Role of Phospholipase D in the cAMP Signal Transduction Pathway Activated during Fibroblast Contraction of Collagen Matrices

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Abstract. Fibroblast contraction of stressed collagen matrices results in activation of a cAMP signal transduction pathway. This pathway involves influx of extracellular Ca^{2+} ions and increased production of arachidonic acid. We report that within 5 min after initiating contraction, a burst of phosphatidic acid release was detected. Phospholipase D was implicated in production of phosphatidic acid based on observation of a transphosphatidylation reaction in the presence of ethanol that resulted in formation of phosphatidylethanol at the expense of phosphatidic acid. Activation of phospholipase D required extracellular Ca^{2+} ions and was regulated by protein kinase C. Ethanol treatment of cells also inhibited by 60–70% contraction-dependent release of arachidonic acid and cAMP but had no effect on increased cAMP synthesis after addition of exogenous arachidonic acid or on phospholipase A2 activity measured in cell extracts. Moreover, other treatments that inhibited the burst of phosphatidic acid release after contraction—chelating extracellular Ca^{2+} or downregulating protein kinase C—also blocked contraction activated cyclic AMP signaling. These results were consistent with the idea that phosphatidic acid production occurred upstream of arachidonic acid in the contraction-activated cAMP signaling pathway.

LIVING organisms can sense and react to mechanical stimuli, although the underlying regulatory mechanisms are not yet well understood. A variety of studies have shown that many types of cells respond to mechanical stress by an increase in cell mass or number, and that mechanical signals are important determinants of cell differentiation (Ryan, 1989; Thyberg et al., 1990; Heidemann and Buxbaum, 1990; Erdos et al., 1991; Daniels and Solursh, 1991; Watson, 1991; Vandenburgh, 1992; Davies and Tripathi, 1993; Ingber, 1993; Grinnell, 1994; Simpson et al., 1994; Reinhart, 1994). From an evolutionary perspective, the capacity of cells to recognize mechanical stimuli may have developed first as a requirement for unicellular organisms to control cell volume in changing osmotic environments (Hamill and McBride, 1995).

In some cases, mechanosensitive regulation has been attributed to autocrine signaling mechanisms (Sadoshima et al., 1993; Wilson et al., 1993), but direct cytoskeletal modulation (Wang and Ingber, 1994) and mechanoreceptive membrane channels (Hamill and McBride, 1995) also may be involved. Studies on mechanoregulated control of transcription when endothelial cells are subjected to fluid shear have led to identification of distinct mechanical stress-responsive promoters that upregulate the PDGF B chain gene and downregulate the endothelin 1 gene (Resnick et al., 1993; Malek et al., 1993). On the other hand, induction of c-*fos* by cardiac myocytes subjected to mechanical stretch was reported to occur primarily through the serum response element (Sadoshima and Izumo, 1993).

Most experiments on mechanoregulation have measured how cells react when they are subjected to increased mechanical stress. Equally important but less well studied is the cellular reaction to decreased stress. We have been particularly interested in the latter because of its potential role in promoting quiescence and regression of wound tissue fibroblasts after wound contraction (Arem and Madden, 1976; Burgess et al., 1990).

An in vitro model using fibroblasts cultured in collagen matrices has been developed for studying relaxation of mechanical stress (Grinnell, 1994). In this model, cells in anchored matrices reorganize collagen fibrils as a consequence of cell migration (Harris et al., 1981). Subsequently, when the stressed matrices are released experimentally, the cells undergo a smooth muscle–like contraction as a result of which stress dissipates. During contraction, actin stress fibers shorten and eventually disappear, fibronectin is released from binding sites on the cell surface, and transient budding (ectocytosis) of 200 nm plasma membrane

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vesicles occurs (Mochitate et al., 1991; Tomasek et al., 1992; Lee et al., 1993).

After fibroblasts contract stressed collagen matrices, cells become quiescent (Mochitate et al., 1991). Development of quiescence has been found to occur, at least in part, because the cells become less responsive to PDGF (Nakagawa et al., 1989; Nishiyama et al., 1991). They show decreased PDGF-induced receptor aggregation (Tingstrom et al., 1992) and loss of PDGF receptor autophosphorylation (Lin and Grinnell, 1993).

Recently, we found that fibroblast contraction of stressed collagen matrices also triggers a signal transduction pathway resulting within minutes in a 10–20-fold increase in cellular cAMP levels and activation of protein kinase A. This contraction-activated signal transduction pathway requires extracellular Ca^{2+} ions and involves increased production of arachidonic acid (He and Grinnell, 1994).

Increased production of arachidonic acid could have resulted directly from increased activity of phospholipase A2 or indirectly from increased activity of phospholipase C or phospholipase D (Burgoyne and Morgan, 1990). In this report, we present evidence that fibroblast contraction of stressed collagen matrices results in activation of phospholipase D. The findings suggest that phospholipase D activation is an early step in the contraction-activated cAMP signal transduction pathway, and that this pathway is regulated by protein kinase C.

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 (without serum) were mixed with neutralized collagen (Vitrogen 100; Celtrix Labs, Santa Clara, CA) (1.5 mg/ml). The mixtures were 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 with a 12-mm-diam circular score. The precise time of warming varied somewhat with different lots of collagen, but was always selected to ensure that the cells were dispersed throughout the matrix after collagen polymerization. The attached matrices were cultured for 48 h in culture medium (DME supplemented with 10% FBS and 50 μg/ml ascorbic acid).

