean ± SE percent radioactivity in the arachidonic acid or phospholipid fractions relative to the total lipid radioactivity spotted onto thin layer chromatography plates. In control cultures, 0.3 - 0.6% of ^{3}H is in the free arachidonic acid fraction and 60 - 63% in the phospholipid fraction and the cells incorporated 267,000 to 580,000 total lipid DPMS per culture well. Each point represents 5-10 values and statistical analyses are by t-tests for unpaired values. Standard error bars are smaller than symbols where not shown.

Figure 4. **Mechanical stimulation activates PLA_2.** The cells are labelled with ^{3}H-arachidonic acid and [methyl-^{14}C] choline as outlined in Figure 3 legend. The cells are mechanically stimulated for 18 min and lysophosphatidylcholine isolated from the total lipid fraction as outlined in Materials and Methods. The results are expressed as mean ± SE (n=6 to 12) of the per cent total lipid ^{14}C radioactivity found in the lysophosphatidylcholine fraction isolated by thin layer chromatography (left graph) and as the ratio of ^{3}H/^{14}C in the lysophosphatidylcholine fraction. The cells incorporated 26,000 to 88,000 total lipid ^{3}H and ^{14}C DPMS per

culture well. Statistical analyses are by t-tests for paired (left graph) or unpaired (right graph) values.

Figure 5. **Mechanical activity increases the formation of ^{3}H-arachidonic acid labelled monoacylglycerol and diacylglycerol.** Experiments are performed as outlined in Figure 3 legend. The neutral lipids are isolated as outlined in Materials and Methods and the results are expressed as the mean ± SE percent stretch-induced change in the (A) diacylglycerol (DAG) and (B) monoacylglycerol (MAG) fractions relative to time matched static controls. The data are calculated as outlined in Figure 3 legend. In control cultures 0.73 - 2.24% of ^{3}H is in the DAG fraction and 0.04 - 0.16% in the MAG fraction. DPMS incorporated into the total lipid fraction are 168,000 - 390,000 per culture well. Each point represents 6-14 values and statistical analyses are by t-tests for unpaired or paired values. Standard error bars are smaller than symbols where not shown.

Figure 6. **Total inositol phosphates, myo-inositol levels, and phospholipase D activity are increased by mechanical stimulation.** Day 6 muscle cultures are labelled overnight with either myo-[2-^{3}H]inositol (A) or ^{3}H-arachidonic acid (B), extensively rinsed and mechanical stimulation initiated at time 0 as outlined in Materials and Methods. The intracellular levels of ^{3}H-inositol phosphates and myo-[2-^{3}H]inositol are determined by Dowex 1-X8 column chromatography 2 h after initiating mechanical activity.

Phospholipase D activity is measured as the intracellular levels of ^{3}H-arachidonic acid labelled phosphatidylethanol at 30 and 120 min after initiating mechanical activity. Results are expressed as the mean ± SE percent stretch-induced change compared to time matched static controls. Data are calculated as the radioactivity in the various fractions relative to the total radioactivity applied to the column (inositol phosphate assay) or thin layer plate (phosphatidylethanol fraction). For (A), 15 - 20% of the ^{3}H is in the total inositol phosphate fractions and 75% in the myo-inositol fraction. For (B), 0.23 - 2.7% of the ^{3}H is in the phosphatidylethanol fraction. Total DPMS incorporated per culture well are 50,000 - 260,000. The bars represent the combined results from three separate experiments with 2-4 samples per group in each experiment.

Figure 7. **Tetrodotoxin inhibits stretch-induced inositol phosphate formation but not ^{3}H-arachidonic acid labelled phospholipid breakdown, ^{3}H-arachidonic acid labelled diacylglycerol formation, or phospholipase D activity.** Experiments are performed as outlined in Figures 3 to 6 legends. When tetrodotoxin (1 μg/ml) is used, the cells are exposed for 30 min to the drug before initiating mechanical stimulation. The data are calculated as outlined in Materials and Methods and the results expressed as mean percent stretch-induced changes compared to time matched static controls. Abbreviations are: PL, phospholipids; DAG, diacylglycerol; IP, inositol phosphates; PLD, phospholipase D. Each bar represents 6 to

22 values and statistical analyses are by t-tests for either unpaired or paired values.

