Experimental Cell Research **254,** 210–220 (2000)
doi:10.1006/excr.1999.4754, available online at http://www.idealibrary.com on IDEAL®

Regulation of LPA-Promoted Myofibroblast Contraction: Role of Rho, Myosin Light Chain Kinase, and Myosin Light Chain Phosphatase[1]

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Myofibroblasts generate the contractile force responsible for wound healing and pathological tissue contracture. In this paper the stress-relaxed collagen lattice model was used to study lysophosphatidic acid (LPA)-promoted myofibroblast contraction and the role of the small GTPase Rho and its downstream targets Rho kinase and myosin light chain phosphatase (MLCPPase) in regulating myofibroblast contraction. In addition, the regulation of myofibroblast contraction was compared with that of smooth muscle cells. LPA-promoted myofibroblast contraction was inhibited by the myosin light chain kinase (MLCK) inhibitors KT5926 and ML-7; however, in contrast to that observed in smooth muscle cells, elevation of intracellular calcium alone was not sufficient to promote myofibroblast contraction. These results suggest that Ca^{2+}-mediated activation of MLCK, while necessary, is not sufficient to promote myofibroblast contraction. The specific Rho inactivator C3-transferase and the Rho kinase inhibitor Y-27632 inhibited LPA-promoted myofibroblast contraction, suggesting that contraction depends on activation of the Rho/Rho kinase pathway. Calyculin, a type 1 phosphatase inhibitor known to inhibit MLCPPase, could promote myofibroblast contraction in the absence of LPA, as well as restore contraction in the presence of C3-transferase or Y-27632. Together these results support a model whereby Rho/Rho kinase-mediated inhibition of MLCPPase is necessary for LPA-promoted myofibroblast contraction, in contrast to smooth muscle cells in which Ca^{2+} activation of MLCK alone is sufficient to promote contraction.

Key Words: **myofibroblast; LPA; myosin light chain phosphatase.**

INTRODUCTION

Myofibroblasts are proposed to be the cells responsible for generation of contractile forces in granulation tissue and pathologic contractures [21, 53]. These nonmuscle, fibroblastic cells are characterized by the presence of large intracellular bundles of actin microfilaments, termed stress fibers, and fibronexus adhesion complexes linking intracellular stress fibers and extracellular fibronectin fibrils [52, 53, 58]. Cultured myofibroblasts can generate contractile force and transmit this force to the surrounding extracellular matrix [21, 58, 59], similar to tissue contraction *in vivo.* Force generation in the myofibroblast results from actin–myosin interaction in the intracellular stress fibers. Stress fibers which contain actin and myosin II [7, 38] can contract upon addition of ATP [31, 36]. Although stress fibers can generate contractile force in myofibroblasts, the intracellular signaling pathways regulating the actin–myosin interaction responsible for contraction are not fully understood. In fact, it has been assumed that myofibroblast contraction and smooth muscle cell contraction are regulated in similar manners; however, the distinct pathways regulating the contractility of myofibroblasts have not yet been defined.

In smooth muscle cells, the level of intracellular Ca^{2+} is the dominant system regulating contraction. Increased intracellular Ca^{2+} activates the Ca^{2+}-dependent myosin light chain kinase (MLCK), which results in increased phosphorylation of myosin light chain (MLC-P) and increased contractility [24, 30, 56]. In nonmuscle cells increased MLC-P has been correlated with increased contractility [9, 35]. Whether increased intracellular Ca^{2+} is the dominant regulatory system in myofibroblasts is unclear. Recent studies have demonstrated that elevation of intracellular Ca^{2+} does not promote stress fiber assembly in fibroblasts [6, 46, 49], an event correlated with increased contractility [9]. Similarly, increased intracellular Ca^{2+} does not promote contraction of endothelial cells [17].

Activation of the small GTPase Rho could provide an additional mechanism regulating myofibroblast con-

[1] This work was supported by the Oklahoma Center for the Advancement of Science and Technology (H97-089).
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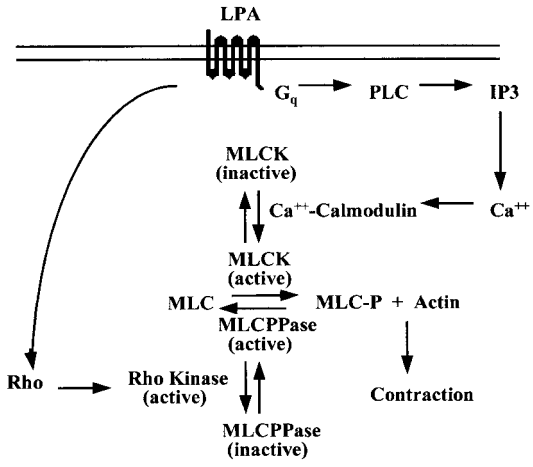

FIG. 1. Model for regulation of LPA-promoted myofibroblast contraction. LPA is proposed to promote myofibroblast contraction by activation of two separate signaling pathways. The first is a Ca^{2+}-dependent pathway leading to release of intracellular Ca^{2+} and activation of MLCK. The second is a Rho-dependent pathway in which Rho is activated, leading to activation of Rho kinase and inactivation of MLCPPase. Acting together these two signaling pathways elevate MLC phosphorylation, resulting in myosin–actin interaction and myofibroblast contraction. Unlike smooth muscle cells in which activation of the Ca^{2+}-dependent pathway is sufficient to promote contraction, Rho-dependent inactivation of MLCPPase is required before myofibroblast contraction can occur in response to Ca^{2+} activation of MLCK.

traction. Recent studies have demonstrated that activation of Rho can promote stress fiber assembly and contraction in fibroblasts [22, 45]. Activation of Rho could promote contraction by affecting its downstream targets Rho kinase and myosin light chain phosphatase (MLCPPase) [32, 37]. Dephosphorylation of MLC by MLCPPase provides an additional target for regulating MLC phosphorylation level and thereby contraction [24, 30, 54]. In smooth muscle cells, MLCPPase activity modulates smooth muscle cell contractility by regulating cellular responsiveness to Ca^{2+} [20]. It is possible that in myofibroblasts the activation of Rho and Rho kinase will inactive MLCPPase and subsequently increase generation of contractile force (Fig.1).

