MECHANICAL STIMULATION OF SKELETAL MUSCLE INCREASES PROSTAGLANDIN $F_{2\alpha}$ SYNTHESIS AND CYCLOOXYGENASE ACTIVITY BY A PERTUSSIS TOXIN SENSITIVE MECHANISM

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Running Head: Stretch and Skeletal Muscle Cyclooxygenase

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ABSTRACT

Repetitive mechanical stimulation of differentiated skeletal muscle in tissue culture increases the production of prostaglandin $F_{2\alpha}$, an anabolic stimulator of myofiber growth. Within 4 h of initiating mechanical activity, the activity of cyclooxygenase, a regulatory enzyme in prostaglandin synthesis, was increased 82% ($P<.005$), and this increase was maintained for at least 24 h. Kinetic analysis of the stretch-activated cyclooxgenase indicated a two to three-fold decrease in the enzyme's K_m with no change in V_{max}. The stretch-induced increase in enzymatic activity was not inhibited by cycloheximide, was independent of cellular electrical activity (tetrodotoxin-insensitive), but was prevented by the G protein inhibitor pertussis toxin. Pertussis toxin also inhibited the stretch-induced increases in $PGF_{2\alpha}$ production, and cell growth. It is concluded that stretch of skeletal muscle increases the synthesis of the anabolic modulator $PGF_{2\alpha}$ by a G protein-dependent process which involves activation of cyclooxgenase by a posttranslational mechanism.

Keywords: prostaglandin GH synthase; stretch; protein synthesis; growth; G proteins.

INTRODUCTION

Prostaglandins (PGs) are important regulators of skeletal muscle growth both during development and in the adult animal [reviewed in (19)]. PGE_1 stimulates myoblast fusion into multinucleated myofibers (3,7,17) while PGE_2 and $PGF_{2\alpha}$ regulate skeletal muscle protein turnover rates (22,23,29,34). Skeletal muscles synthesize and efflux prostaglandins such as PGE_1, PGE_2, and $PGF_{2\alpha}$ *in vivo* (40,42), in organ culture (20,22), and in tissue culture (15,34), and these compounds act as both autocrine and paracrine growth modulators in this tissue. The mechanism by which PGs regulate skeletal muscle growth is not known.

Increased passive muscle tension induced by stretch is an important growth stimulus in skeletal muscle, while decreased tension from muscle shortening leads to muscle atrophy (31). PGs have been shown to act as autocrine/paracrine second messengers in regulating stretch/tension-induced muscle protein turnover rates *in vivo* (9,18), and *in vitro* (20,27,34). Repetitive mechanical stimulation of tissue cultured skeletal muscle cells at specific frequencies results in an increase in the production of PGE_2 and $PGF_{2\alpha}$ while the stable derivative of prostacyclins (6 keto - $PGF_{1\alpha}$) is not altered (34). Increased PGE_2 synthesis in the cultured muscle cells occurs within 4-6 h after initiating mechanical activity, returns to baseline levels by 24 h, and is associated with initial muscle damage (34). $PGF_{2\alpha}$ synthesis increases by 24 h of continued mechanical stimulation, is maintained at elevated levels for days, and is associated with long term stretch-induced

muscle cell growth (34). These data are compatible with the notion that PGE_2 stimulates catabolic processes while $PGF_{2\alpha}$ stimulates anabolic processes in skeletal muscle (22,23).

There are two main mechanisms which control the synthesis rates of PGs - activity of the various phospholipases which provide the immediate precursor arachidonic acid for prostaglandin synthesis (8), and the activity of the enzyme which converts arachidonic acid into PGs, prostaglandin endoperoxide synthase, EC 1.14.99.1 [also called cyclooxgenase (2)]. Mechanical stimulation of the cultured skeletal muscle cells increases the breakdown rate of arachidonic acid-containing phospholipids by activating phospholipases A_2 and D but not phosphatidylinositol specific phospholipase C (38). In this paper, we examined whether stretch also increases cyclooxgenase activity, the second regulatory step in PG synthesis. Cyclooxygenase is a rapidly turning over protein with a half-life of 60 minutes in fibroblasts (21) and less than 10 min in skeletal muscle (4). One isoform of cyclooxgenase (mitogen-inducible) is an immediate early gene product activated at the transcriptional level during growth factor-stimulated cell growth (5,26,41). We show in this paper that mechanical stimulation of tissue cultured skeletal muscle cells increases cyclooxgenase activity by a posttranslational, G-protein dependent mechanism. The mitogen-inducible, immediate early gene isoform of cyclooxygenase was immunocytochemically localized to the skeletal myofibers but its contribution to total enzymatic activity in the stretch-stimulated skeletal muscle cultures is not presently known. Parts

of this work have appeared in abstract form (39).

MATERIALS AND METHODS

Cell Cultures

Avian myoblasts were isolated from 11-12 days in ovo pectoralis muscle by standard dissection techniques (35). Fertilized chicken eggs were obtained from Beaver River Farm (West Kingston, RI). The isolated cells were plated at a very high density of 5,700-7,900 cells/mm^2 in 1 ml of growth medium and maintained in a humidified 5.0% CO_2 incubator at 37°C. Growth medium was Eagle's basal medium containing 50 U/ml penicillin, 10% horse serum, and 5% chicken embryo extract (85/10/5 medium), and was changed every 24 h. Cells were plated and grown in culture wells of a Mechanical Cell Stimulator device (Cell Kinetics, Inc., P.O. Box 40643, Providence, RI 02940) described previously (33) with minor modifications. The cell growth chamber of the modified Mechanical Cell Stimulator (Figure 1A) was made of stainless steel and contains 36 - 15 mm diameter teflon-lined culture wells. Use of stainless steel for the cell growth chamber rather than the previously used teflon material allowed rapid sterilization of the chamber by autoclaving rather than by gas sterilization. Sandwiched between the two halves of the cell growth chamber was a collagen-coated (Type I, Collaborative Research, Bedford, MA) elastic membrane (0.01" thick SILASTIC™, Dow Corning, Corp., Midland, MI) to which the muscle cells attach and grow. A circular piece of stainless steel wire cloth was located around the periphery of each well and acted as an "artificial tendon" by providing a surface to which the differentiating cells attach and are stretched (Figure

1B). At 48-54 h postplating, the newly formed multinucleated myofibers were embedded in a Type I collagen gel matrix as described previously to strengthen the cells for subsequent mechanical stimulation (6). By Day 7-8 postplating, the cultures consisted of a thin 10-50 μm thick "diaphragm-like" multilayer of cells containing a high density of well-striated, spontaneously contracting neonatal-like myofibers, unfused myoblasts, and interstitial fibroblasts. Approximately forty percent of the total culture nuclei were in unfused myoblasts and interstitial fibroblasts while 85-90% of the total noncollagenous protein in the cultures was located in the much larger multinucleated skeletal myofibers (33). The fibroblasts are essential in the culture model system to integrate the myofibers with the extracellular matrix material to form an organ-like tissue capable of being subjected to long-term mechanical stimulation without detachment or long-term damage (6).

Mechanical Stimulation

Day 7-8 muscle cultures were mechanically stimulated in a 37°C, 5% CO_2 incubator by placing the cell growth chamber above a computer controlled stepper motor attached to a moveable prong plate as described previously (32). Prior to mechanical stimulation, the cells were rinsed extensively over a 1-2 h period with Eagle's basal medium and then incubated with 0.5 ml/well of either Eagle's basal medium only or supplemented with bovine serum albumin (0.5 g/L), selenous acid (4.0 μg/L) and ferrous sulfate (0.8 mg/L). Results in both media were similar. Two mm diameter

prongs positioned below 18 of the 36 culture wells place a radial stretch on the cell layer when they move upward, and the cell layer returns to its initial position when the prongs move downward (Figure 1C). The cells were repetitively stretched and relaxed by the prong/stepper motor assembly using a software activity program which was shown previously to optimally increase PG synthesis in the muscle cells (34). The rounded prongs used in the modified mechanical cell stimulator place a nonuniform stretch on the cells, with the greatest stretch in the center of each well and the least stretch near the periphery. For example, in the activity program used in the present experiments (TRIAL 51.PGM), the prong moves up and down by 5 mm and the substratum stretches and relaxes by 25% in the inner third, 10% in the middle third, and 6% in the outer third of each well (mean stretch of all areas equals 12%). The cells are stretched and relaxed five times during a 20 s period followed by a 10 s rest; this activity is repeated twice more and is followed by a 15 min rest period (Figure 1D). The cells are thus mechanically stimulated for 6.7% of the experimental time. The rate of prong movement is 2.5 mm/s. In experiments where the percent stretch was varied from 2-23%, prong distance movement was varied in the same experiment by using prongs of different heights. Unstimulated controls were maintained in the same setup and on the same elastic substratum as the cells that were mechanically stimulated. The setup was placed on a rotary shaker (20-40 rpm) to eliminate any medium stirring differences between the control and stretch groups. All experiments were repeated with at least two

different preparations of primary muscle cells.

Biochemical Assays

PGE_2 and $PGF_{2\alpha}$ levels in the medium (the major eicosanoids produced by these cells) were measured using commercial radioimmunoassay kits (Advanced Magnetics, Cambridge, MA) as outlined previously (34) or by ELISA ($PGF_{2\alpha}$ only, Cayman Chemical Co., Ann Arbor, MI). The cross reactivity of the RIA antibodies at 50% binding (B)/initial binding (B_o) ratio were PGE_2 antibody: 1.3% for $PGF_{2\alpha}$, <1.0% for 6-keto-$PGF_{1\alpha}$; $PGF_{2\alpha}$ antibody, <0.3% for PGE_2, <2.0% for 6-keto-$PGF_{1\alpha}$. In the ELISA assay, $PGF_{2\alpha}$ cross-reacted <0.1% with PGE_2 and <2% with 6-keto-$PGF_{1\alpha}$.

Cyclooxgenase (prostaglandin GH synthase) activity was assayed in situ (24) b following the conversion of exogenously added arachidonic acid to PGE_2 or $PGF_{2\alpha}$. Activity was measured by adding 30 μM arachidonic acid to the culture medium (Eagle's basal medium) of the cells for 30 min at 37°C as described by Rosen et al (24). The concentration of newly synthesized $PGF_{2\alpha}$ in the medium after this 30 min incubation was assayed by either RIA or ELISA as outlined above. Without exogenous arachidonic acid, no $PGF_{2\alpha}$ was detected in the cells' culture medium after 30 min, and incubation of the arachidonic acid containing medium for 30 min at 37°C in the absence of cells did not lead to $PGF_{2\alpha}$ formation (data not shown). Similar results were obtained if PGE_2 was assayed.

Total noncollagenous protein content was measured by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL) as described previously (33). All other chemicals were reagent

grade and purchased from Sigma Chemical Co. (St. Louis, MI).