During the 48-h culture period, stress developed in the attached collagen matrices. Contraction was then initiated by releasing the attached matrices from the substratum with a thin spatula. After releasing the matrices, few cells were left behind on the plastic surface. In some experiments, extracts were prepared from the cells, in which case aliquots of the extracts were used to measure lactate dehydrogenase (LDH)[1] activity (diagnostic kit; Sigma Chemical Co., St. Louis, MO), and other data collected were normalized to LDH units as described previously (Lin and Grinnell, 1993).

Phospholipase A2 Activity

Phospholipase A2 activity was measured using a modification of the method described by Diez and Mong (1990). To prepare substrate vesicles, 1 μl (0.88 nmol) of 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine (sp act, 57 mCi/mmol) (New England Nuclear, Boston, MA) was dried under N_2, suspended in 10 μl H_2O by vortexing for 1 min, sonicated for 5 min (model 2210; Branson Ultrasonics Corp., Danbury, CT), and then vortexed a second time. Reactions were carried out in 50-μl volume containing 10-μl aliquots of cell extracts (see below), substrate vesicles, 50 mM Tris HCl (pH 8.0), 100 mM NaCl, 2 mM $CaCl_2$, and 15% glycerol. After 30 min at 37°C, the reactions were stopped by addition of 50 μl of chloroform/methanol (1:2, vol/vol, containing 0.05 N hydrochloric acid and 20 μg arachidonic acid). To extract lipids, the samples were mixed with 50 μl chloroform and 50 μl of 4 M KCl, centrifuged at 14,000 rpm for 1 min, and the organic lower phase (50 μl) was spotted onto the preadsorbent layer of pregrooved plates (LK6D; Whatman Inc., Clifton, NJ). The plates were developed in petroleum ether/diethyl ether/acetic acid (75:25:1, vol/vol). The region corresponding to free arachidonic acid was visualized by exposure to I_2 vapor, scraped into scintillation vials, and mixed with 10 ml of solvent (Budget-solve; Research Products International Corp., Mount Prospect, IL). Radioactivity was determined in a scintillation counter (model LS3801; Beckman Instruments, Inc., Fullerton, CA). Each data point represents extracts combined from 6 matrices. LDH activity also was measured (see above), and phospholipase A2 activity was normalized to picomoles per minute per 10^6 LDH units.

Cell extracts were prepared as follows. Collagen matrix cultures that were attached or released to initiate contraction were placed in 180 μl (30 μl/matrix) of ice-cold homogenization buffer (20 mM Tris HCl, pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM 4-(2-aminoethyl)-benzene-sulfonylfluoride HCl, 5 μg/ml leupeptin, 5 μg/ml pepstatin A). Samples were homogenized (50 strokes) with a 1-ml Dounce homogenizer (B pestle; Wheaton Scientific, Millville, NJ) at 4°C and then centrifuged at 100,000 *g* for 1 h at 4°C. The supernatant fraction and particulate fraction resuspended in extraction buffer were assayed separately as indicators of cytoplasmic and membrane-bound phospholipase A2. Extracting cells under conditions where Ca^{2+} is chelated results in maximal enzyme recovery and prevents translocation of soluble phospholipase A2 to the particulate fraction, while only partially causing translocation of membrane-bound phospholipase A2 to the soluble fraction (Channon and Leslie, 1990).

Synthesis of Inositol Phosphates

Inositol phosphates were assayed similarly as described by Brown et al. (1991). Fibroblasts in attached collagen matrices were cultured for 2 d in 0.5 ml of inositol-free culture medium containing 4 μCi/ml [^{3}H]myoinositol (sp act, 10–20 Ci/mmol) (New England Nuclear). Subsequently, the cultures were rinsed briefly and preincubated in culture medium containing 10 mM LiCl for 20 min before releasing the matrices or adding bradykinin. After removing excess medium, reactions were terminated by addition of 500 μl of ice-cold 5% PCA. After 1 h, the extracts were diluted to 1 ml with H_2O and extracted with an equal volume of tri-*n*-octylamine/1,1,2-trichlorotrifluoroethane (Sigma Chemical Co.). The samples were mixed thoroughly and centrifuged at 2,500 rpm for 5 min. Then 800 μl of the aqueous phase was applied to a 1-ml Dowex AG1-X8 anion exchange column (mesh size 100–200, formate form) (Bio-Rad Laboratories, Richmond, CA). The columns were washed twice with 10 ml H_2O and once with 8 ml 50 mM ammonium formate. Inositol phosphates were bulk eluted with 6 ml 1.2 M ammonium formate/100 mM formic acid. Eluates were mixed with 10 ml of Budget-solve, and radioactivity was determined as above. Each data point represents the extract from one matrix.

Synthesis of 1,2-Diacylglycerol, Phosphatidic Acid, and Phosphatidylethanol

Fibroblasts in attached collagen matrices were cultured overnight in 0.5 ml culture medium containing 10 μCi/ml [^{3}H]palmitic acid (sp act, 39 Ci/mmol) (New England Nuclear) or 2.5 μCi/ml [^{3}H]arachidonic acid (sp act, 100 Ci/mmol) (New England Nuclear) as indicated. Subsequently, the cultures were washed with four changes of fresh culture medium during 1 h, after which matrices were released to initiate contraction. For phosphatidic acid and phosphatidylethanol measurement, reactions were stopped using concentrated HCl, and lipids were extracted with 1-butanol (Bremer, 1963; Bjerve et al., 1974). For 1,2-diacylglycerol measurement, reactions were stopped, and cellular lipids were extracted by the Bligh and Dyer (1959) procedure. Phosphatidic acid separation was accomplished by double one-dimensional TLC (Gruchalla et al., 1990) or two-dimensional TLC (Thomas and Holub, 1991), and phosphatidylethanol separation was carried out by one-dimensional TLC (Gruchalla et al., 1990). After drying, thin-layer plates were sprayed with EN^{3}HANCE (Dupont-New England Nuclear) and autoradiography performed by using XAR film (Eastman Kodak Co., Rochester, NY) for 2–3 d. The identity of radiolabeled bands was based on chromatography of authentic lipid standards (Avanti Polar Lipids, Inc., Alabaster, AL) visualized by iodine vapor. To quantify phosphatidic acid levels, the autoradiographs were analyzed using a laser scanning densitometer (Ultrascan XL; LKB Instruments, Inc., Bromma, Sweden).

