Stress Relaxation of Fibroblasts Activates A Cyclic AMP Signaling Pathway

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Abstract. Mechanical force regulates gene expression and cell proliferation in a variety of cell types, but the mechanotransducers and signaling mechanisms involved are highly speculative. We studied the fibroblast signaling mechanism that is activated when cells are switched from mechanically stressed to mechanically relaxed conditions, i.e., stress relaxation. Within 10 min after initiation of stress relaxation, we observed a transient 10–20-fold increase in cytoplasmic cyclic AMP (cAMP) and a threefold increase in protein kinase A activity. The increase in cAMP depended on stimulation of adenylyl cyclase rather than inhibition of phosphodiesterase. Generation of cAMP was inhibited by indomethacin, and release of arachidonic acid was found to be an upstream step of the pathway. Activation of signaling also depended on influx of extracellular Ca^{2+} because addition of EGTA to the incubations at concentrations just sufficient to exceed Ca^{2+} in the medium inhibited the stress relaxation-dependent increase in free arachidonic acid and cAMP. This inhibition was overcome by adding $CaCl_2$ to the medium. On the other hand, treating fibroblasts in mechanically stressed cultures with the calcium ionophore A23187-stimulated arachidonic acid and cAMP production even without stress relaxation. In summary, our results show that fibroblast stress relaxation results in activation of a Ca^{2+}-dependent, adenylyl cyclase signaling pathway. Overall, the effect of stress relaxation on cAMP and PKA levels was equivalent to that observed after treatment of cells with forskolin.

Cells in tissues constantly are subjected to mechanical stimuli, and mechanical force is important for cell function in a wide range of biological organisms including bacteria and plants. Mechanical stress has been shown to regulate expression of numerous gene products, and a positive correlation between stress and cell proliferation has been demonstrated (Ingber and Folkman, 1989; Ryan, 1989; Heidemann and Buxbaum, 1990; Erdos et al., 1991; Watson, 1991; Vandenburgh, 1992; Davies and Tripathi, 1993). In the case of endothelial cells subjected to fluid shear, stress–specific promoter elements have been implicated in upregulation of PDGF-B and downregulation of endothelin 1 (Resnick et al., 1993; Malek et al., 1993).

In general, initial signaling mechanisms involved in mechanical regulation of cell function are just beginning to be understood. A variety of cells and tissues subjected to fluid shear or mechanical stress have been shown to respond by activation of the phosphatidylinositol turnover pathway (von Harsdorf et al., 1989; Sandy et al., 1989; Nollert et al., 1990; Jones et al., 1991; Reich and Frangos, 1991; Prasad et al., 1993), and most investigators report that stressed cells contain increased levels of protein kinase C (PKC)[1] (Homma et al., 1992; Rosales and Sumpio, 1992; Kroll et al., 1993). Some studies also indicate increased production of cyclic AMP (cAMP) (Watson, 1990; Ngan et al., 1990).

Fibroblast responses to mechanical stress can be studied in vitro using fibroblasts cultured in collagen matrices (Elsdale and Bard, 1972; Bell et al., 1979). Matrices that are attached to a rigid support become mechanically stressed during culture, whereas matrices that are floating in culture medium remain mechanically relaxed. Strain gauge measurements have shown that the force exerted by fibroblasts in attached collagen matrices is comparable to that generated in contracting skin wounds or during tooth eruption (Kasugai et al., 1990; Delvoye et al., 1991; Kolodney and Wysolmerski, 1992).

In floating collagen matrices, fibroblasts become quiescent within 1–2 d, whereas cells in attached matrices continue to proliferate (Sarber et al., 1981; Nishiyama et al., 1989; Nikagawa et al., 1989). Also, cells in floating matrices show low levels of collagen synthesis (Nusgens et al., 1984; Paye et al., 1987) and high levels of collagenase production (Unemori and Werb, 1986; Mauch et al., 1989; Lambert et al., 1992) compared to cells in attached matrices. These differences in cell proliferation and gene expression by fibroblasts in stressed vs relaxed matrices have important implications regarding wound repair because cell proliferation and biosynthetic activity will persist as long as granulation (wound) tissue is under mechanical stress. Once mechanical stress is relieved, usually by a combination of wound contraction and tissue replacement through biosynthetic activity,

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1. *Abbreviations used in this paper*: cAMP, cyclic AMP; cPLA2, cytoplasmic phospholipase A2; IBMX, 3-isobutyl-1-methoxanthine; LDH, lactic dehydrogenase; PGE2, prostaglandin E2; PKA, protein kinase A.

cells become quiescent and tissue remodeling begins (Grinnell, 1994).