In this paper we have tested the model illustrated in Fig. 1 for regulation of myofibroblast contraction. We used stress-relaxed collagen lattices to analyze the generation of contractile force by myofibroblasts in response to lysophosphatidic acid (LPA) [21, 44, 58, 59]. We found that the MLCK inhibitor KT5926 blocked LPA-promoted myofibroblast contraction; however, in contrast to smooth muscle cells, Ca^{2+} activation of MLCK was not sufficient to promote myofibroblast contraction, but required an additional Ca^{2+}-independent regulatory mechanism. On the other hand, the phosphatase inhibitor calyculin was sufficient to promote myofibroblast contraction. In addition, inactivation of Rho with C3-transferase inhibited LPA-promoted myofibroblast contraction, which could be restored upon addition of calyculin. The Rho kinase inhibitor Y-27632 also blocked LPA-promoted myofibroblast contraction, which could be restored upon addition of calyculin. Together our data suggest a model whereby myofibroblast contraction is dependent on the activation of two signaling cascades: a Ca^{2+}-dependent pathway activating MLCK and a Rho/Rho kinase-dependent pathway inhibiting MLCPPase. In addition, unlike smooth muscle cell contraction, myofibroblast contraction requires inhibition of the MLCPPase before the cells can respond to Ca^{2+}-activated MLCK.

MATERIALS AND METHODS

Cell culture. Human myofibroblasts were obtained as explant cultures or by collagenase digestion of palmar aponeurosis from patients with Dupuytren's disease [44, 59]. Cells were cultured in complete culture medium composed of M-199 supplemented with 10% fetal bovine serum (FBS) (Irvine Scientific, Santa Ana, CA), 2 mM glutamine, and 1% antibiotic–antimycotic and used in all experiments between passages 2 and 15. Rat aortic smooth muscle cells (a gift from Dr. Paula Grammas, University of Oklahoma Health Sciences Center) were isolated as previously described [13].

Collagen lattice contraction assay. Cells were cultured within stabilized type I collagen lattices as previously described [42, 44, 58, 59], such that the final collagen concentration was 0.65 mg/ml and the cell concentration was 1.25×10^5 cells/ml. A 250-μl drop of the collagen/cell suspension was plated onto 35-mm tissue culture dishes and after 1 h covered with complete culture medium. After 5 days in culture, generation of contractile force was measured. First, stabilized lattices were washed with unsupplemented M-199 twice for 2.5 min each, followed by addition of 10% FBS, unsupplemented M-199, or LPA (L-α-lysophosphatidic acid, oleoly (C18:1,[*cis*]-9); Sigma Chemical Company, St. Louis, MO). Immediately after addition of agonist, lattices were mechanically released from the bottom of the tissue culture dishes by gently pipetting medium at the lattice–dish interface. Rapid contraction was analyzed by measuring the lattice diameter before release and at specific times after release using a Nikon SMZ-1 stereoscope. The initial collagen lattice diameters ranged from 14 to 16 mm. Relative lattice diameter was obtained by dividing the diameter at each time point by the initial diameter of the lattice. All data are from experiments using at least three sets of three collagen lattices and are expressed as the means ± SEM.

Inhibitors and agonists affecting contractility. In some experiments FBS was incubated with 6 U/ml phospholipase B (Sigma) for 1 h at 37°C before being added to serum-free collagen lattices immediately prior to release. The kinase inhibitors were used as follows: KT5926 (Calbiochem Corp., La Jolla, CA) at 0.2 μM and 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine · HCl (ML-7; Biomol, Plymouth Meeting, PA) at 0.22–22 μM. An inhibitor of actin–myosin interaction, 2,3-butanedione 2-monoxime (BDM; Sigma), was used at 10 mM. The kinase inhibitors and BDM were added to serum-free collagen lattices immediately prior to addition of 1 μM LPA and release. To determine the effect of increased intracellular Ca^{2+} on contraction, the Ca^{2+} ionophores A23187 (0.01–10 μM) (Sigma) or ionomycin (10 μM) (Sigma) were added to serum-free collagen lattices immediately prior to release. To determine the effect of phosphatase inhibitors on contraction, serum-free collagen lattices were incubated with the phosphatase inhibitors calyculin (4–10 nM) (Calbiochem) or okadaic acid (10–100 nM) (Calbiochem) for 20 min prior to release. The kinase inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7; Sigma) was used at 0.2–200 μM and added to serum-free collagen lattices immediately prior to addi-

tion of 1 μM LPA or after 20-min incubation with 10 nM calyculin followed by release of lattice. To determine the involvement of Rho in myofibroblast contraction, serum-free collagen lattices were incubated with 30 μg/ml C3-transferase for 3 h prior to addition of 1 μM LPA or 10 nM calyculin. The Rho kinase inhibitor Y-27632 (Yoshitomi Pharmaceutical Industries, Ltd., Osaka, Japan) was used at 10^{-8}–10^{-5} M and added to serum-free collagen lattices immediately prior to addition of 1 μM LPA or after a 20-min incubation with 10 nM calyculin followed by release of lattice.

Ca^{2+}-depleted collagen lattices were obtained as follows: lattices were washed with Ca^{2+}-free Dulbecco's phosphate-buffered saline (Ca^{2+}-free DPBS) (3×, 2 min each), followed by Ca^{2+}-free DPBS + 3 mM EGTA (2×, 5 min each, then 14 min) and a final set of washes with Ca^{2+}-free DPBS + 3 mM EGTA + 10 μM ionomycin (3×, 2 min each). The final set of washes contained ionomycin to permit depletion of intracellular Ca^{2+}. Lattices were released following the final set of washes. In some of the lattices 10 nM calyculin was added to the washes for the final 20 min prior to release. To obtain Ca^{2+}-containing collagen lattices, Ca^{2+}-depleted collagen lattices were prepared as described above except that the final set of washes contained DPBS + 10 μM ionomycin.