1. *Abbreviations used in this paper*: LDH, lactate dehydrogenase; TPA, 12-*o*-tetradecanoylphorbol-13-acetate.

Synthesis of [^{3}H]Arachidonic Acid Metabolites

Arachidonic acid production was measured as described previously (He and Grinnell, 1994). Attached collagen matrix cultures were cultured overnight in 0.5 ml culture medium containing 1 μCi/ml [^{3}H]arachidonic acid (sp act, 210 Ci/mmol) (New England Nuclear). Subsequently, the cultures were washed with four changes of fresh culture medium over 1 h, after which matrices were released to initiate contraction. At the times indicated, 0.4-ml aliquots of the culture medium were mixed with 10 ml of Budget-solve, and radioactivity was determined as above.

Synthesis of cAMP

cAMP levels were measured using the two-column method (Salomon, 1991) as described previously (He and Grinnell, 1994). Attached collagen matrix cultures were cultured for 2 h in 0.5 ml culture medium containing 8 μCi/ml [^{3}H]adenine (sp act, 36 Ci/mmol) (ICN Biomedicals, Irvine, CA). Subsequently, cultures were rinsed, 0.5 ml fresh culture medium (supplemented with 0.1 mM IBMX to block cAMP degradation) was added, and matrices were released to initiate contraction. To extract nucleotides from the cells, 0.5 ml ice-cold 10% TCA 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 Chemical Co.). 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 Chemical Co.). ^{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 Budget-solve, and radioactivity was determined as above. Efficiency of cAMP recovery was ~50% based on OD_{260} measurements of carrier cAMP, and data presented in the figures were normalized to recovery. Each data point represents extract from one matrix.

Results

Activity of Phospholipase A2 and Phospholipase C during Fibroblast Contraction of Stressed Collagen Matrices

Previous studies implicated influx of extracellular Ca^{2+} in contraction-activated increases in arachidonic acid release and cAMP production (He and Grinnell, 1994). Influx of Ca^{2+} has been reported to activate cytoplasmic phospholipase A2 (Brooks et al., 1989), presumably by promoting binding of cytoplasmic enzyme molecules to the plasma membrane (Channon et al., 1990; Kramer et al., 1991; Clark et al., 1991). Therefore, we measured phospholipase A2 before and during contraction to learn whether there were overall changes in enzyme activity or a shift in enzyme location from the cytoplasmic fraction to the membrane-bound fraction. In these experiments, cells were extracted under conditions where Ca^{2+} was chelated, which results in maximal enzyme recovery and prevents binding of cytoplasmic phospholipase A2 to the membrane during extraction, but causes partial release of membrane-bound enzyme (Channon and Leslie, 1990). Fig. 1 shows that during contraction there was no detectable change in membrane-bound phospholipase A2, but we did observe a 2.5-fold increase in the activity of soluble phospholipase A2. Despite the increase in phospholipase A2 activity, there were no obvious changes in metabolites produced by phospholipase A2. That is, as shown in Fig. 2, lysophosphatidic acid was undetectable before or during contraction. Also, lysophosphatidylcholine was detected in fibroblasts in attached matrices but showed no increase during contraction.

Activity of phospholipase C during contraction was

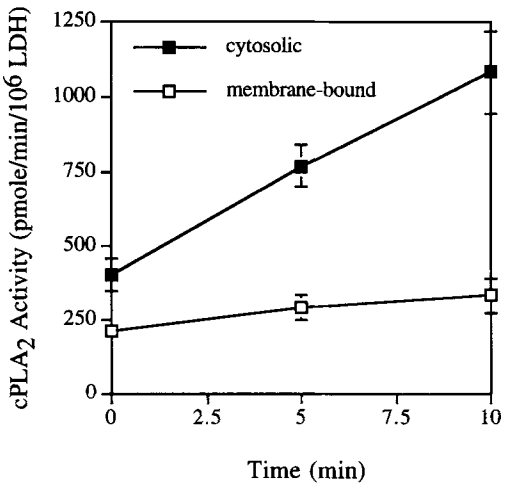

Figure 1. Effect of fibroblast contraction on membrane-bound and cytoplasmic phospholipase A2 ($cPLA_2$) activity. Stressed collagen matrices were released to initiate contraction. At the times indicated after release, membrane-bound and cytoplasmic phospholipase A2 levels were measured. Activity was normalized to LDH units extracted from the cells. Data presented are from duplicate samples.

measured by analyzing metabolite production. Fig. 3 shows that there was an approximately twofold increase in 1,2-diacylglycerol levels after attached matrices were released. This increase could be detected within 1 min after initiating contraction and remained constant over the next hour (data not shown). The results shown in Fig. 4, on the other hand, demonstrated that no increase of inositol phosphates occurred during fibroblast contraction. As a positive control, cells in attached matrices were treated with bradykinin, which elicited a predictable activation of phospholipase C resulting in a rapid rise in inositol phosphates (Burch and Axelrod, 1987; Etscheid and Villereal, 1989). Taken together, these results indicate that phosphoinositide-specific phospholipase C was not activated during fibroblast contraction. Release of 1,2-diacylglycerol may have occurred as a result of phospholipase C action on phosphatidylcholine (van Blitterswijk et al., 1991; Fisher et al., 1991; Carnero et al., 1994).