Morphometric Measurements

Cells were stained after fixation in either ethanol:acetic acid (2:1,v/v) for hematoxylin-eosin staining, or -20° methanol for enzyme immunocytochemistry using a monoclonal antibody specific for avian embryonic fast myosin heavy chain (EB165) [kindly provided by Dr. Everett Bandman] together with avidin-biotinylated secondary antibody reaction coupled to horseradish peroxidase (Vectastain ABC, Vector Labs., Burlingame, CA). The fixed and stained cells were coverslipped, and observed under a Zeiss light microscope. For cyclooxgenase immunocytochemical staining, cells were fixed in 3.7% formaldehyde for 10 min and stained using either polyclonal rabbit anti-mammalian cyclooxygenase antibody against the constitutive isoform (Oxford Biomedical, PG20) or polyclonal rabbit anti-chicken cyclooxygenase antibody against the mitogen-inducible isoform (Oxford Biomedical, PG25).

All data were analyzed statistically by PCStatistician software (Human Systems Dynamics, Northridge, CA).

RESULTS

Intermittent mechanical stimulation of differentiated myofiber cultures in the modified mechanical cell stimulator for 24 h significantly increased $PGF_{2\alpha}$ production as previously reported for the original system (34,38). In three separate experiments, the percent stretch-induced increase in $PGF_{2\alpha}$ efflux after 24 h varied between 45% and 173% with different cell preparations as previously found (34). Twelve to fifteen percent repetitive cell stretches were found to be optimal for increased $PGF_{2\alpha}$ production and percent stretches less than or greater than this were not as effective (Figure 2). These results are similar to those reported elsewhere (34).

The muscle cultures contain two main cell types, multinucleated striated myofibers and interstitial fibroblasts. Cyclooxgenase specific antibodies were used to identify the cyclooxygenase isoform and cell type in which the enzyme is located. Staining with antibody PG20 which is specific for the constitutive isoform of cyclooxgenase gave very weak general staining of both myofibers and fibroblasts (data not shown), while antibody PG25 which is specific for the mitogen-inducible isoform of cyclooxgenase stained much more intensely and specifically stained the cytoplasm of the differentiated myofibers without any indication of fibroblast staining (Figure 3). PG25 antibody diluted 1:100 reacted with a single protein band in avian muscle culture extracts on Western blots while the PG20 antibody did not detect any protein bands at a 1:100 dilution (data not shown).

To determine whether the stretch-induced increase in $PGF_{2\alpha}$ production resulted from altered cyclooxgenase activity, enzymatic activity was measured in intact cells by adding 30 μM exogenous arachidonic acid during the last 30 min of mechanical activity. Control experiments indicated that $PGF_{2\alpha}$ synthesis from exogenously added arachidonic acid was linear for at least 60 min (Figure 4 upper left insert) and a 30 μM concentration was found to optimally stimulate $PGF_{2\alpha}$ synthesis (Figure 4, upper right insert). Cyclooxygenase activity in the cultured muscle cells by this assay represents true enzyme activity since it is inhibitable by three well-known cyclooxgenase inhibitors - indomethacin, aspirin, and ibuprofen (Figure 4, lower insert). Intermittent mechanical stimulation for either 4-5 h or 24 h significantly increased cyclooxgenase activity compared to static controls (Figure 4). After 4-5 h of mechanical stimulation by TRIAL51.PGM, cyclooxgenase activity was increased 98% ($P<.003$) and by 178% ($P<.002$) after 24 h. Similar results were obtained in two additional experiments with different cell preparations (data not shown).

For kinetic analysis of the stretch-induced increase in cyclooxygenase activity, the cells were mechanically stimulated for 4 or 24 h and incubated with 5-50 μM exogenous arachidonic acid during the last 30 min of the experiment. $PGF_{2\alpha}$ production was assayed from an aliquot of the medium. Plotting the data as double reciprocal plots and calculating K_M and V_{max} values for cyclooxygenase indicated that stretch-induced increases in enzyme activity at both time points were due to a two-to-three-fold

decrease in the K_M with little effect on the enzyme's V_{max} (Figure 5).

To determine whether protein synthesis was required for the stretch-induced increase in cyclooxygenase activity, the stretch experiments were repeated in the presence of 70 μM cycloheximide. This concentration of cycloheximide inhibited total protein synthesis rates by 87-92%, based on reduced incorporation of ^{14}C-phenylalanine into trichloroacetic acid precipitable material (data not shown). Total noncollagenous cellular protein levels were reduced 12% after 4-5 h in cycloheximide (mean ± S.E.: 363 ± 26 vs. 321 ± 33 μg/well, $p < .04$) and 33% after 20-22 h (mean ± S.E. 444 ± 26 vs. 299 ± 34, $p < .001$). Surprisingly, cyclooxygenase activity was significantly elevated in static control cultures by cycloheximide after both 4-5 h and 20-22 h of incubation (Table 1). In 4 separate experiments, cycloheximide increased cyclooxygenase activity by 56% to 220%. Similar results were obtained when RNA synthesis was inhibited with 1.0 μM actinomycin D (data not shown). This elevated enzymatic activity was sensitive to inhibition by the cyclooxgenase inhibitor indomethacin (Table 1, Experiment 3). $PGF_{2\alpha}$ production from endogenous arachidonic acid over a 12 h period was increased two-fold by 70 μM cycloheximide. $PGF_{2\alpha}$/mg protein increased from a mean ± S.E. of 71 ± 12 to 220 ± 55 ($n=4$, $P < .04$).

Following a 1 h preincubation in 70 μM cycloheximide at 37°C, the cells were mechanically stimulated for 4-24 h in the presence of cycloheximide and cyclooxygenase activity measured during the last 30 min with 30 μM exogenous arachidonic acid. After 4-5 h of

mechanical stimulation, cyclooxygenase activity was still significantly increased compared to static controls (Figure 6). At 20-24 h, cyclooxygenase activity was not significantly greater than static control levels, but this was primarily due to the elevated control values in the cycloheximide-treated cultures at the longer incubation periods (Figure 6).

Since the differentiated myofibers are spontaneously contractile, it is important to distinguish between a direct mechanical effect on cyclooxgenase activity and an indirect mechanically-induced alteration in myofiber electrical activity. We have shown previously that stretch-induced $PGF_{2\alpha}$ production is not inhibited by tetrodotoxin, a voltage-sensitive sodium channel inhibitor which completely blocks all contractile activity in the cultures. Tetrodotoxin also had no significant effect on either control or stretch-activated cyclooxygenase activity (Figure 7). Whereas tetrodotoxin was ineffective in preventing stretch-induced prostaglandin production (34) or cyclooxygenase activity, the G protein inhibitor pertussis toxin inhibited both stretch-induced $PGF_{2\alpha}$ production (Figure 8A) and cyclooxygenase activation (Figure 8B). In addition, pertussis toxin inhibited the stretch-induced increase in noncollagenous protein (Figure 8C) associated with myofiber hypertrophy (33).

DISCUSSION

The major finding in this report is that repetitive mechanical stretching of differentiated skeletal muscle cultures increased the production of $PGF_{2\alpha}$, an anabolic growth modulator, by increasing the activity of cyclooxygenase, an important regulatory enzyme in prostaglandin synthesis. Stretch activation of cyclooxygenase occurred by a posttranslational mechanism based on two lines of evidence: (1) decreased cyclooxygenase K_M with no change in V_{max}; and (2) cycloheximide insensitivity. The stretch-induced increases in $PGF_{2\alpha}$ production and cyclooxygenase activity, as well as the increase in protein accumulation, were inhibited by pertussis toxin. Thus, these stretch-related growth responses involve mechanically-activated G proteins of the G_i or G_o subtype. Repetitive stretch of these cells also activates several specific phospholipases (A2 and D) (38) which provide endogenous arachidonic acid for cyclooxygenase conversion into prostaglandins. Thus, mechanical cell stretching appears to activate a cascade of steps involved in the generation of lipid-related mechanogenic second messengers which regulate skeletal muscle growth.

Cyclooxygenase exists in two isoforms (24,41), one of which is a mitogen-inducible, immediate-early gene product (5,26). The cyclooxygenase isoform which is activated by stretch in the skeletal muscle cultures is not known, but may be the mitogen-inducible isoform, based on several lines of evidence. First, the mitogen-inducible isoform is approximately 25 fold less sensitive

to indomethacin inhibition than the constitutive isoform[1], and high concentrations of indomethacin are required to inhibit stretch-activated cyclooxygenase activity in the skeletal muscle cultures (34). Secondly, greater than 90% of the stretch-induced prostaglandin production in these cultures comes from the skeletal myofibers rather than the fibroblasts (38). Immunocytochemical staining of the cultured skeletal muscle cells indicate that the skeletal myofibers contain high levels of the mitogen-inducible cyclooxygenase isoform when compared to neighboring mononucleated fibroblasts (Figure 4). Other immediate-early gene products, especially the protooncogenes, have been shown to be associated with cardiac muscle hypertrophy (28) and are stretch-activated (14,25). If the mitogen-inducible isoform of cyclooxygenase is stretch-activated in skeletal muscle and responsible for increased $PGF_{2\alpha}$ production, it would be the first demonstration of an immediate early gene product involved in cell hypertrophy with a well defined function.

Cyclooxygenase in adult rat skeletal muscle has been reported to be an extremely rapidly turning over protein, with a half-life of less than 10 min, based on rapid inhibition of enzymatic activity in organ cultures with either cycloheximide or actinomycin D (4). In contrast, other studies with adult rat skeletal muscle organ cultures have reported no effect of cycloheximide on cyclooxygenase activity for at least 2 h (16). Direct measurement of human dermal fibroblast cyclooxygenase turnover indicated a one

[1]Cayman Chemical Co. product literature.

hour half-life (21). In the present study, it was surprising that neither cycloheximide nor actinomycin D inhibited cyclooxygenase activity in cultured skeletal muscle over a 20-24 h incubation period under conditions where protein synthesis was inhibited by 87-93% (Table 1). In fact, cyclooxygenase activity was significantly elevated by an indomethacin-sensitive mechanism when protein synthesis was inhibited. If the enzyme in these cells had a half-life of one hour or less, little activity should remain after 4 h or longer incubations in cycloheximide. The reason for the discrepancy in cyclooxygenase half life and the effects of protein synthesis inhibition in different experiments is not known, but may reflect greater cyclooxygenase stability in skeletal muscle versus fibroblasts (21), age-related differences in skeletal muscle [neonatal muscle in this study versus adult muscle in (4)] or the conditions under which the cyclooxygenase was assayed [isometric in this study versus hypercontracted organ culture in (4)]. More direct measurement of cyclooxygenase half-lives in the myofibers will be required to resolve this question. Cycloheximide activation of cycooxygenase activity in these cells may occur by inhibition of a rapidly turning over negative regulator of the enzyme.