Purification of recombinant C3-transferase. Recombinant C3-transferase was expressed as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli* (a gift from Dr. Keith Burridge, University of North Carolina). C3-transferase was purified as previously described [67]. Briefly, the protein was purified on glutathione-agarose beads and cleaved from GST with thrombin (Sigma) in a buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, 5 mM DTT), which was then removed by three subsequent incubations with *p*-aminobenzamidine agarose (Sigma). The final supernatant was collected and dialyzed overnight in a Tris buffer (50 mM Tris–HCl, pH. 7.5, 30 mM NaCl, 0.1 mM DTT) followed by two changes in phosphate-buffered saline (PBS). An aliquot of the final product was analyzed by SDS–PAGE. Purified C3-transferase was added to stabilized collagen lattices as described above.

Fluorescence microscopy. Collagen lattices were fixed in 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4, for 20 min and washed with 0.1 M phosphate buffer, pH 7.4, followed by 0.05 M Tris buffer, pH 7.4. Lattices were cut into pieces and cells were opened with 0.5% Triton X-100 in PBS for 10 min, rinsed with PBS, and stained for 30 min with a 1:40 dilution of the F-actin probe rhodamine phalloidin (Molecular Probes, Eugene, OR). After staining, lattices were rinsed with PBS, mounted as whole mounts in glycerol:PBS (9:1), and observed and photographed using an Olympus Vanox microscope equipped with epifluorescence optics.

Fluo-3 visualization of intracellular calcium. Myofibroblasts were grown on glass coverslips for 3 days in complete culture medium. Coverslips were washed with DPBS + 1 mg/ml bovine serum albumin (BSA) (3×, 5 min each) followed by incubation for 90 min at room temperature with 5 μM Fluo-3 (Molecular Probes) + 0.025% pleurionic (Molecular Probes) in DPBS + 1 mg/ml BSA. Coverslips were then washed for 5 min with DPBS + 1 mg/ml BSA and placed in a Dvorak–Stotler chamber (Nicholson Precision Instruments, Gaithersburg, MD). Coverslips were examined using an Olympus IMT-2 inverted microscope equipped with epifluorescence optics and a CCD camera (RS-170; Cohu, San Diego, CA) connected to a Power MacIntosh 7100/80 with NIH Image 1.61 software. Cells were observed and photographed prior to and after addition of either 10 μM ionomycin or 10 μM A23187.

RESULTS

LPA Is a Major Agonist in Fetal Bovine Serum That Promotes Myofibroblast Contraction

We have previously reported that FBS contains factors necessary for the contraction of cultured human myofibroblasts [44, 59]. To explore whether LPA, a small phospholipid released upon platelet activation and present in FBS [14, 57], is a major agonist in FBS that promotes contraction of myofibroblasts, we used the stressed-relaxed collagen lattice model [58]. Myofibroblasts cultured for 5 days within stabilized collagen lattices followed by removal of FBS and addition of 1 μM LPA retained large bundles of actin microfilaments and appeared similar to myofibroblasts *in vivo* (Fig. 2A). Myofibroblasts rapidly contracted released collagen lattices in the presence of either 10% FBS or 1 μM LPA (Fig. 2B). Most of the rapid contraction occurred within the first 10 min after mechanical release of the collagen lattice. In contrast, myofibroblasts promoted only a small amount of spontaneous contraction upon release of collagen lattices in the absence of any agonist (Fig. 2B). LPA-promoted contraction was dose dependent, with an ED_{50} of 0.1 μM (Fig. 2C). This correlates well with the amount of LPA present in freshly prepared mammalian FBS, which is in the range of 2–20 μM [14, 57]. Next, we hydrolyzed the LPA in FBS by treatment with phospholipase B [66]. Phospholipase B pretreatment of FBS reduced collagen lattice contraction by approximately 50% compared to untreated FBS (Fig. 2C). These results demonstrate that LPA promotes contraction of myofibroblasts and that it is a major agonist present in FBS.

Increased Intracellular Calcium Is Not Sufficient for Myofibroblast Contraction

In smooth muscle cells, an increase in intracellular Ca^{2+} is by itself sufficient to promote contraction through the activation of Ca^{2+}-dependent MLCK and subsequent phosphorylation of MLC [24, 30, 56]. To investigate whether increased intracellular Ca^{2+} is sufficient to promote myofibroblast contraction, we examined the effect of the Ca^{2+} ionophores A23187 and ionomycin on myofibroblast contraction. The addition of 0.01 to 10 μM A23187 or 10 μM ionomycin had minimal effects on myofibroblast contraction of collagen lattices (Fig. 3A). In contrast, rat aortic smooth muscle cells, cultured under identical conditions, contracted in response to A23187 with efficacy similar to that of contraction promoted by LPA (Fig. 3B). In addition, rat, dog, and human bladder smooth muscle cells also contracted in response to elevated intracellular Ca^{2+} (not illustrated). To be certain that the ionophores were increasing intracellular Ca^{2+} in myofibroblasts, cells were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-3 prior to addition of the ionophore. A significant increase in fluorescence intensity was observed within 30 s after addition of the ionophore, indicating an increase in intracellular Ca^{2+} levels (Fig. 4). These results suggest that, in contrast to