Activity of Phospholipase D during Fibroblast Contraction of Stressed Collagen Matrices

Unlike the modest changes in phospholipase A2 and phospholipase C described above, there appeared to be marked activation of phospholipase D initiated by matrix contraction. This was shown first by analyzing phosphatidic acid, which occurred at low levels in fibroblasts in attached matrices and increased after attached matrices were released (Fig. 2 and Fig. 3 *b*). Elevated phosphatidic acid levels could be detected within 3 min but not 1 min (data not shown). Fig. 5 shows that the increase in phosphatidic acid peaked 5–10 min after initiating cell contraction and declined to baseline levels by 60 min. Similar results were obtained regardless of whether cells had been radiolabeled with [^{3}H]palmitic acid or [^{3}H]arachidonic acid.

To show more directly that phospholipase D was activated, we took advantage of the enzyme's known capacity

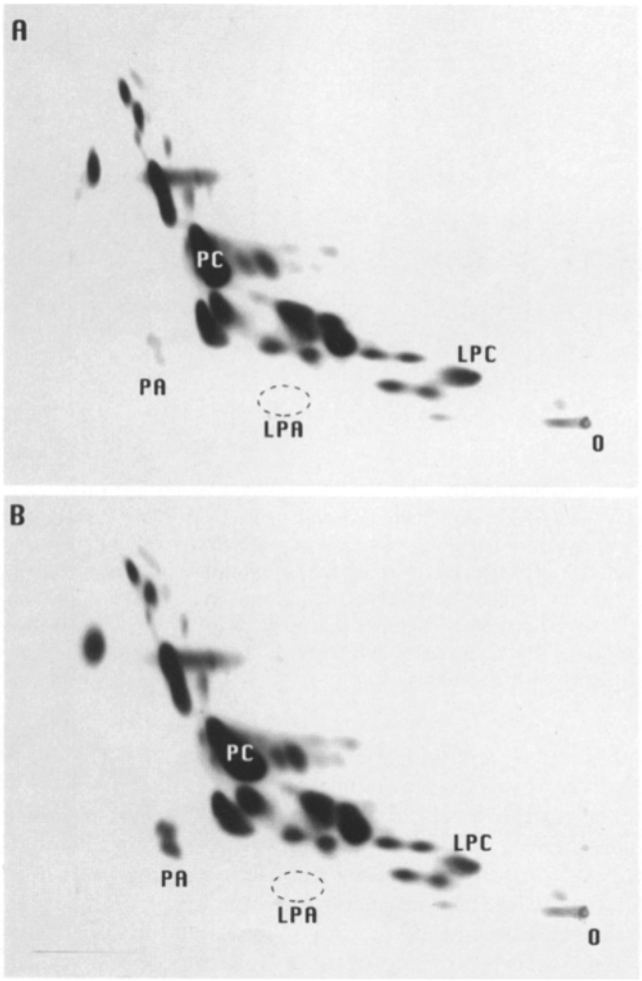

Figure 2. Effect of fibroblast contraction on release of phosphatidylcholine, lysophosphatidylcholine, and phosphatidic acid. Fibroblasts in attached collagen matrices (*A*) were prelabeled overnight with [^{3}H]palmitic acid. Matrices were released (*B*) to initiate contraction. After 10 min of contraction, lipids were extracted and subjected to two-dimensional TLC. *PA*, phosphatidic acid; *PC*, phosphatidylcholine; *LPA*, lysophosphatidic acid; *LPC*, lysophosphatidylcholine. *O*, origin.

to preferentially catalyze a transphosphatidylation reaction in the presence of primary alcohols (Thompson et al., 1991; Moehren et al., 1994). Fig. 6 shows that addition of ethanol (0.5–2.5%) resulted in formation of phosphatidylethanol after attached collagen matrices were released. In parallel experiments, as shown in Fig. 7, addition of ethanol inhibited contraction-dependent phosphatidic acid production. On the other hand, 2.5% ethanol had no effect on the time course of collagen matrix contraction or cellular release of LDH activity, indicating that ethanol was not generally toxic for the cells. These results showed that fibroblast contraction resulted in activation of phospholipase D, leading to increased production of phosphatidic acid.

Effect of Ethanol on Contraction-activated Production of Arachidonic Acid and cAMP