Stretch-induced myofiber hypertrophy and prostaglandin production were shown previously to be independent of cell electrical activity i.e. tetrodotoxin-insensitive (33,34). Likewise, the present paper indicates that stretch-activated cyclooxygenase activity is tetrodotoxin-insensitive (Figure 7). In contrast, stretch-induced increases in protein content,

prostaglandin production, and cyclooxygenase activity are inhibited by pertussis toxin (Figure 8), indicating involvement of stretch-sensitive G proteins of the α_1 or α_o subclass. While G proteins are known to regulate prostaglandin synthesis through their control of phospholipase activities (12), this is the first report of their involvement in also regulating cyclooxygenase enzymatic activity.

There are several mechanisms by which G proteins could regulate mechanically-induced skeletal muscle growth and prostaglandin synthesis. Stretch increases the sensitivity of muscle cells to exogenous anabolic growth factors such as insulin and insulin-like growth factor-1 (30,36) whose receptors are G protein-coupled (11). Pertussis toxin may uncouple this signal transduction pathway. Secondly, muscle cell growth is influenced by calcium levels (10), and stretch is known to regulate ionic fluxes in skeletal muscle (37). G proteins may regulate stretch-activated ionic channels important for regulating intracellular calcium levels (1,12,13). Stretch-activated PG production in these cells is partially dependendent on extracellular calcium (38).

In summary, mechanical stimulation of tissue cultured skeletal muscle stimulates the activity of cyclooxgenase, the major regulatory enzyme in $PGF_{2\alpha}$ production, an anabolic growth factor for skeletal muscle cells. This activation occurs by a posttranslational, G-protein dependent mechanism. Understanding which G proteins are mechanically-sensitive and involved in regulating anabolic growth modulators such as prostaglandins in skeletal muscle will aid in understanding how muscle

stretch/tension/exercise regulates muscle cell size.

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TABLE I

Protein Synthesis Inhibition Stimulates Cyclooxygenase Activity In Skeletal Muscle Cultures

periment	Incubation Time (hours)	Cycloheximide (70 μM)	Indomethacin	Activity (ng $PGF_{2\alpha}$/mg protein)	%Δ
1	4	-	-	0.24 ± 0.03	
		+	-	0.44 ± 0.07*	85%
2	21	-	-	0.30 ± 0.04	
		+	-	0.81 ± 0.15**	165%
3	22	-	-	0.83 ± 0.09	
		+	-	2.65 ± 0.12***	220%
		+	+	0.54 ± 0.03	35%

*P<.01 **P<.02 ***P<.001

Day 6-7 muscle cultures were incubated for 4-22 h with or without 70 μM cycloheximide and cyclooxygenase activity measured over a 30 min period with 30 μM exogenous arachidonic acid as outlined in the text. In experiment 3, 100 μM indomethacin was added 3 h prior to the addition of cycloheximide. Cycloheximide did not interfere with the ELISA assay used to measure $PGF_{2\alpha}$ (data not shown). Each value is the mean ± S.E.of 4-6 samples.

FIGURE LEGENDS

Figure 1. Diagrammatic representation of the modified mechanical cell stimulator. (A) The stainless steel cell growth chamber contains 36 teflon-lined culture wells 15 mm in diameter; (B) 2 mm diameter prongs move up and down to stretch and relax the cells; (C) "diaphragm-like" tissue samples attach and are stretched while attached to stainless steel wire cloth at the periphery of each well; (D) the computer-generated pattern of mechanical activity used to stimulate PG synthesis and cyclooxygenase activity. Bar in A equals 3.0 cm.

Figure 2. Intermittent mechanical stimulation increases $PGF_{2\alpha}$ production in skeletal muscle cultures. Seven days postplating cultures were mechanically stimulated by TRIAL 51.PGM activity program (Figure 1D) at different intensities (percent stretch) in serum-free defined medium for 24 h. Percent stretch was varied between groups of wells in the same experiment by using prongs of different heights. $PGF_{2\alpha}$ levels in the medium were assayed as described in MATERIALS AND METHODS. Results are expressed as pg/mg total noncollagenous protein and each point represents the mean $\pm$ SE of 5-6 samples. Statistical analysis was by t-tests for unpaired values.

Figure 3. Cyclooxygenase immunocytochemical localization in skeletal myofibers. Day 7-8 cultured cells were fixed and stained with either hematoxylin and eosin (upper), preimmune serum

(middle), or anti-cyclooxygenase antibody (mitogen-inducible) as outlined in MATERIALS AND METHODS (lower).

Figure 4. Intermittent mechanical stimulation increases cyclooxygenase activity in intact cells. Seven to eight day postplating cultures were mechanically stimulated by TRIAL 51.PGM activity program for 5-24 h in serum-free medium. Thirty μM exogenous arachidonic acid was added in fresh serum-free medium for the last 30 min of incubation to either static control wells ($\circ$) or mechanically stimulated wells ($\bullet$). $PGF_{2\alpha}$ levels were measured in an aliquot of the medium. Total noncollagenous protein concentrations are within 10% of each other in control and mechanically stimulated cultures. The upper left insert shows that $PGF_{2\alpha}$ synthesis from 30 μM arachidonic acid in static muscle cultures was linear for at least 60 min ($\bullet$) and that no detectable $PGF_{2\alpha}$ was synthesized during the 30 min incubation in the absence of exogenous arachidonic acid (∇). The upper right insert shows that 30 μM exogenous arachidonic acid maximally stimulated $PGF_{2\alpha}$ synthesis during a 30 min assay period. The bottom left insert shows that cyclooxygenase inhibitors indomethacin ($\circ$), aspirin ($\triangle$), or ibuprofen ($\square$) inhibit cyclooxygenase activity in a dose-dependent manner in this in situ assay. Each point is the mean $\pm$ SE of 4-6 samples and statistical analyzing was by unpaired t-tests. *P<.003; **P<.002.

Figure 5. Kinetics of stretch-induced alterations in cyclooxygenase activity. Seven to eight day postplating cultures were mechanically stimulated by TRIAL 51.PGM activity program for 4 (A) or 24 h (B) in serum-free medium. Varying concentrations of exogenous arachidonic acid were added in fresh serum-free medium for the last 30 min of incubation to either static control wells (O) or mechanically stimulated wells (●). Each point is the mean ± SE of 4-6 samples and statistical analyzing was by unpaired t-tests.

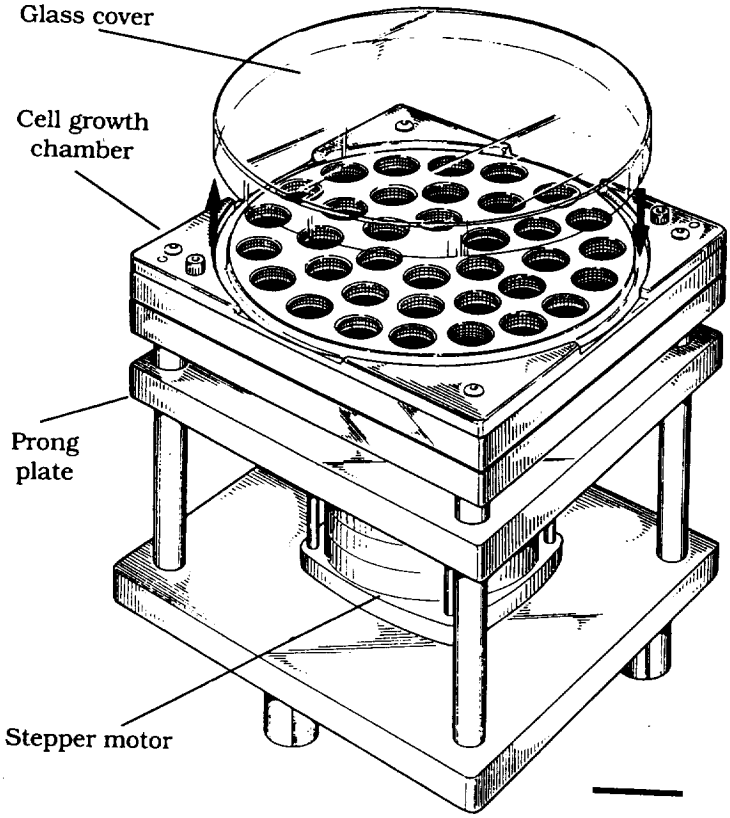
Figure 6. Stretch increases cyclooxygenase activity in the presence of cycloheximide. Day 7-8 postplating muscle cultures were rinsed in serum-free medium for 1-2 h as outlined in MATERIALS AND METHOD with or without 70 μM cycloheximide (CHX). Mechanical stimulation by TRIAL51.PGM was initiated for 4-24 h ± CHX. Fresh serum-free medium was added ± CHX containing 30 μM arachidonic acid for 30 min and an aliquot of the medium assayed for $PGF_{2\alpha}$. The values are the mean ± SE of 4-6 samples per group and statistical analyses were by unpaired t-tests.

Figure 7. Intermittent mechanical stimulation increases cyclooxygenase activity in electrically quiescent muscle cultures. Day 7-8 postplating muscle cultures were rinsed in serum-free medium for 2 h as outlined in MATERIAL AND METHODS with 1 μg/ml tetrodotoxin (TTX) in some wells. Mechanical stimulation by TRIAL51.PGM activity program was initiated for 24 h ± TTX. Fresh serum-free medium was added ± TTX containing 30 μM arachidonic acid

for 30 min and an aliquot of the medium assayed for $PGF_{2\alpha}$. Total noncollagenous protein concentrations were within 20% in the different experimental groups. The values are the mean ± SE of 5-6 samples and statistical analyses were by unpaired t-tests.