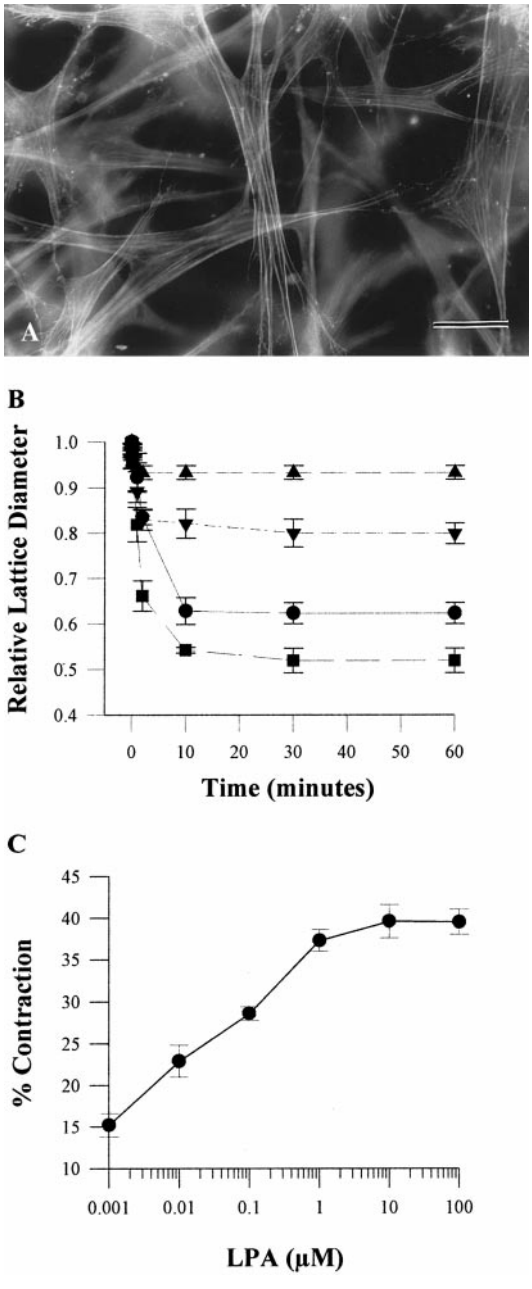

FIG. 2. (A) Myofibroblasts, when treated with LPA, exhibit large bundles of actin microfilaments or stress fibers. Myofibroblasts were cultured within stabilized collagen lattices for 5 days. Lattices were washed with serum-free medium prior to addition of 1 μM LPA. Lattices were fixed with 4% paraformaldehyde immediately following LPA addition and stained with rhodamine phalloidin to visualize the actin cytoskeleton. Bar, 40 μM. (B and C) LPA promotes myofibroblast contraction. Myofibroblasts within serum-free stabilized collagen lattices were treated with the following immediately prior to release: (B) 10% FBS (■), 1 μM LPA (●), 10% FBS previously incubated with 6 U/ml phospholipase B for an hour (▼), or serum-free medium with no agonist (▲), or (C) increasing doses of LPA. Percentage collagen lattice contraction (C) was determined at 10 min after release. All data are averages ± SEM of three sets of three collagen lattices.

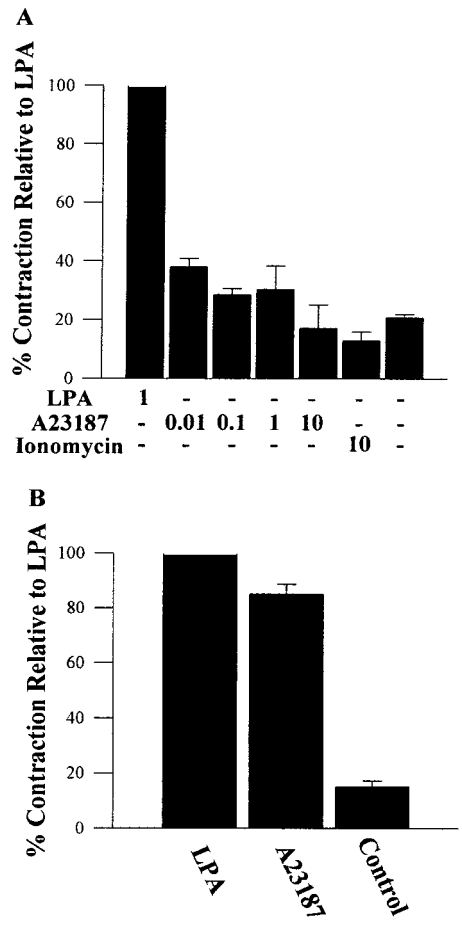

FIG. 3. Increased intracellular Ca^{2+} promotes smooth muscle, but not myofibroblast, contraction. Myofibroblasts (A) or rat aortic smooth muscle cells (B) were cultured in stabilized collagen lattices for 5 days. Lattices were washed with serum-free medium and treated with the following immediately prior to release: (A) 1 μM LPA, various concentrations of the Ca^{2+} ionophore A23187, 10 μM ionomycin, or serum-free medium with no agonist or (B) 1 μM LPA, 10 μM A23187, or serum-free medium with no agonist. Percentage collagen lattice contraction relative to LPA was determined 10 min after release. All data are averages ± SEM of three sets of three collagen lattices.

smooth muscle cells, increased intracellular Ca^{2+} is not sufficient to promote contraction of myofibroblasts.

This lack of responsiveness to Ca^{2+} appears to occur in a wide variety of fibroblasts. In addition to myofibroblasts, human palmar aponeurosis fibroblasts and WI-38 human fetal lung fibroblasts did not respond to these Ca^{2+} ionophores (not illustrated). These results suggest a significant difference in the Ca^{2+} regulation of contraction between smooth muscle and fibroblastic cells.

LPA-Promoted Contraction Is Blocked by MLCK Inhibitor KT5926

We have previously demonstrated that FBS-promoted contraction of myofibroblasts requires an intact

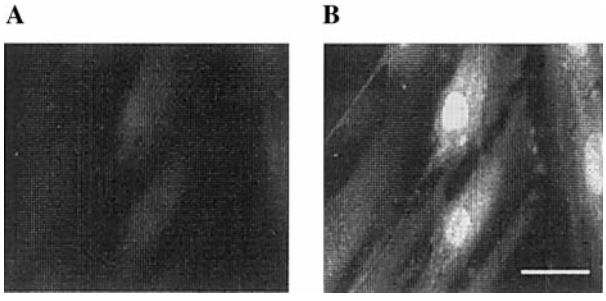

FIG. 4. Ionomycin induces increased intracellular Ca^{2+} in myofibroblasts. Myofibroblasts were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-3 and examined under epifluorescence microscopy. (A) Fluorescence photomicrograph of myofibroblasts incubated with DPBS. (B) The same field as in A, 30 s after addition of 10 μM ionomycin. Bar, 50 μm.

actin cytoskeleton [58]. To determine whether LPA-promoted myofibroblast contraction requires actin–myosin interaction, we used the inhibitor BDM, which inhibits the ATPase activity of nonmuscle myosin II [10]. Treatment of myofibroblasts with 10 mM BDM immediately prior to LPA stimulation completely inhibited contraction (Fig. 5A). These results suggest that although increased intracellular Ca^{2+} is not sufficient to promote contraction, myofibroblast contraction requires actin–myosin interaction.