Recently, we found that changes in cell proliferation and collagen synthesis could be detected within hours after mechanically stressed collagen matrices are released from their attachment sites, thereby allowing stress to dissipate, i.e., "stress relaxation" (strain recovery in engineering jargon) (Mochitate et al., 1991). During this time, PDGF receptors lose their signaling capacity (Lin and Grinnell, 1993). In studies on the initial events that accompany stress relaxation, we observed that cells within the matrices rapidly withdraw their pseudopodia resulting in "hypercontraction" of the matrix. Actin stress fibers shorten and disappear, cell surface fibronectin is released from its cell surface binding sites, and transient budding of cell surface vesicles occurs (Mochitate et al., 1991; Tomasek et al., 1992; Lee et al., 1993). Now, we have discovered that stress relaxation activates cAMP/PKA signaling in fibroblasts. This mechanoregulated pathway requires influx of extracellular Ca^{2+} followed by release of arachidonic acid. Details are reported herein.

Materials and Methods

Cell Culture

Human foreskin fibroblasts were cultured in collagen matrices as described previously (Mochitate et al., 1991; Lee et al., 1993). Briefly, early passage cells (10^6/ml matrix) in DME medium (without serum) were mixed with neutralized collagen (Vitrogen 100; Celtrix Labs, Palo Alto, CA) (1.5 mg/ml). The mixtures were briefly warmed to 37°C, and then 0.2-ml aliquots were polymerized for 1 h in 24-well culture plates that had been inscribed previously within a 12-mm diameter circular score. The precise time of warming varied somewhat with different lots of collagen, but was always selected to insure that the cells were dispersed throughout the matrix after collagen polymerization. The attached matrices were cultured 48 h in culture medium (DME supplemented with 10% fetal bovine serum and 50 μg/ml ascorbic acid). To initiate stress relaxation, the attached matrices were dislodged from the substratum with a thin spatula. Few if any cells were left behind on the plastic surface after collagen matrices were released.

Measurement of cAMP

cAMP levels were measured using the two column method (Salomon, 1991). Attached collagen matrix cultures were cultured for 2 h in 0.5 ml culture medium containing 8–40 μCi/ml [^{3}H]adenine (ICN, 36 Ci/mmol). Subsequently, cultures were rinsed, 0.5 ml fresh culture medium (supplemented with 0.1 mM 3-isobutyl-1-methoxanthine [IBMX] to block cAMP degradation) was added, and stress relaxation was initiated. In the experiment described in Fig. 1, IBMX was added only to the cultures as indicated in the figure legend. To extract nucleotides from the cells at the times indicated, 0.5 ml ice-cold 10% trichloroacetic acid containing 0.2 mM cAMP as carrier was added to the cultures, and the samples were incubated on ice for 1 h. Acid extracts (800 μl) were applied to 1-ml Dowex-50w columns (mesh size-200–400) (Sigma Immunochemicals, St. Louis, MO). The columns were washed twice with 1 ml H_2O and then eluted with an additional 4 ml of H_2O. The eluted Dowex columns were drained completely, and the eluates were applied to 0.75-g alumina columns (Sigma Immunochemicals). [^{3}H]cAMP was eluted from the alumina columns with 3 ml of 100 mM imidazole buffer (pH = 7.3). Eluates were mixed with 10 ml of Budgetsolve (Research Products Int., Mt. Prospect, IL), and radioactivity was determined in a scintillation counter (LS3801; Beckman Instruments, Inc., Fullerton, CA). Efficiency of adenine recovery was ~50% based on OD_{260} measurements of carrier cAMP, and data presented in the figures were normalized to recovery. Data presented in the figures, averages ±SD of duplicate samples, are from representative experiments.