Figure 8. Stretch-stimulated $PGF_{2\alpha}$ production, cyclooxygenase activity and protein accumulation are pertussis toxin-sensitive. Six to seven day postplating cultures were preincubated overnight with or without 100 ng/ml pertussis toxin, rinsed, and mechanical stimulation (TRIAL 51.PGM) initiated for 24 h with or without pertussis toxin. (A) $PGF_{2\alpha}$ production. The 24 h culture medium was collected and assayed for $PGF_{2\alpha}$ production by ELISA. (B) Cyclooxygenase activity. Fresh medium containing 30 μM arachidonic acid was added for the last 30 min of incubation and $PGF_{2\alpha}$ production assayed. (C) Total noncollagenous cellular protein in the different groups was assayed as outlined in MATERIALS AND METHODS. Each bar represents 4-6 samples ± S.E. and statistical analyses are by unpaired t-tests. IAP, pertussis toxin

Fig. 1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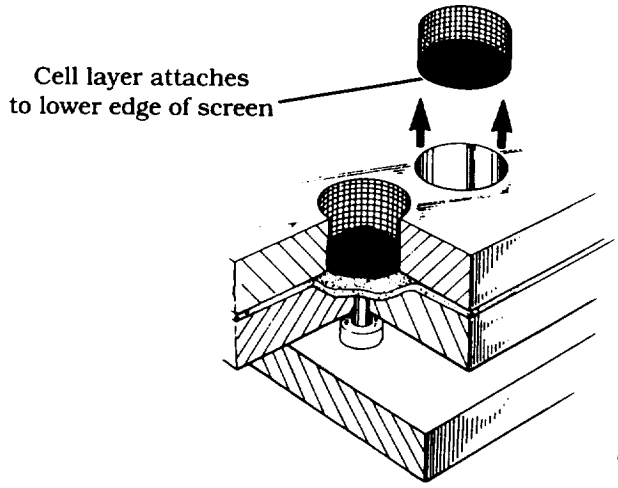
Prong plate
Stepper motor

C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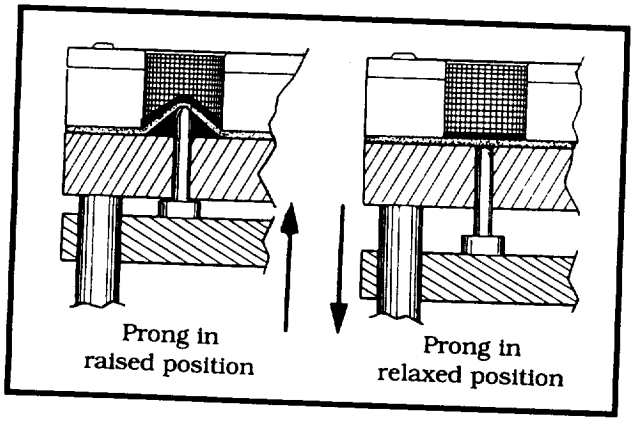
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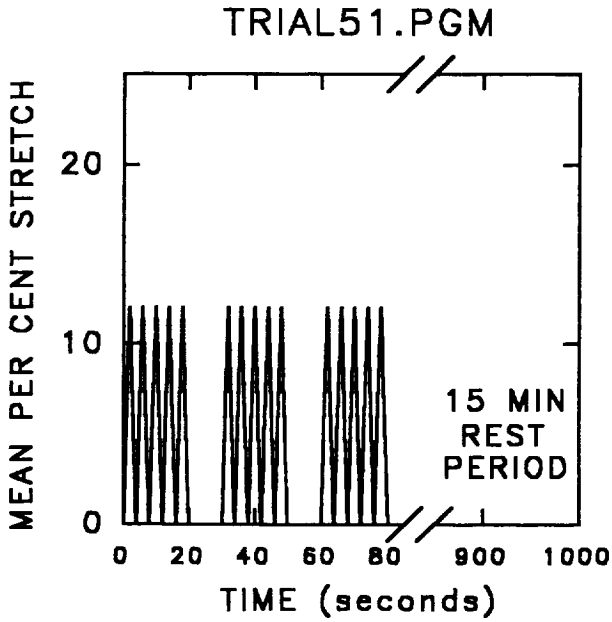
Prong in relaxed position

D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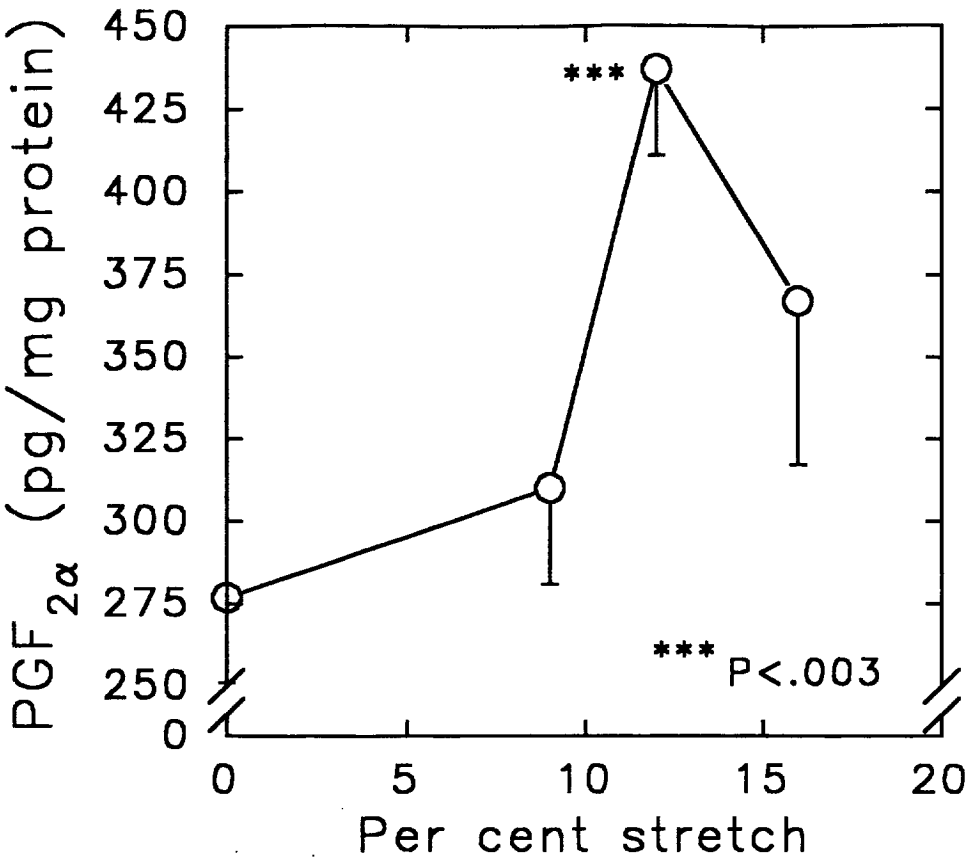
20
10
0
15 MIN REST PERIOD
0 20 40 60 80 900 1000
TIME (seconds)

Fig 2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425
400
375
350
325
300
275
250
0
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***
***
P<.003
0
5
10
15
20
Per cent stretch

MCS3-2C

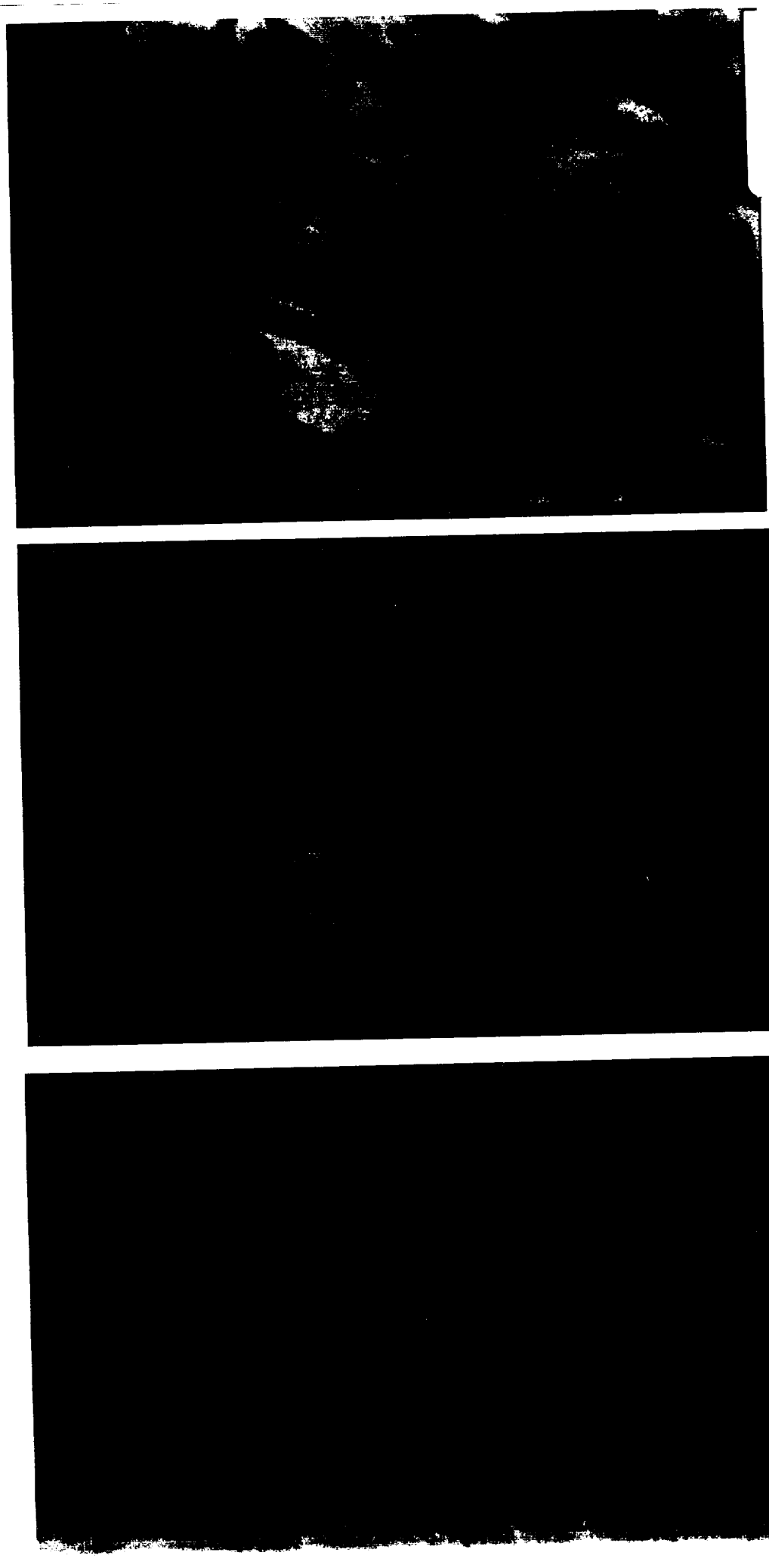
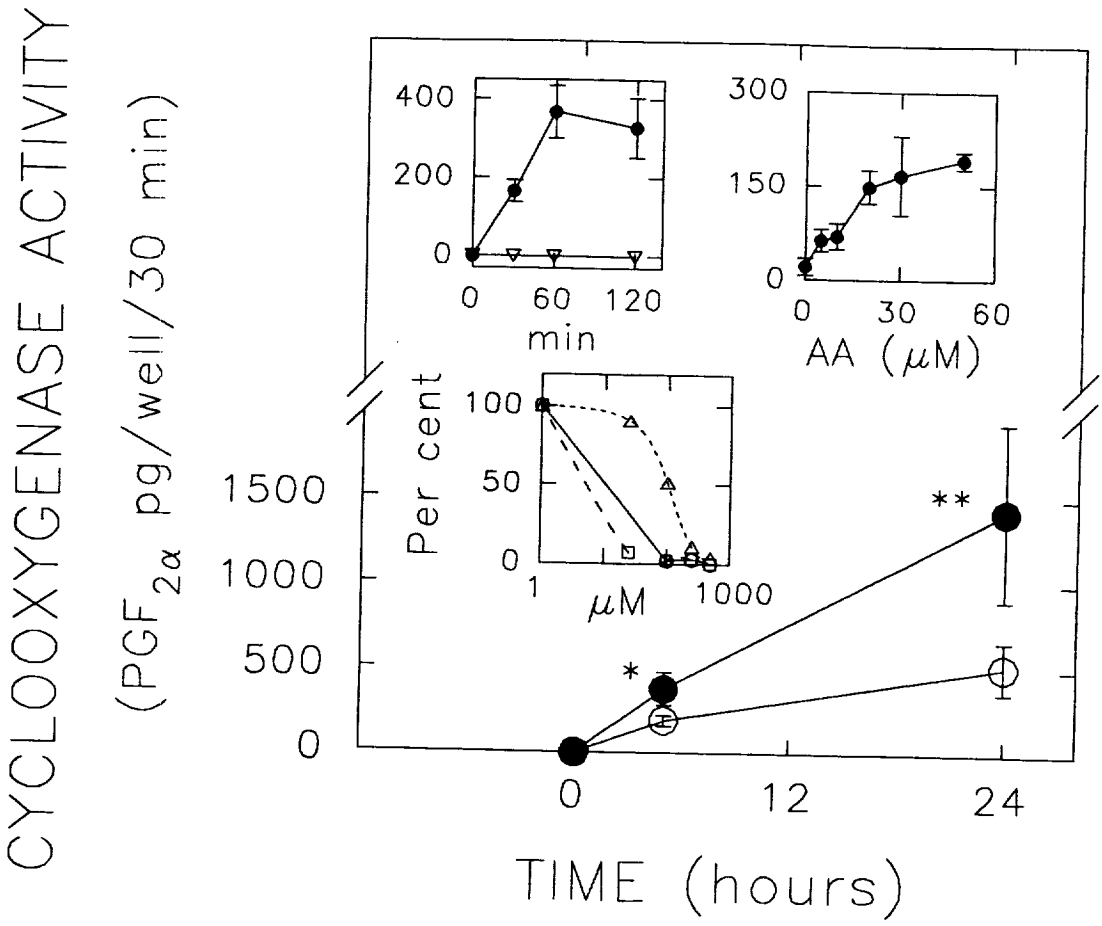