To study the possibility that phosphatidic acid production

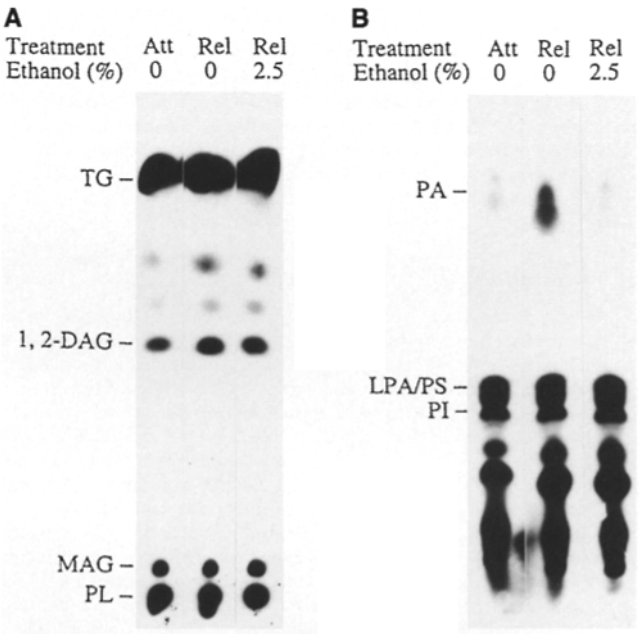

Figure 3. Effect of fibroblast contraction on release of 1,2-diacylglycerol and phosphatidic acid. Fibroblasts in attached collagen matrices (*Att*) were prelabeled overnight with [^{3}H]palmitic acid. Ethanol (2.5%) was added to the cultures where indicated. 10 min later, matrices were released (*Rel*) to initiate contraction. After 10 min of contraction, lipids were extracted. Phospholipids were resolved using one-dimensional TLC (*a*) or double one-dimensional TLC (*b*). *TG*, triglyceride; *1,2-DAG*, 1,2-diacylglycerol; *MAG*, monoacylglyceride; *PL*, phospholipid; *PA*, phosphatidic acid; *LPA/PS*, lysophosphatidic acid phosphatidylserine; *PI*, phosphatidylinositol.

was an upstream event in the contraction-activated cAMP signal pathway, we also measured the effect of ethanol on production of arachidonic acid and cAMP. Fig. 8 shows that the dose response was similar for ethanol inhibition of

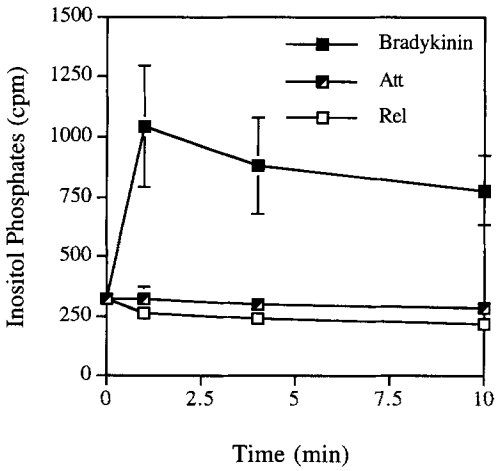

Figure 4. Effect of fibroblast contraction on release of inositol phosphates. Fibroblasts in attached collagen matrices (*Att*) were prelabeled for 2 d with [^{3}H]myoinositol. Bradykinin (100 nM) was added to the cultures, or matrices were released (*Rel*) to initiate contraction. At the times indicated after addition of bradykinin or release, cultures were extracted, and inositol phosphate levels were analyzed. Data presented are averages and standard deviations based on duplicate samples.

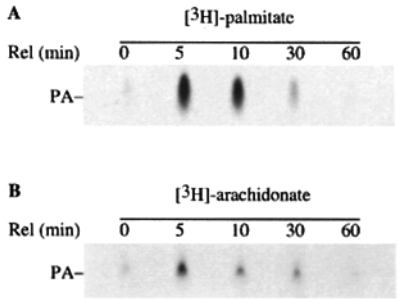

Figure 5. Fibroblast contraction triggers a transient increase in phosphatidic acid release. Fibroblasts in attached collagen matrices were prelabeled overnight with [^{3}H]palmitic acid (*A*) or [^{3}H]arachidonic acid (*B*). Matrices were released (*Rel*) to initiate contraction. At the times indicated after release, lipids were extracted, and phospholipids were resolved using double one-dimensional TLC. *PA*, phosphatidic acid.

increased production of phosphatidic acid, arachidonic acid, and cAMP. In control experiments (not shown), we found that treatment of cells with ethanol had no effect on activation of cAMP production in response to exogenously added arachidonic acid or on phospholipase A2 activity subsequently measured in cell extracts. These results were consistent with the idea that activation of phosphatidic acid production was required for increased arachidonic acid production in the contraction-activated signal transduction pathway. It should be noted, however, that the contraction-activated increase in arachidonic acid and cAMP production was not completely blocked even when phosphatidic acid production was totally inhibited by ethanol (Fig. 8), suggesting that at least some arachidonic acid was produced independently of phosphatidic acid.

Whether arachidonic acid derived from phosphatidic acid was released directly or first converted to 1,2-diacylglycerol could not be clearly ascertained. Fig. 3 *a* shows that ethanol did not inhibit increased production of diacylglycerol during contraction under conditions that blocked phosphatidic acid release, which seemed to favor the possibility of direct release. On the other hand, lysophosphatidic acid was undetectable in the cells or medium at any time, as already mentioned.

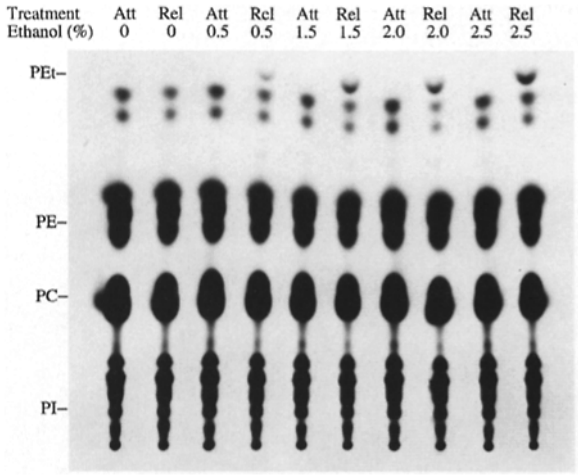

Figure 6. Fibroblast contraction in the presence of ethanol triggers production of phosphatidylethanol. Fibroblasts in attached collagen matrices (*Att*) were prelabeled overnight with [^{3}H]palmitic acid. Ethanol was added to the cultures at the concentrations indicated for 10 min. Matrices were released (*Rel*) to initiate contraction. After 10 min, lipids were extracted and resolved by one dimensional TLC. PEt, phosphatidylethanol; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *PI*, phosphatidylinositol.