Measurement of Protein Kinase A Activity

The general method for extracting cellular components from collagen matrices and normalizing recovery to lactic dehydrogenase (LDH) activity was described previously (Lin and Grinnell, 1993). Collagen matrix cultures (5 matrices/data point) that were attached or undergoing stress relaxation were placed in 150 μl of ice-cold PKA homogenization buffer (50 mM Tris, 5 mM EDTA, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzene-sulfonylfluoride HCl, 0.5 mM IBMX, pH 7.5). Samples were homogenized (50 strokes) with a 1-ml Dounce homogenizer (Wheaton, B pestle) at 4°C, and collagen fibrils and nuclei were removed by centrifugation at 12,000 *g* (Microfuge; Beckman Instruments, Inc.) for 2 min. PKA activity in aliquots of the supernatants was measured using a protein kinase A assay system (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Assays contained 20 mM Tris pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μM ATP, 50 μM Ac-MBP (4-14), and ~5 μCi/ml [γ-^{32}P]ATP (3,000 Ci/mmole; New England Nuclear, Boston, MA). Total activity was measured after addition of 10 μM cAMP. Aliquots of the supernatants also were used to measure LDH activity (LD diagnostic kit; Sigma Immunochemicals), and PKA activity was normalized to LDH units, pmol/min per 10^3 LDH units.

Measurement of [^{3}H]Arachidonic Acid Metabolite Release

Attached collagen matrix cultures were cultured overnight in 0.5 ml culture medium containing 1 μCi/ml [^{3}H]arachidonic acid (specific activity 210 Ci/mmol; New England Nuclear). Subsequently, the cultures were washed with four changes of fresh culture medium during 1 h, after which stress relaxation was initiated. At the times indicated, 0.4-ml aliquots of the culture medium were mixed with 10 ml of Budgetsolve (RPI), and radioactivity was determined in a scintillation counter (LS3801; Beckman Instruments, Inc.).

Results

cAMP Elevation during Stress Relaxation

Fibroblasts were cultured in attached collagen matrices for 2 d, during which time cells reorganized collagen in the matrices and mechanical stress developed. Subsequently, stress relaxation was initiated by releasing the matrices from the culture dishes. Fig. 1 shows that cellular cyclic AMP (*cAMP*) levels increased >10-fold within 10 min after attached collagen matrices were released (*REL*). Subsequently, cAMP levels declined gradually. Control incubations showed that throughout the same time period, cAMP levels of fibroblasts in attached collagen matrices (*ATT*) remained constant. Although cAMP concentrations were not measured directly, the cAMP elevation observed 10 min after initiating stress relaxation was comparable to that obtained 10 min after treating cells in attached collagen matrices with 10 μM forskolin (see Table I below).

Stress relaxation–dependent cAMP elevation could have resulted from an activation of adenylyl cyclase or an inhibition in phosphodiesterase activity. Fig. 1 shows that addition of the phosphodiesterase inhibitor IBMX at concentrations ≤2 mM (the highest tested) did not result in increased production of cAMP by fibroblasts in attached matrices. Also, IBMX did not change the overall pattern of the cAMP response after release, although with IBMX present, the response was higher and more sustained. These results indicate that stress relaxation–dependent cAMP elevation involves activation of adenylyl cyclase rather than inhibition of phosphodiesterase.

During the first 10 min of stress relaxation, fibroblast stress fibers observed by immunofluorescence or transmission electron microscopy shorten and disappear as the cells contract the collagen matrix (Mochitate et al., 1991; Tomasek et al., 1992; Lee et al., 1993). Therefore, we analyzed

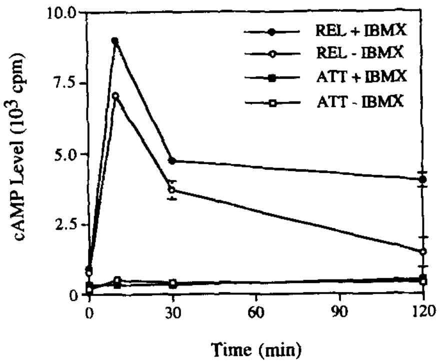

Figure 1. Effect of stress relaxation on cellular cAMP levels. cAMP levels were measured at the times indicated in attached collagen matrix cultures (*ATT*) or released cultures undergoing stress relaxation (*REL*). The phosphodiesterase inhibitor 3-isobutyl-1-methoxanthine (*IBMX*) (0.1 mM) was added where indicated. The culture medium contained 8 μCi/ml [^{3}H]adenine. Data presented are from duplicate samples.