The protein kinase inhibitor KT5926 has been previously used as a relatively specific MLCK inhibitor for both smooth muscle and nonmuscle cells [9]. We have found that KT5926 will inhibit LPA-promoted myofibroblast contraction (Fig. 5A). We have also found that the relatively nonspecific protein kinase inhibitor ML-7 inhibited LPA-promoted contraction (Figs. 5A and 5B). The ability of KT5926 and ML-7 to block contraction is not due to their disruption of the actin stress fibers. Myofibroblasts treated with ML-7 (Fig. 5C) or KT5926 (not illustrated) followed by LPA retained their stress fibers. Together, these findings indicate that activation of MLCK by LPA is necessary for promotion of myofibroblast contraction, even though increased intracellular Ca^{2+} is not sufficient to promote contraction.

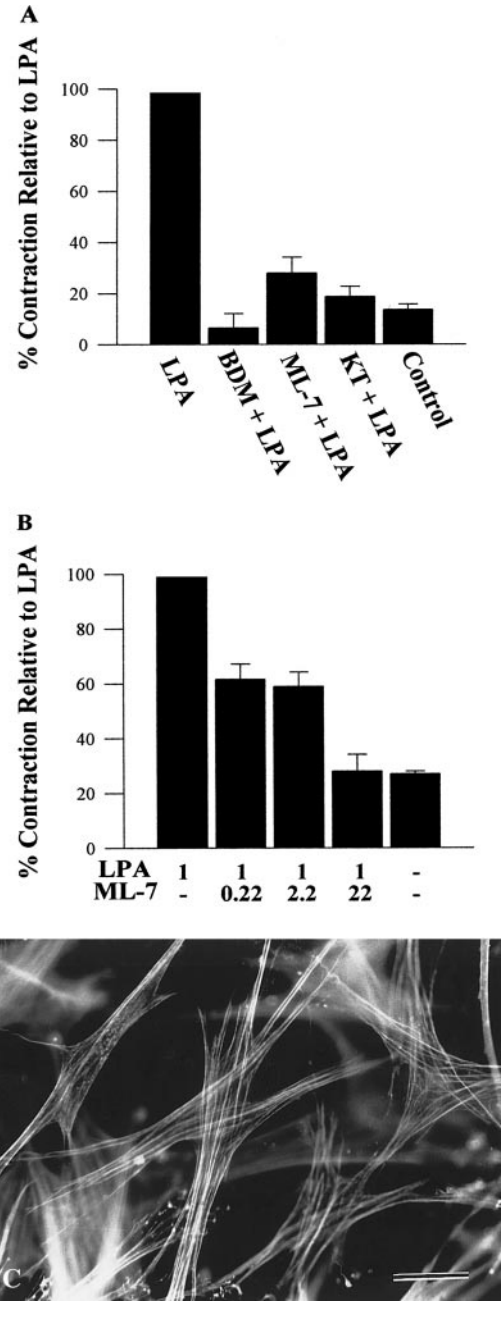

FIG. 5. Inhibitors of actin–myosin interaction or MLCK block LPA-promoted myofibroblast contraction. Myofibroblasts within serum-free stabilized collagen lattices were treated with the following immediately prior to release: (A) 1 μM LPA, 10 mM BDM + 1 μM LPA, 22 μM ML-7 + 1 μM LPA, 0.2 μM KT5926 + 1μM LPA, or serum-free medium with no agonist or (B) 1 μM LPA, various concentrations of ML-7 + 1 μM LPA, or serum-free medium with no agonist. Percentage collagen lattice contraction relative to LPA was determined at 10 min after release. All data are averages ± SEM of three sets of three collagen lattices. (C) Myofibroblasts within serum-free stabilized collagen lattices were treated with 22 μM ML-7 + 1 μM LPA and immediately fixed in paraformaldehyde and stained with rhodamine phalloidin to visualize actin microfilaments. Bar, 40 μm.

The Type 1 Phosphatase Inhibitor Calyculin Is Sufficient to Promote Myofibroblast Contraction

MLCPPase, the enzyme responsible for dephosphorylation of MLC, is a member of the family of type 1 phosphatases that can be inhibited by the phosphatase inhibitor calyculin [27, 32, 50]. We tested whether calyculin itself was sufficient to promote myofibroblast contraction. The addition of calyculin alone promoted myofibroblast contraction, and this was dose-dependent (Figs. 6A and 6B); however, the maximal amount

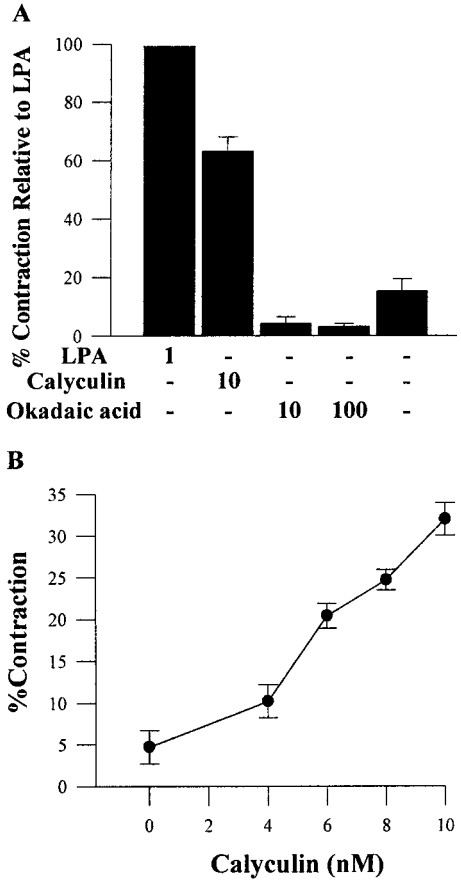

FIG. 6. Calyculin, but not okadaic acid, can promote myofibroblast contraction. Myofibroblasts within serum-free stabilized collagen lattices were treated with the following immediately prior to release: (A) serum-free medium for 20 min followed by 1 μM LPA, 10 nM calyculin for 20 min, 10 nM okadaic acid for 20 min, 100 nM okadaic acid for 20 min, or serum-free medium with no agonist for 20 min or (B) various concentration of calyculin for 20 min. Percentage contraction relative to LPA (A) or percentage collagen lattice contraction (B) was determined at 10 min after release. All data are averages ± SEM of three sets of three collagen lattices.

of contraction observed with calyculin was approximately 70% of that which occurred in response to LPA. We found that concentrations of calyculin greater than 10 nM resulted in a loss of contraction (not illustrated). At these higher concentrations myofibroblasts rapidly became rounded and lost their attachments to the collagen matrix (not shown). Calyculin inhibits both type 1 and type 2A phosphatases with almost equal potency [27]. To discriminate between the inhibition of these two phosphatases, we used the phosphatase inhibitor okadaic acid, which is 100-fold more potent an inhibitor of type 2A than type 1 phosphatases [27]. Okadaic acid did not promote myofibroblast contraction, even at concentrations 10-fold higher than those of calyculin (Fig. 6A). These results demonstrate that inhibition of a type 1 phosphatase with calyculin is sufficient to promote contraction of myofibroblasts and suggest that MLCPPase plays an important role in the regulation of myofibroblast contraction.