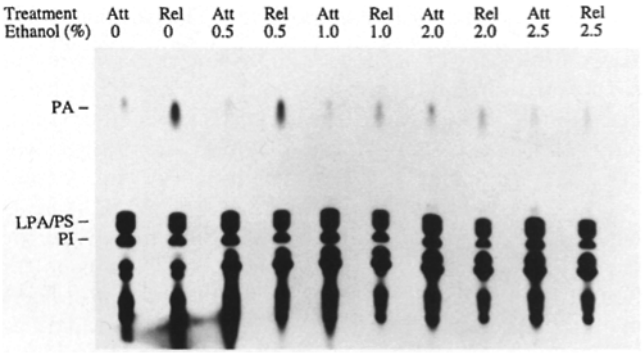

Figure 7. Fibroblast contraction in the presence of ethanol inhibits its production of phosphatidic acid. Fibroblasts in attached collagen matrices (*Att*) were prelabeled overnight with [^{3}H]palmitic acid. Ethanol was added to the cultures at the concentrations indicated for 10 min. Matrices were released (*Rel*) to initiate contraction. After 10 min, lipids were extracted and resolved using double one-dimensional TLC. *PA*, phosphatidic acid; *LPA/PS*, lysophosphatidic acid/phosphatidylserine; *PI*, phosphatidylinositol.

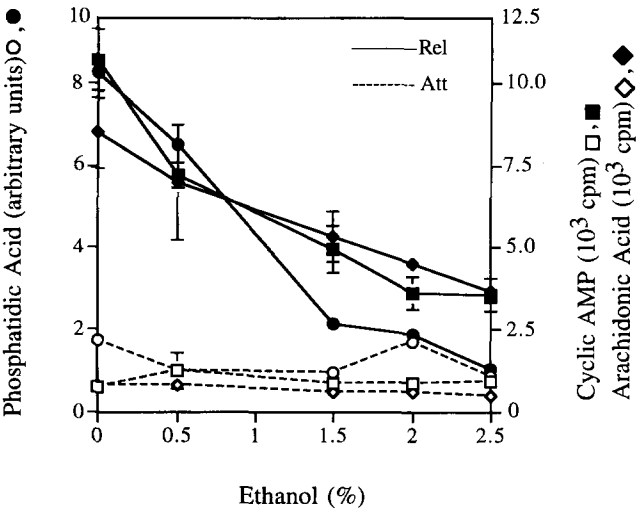

Figure 8. Parallel inhibition of phosphatidic acid, arachidonic acid, and cyclic AMP production by ethanol. Fibroblasts in attached collagen matrix cultures (*Att*; *open symbols*) were prelabeled overnight with [^{3}H]palmitic acid or [^{3}H]arachidonic acid, or for 2 h with [^{3}H]adenine. Ethanol was added to the cultures at the concentrations indicated for 10 min. Matrices were released (*Rel*, *solid symbols*) to initiate contraction. After 10 min, samples were treated by one of the following protocols: (*a*) lipids were extracted and resolved using double one-dimensional TLC, and phosphatidic acid production was quantified by scanning densitometry; (*b*) arachidonic acid release was measured; or (*c*) samples were extracted and used to measure cAMP production. Data shown are averages and standard deviations from duplicate samples.

Ca^{2+} Dependence of Phospholipase D Activation

Several different phospholipase D isoenzymes varying in cellular location and regulatory features have been identified (Billah, 1993; Exton, 1994). To learn whether phospholipase D activated during fibroblast contraction requires extracellular Ca^{2+}, studies with EGTA were carried out. Fig. 9 shows that addition of 3 mM EGTA to the incubations inhibited phosphatidic acid production when attached collagen matrices were released. This concentration of EGTA slightly exceeds the concentration of Ca^{2+} in 10% FBS–containing medium and blocks contraction-activated cAMP signaling (He and Grinnell, 1994). Fig. 9 also shows that inhibition of phosphatidic acid production by EGTA was overcome by addition to the medium of 2 mM $CaCl_2$ but not 5 mM $MgCl_2$. Moreover, addition of calcium ionophore A23187 resulted in increased phosphatidic acid production by fibroblasts in attached matrices even in the absence of contraction. This increase also was prevented, at least partially, by addition of 3 mM EGTA to the medium. These studies indicate that contraction-activation of phospholipase D requires extracellular Ca^{2+} influx.

Protein Kinase C Dependence of Phospholipase D Activation and cAMP Signaling

In other experiments, we tested whether contraction activation of phospholipase D is regulated by protein kinase C (Billah, 1993; Exton, 1994). Fibroblasts in attached collagen matrices were incubated overnight with 100 nM 12-*o*-tetradecanoylphorbol-13-acetate (TPA), which decreased protein kinase C activity from ~1,200 to ~60 pmol/min/10^5 LDH units (measured using an assay system purchased from GIBCO BRL [Gaithersburg, MD]). As shown in Fig. 10, this downregulation of protein kinase C blocked contraction-activated phosphatidic acid production. Moreover, as shown in Fig. 11, inhibition was not overcome by