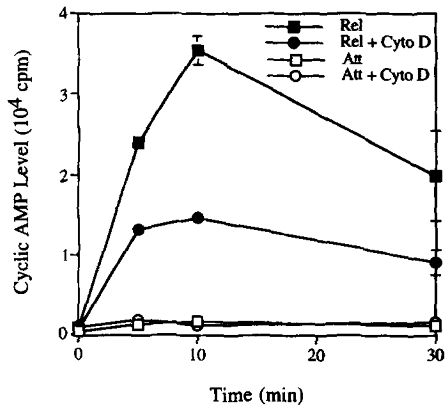

Figure 3. Effect of cytochalasin D on cellular cAMP levels. Attached collagen matrix cultures were incubated in culture medium containing 10 μM cytochalasin D (Sigma Immunochemicals) for 20 min, after which stress relaxation was initiated. cAMP levels were measured at the times indicated. The culture medium contained 10 μCi/ml [^{3}H]adenine. Data presented are from duplicate samples.

the possibility that disruption of the actin cytoskeleton resulted in cAMP elevation. Addition of cytochalasin D to fibroblasts in attached collagen matrices was found to cause disruption of stress fibers (Tomasek et al., 1992), and to partially inhibit matrix contraction as shown in Fig. 2 (see also Tomasek, 1992). Fig. 3 shows that cytochalasin D had no effect on cAMP levels of fibroblasts in attached matrices, but reduced stress relaxation–dependent cAMP elevation. These results suggest that cAMP elevation cannot be initiated simply by disruption of the actin cytoskeleton, but does require matrix contraction.

To assess whether cAMP elevation had downstream physiological consequences, we measured changes in cAMP-dependent PKA activity before and after stress relaxation. Table I shows that cellular PKA activity increased threefold within 10 min after attached matrices were released (*Rel*), and it was still elevated 30 min later. Total PKA (extract + added cAMP) decreased slightly in released matrices for unknown reasons. Table I also shows that the extent of PKA activation induced by stress relaxation was comparable to that observed after treatment of fibroblasts in attached matrices for 10 min with 10 μM forskolin, which directly activates adenylyl cyclase.

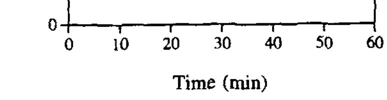

Figure 2. Effect of cytochalasin D on collagen matrix contraction. Attached collagen matrix cultures were incubated in culture medium containing 10 μM cytochalasin D (Sigma Immunochemicals) for 20 min, after which stress relaxation was initiated. At the times indicated, samples were fixed 10 min with 3% paraformaldehyde in phosphate-buffered saline (150 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.2). Matrices were then removed from the culture dishes, placed on a glass slide, and diameters were measured with a ruler. Data presented are from two experiments each with triplicate samples.

Arachidonic Acid Production during Stress Relaxation

Prostaglandin E2 (PGE2) is a known activator of adenylyl cyclase (Brunton et al., 1976), and arachidonic acid and PGE2 levels were reported to be higher in fibroblasts cultured in floating collagen matrices compared to cells in monolayer culture (Pentland, 1989). Therefore, we tested the possibility that cAMP elevation during stress relaxation involved the arachidonic acid–prostaglandin pathway. Indomethacin blocks conversion of arachidonic acid to prostaglandins (Vane, 1971), and Fig. 4 shows that indomethacin inhibited stress relaxation–dependent elevation of cAMP $\leqslant$65% in a dose-dependent manner. Control incubations showed that indomethacin had no effect on cAMP levels of cells in attached matrices. Also, indomethacin had no effect on collagen matrix contraction accompanying stress relaxation.

Table I. Effect of Stress Relaxation on Protein Kinase A Activity

		PKA activity (Average ± SD)	
Experiment Treatment	Time	Extract	Total (Extract + cAMP)
1 Att	–	6.0 ± 0.3	40.9 ± 2.0
Rel	10 min	17.2 ± 0.7	29.1 ± 0.3
Att + Fors	10 min	20.4 ± 1.2	29.1 ± 0.8
2 Att	–	6.3 ± 0.6	29.0 ± 2.9
Rel	30 min	10.4 ± 0.4	14.5 ± 4.9

PKA activity was measured at the times indicated in fibroblasts in collagen matrices that were mechanically stressed (Att) or undergoing stress relaxation (Rel). Forskolin (Fors, 10 μM) was added as indicated. PKA activity was normalized to LDH units extracted from the cells, i.e., pmol/min per 10^3 LDH units. Data presented are from duplicate samples.