In smooth muscle cells, inhibition of MLCPPase increases the amount of force generated in response to a specific level of Ca^{2+} [19, 34, 54]. The lack of contraction by myofibroblasts in response to increased Ca^{2+} could be due to the activity of MLCPPase (see Fig. 1). To test this hypothesis, myofibroblast contraction was examined under Ca^{2+}-depleted or Ca^{2+}-containing conditions in the presence or absence of calyculin. Myofibroblasts under Ca^{2+}-depleted conditions did not contract either in the absence or in the presence of calyculin (Fig. 7A). Myofibroblast contraction did not occur under Ca^{2+}-containing conditions in the absence of calyculin; contraction occurred only in the presence of both Ca^{2+} and calyculin (Fig. 7A). To determine the

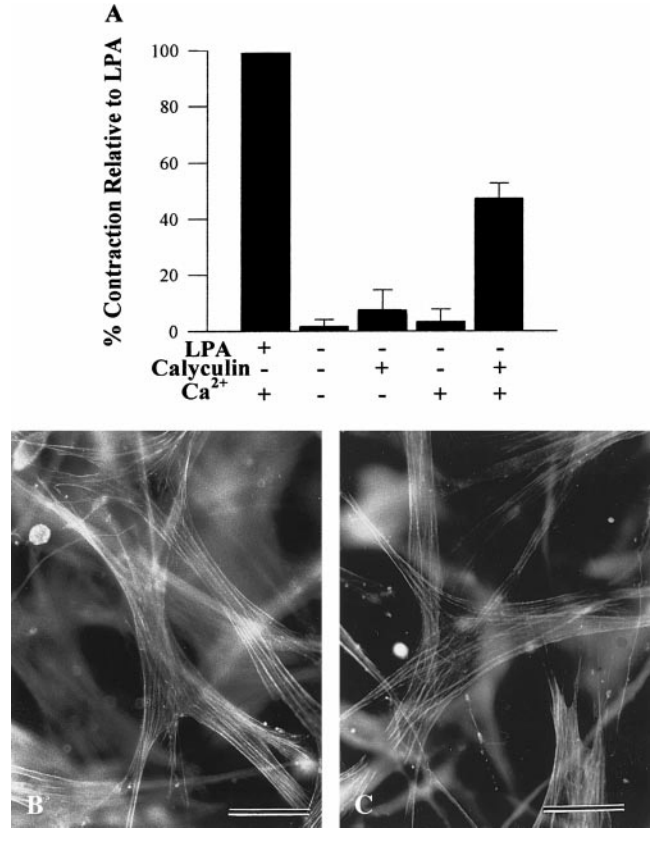

FIG. 7. Calyculin and Ca^{2+} are both necessary for myofibroblast contraction. (A) Myofibroblasts were cultured within stabilized collagen lattices for 5 days. Ca^{2+}-depleted or Ca^{2+}-containing collagen lattices were released either in the presence or in the absence of 10 nM calyculin. Percentage collagen lattice contraction relative to LPA was determined at 10 min after release. All data are averages ± SEM of three sets of three collagen lattices. (B and C) Myofibroblasts were cultured within stabilized collagen lattices for 5 days. Lattices were depleted of calcium, fixed with paraformaldehyde in the absence (B) or presence (C) of 10 nM calyculin, and stained with rhodamine phalloidin to visualization of the actin microfilaments. Bar, 40 μm.

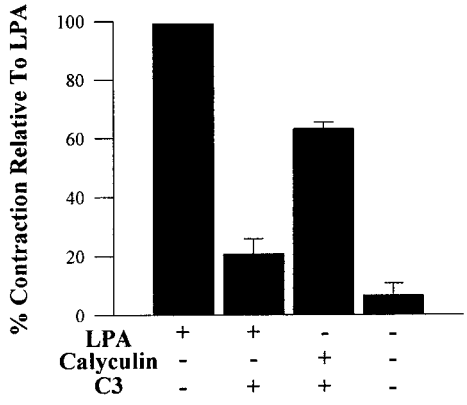

FIG. 8. C3-transferase inhibits LPA-promoted myofibroblast contraction. Myofibroblasts within serum-free stabilized collagen lattices were treated with the following immediately prior to release: serum-free medium for 3 h followed by 1 μM LPA, 30 μg/ml C3 for 3 h followed by 1 μM LPA, 30 μg/ml C3 for 3 h followed by 10 nM calyculin, or serum-free medium with no agonist for 3 h. Percentage collagen lattice contraction relative to LPA was determined at 10 min after release. All data are averages ± SEM of three sets of three collagen lattices.

possible effect that removal of Ca^{2+} might have on integrin-mediated extracellular matrix attachments, myofibroblasts in Ca^{2+}-depleted collagen lattices were fixed and examined for their shape and actin cytoskeletal organization. Myofibroblasts in Ca^{2+}-depleted collagen lattices remained spread within the collagen lattice and retained their stress fibers (Figs. 7B and 7C). Therefore, the lack of response to calyculin by myofibroblasts in Ca^{2+}-depleted collagen lattices was not due to loss of stress fibers or cell–matrix attachments. These results demonstrate that both the presence of Ca^{2+} and the inhibition of a type 1 phosphatase by calyculin are required for myofibroblast contraction. Therefore, both inactivation of MLCPPase and activation of MLCK are required for myofibroblast contraction (Fig. 1).