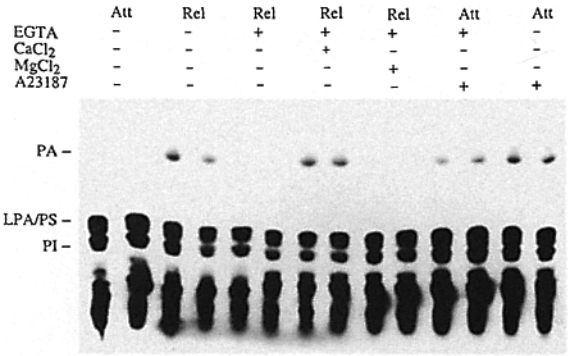

Figure 9. Regulation of phosphatidic acid production by extracellular Ca^{2+}. Fibroblasts in attached collagen matrix cultures (*Att*) were prelabeled overnight with [^{3}H]palmitic acid. Cultures were incubated 10 min in medium containing 3.0 mM EGTA, followed by addition of 2.0 mM $CaCl_2$ or 5.0 mM $MgCl_2$ to the designated samples. Matrices were released (*Rel*) to initiate contraction. Alternatively, 50 μM A23187 was added to attached cultures. 5 min after initiating contraction or 10 min after adding A23187, lipids were extracted, and phospholipids were resolved using double one-dimensional TLC. *PA*, phosphatidic acid; *LPA/PS*, lysophosphatidic acid/phosphatidylserine; *PI*, phosphatidylinositol.

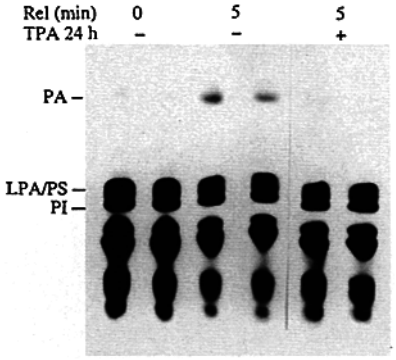

Figure 10. Stress-sensitive activation of phospholipase D is inhibited by downregulation of protein kinase C. Fibroblasts in attached collagen matrix cultures were prelabeled overnight with [^{3}H]palmitic acid. Designated samples also contained 100 nM TPA. Matrices were released (*Rel*) to initiate contraction for the times shown, and then lipids were extracted and resolved using double one-dimensional TLC. *PA*, phosphatidic acid; *LPA/PS*, lysophosphatidic acid/phosphatidylserine; *PI*, phosphatidylinositol.

stimulating cells with Ca^{2+} ionophore. Therefore, downregulation of protein kinase C prevented phospholipase D activation by Ca^{2+} influx.

If phosphatidic acid production was an upstream event in contraction-activated cAMP signaling, then downregulation of protein kinase C should also have blocked increased production of cAMP during fibroblast contraction. Table I shows that this was the case. That is, overnight treatment of fibroblasts in attached matrices with phorbol ester had little effect on basal cAMP levels but markedly inhibited the increase in cAMP that occurred when the matrices were released. Downregulation of protein kinase C also inhibited the increase in arachidonic acid production during contraction (data not shown). Control experiments ruled out the possibility that downregulation of protein kinase C resulted from nonspecific inhibition of cAMP syn-

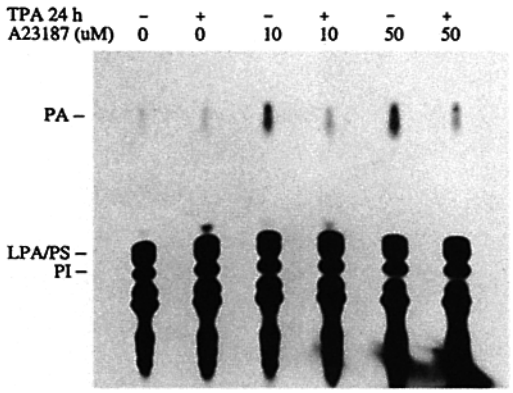

Figure 11. Ca^{2+} influx is unable to overcome the inhibition of stress-sensitive signaling after protein kinase C downregulation. Fibroblasts in attached collagen matrix cultures were prelabeled overnight with [^{3}H]palmitic acid. Designated samples also contained 100 nM TPA. A23187 was added at concentrations indicated for 10 min, and then lipids were extracted and resolved using double one-dimensional TLC. *PA*, phosphatidic acid; *LPA/PS*, lysophosphatidic acid/phosphatidylserine; *PI*, phosphatidylinositol.

thesis since the inhibitory effect of overnight TPA treatment could be overcome by adding arachidonic acid to the cells (Table I).

Discussion

The goal of our research has been to characterize mechanoregulated events that occur when fibroblasts in stressed collagen matrices contract the matrix. Previously, we described a contraction-activated signal transduction pathway. This pathway involves influx of extracellular Ca^{2+} ions and increased production of arachidonic acid resulting within min in a 10–20-fold increase in cellular cAMP levels and activation of protein kinase A (He and Grinnell, 1994). Here we report that within 5 min after initiating contraction, a burst of phosphatidic acid release was detected in fibroblasts that had been radiolabeled with [^{3}H]palmitic acid or [^{3}H]arachidonic acid. Phosphatidic acid production was found to depend on activation of phospholipase D. Activation was regulated by extracellular Ca^{2+} ions and protein kinase C, and in this regard resembled a number of other instances of agonist-induced activation of phospholipase D (Billah, 1993; Exton, 1994).

Phospholipase D was implicated in production of phosphatidic acid based on our observation that a transphosphatidylation reaction occurred in the presence of ethanol (Thompson et al., 1991; Moehren et al., 1994). Transphosphatidylation resulted in formation of phosphatidylethanol at the expense of phosphatidic acid. Ethanol treatment of cells also inhibited by 60–70% contraction-dependent release of arachidonic acid and cAMP but had no effect on increased cAMP synthesis after addition of exogenous arachidonic acid or on phospholipase A2 activity subsequently found in cell extracts. Other treatments that blocked contraction-activated cAMP signaling—chelating extracellular Ca^{2+} (He and Grinnell, 1994) or downregulating protein kinase C (this work)—also inhibited the burst of phosphatidic acid release after contraction. Taken together, these results suggest that phosphatidic acid production was upstream of arachidonic acid in the contraction-activated cAMP signaling pathway.