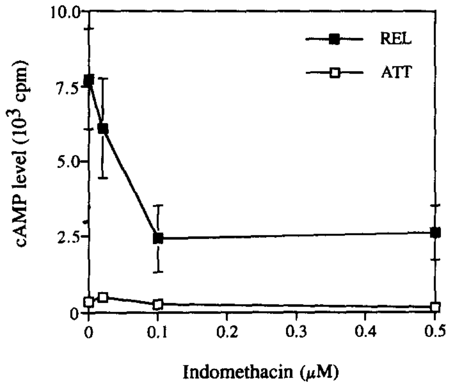

Figure 4. Effect of indomethacin on cellular cAMP increase after stress relaxation. Attached collagen matrix cultures were incubated in culture medium containing indomethacin as indicated for 1 h before, after which stress relaxation was initiated. cAMP levels were measured 10 min later. The culture medium contained 10 μCi/ml [^{3}H]adenine. Data presented are from duplicate samples.

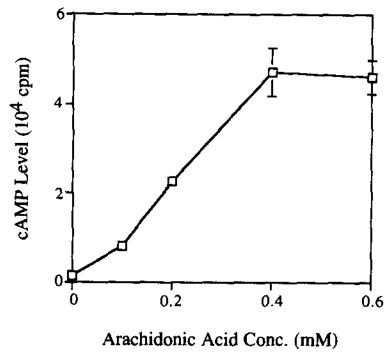

Figure 6. Effect of arachidonic acid on cAMP levels in mechanically stressed cultures. Attached collagen matrix cultures were incubated in culture medium with arachidonic acid at the concentrations shown. At the times indicated cAMP levels were measured. The culture medium contained 10 μCi/ml [^{3}H]adenine. Data presented are from duplicate samples.

The above results were consistent with the idea that the arachidonic acid–prostaglandin pathway plays a role in stress relaxation–dependent activation of adenylyl cyclase. Experiments were then carried out to measure directly the release of free arachidonic acid and its metabolites by cells in attached vs released collagen matrices. Fig. 5 shows that production of free arachidonic acid increased when attached matrices (*ATT*) were released (*REL*), and the released/attached ratio (*dotted line*) indicated that a peak in arachidonic acid metabolite production occurred by 10 min after release. These data provide direct evidence that stress relaxation induces elevation of free arachidonic acid and its metabolites with kinetics similar to the increase in cAMP.

In a final series of studies on the role of the arachidonic acid–prostaglandin pathway in activation of adenylyl cyclase, experiments were carried out in which arachidonic acid was added to the cultures. Arachidonic acid is known to stimulate cAMP production by fibroblasts in monolayer culture, and the dose-response curve in Fig. 6 shows directly that arachidonic acid can stimulate cAMP production by fibroblasts in collagen matrices.

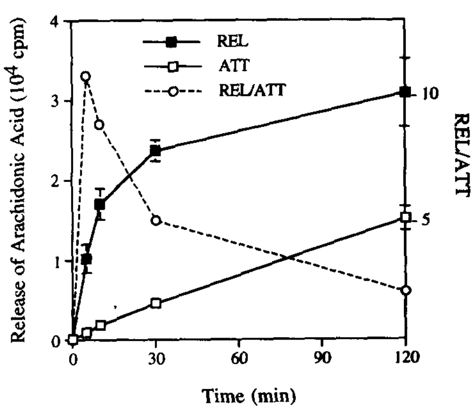

Figure 5. Effect of stress relaxation on release of arachidonic acid. Appearance of arachidonic acid in the culture medium was measured at the times indicated in attached collagen matrix cultures (*ATT*) or released cultures undergoing stress relaxation (*REL*). Data presented are duplicate samples.

Initiation of Arachidonic Acid Production and cAMP Elevation by Calcium Influx

Experiments were also carried out to identify other signaling molecules that participated in stress relaxation–dependent cAMP elevation. In preliminary studies, we found that addition of EGTA to the incubation medium at 3 mM or higher inhibited cAMP elevation by >80% (see below). Since the concentration of total Ca^{2+} in 10% FBS-containing DME medium is ~3 mM, this result suggested that extracellular Ca^{2+} was required for cAMP elevation. At 3 mM, EGTA did not inhibit fibroblast contraction of collagen matrices, showing that cAMP elevation was not required for matrix contraction. At higher EGTA concentrations, inhibition of matrix contraction was observed, ~35% at 10 mM EGTA and ~50% at 20 mm EGTA, possibly resulting from sequestration of intracellular Ca^{2+}.