LPA-Promoted Myofibroblast Contraction Is Blocked by the Rho Inhibitor C3-Transferase and the Rho Kinase Inhibitor Y-27632

The soluble G protein Rho promotes stress fiber assembly in fibroblasts, as well as increased contractility in smooth muscle cells and fibroblasts [9, 22, 25, 32, 33, 45]. To determine whether Rho regulates myofibroblast contraction, we used C3-transferase, which ADP-ribosylates and inactivates Rho [47]. Since C3-transferase is taken up slowly from the medium by fibroblasts [65, 67], cells in collagen lattices were incubated with 30 μg/ml C3-transferase for 3 h prior to addition of LPA. C3-transferase almost completely inhibited LPA-promoted contraction of myofibroblasts (Fig. 8). It has been demonstrated in both smooth muscle cells and fibroblasts that Rho can regulate the activity of MLCPPase [32, 37]. We found that calyculin could promote myofibroblast contraction even after inhibition of Rho with C3-transferase (Fig. 8). These results suggest that LPA may promote myofibroblast contraction, at least in part, by decreasing the activity of MLCPPase through a Rho-mediated signaling pathway.

Rho kinase is one of the targets of Rho and has been implicated as the protein kinase responsible for phosphorylating and thereby inactivating MLCPPase [32, 37, 61]. To evaluate Rho kinase in myofibroblast contraction we used the inhibitor Y-27632 [61]. Y-27632 inhibited LPA-promoted contraction with a half-maximal inhibition of approximately 7.5×10^{-7} M (Fig. 9). In contrast, 10^{-5} M Y-27632, which inhibited LPA-promoted contraction by greater than 80%, inhibited calyculin-promoted contraction by no more than 35% (not illustrated). These results are consistent with the hypothesis that calyculin is inhibiting a phosphatase downstream of Rho kinase.

We have also used the protein kinase inhibitor H7 to inhibit Rho kinase. H7 is approximately 10-fold more potent at inhibiting Rho kinase than inhibiting protein kinase C (PKC) or cAMP-dependent protein kinase and 100-fold more potent at inhibiting Rho kinase than inhibiting MLCK [61]. H7 inhibited both LPA- and calyculin-promoted myofibroblast contraction, but at different concentrations (Fig. 10). In contrast, ML-7 inhibited both LPA and calyculin at the same concentration (not illustrated). In addition, the PKC inhibitor GF109203X [60] did not inhibit contraction (not illustrated). These results suggest that H7 inhibits LPA-promoted myofibroblast contraction by inhibiting Rho kinase and inhibits calyculin-promoted myofibroblast contraction by inhibiting MLCK (Fig. 1). These results

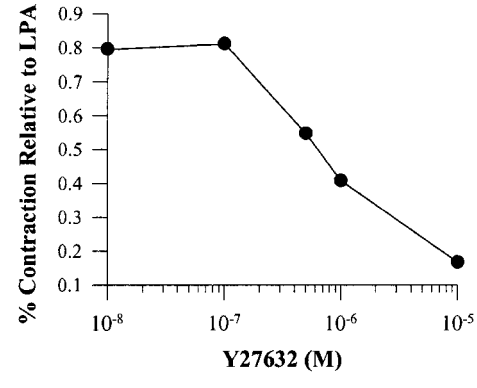

FIG. 9. Y-27632 inhibits LPA-promoted myofibroblast contraction. Myofibroblasts within serum-free stabilized collagen lattices were treated with the following immediately prior to release: various concentrations of Y-27632 followed by 1 μM LPA (●). Percentage collagen lattice contraction was determined at 10 min after release. All data are averages of duplicate collagen lattices and are representative of three different experiments.

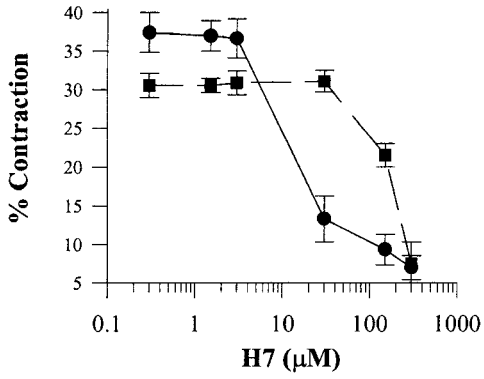

FIG. 10. H7 differentially inhibits LPA-promoted and calyculin-promoted myofibroblast contraction. Myofibroblasts within serum-free stabilized collagen lattices were treated with the following immediately prior to release: various concentrations of H7 followed by 1 μM LPA (●) or incubation for 20 min in 10 nM calyculin followed by various doses of H7 (■). Percentage collagen lattice contraction was determined at 10 min after release. All data are averages ± SD of three collagen lattices and are representative of three different experiments.

are consistent with the hypothesis that MLCPPase must be inhibited in order for myofibroblasts to contract.

DISCUSSION

Myofibroblasts are responsible for generating the force for normal wound healing, as well as tissue contraction in various fibrotic diseases, including Dupuytren's disease [21, 53]. To better understand the mechanisms of tissue contraction an understanding of the intracellular signaling mechanisms responsible for myofibroblast contraction is needed. We present evidence that there is a fundamental difference between the regulation of myofibroblast and that of smooth muscle cell contraction. Similar to regulation of smooth muscle cell contraction, myofibroblast contraction is regulated by the activity of MLCK and MLCPPase, two enzymes that regulate the phosphorylation of MLC. However, in contrast to smooth muscle cells, in which the level of intracellular Ca^{2+} is the dominant system regulating contraction, we found in myofibroblasts that increasing the level of intracellular Ca^{2+} was not sufficient to promote contraction. In addition, we present evidence that myofibroblast contraction requires activation of the small GTPase Rho and the associated Rho kinase with subsequent inactivation of MLCPPase. In contrast, in smooth muscle cells, MLCPPase activity regulates only the sensitivity of the contractile response to Ca^{2+}. These results suggest that Rho/Rho kinase-mediated inhibition of MLCPPase is the critical pathway regulating myofibroblast contraction, not the level of intracellular calcium and its regulation of MLCK activity as occurs in smooth muscle cells (Fig. 1).