Fig 3

Fig. 4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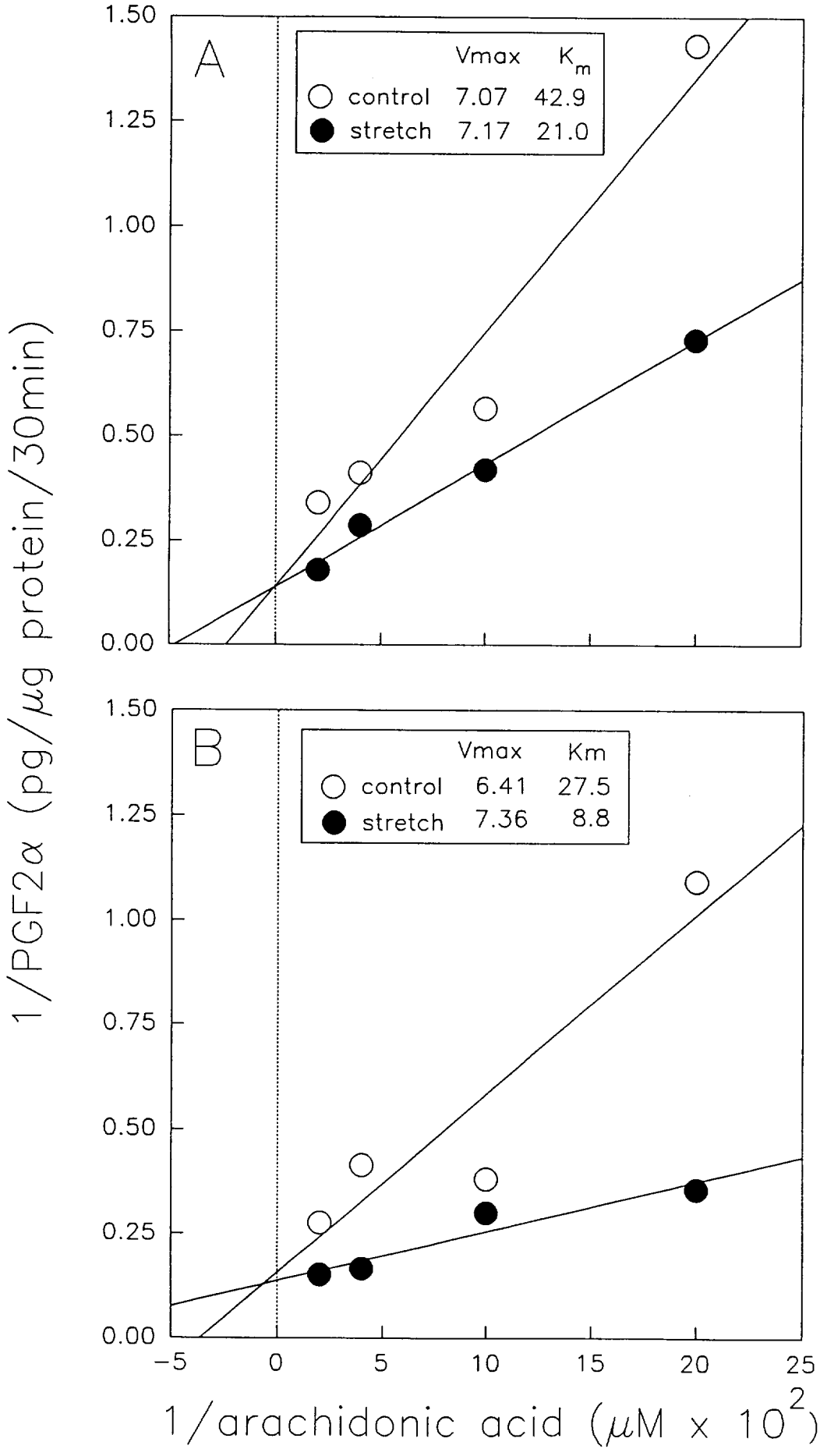
(PGF2α pg/well/30 min)
1500
1000
500
0
0
12
24
TIME (hours)
*
**
400
200
0
0
60
120
min
300
150
0
0
30
60
AA (μM)
Per cent
100
50
0
1
μM
1000

Fig. 5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1.25
1.00
0.75
0.50
0.25
0.00
−5
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5
10
15
20
25
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1/arachidonic acid (μM x 10²)