Table 1

Mechanical Stimulation Increases Prostaglandin Production in Electrically Quiescent Muscle Cells

Group	Tetrodotoxin (1 μg/ml)	PGE_2 (pg/mg protein)	Δ	$PGF_{2\alpha}$ (pg/mg protein)	Δ
Control	-	297 ± 45		415 ± 142	
Stretch	-	457 ± 40*	54%	591 ± 49	42%
Control	+	342 ± 46		437 ± 30	
Stretch	+	554 ± 31**	62%	712 ± 45***	63%

Each value is the mean ± SE of 4-6 samples and statistical analyses are by t - tests for unpaired values. Day 7 postplating muscle cells are mechanically stimulated for 24 h as outlined in Materials and Methods. Data are expressed as pg PG/mg total noncollagenous cellular protein. Cultures treated with tetrodotoxin are pretreated for 30 min before mechanical stimulation is initiated. Media are collected at end of the stimulation period and assayed for PGE_2 and $PGF_{2\alpha}$ as outlined in Materials and Methods. *P<.01 **P<.03 ***P<.02.

Table 2

Lipase Inhibitors Reduce Mechanically Induced Prostaglandin Production

Group	Lipase Inhibitors	PGE_2 (pg/mg protein)	Δ	$PGF_{2\alpha}$ (pg/mg protein)	Δ
Control	-	352 ± 31		819 ± 74	
Stretch	-	516 ± 78	47%	1852 ± 323**	126%
Control	+	379 ± 26		755 ± 51	
Stretch	+	409 ± 35	8%	1014 ± 106***	34%

Each value is the mean ± SE of 5-6 samples and statistical analyses are by t - tests for unpaired values. Day 7 postplating muscle cells are mechanically stimulated for 24 h as outlined in Materials and Methods. Data are expressed as pg PG/mg total noncollagenous cellular protein. Cultures treated with a combination of lipase inhibitors (bromophenacylbromide, 20-25 μM, and RHC80267, 10 μM) were pretreated for 30 - 60 min before mechanical stimulation is initiated. Media are collected at end of the stimulation period and assayed for PGE_2 and $PGF_{2\alpha}$ as outlined in Materials and Methods. **P<.03 ***P<.02.