Fig. 7 shows an experiment in which fibroblasts in at-

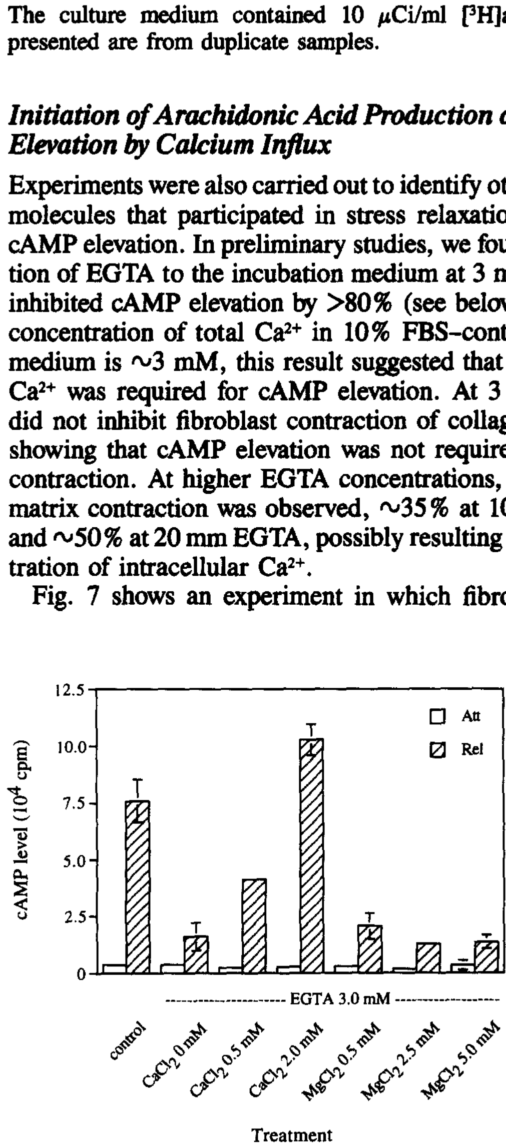

Figure 7. Effect of EGTA, Ca^{2+}, and Mg^{2+} on cAMP levels after stress relaxation. Attached collagen matrix cultures were incubated 15 min in culture medium containing 3.0 mM EGTA. Cultures were then switched to fresh culture medium containing 3.0 mM EGTA and $CaCl_2$ or $MgCl_2$ at the concentrations shown. Stress relaxation was initiated, and cAMP levels were measured 10 min later. The culture medium contained 40 μCi/ml [^{3}H]adenine.

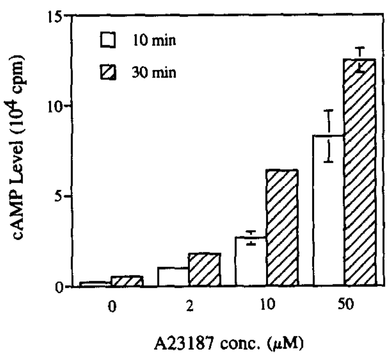

Figure 8. Effect of calcium ionophore A23187 on cAMP levels in mechanically stressed cultures. Attached collagen matrix cultures were incubated in culture medium with A23187 at the concentrations shown. At the times indicated, cAMP levels were measured. The culture medium contained 40 μCi/ml [^{3}H]adenine. Data presented are from duplicate samples.

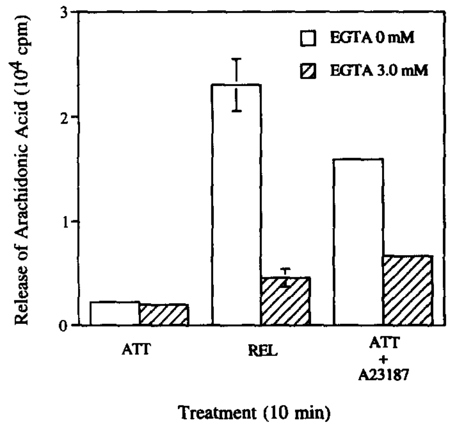

Figure 9. Effect of EGTA on stress relaxation induced or A23187 induced release of arachidonic acid. Appearance of arachidonic acid in the culture medium was measured at the times indicated in attached collagen matrix cultures (*ATT*), released cultures undergoing stress relaxation (*REL*), or attached cultures treated with 50 μM A23187. EGTA was added as indicated. Data presented are from duplicate samples.