Fig 6

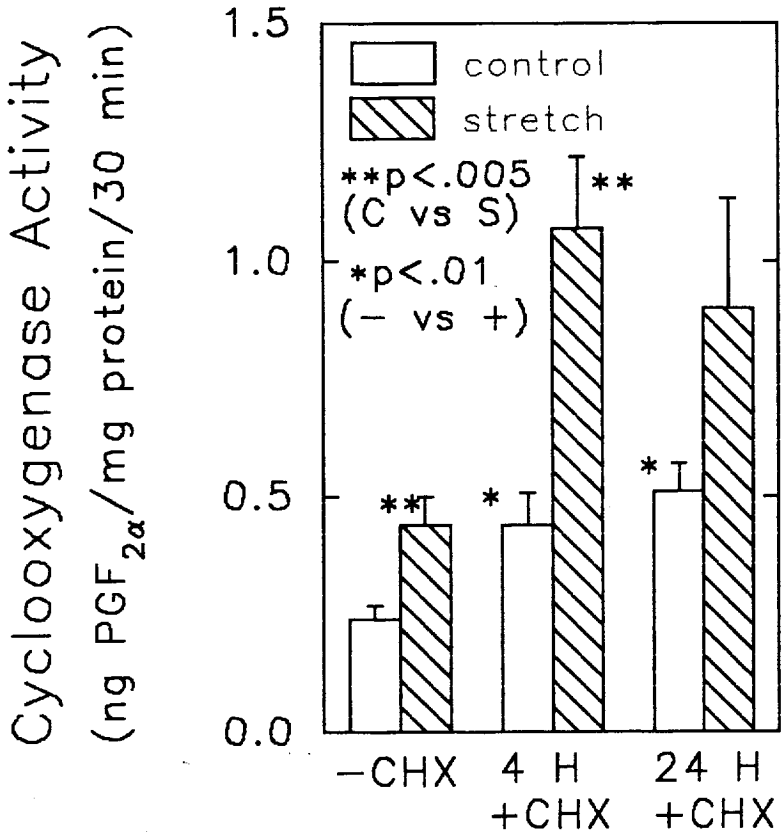

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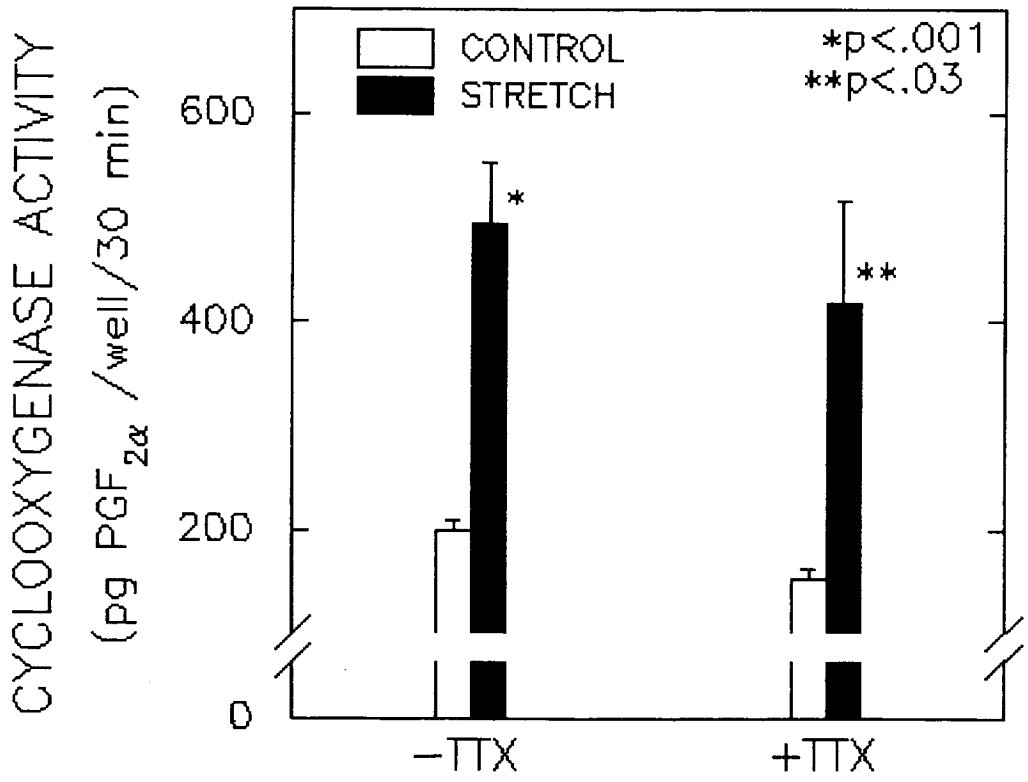
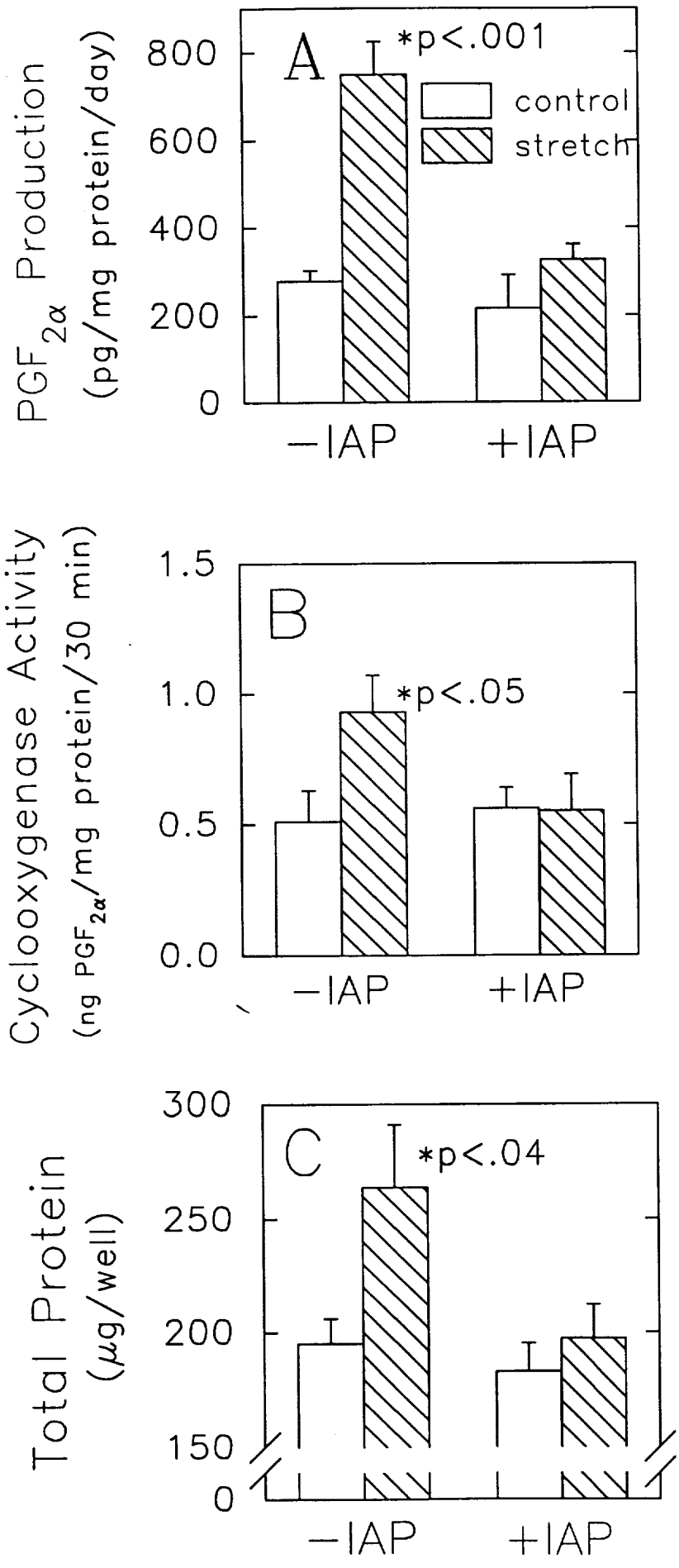
Fig 7



COXFIG4.SPG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200
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+IAP
B
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Cyclooxygenase Activity
(ng PGF2α/mg protein/30 min)
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1.0
0.5
0.0
−IAP
+IAP
C
*p<.04
Total Protein
(μg/well)
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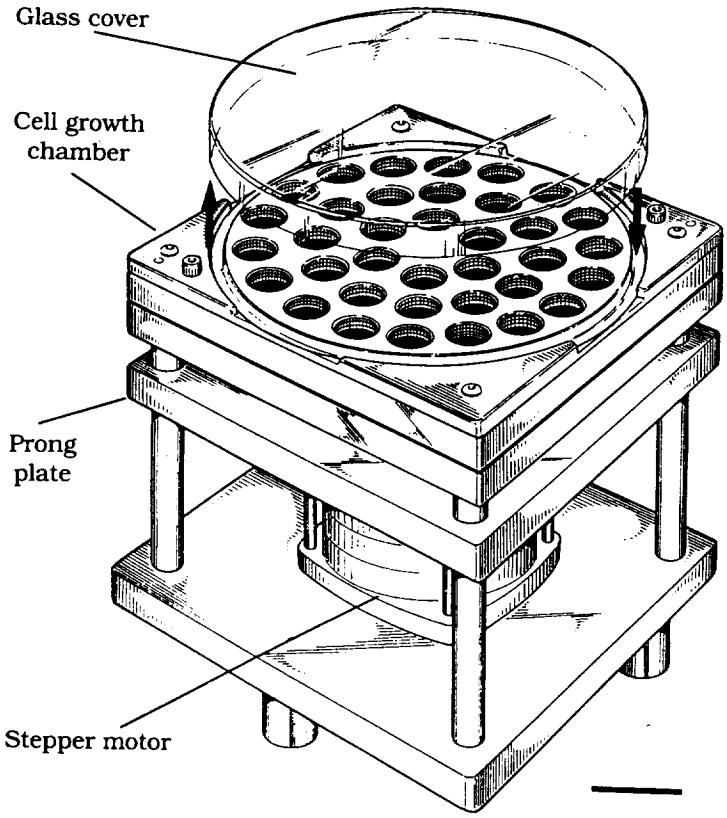
200
150
0
−IAP
+IAP

Fig. 8

Fig. 1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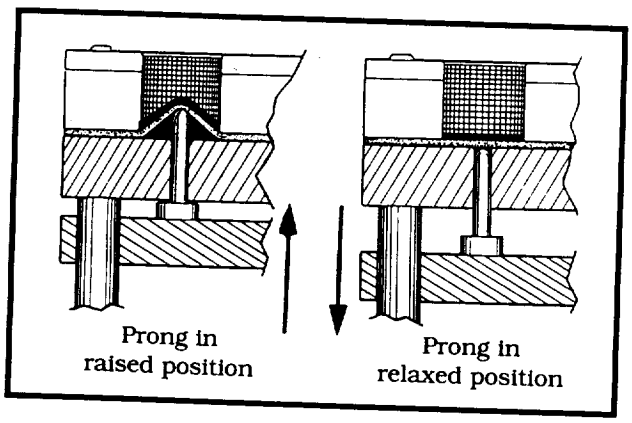
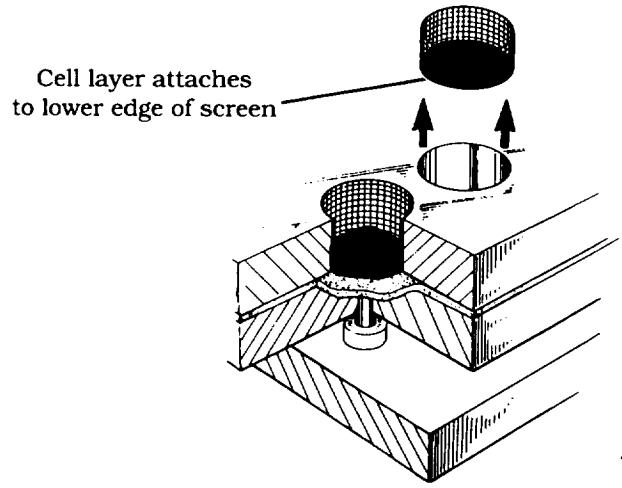
Cell growth chamber
Prong plate
Stepper motor


Prong in raised position
Prong in relaxed position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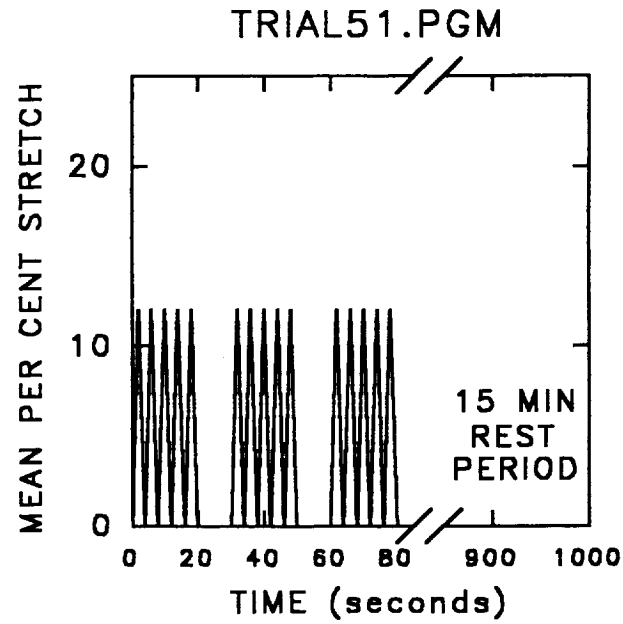
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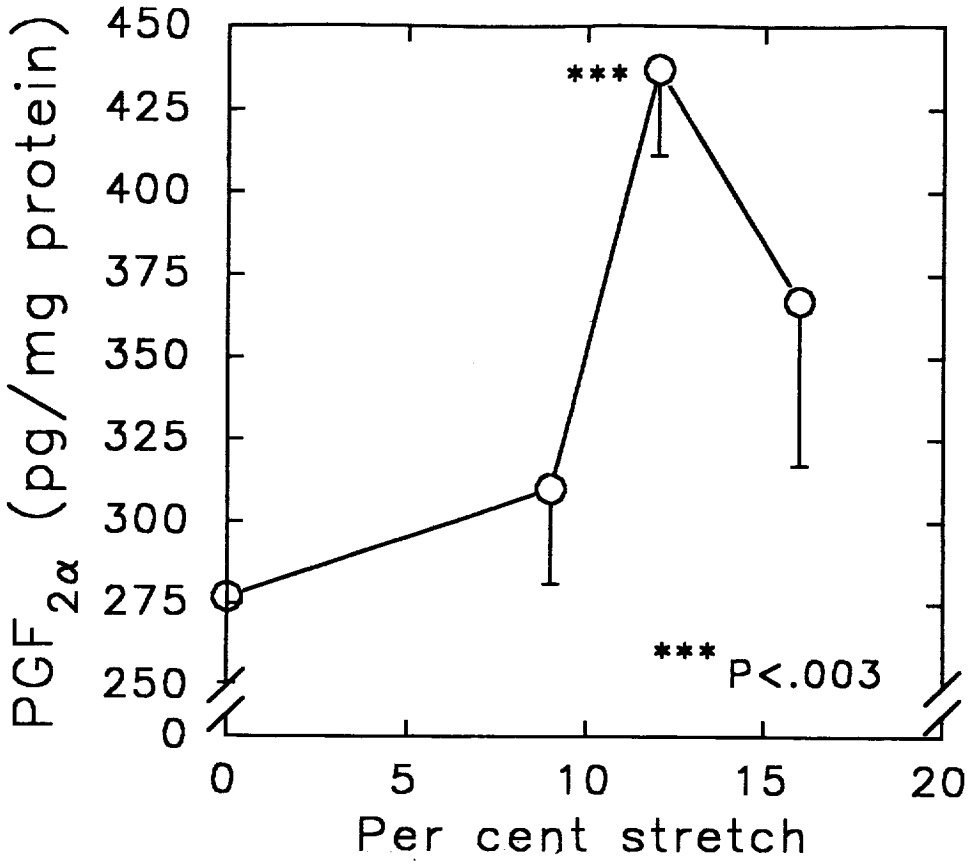
0
15 MIN REST PERIOD
0 20 40 60 80 900 1000
TIME (seconds)