FIGURE 1

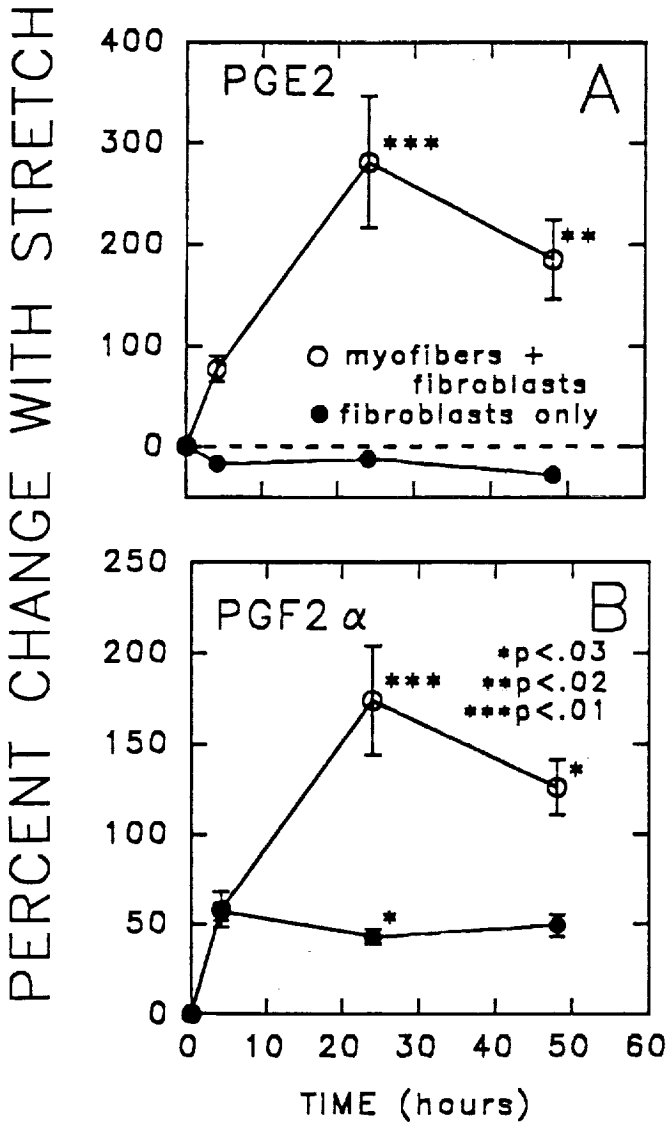

PERCENT CHANGE WITH STRETCH
PGE2
A
400
300
200
100
0
***
**
O myofibers + fibroblasts
● fibroblasts only
PGF2 α
B
250
200
150
100
50
0
*p<.03
**p<.02
***p<.01
***
*
*
0 10 20 30 40 50 60
TIME (hours)

FIGURE 2

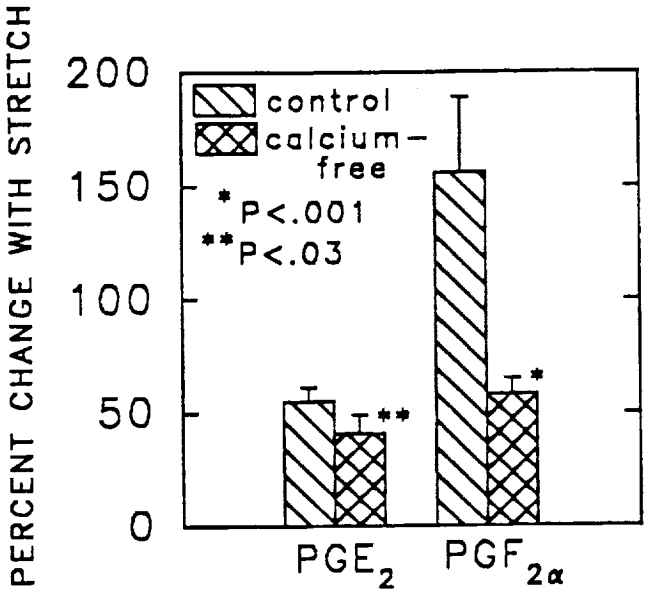

PERCENT CHANGE WITH STRETCH
200
150
100
50
0
control
calcium-free
*P<.001
**P<.03
**
*
PGE2
PGF2α