tached collagen matrices were treated 15 min with 3 mM EGTA, after which increasing concentrations of $CaCl_2$ or $MgCl_2$ were added to the incubations and half the samples were released. Attached matrices showed no changes in cAMP levels under any of the experimental conditions. Released matrices showed typical stress relaxation–dependent cAMP elevation in the absence of EGTA and marked inhibition of cAMP elevation in the presence of EGTA. EGTA inhibition was overcome by adding back $CaCl_2$ but not $MgCl_2$, further demonstrating that the response was Ca^{2+} dependent. Partial restoration of activity occurred with 0.5 mM $CaCl_2$ added to the incubations, and complete activity occurred with 2.0 mM $CaCl_2$, which showed that there is a close association between the level of extracellular Ca^{2+} ions and cAMP elevation during stress relaxation. Addition of 3 mM EGTA also inhibited release of arachidonic acid and its metabolites, and inhibition was overcome by 2 mM $CaCl_2$ but not by 2 mM $MgCl_2$.

The above results implicated extracellular Ca^{2+} in cAMP elevation. An attractive hypothesis was that stress relaxation resulted in Ca^{2+} influx, which triggered subsequent events. To analyze this possibility further, fibroblasts in attached cultures were treated with the calcium ionophore A23187 for 10 or 30 min, after which cAMP levels were measured. Fig. 8 shows that addition of the ionophore resulted in a dose-dependent cAMP elevation. This increase was inhibited 58% by 3 mM EGTA. Fig. 9 shows similar findings for arachidonic acid and its metabolite. That is, treating fibroblasts in attached matrices with calcium ionophore (ATT + A23187) resulted in an increase in production of arachidonic acid comparable to that obtained by stress relaxation, and in either case, addition of 3 mM EGTA inhibited arachidonic acid production. Taken together, these results indicated that Ca^{2+} influx alone is sufficient to activate the cAMP signaling pathway.

Discussion

The goal of our studies was to identify mechanoregulated signaling mechanisms in fibroblasts. Previous studies on mechanoregulation of cell function by other laboratories used systems in which cells were subjected to increased or intermittent stress (Erdos et al., 1991; Vandenburgh, 1992; Davies and Tripathi, 1993). The stress relaxation model is unique in that it allows studies on cells switched from mechanically stressed to relaxed conditions. Because the mechanical changes occur rapidly and synchronously in the cell population, stress relaxation can be used to determine initial cellular responses to mechanical change.

The major finding of our studies was that stress relaxation triggers a cAMP signaling pathway. The earliest step of the pathway identified was influx of extracellular Ca^{2+}. The rise in cytosolic Ca^{2+} appeared to be followed by generation of free arachidonic acid. Elevated arachidonic acid was found to stimulate cAMP synthesis by an indomethacin-sensitive mechanism. Finally, increased levels of cAMP resulted in activation of PKA. Overall, the effect of stress relaxation on PKA levels was equivalent to that observed after treatment of cells with forskolin.

Arachidonic acid, cAMP, and PKA were directly measured and shown to increase dramatically within 10 min after initiating stress relaxation. cAMP elevation could have resulted from activation of adenylyl cyclase or inhibition in phosphodiesterase. Since addition of IBMX to the incubations did not increase cAMP levels of fibroblasts in stressed matrices, and it did not change the overall pattern of the cAMP elevation after release of stressed matrices, it could be concluded that stress relaxation resulted in activation of adenylyl cyclase rather than inhibition of phosphodiesterase. Inhibition of the signaling pathway by indomethacin suggested a role for prostaglandins because indomethacin blocks cyclooxygenase-mediated conversion of arachidonic acid to prostaglandin (Vane, 1971), and prostaglandins are known activators of adenylyl cyclase (Brunton et al., 1976; Kerins et al., 1991). Nevertheless, maximal inhibition of cAMP production by indomethacin only reached 65–70%, so we cannot rule out participation of a second pathway leading to cAMP synthesis, such as direct Ca^{2+} activation of adenylyl cyclase (Tang et al., 1991; Choi et al., 1992).