Fig 2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400
375
350
325
300
275
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PGF2α (pg/mg protein)
***
*** P<.003
0
5
10
15
20
Per cent stretch

MCS3-2C

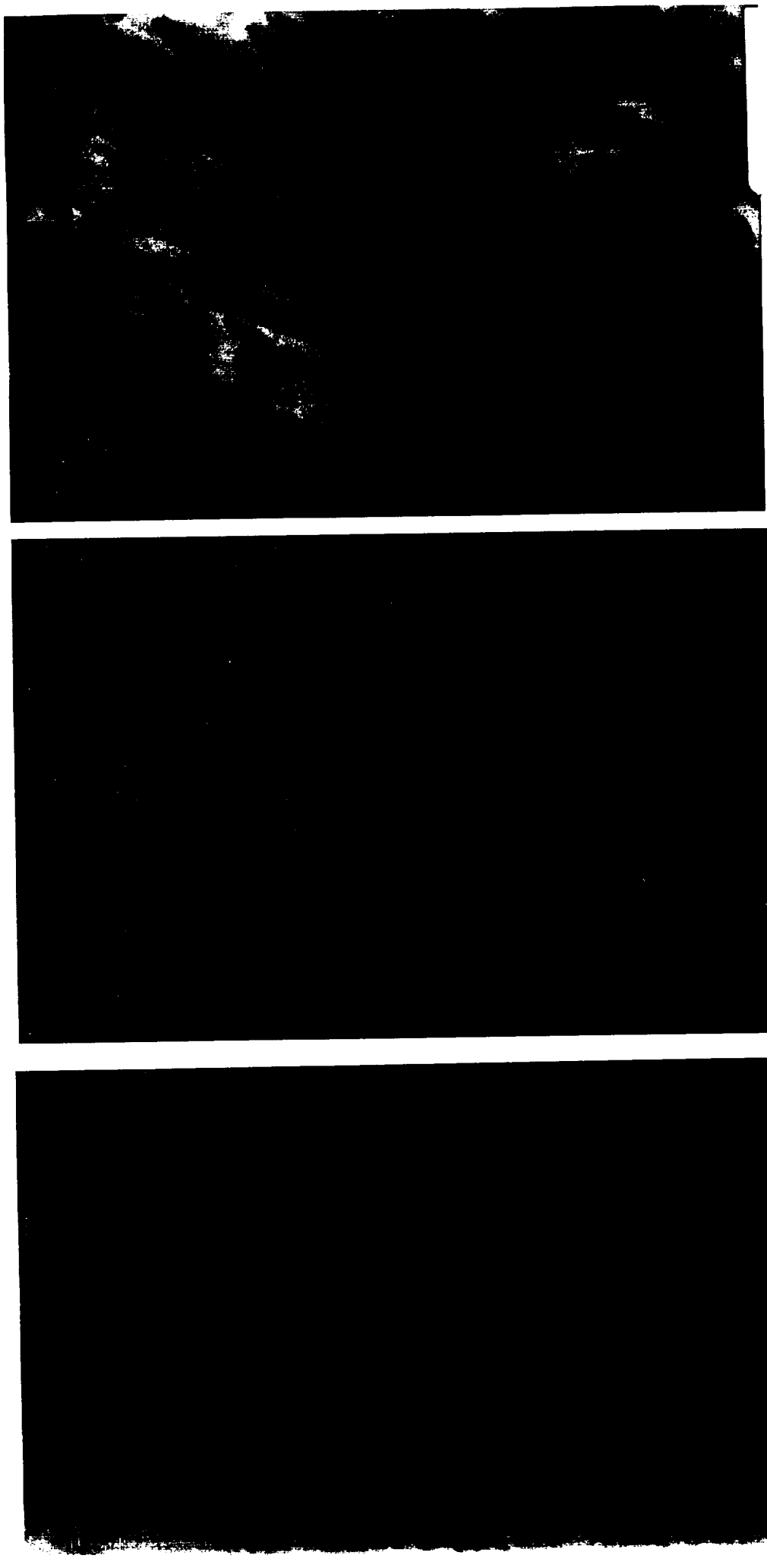
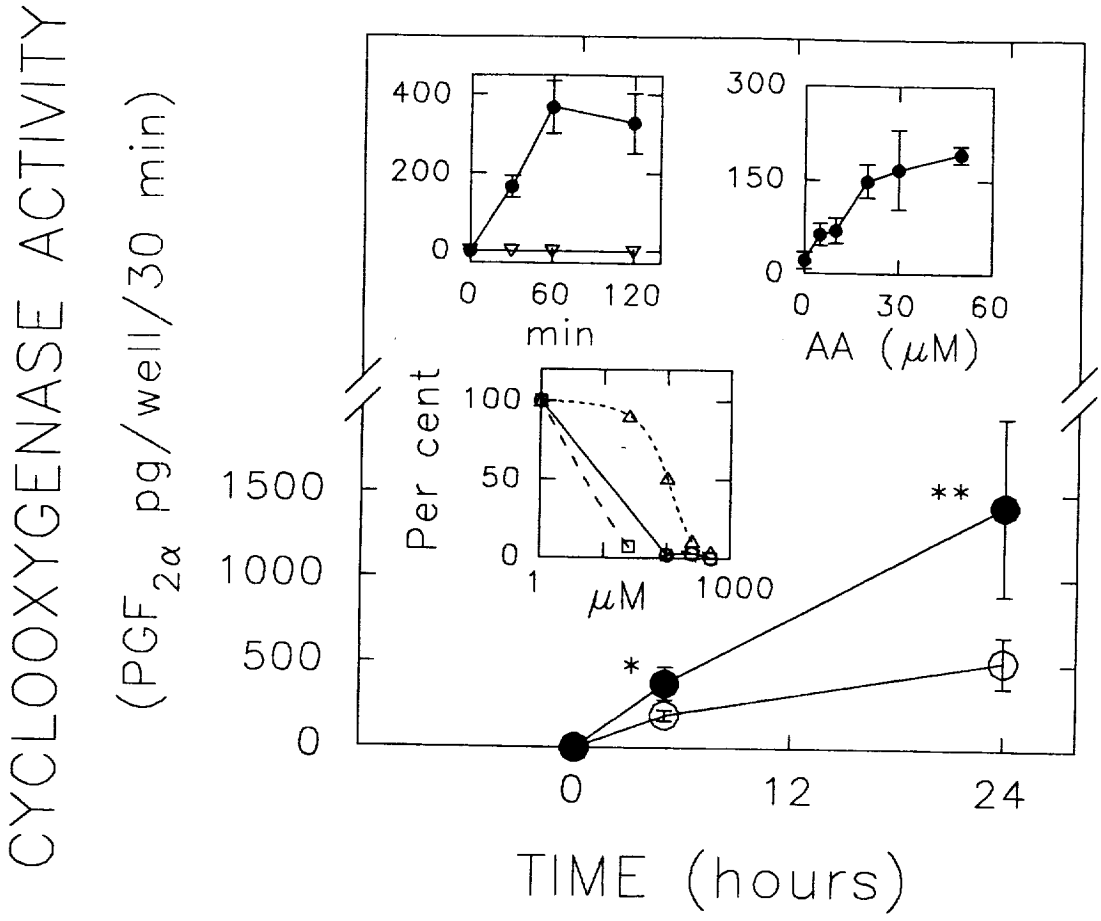


Fig 3

Fig. 4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(PGF2α pg/well/30 min)
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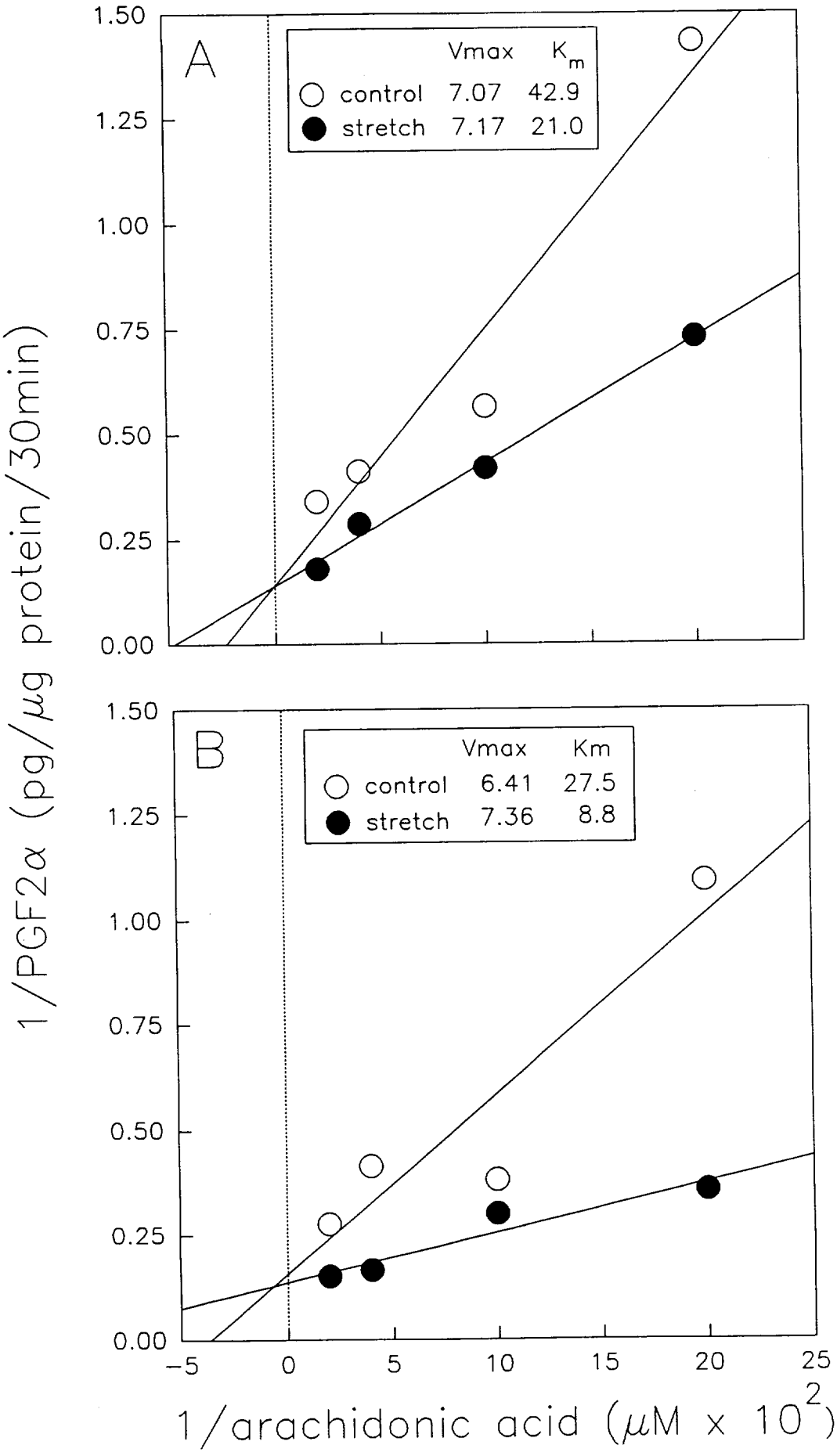
1000
500
0
0
12
24
TIME (hours)
*
**
400
200
0
0
60
120
min
300
150
0
0
30
60
AA (μM)
Per cent
100
50
0
1
μM
1000