FIGURE 3

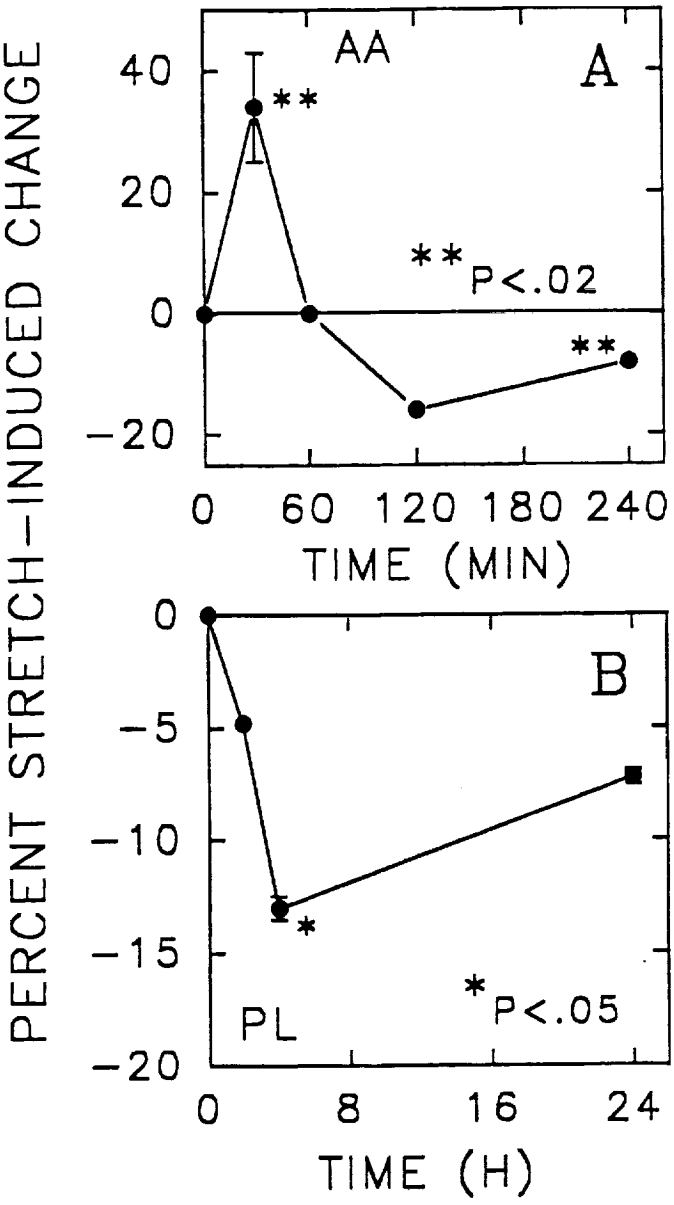

AA
A
40
20
0
−20
**
**P<.02
**
0
60
120
180
240
TIME (MIN)
PERCENT STRETCH−INDUCED CHANGE
0
−5
−10
−15
−20
B
*
PL
*P<.05
0
8
16
24
TIME (H)

FIGURE 4

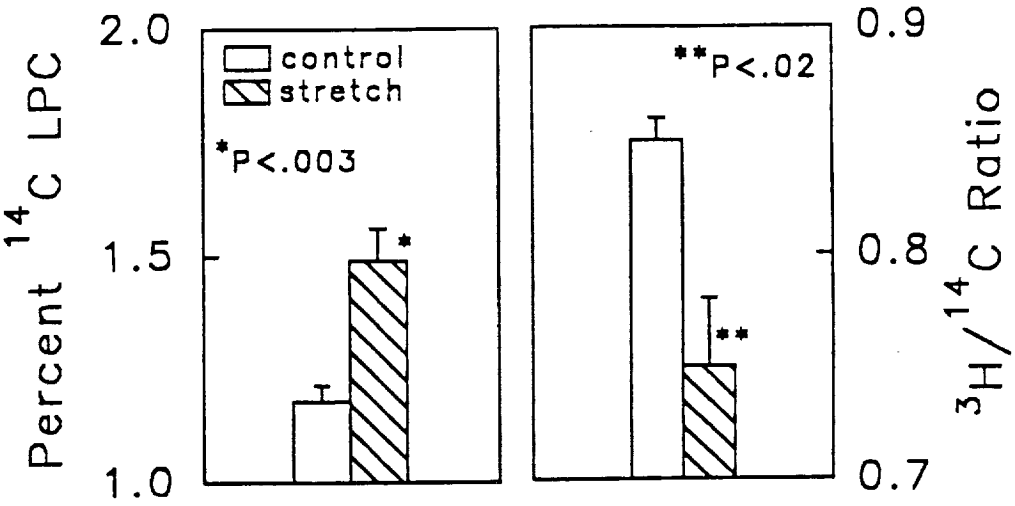

Percent ^{14}C LPC
2.0
1.5
1.0
control
stretch
*P<.003
*
**P<.02
**
0.9
0.8
0.7
^{3}H/^{14}C Ratio