In this paper, we present four primary findings related to regulation of myofibroblast contractility. First, we have demonstrated that although MLCK activity is necessary for myofibroblast contraction, elevation of intracellular Ca^{2+} by itself is not sufficient to promote myofibroblast contraction. This does not appear to be due to cell culture as myofibroblasts in rat granulation tissue do not contract in response to Ca^{2+} ionophore A23187 (Tomasek and Vaughan, unpublished results). Similarly, inhibition of MLCK will block LPA-promoted stress fiber assembly [9]; however, increased intracellular Ca^{2+} has no effect [6, 46, 49]. These results are in contrast to regulation of smooth muscle cell contraction in which elevation of intracellular Ca^{2+} promotes contractility. The inability of increased intracellular Ca^{2+} to promote contraction suggests that myofibroblasts have a mechanism in addition to Ca^{2+}-activated MLCK that is critical in regulating contractility.

Second, our evidence suggests that inhibition of MLCPPase is the additional and critical component regulating myofibroblast contraction (Fig. 1). MLCPPase is a type 1 phosphatase that dephosphorylates MLC and thereby counteracts MLCK [34, 51]. We have demonstrated that the phosphatase inhibitor calyculin can promote myofibroblast contraction. Although it has been demonstrated that a type 1 phosphatase regulates organization of the actin cytoskeleton in fibroblasts [15], this is the first demonstration that calyculin can promote the generation of contractile force in fibroblastic cells. While calyculin has been demonstrated to promote contraction of smooth muscle cells [27, 28], it should be stressed that in myofibroblasts, unlike smooth muscle cells, elevation of Ca^{2+} is unable to promote contraction in the absence of calyculin. These results are consistent with our hypothesis that, unlike smooth muscle cells, inhibition of MLCPPase is the critical regulator of myofibroblast contractility (Fig. 1).

Third, we present evidence that in myofibroblasts, LPA promotes contraction by regulating MLCPPase activity through a Rho/Rho kinase-mediated pathway (Fig. 1). Previous studies have demonstrated the involvement of Rho in LPA-mediated events thought to be associated with increased fibroblast contractility including assembly of stress fibers in serum-starved 3T3 fibroblasts, wrinkling of a silastic substratum, and fibronectin fibril assembly [45, 67]. Here we demonstrate that myofibroblast contraction, in response to LPA, is inhibited by the specific Rho inhibitor C3-transferase and the Rho kinase inhibitors Y-27632 and H7. These results demonstrate a necessity for Rho/Rho kinase in LPA-promoted myofibroblast contraction. Consistent with the hypothesis that MLCPPase is

downstream of Rho/Rho kinase (Fig. 1), we have found that calyculin can still promote myofibroblast contraction in the presence of C3-transferase or Y-27632. Although Rho kinase may be able to directly phosphorylate MLC [2, 37], it does not appear to be able to substitute for MLCK in myofibroblasts. We speculate that Rho kinase regulates myofibroblast contractility by regulating the activity of MLCPPase; however, further studies are necessary to determine whether Rho kinase can directly phosphorylate MCL, resulting in myofibroblast contraction. Previous studies have demonstrated that neurite retraction in response to LPA is induced through a Rho/Rho kinase signaling pathway [3, 26, 29]. Neurite retraction appears to result from generation of contractile, actomyosin-based forces in the cortical cytoskeleton [29], unlike myofibroblasts in which the force appears to be generated by actomyosin-based stress fiber contraction [31]. These results suggest that whether the actin and myosin are organized as a cortical cytoskeleton, as in the neurite, or as a stress fiber contractile element, as in the myofibroblast, there appears to be a similar regulation of actomyosin force generation.

Fourth, we have identified LPA as a major agonist in serum, which promotes myofibroblast contractility. We have previously reported that factors present in FBS can promote myofibroblast contraction [44, 59]. LPA is released by platelets during FBS production and is present at approximately 2–20 μM [14, 57]. Here we demonstrate that pretreatment of FBS with phospholipase B reduces the contractile activity of FBS by greater than 50%. However, even after phospholipase B treatment, FBS maintained some contractile-promoting activity. This may be the result of additional agonists in FBS, such as thrombin, which has previously been demonstrated to promote fibroblast contraction [35, 42].

It should be stressed that the regulation of contraction proposed for myofibroblasts appears to be similar in fibroblasts that have developed stress fibers and become contractile. We have found that human palmar aponeurosis fibroblasts, human fetal lung (WI-38) fibroblasts, and NIH 3T3 fibroblasts will form stress fibers in stabilized collagen lattices and behave similar to myofibroblasts in the stress-relaxed collagen lattice model [44, 58, 59]. Similarly, human foreskin fibroblasts appear to contract stress-relaxed collagen lattices similar to Dupuytren's myofibroblasts [21, 22, 39]. A pathway similar to that proposed here for regulation of myofibroblast contraction may be responsible for regulating LPA-mediated events associated with increased fibroblast contractility, including assembly of stress fibers in serum-starved 3T3 fibroblasts, wrinkling of a silastic substratum, and fibronectin fibril assembly [45, 67]. The major difference between myofibroblasts and fibroblasts is the expression of α-smooth muscle actin [11, 12]. We have found a correlation between increased expression of α-smooth muscle actin and force generation [59]; however, the mechanism by which contraction is regulated does not appear to be altered with increased α-smooth muscle actin expression. Together, these results suggest that myofibroblasts and fibroblasts regulate generation of contractile force similarly.

In summary, we present evidence that myofibroblast contraction is dependent on two events, inhibition of MLCPPase and Ca^{2+}-mediated activation of MLCK. In contrast to smooth muscle cells, myofibroblast contraction does not occur in response to increased intracellular Ca^{2+} concentrations. Rather, the critical component regulating myofibroblast contraction is the regulation of MLCPPase activity by Rho and Rho kinase.

The authors thank Joel McRae for technical assistance. The authors are grateful for the gift of rat aortic smooth muscle cells from Dr. Paula Grammas (University of Oklahoma Health Sciences Center), C3 transferase as a GST fusion protein in *E. coli* from Dr. Keith Burridge (University of North Carolina), and Y-27632 from Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan). The authors are grateful to Mr. Melville Vaughan for helpful discussions and critical comments and thank Mr. Ben Han for assistance in photography.

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Received June 9, 1999
Revised version received November 1, 1999