Fig. 5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stretch 7.17 21.0
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Vmax
Km
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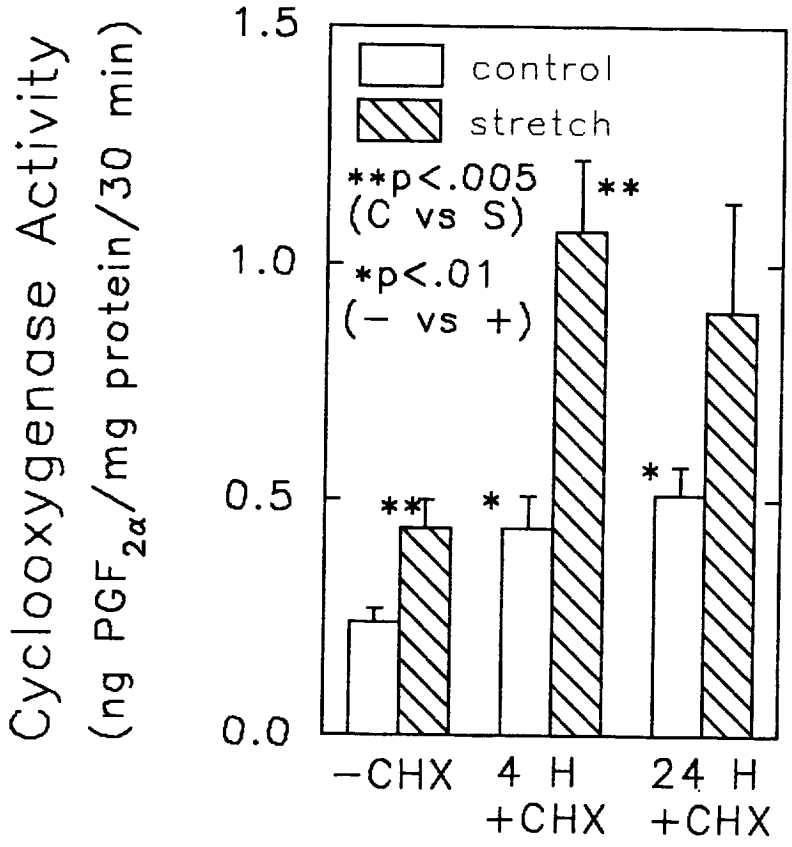
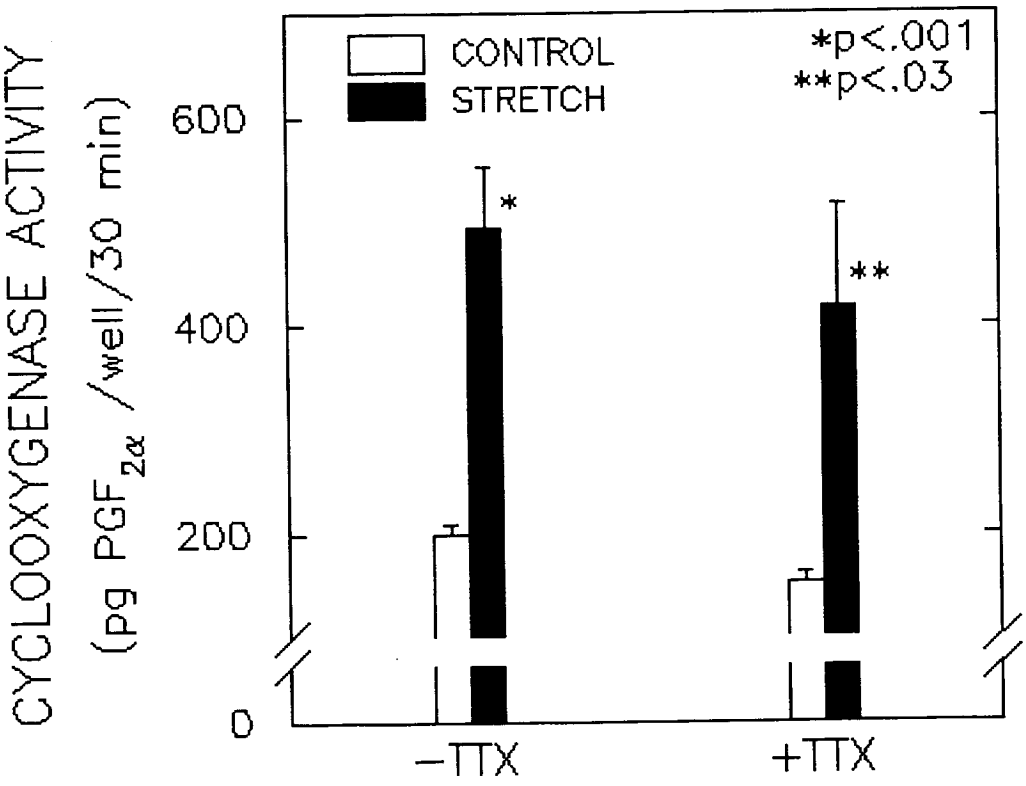
stretch 7.36 8.8
1.50
1.25
1.00
0.75
0.50
0.25
0.00
−5
0
5
10
15
20
25
1/PGF2α (pg/μg protein/30min)
1/arachidonic acid (μM x 10^2)

Fig 6

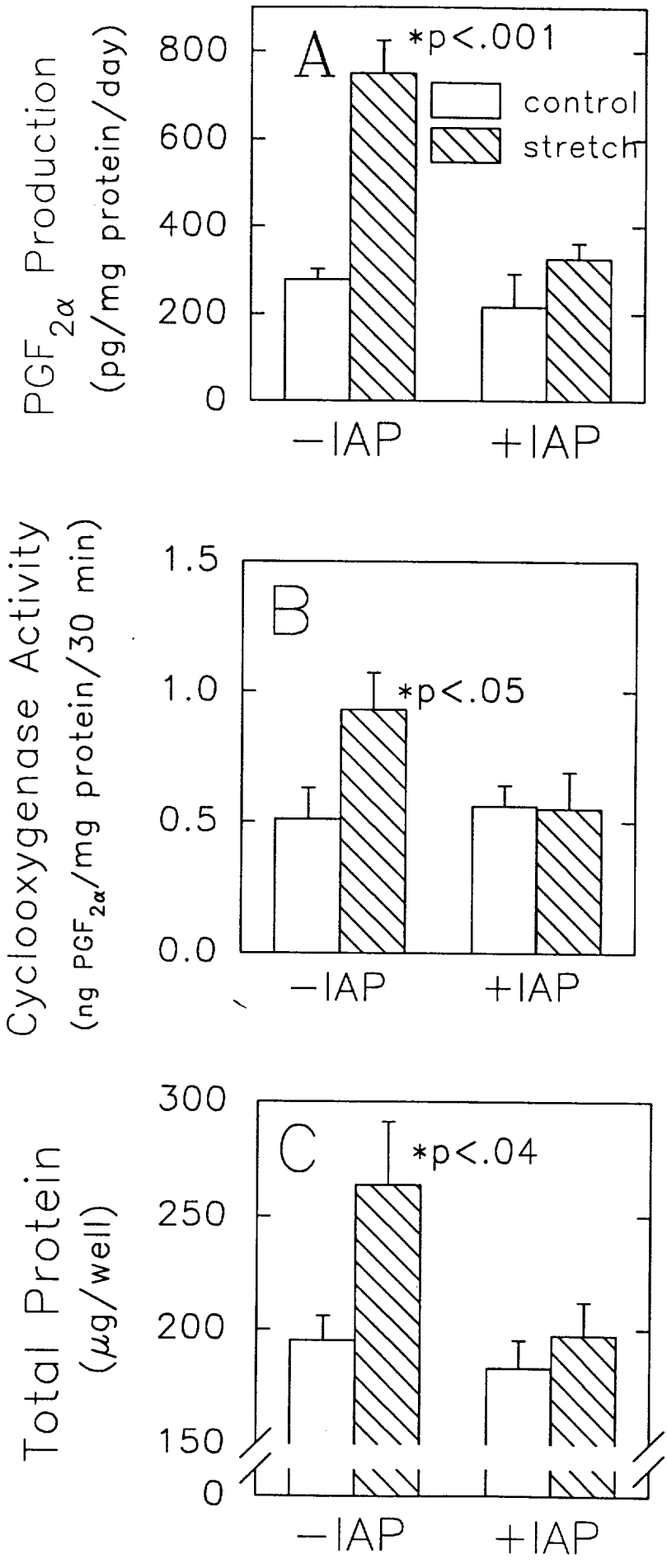


COXCHX4.SPG

Fig 7



COXFIG4.SPG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*p<.001
control
stretch
$PGF_{2\alpha}$ Production
(pg/mg protein/day)
800
600
400
200
0
−IAP
+IAP
B
*p<.05
Cyclooxygenase Activity
(ng $PGF_{2\alpha}$/mg protein/30 min)
1.5
1.0
0.5
0.0
−IAP
+IAP
C
*p<.04
Total Protein
(μg/well)
300
250
200
150
0
−IAP
+IAP

Fig. 8