FIGURE 5

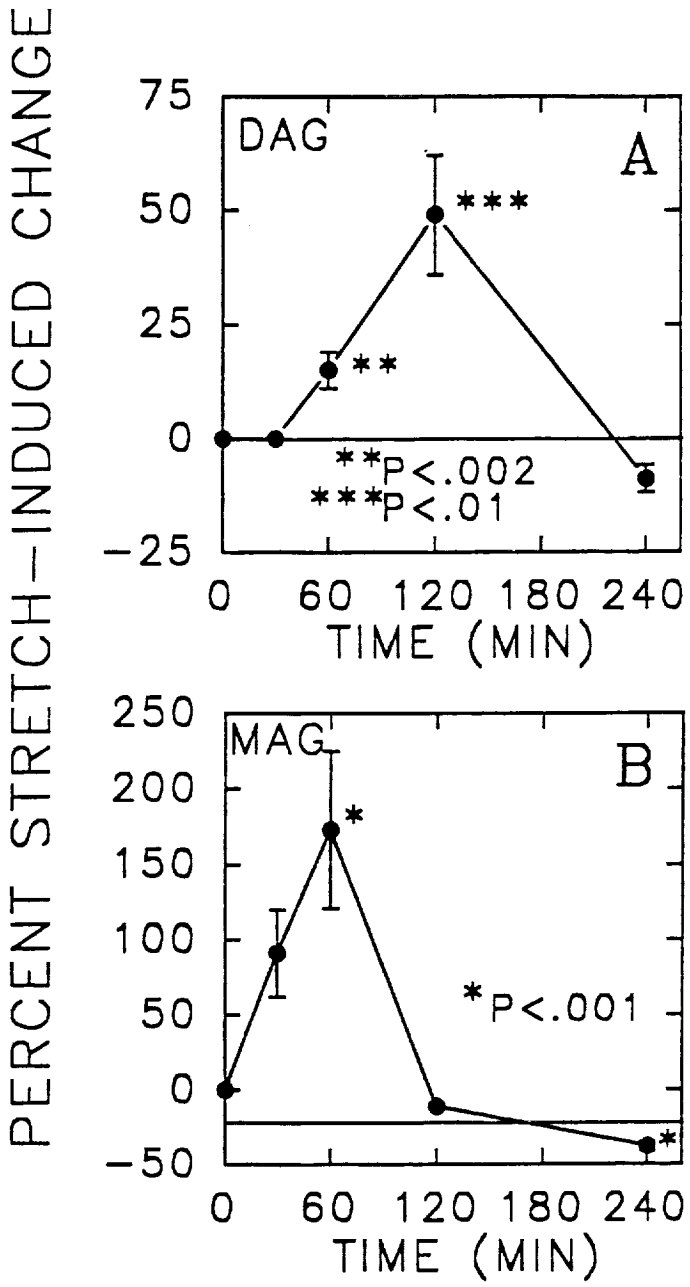

PERCENT STRETCH-INDUCED CHANGE
75
50
25
0
-25
DAG
A
***
**
**P<.002
***P<.01
0 60 120 180 240
TIME (MIN)
250
200
150
100
50
0
-50
MAG
B
*
*P<.001
*
0 60 120 180 240
TIME (MIN)