Arachidonic acid could have been released from phosphatidic acid indirectly through the combined action of diacylglycerol and monoacylglycerol lipases (Rindlisbacher et al., 1987; Balsinde et al., 1991; Allen et al., 1992; Mattila et al., 1993) after conversion of phosphatidic acid to 1,2-diacylglycerol (Billah et al., 1989; Ahmed et al., 1994). Although we observed a twofold increase in 1,2-diacylglycerol during contraction, this increase was not blocked by ethanol. Moreover, the rise in 1,2-diacylglycerol levels took place earlier than the increase in phosphatidic acid and remained constant over the time during which phosphatidic acid dramatically increased and then decreased. Therefore, it seems unlikely that 1,2-diacylglycerol was released from phosphatidic acid. Rather, the early 1,2-diacylglycerol increase may have occurred as a result of phospholipase C action on phosphatidylcholine (van Blitterswijk et al., 1991; Fisher et al., 1991; Carnero et al., 1994).

Table I. Effect of Protein Kinase C Downregulation on Contraction-activated cAMP Signaling

Treatment	cAMP (10^3 cpm)
Att	3.9 ± 1.2
TPA, Att	3.9 ± 0.2
Rel	20.3 ± 3.4
TPA, Rel	7.0 ± 2.5
Att + AA	19.0 ± 2.8
TPA, Att + AA	15.4 ± 4.1

Fibroblasts in mechanically attached collagen matrices (*Att*) were treated as indicated with or without 100 nM TPA for 24 h. Subsequently, the cultures were released to initiated contraction (*Rel*) or treated with arachidonic acid (*AA*) (final concentration = 0.5 mM). cAMP levels were measured 10 min later. The culture medium contained 10 μCi/ml [^{3}H]adenine. Data presented are from three separate experiments.

Another possibility was that arachidonic acid release occurred directly through the action of phospholipase A2 on phosphatidic acid (Billah et al., 1981). Consistent with this possibility, we found that human fibroblasts contained phospholipase A2 activity that liberated arachidonic acid from vesicles prepared with arachidonyl-phosphatidic acid (He, J., and F. Grinnell, unpublished observations), and cellular phospholipase A2 activity increased about twofold during contraction. Nevertheless, we were unable to detect the predicted increase in lysophosphatidic acid release that should have accompanied arachidonic acid production from phosphatidic acid, but lysophosphatidic acid may have been converted rapidly to other metabolites or degraded. We did detect lysophosphatidylcholine in fibroblasts in stressed matrices, but levels of this metabolite did not increase during contraction, which suggests that arachidonic acid was not released by phospholipase A2 activity on phosphatidylcholine. Whatever the mechanism of arachidonic acid release from phosphatidic acid, it should be noted that activation of phospholipase D after contraction was tightly regulated, and phosphatidic acid declined to basal levels within 30–60 min. Since elevated cAMP has been shown to inhibit agonist-induced phospholipase D activation in other systems (Agwu et al., 1991; Tyagi et al., 1991; Garcia et al., 1992), it may be that the increase in phosphatidic acid production after contraction was transient because of a feedback effect of cAMP. Based on previously published studies, the site of cAMP inhibition probably occurs upstream of phospholipase D. This could be at the level of influx of extracellular Ca^{2+}, but also might involve other mechanisms previously shown to regulate phospholipase D such as tyrosine kinases (Uings et al., 1992; Cook and Wakelam, 1992; Dubyak et al., 1993) or small G proteins (Brown et al., 1993; Cockroft et al., 1994; Malcolm et al., 1994), which have yet to be studied in the context of the contraction-activated signaling pathway.

Although regulation of phospholipase D by cAMP as described here is consistent with the data, the results are somewhat paradoxical since previous studies with cultured fibroblasts have shown that phosphatidic acid inhibits adenylyl cyclase (Clark et al., 1980; Murayama and Ui, 1987; Wang et al., 1994). One possible explanation for this paradox is in the extracellular environment of the cells. That is, differences in adhesive interactions of fibroblasts in monolayer culture and in collagen matrices may prepare the cells to react to external signals through different signaling pathways.

Another unique feature of the contraction-activated sig-

naling pathway is that the cells become quiescent. This is in marked contrast to other systems in which phospholipase D activation has been proposed to play a role in cell mitogenesis (Boarder, 1994), although this function of phospholipase D is controversial (Paul and Plevin, 1994). The mechanism by which fibroblasts in collagen matrices become quiescent after matrix contraction can be attributed at least in part to decreased PDGF receptor function (Tingström et al., 1992; Lin and Grinnell, 1993). Increased production of cAMP also may be important in quiescence considering recent findings that cAMP can block the extracellular signal-regulated kinase pathway required for mitogenic activation (Cook and McCormick, 1993; Wu et al., 1993; Graves et al., 1993; Sevetson et al., 1993; Burgering et al., 1993).

Finally, it should be noted that beyond identifying phospholipase D as an early step in contraction-activated cAMP signaling, our studies now show that protein kinase C regulates the overall pathway. That is, downregulation of protein kinase C by overnight treatment with phorbol ester resulted in inhibition of the contraction-dependent increased production of phosphatidic acid, arachidonic acid, and cAMP. Since the effect of protein kinase C downregulation could be overcome by arachidonic acid but not by calcium ionophore, the site of protein kinase C regulation appeared to be upstream of phosphatidic acid production and arachidonic acid production and downstream of Ca^{2+} influx, probably involving not only phospholipase D, but also phospholipase A2 activity (Lin et al., 1993).

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