Several results suggested that extracellular Ca^{2+} influx was an upstream initiator of the stress relaxation signaling pathway. On one hand, addition of EGTA to the incubations at concentrations just sufficient to exceed Ca^{2+} in the medium inhibited the increases in free arachidonic acid and cAMP when stressed matrices were released. This inhibition was overcome by adding $CaCl_2$ but not $MgCl_2$ to the medium. On the other hand, treating fibroblasts in mechanically stressed cultures with the calcium ionophore A23187 stimulated arachidonic acid and cAMP production even without stress relaxation, and this stimulation also was inhibited by adding EGTA as above.

The mechanism accounting for stress relaxation-dependent influx of extracellular Ca^{2+} influx has yet to be studied. One possibility is participation of stretch-activated ion channels such as those that have been described in membrane patches (Sachs, 1989), whose function in whole cells is unknown (Sadoshima et al., 1992). Another possibility is that cell surface Ca^{2+} channels open in response to physical changes in plasma membrane specializations such as caveolae, which contain inositol triphosphate receptors and have been proposed to play a role in Ca^{2+} signaling (Fujimoto et al., 1992). Finally, Ca^{2+} entry may be regulated by changes in occupancy or organization of integrin receptors (Fujimoto et al., 1991; Ng-Sikorski et al., 1991; Schwartz, 1993). Integrin $\alpha 2\beta 1$ adhesion receptors mediate binding between fibroblasts and collagen fibrils in collagen matrices (Gullberg et al., 1990; Schiro et al., 1991; Klein et al., 1991), and collagen organization changes markedly during stress relaxation (Lee et al., 1993). Also, $\alpha 5\beta 1$ receptors bind to fibronectin in mechanically stressed matrices, where cells form fibronexus junctions, but when the matrices are switched to mechanically-relaxed conditions, fibronexus junctions fall apart and the cells release their surface fibronectin (Mochitate et al., 1991; Tomasek et al., 1992).

An attractive hypothesis consistent with the data is participation of Ca^{2+}-activated cytoplasmic phospholipase A2 (cPLA2) (Brooks et al., 1989; Kramer et al., 1991) in stress relaxation-dependent generation of free arachidonic acid. The enzyme contains a Ca^{2+}-dependent translocation domain, and in the presence of Ca^{2+}, it moves from the cytoplasm to the plasma membrane, where arachidonic acid can be released (Clark et al., 1991). It should be noted, however, that generation of free arachidonic acid is influenced by a variety of enzymes, including phospholipase C and PKC, as well as cPLA2 (Burgoyne and Morgan, 1990). Therefore, the possibility that cPLA2 plays a role is the stress relaxation response must remain speculative until further studies are carried out.

Identification of a mechanoregulated cAMP signaling pathway raises the question of whether elevated cAMP can account for the phenotypic changes that accompany stress relaxation, particularly changes in cell proliferation and matrix remodeling. In support of this possibility, a variety of studies have shown that increased levels of cAMP can inhibit proliferation of fibroblasts and other cells in monolayer culture (Pastan et al., 1975). Also, elevated cAMP has been shown to result in decreased collagen synthesis (Baum et al., 1978; Kollros et al., 1987; Perr et al., 1989) and increased collagenase synthesis (Corcoran et al., 1992). The hormone relaxin, normally associated with changes in contractility and matrix remodeling in the reproductive tract (Bryant-Greenwood, 1991), causes decreased collagen synthesis and increased collagenase synthesis by fibroblasts (Unemori and Amento, 1990; Unemori et al., 1993), and relaxin probably works through a PGE2-cAMP signaling pathway (Marshall and Kroeger, 1973; Hsu et al., 1985). Whether fibroblast collagen and collagenase genes contain stress-sensitive promoter elements remains to be determined.

While the above studies support the idea that cAMP signaling can explain phenotypic changes by fibroblasts in floating vs attached matrices, the situation may turn out to be more complex. For instance, there have been reports of cAMP signaling after subjecting some cells to increased mechanical stress, albeit through different signaling pathways than we have observed. In one case, cAMP elevation appeared to depend on disruption of the cytoskeleton (Watson, 1990); in the other, maximal cAMP levels did not occur until 30-60 min after application of stress (Ngan et al., 1990). Also, in both studies, the magnitude of the cAMP response was much smaller than that we observed. Moreover, depending on cell type, timing of addition, and presence of particular growth factors, cAMP can stimulate rather than inhibit cell proliferation (Rozengurt et al., 1981, 1983), and cAMP also has been reported to inhibit collagenase production (Takahashi et al., 1991). These results indicate that there may be other stress-sensitive regulatory mechanisms in addition to cAMP signaling.

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