FIGURE 6

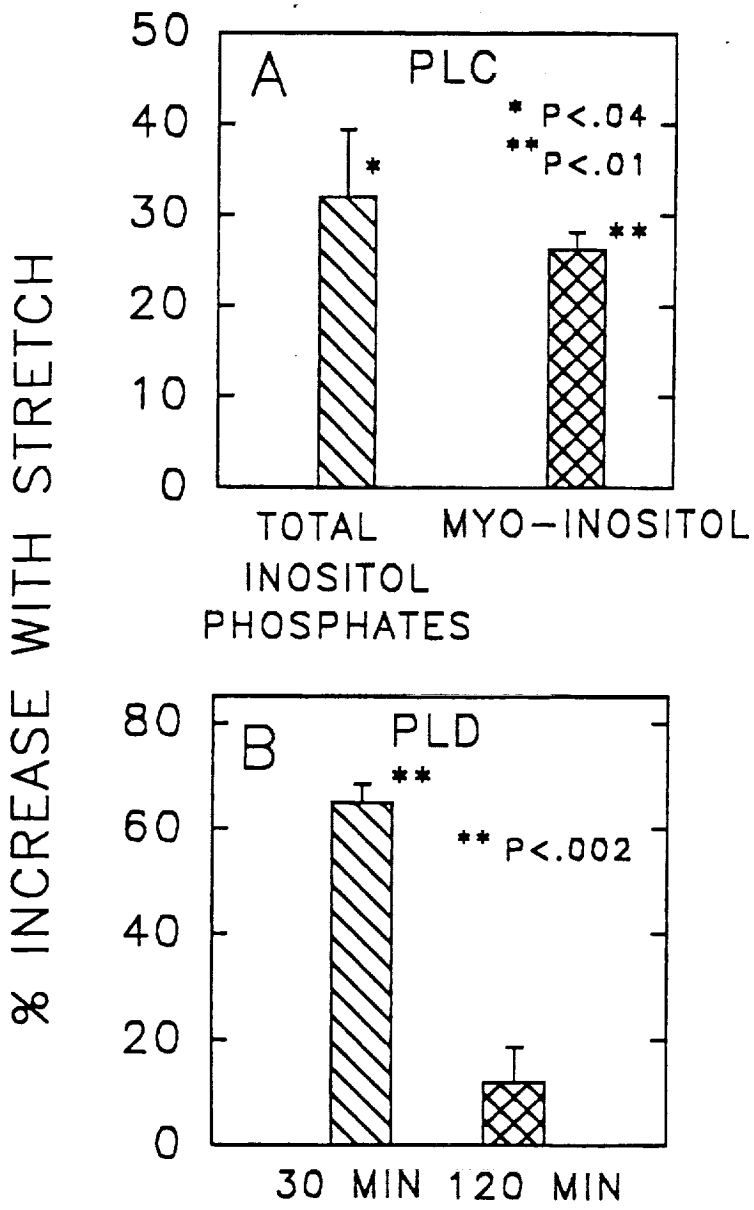
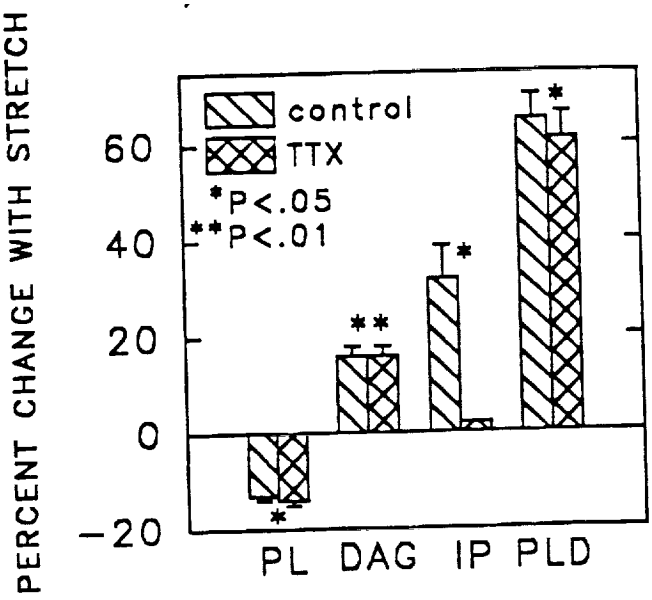

% INCREASE WITH STRETCH
50
40
30
20
10
0
A
PLC
* P<.04
** P<.01
*
**
TOTAL INOSITOL PHOSPHATES
MYO-INOSITOL
80
60
40
20
0
B
PLD
**
** P<.002
30 MIN
120 MIN

FIGURE 7


PERCENT CHANGE WITH STRETCH
60
40
20
0
−20
control
TTX
*P<.05
**P<.01
*
**
*
*
PL DAG IP PLD