Published in final edited form as:
Am J Physiol Cell Physiol. 2004 January ; 286(1): C8–21. doi:10.1152/ajpcell.00428.2002.

Rho-kinase-mediated Ca^{2+}-independent contraction in rat embryo fibroblasts

Daniel A. Emmert[1,*], **Judy A. Fee**[2,*], **Zoe M. Goeckeler**[1], **Jeremy M. Grojean**[1], **Tetsuro Wakatsuki**[2], **Elliot L. Elson**[2], **B. Paul Herring**[3], **Patricia J. Gallagher**[3], and **Robert B. Wysolmerski**[1]

[1]Departments of Pathology, Saint Louis University School of Medicine, St. Louis 63104

[2]Department of Biochemistry & Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

[3]Department of Physiology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Abstract

Thus far, determining the relative contribution of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) and Ca^{2+}-independent Rho-kinase pathways to myosin II activation and contraction has been difficult. In this study, we characterize the role of Rho-kinase in a rat embryo fibroblast cell line (REF-52), which contains no detectable MLCK. No endogenous MLCK could be detected in REF-52 cells by either Western or Northern blot analysis. In the presence or absence of Ca^{2+}, thrombin or lysophosphatidic acid (LPA) increased RhoA activity and Rho-kinase activity, correlating with isometric tension development and myosin II regulatory light chain (RLC) phosphorylation. Resting tension is associated with a basal phosphorylation of 0.31 ± 0.02 mol PO_4/mol RLC, whereas upon LPA or thrombin treatment myosin II RLC phosphorylation increases to 1.08 ± 0.05 and 0.82 ± 0.05 mol PO_4/mol RLC, respectively, within 2.5 min. Ca^{2+} chelation has minimal effect on the kinetics and magnitude of isometric tension development and RLC phosphorylation. Treatment of REF-52 cells with the Rho-kinase-specific inhibitor Y-27632 abolished thrombin- and LPA-stimulated contraction and RLC phosphorylation. These results suggest that Rho-kinase is sufficient to activate myosin II motor activity and contraction in REF-52 cells.

Keywords

myosin light chain kinase; RhoA; myosin II regulatory light chain phosphorylation

MYOSIN II IS THE MAJOR CYTOSKELETAL PROTEIN in muscle and nonmuscle cells responsible for converting chemical energy of ATP into mechanical energy for cell contraction. Myosin II-based contractile activity has been implicated in a wide variety of cellular activities such as cell spreading (52,58,61), migration (31,44,48), stress fiber formation (20,21,23,33,64), neurite outgrowth (1,8,57), endothelial cell contraction (23,69,70), and resistance to mechanical perturbations (8,11,31). These myosin II-based cellular events have been considered to be regulated solely by an increase in cytosolic Ca^{2+} according to the classic smooth muscle model of Ca^{2+}-coupled contraction. However, within the last 10 years myosin II motor activity has

Address for reprint requests and other correspondence: R. B. Wysolmerski, Dept. of Pathology, Saint Louis Univ. School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104 (wysolmer@slucare1.sluh.edu)..

*D. A. Emmert and J. A. Fee contributed equally to this work.

been shown to be activated by Ca^{2+}-independent pathways (39,41,63,68) in addition to Ca^{2+}-dependent pathways (7,23,32,70).

Ca^{2+}-dependent cell contraction results from an influx of Ca^{2+} from the extracellular space or the release of Ca^{2+} from sequestered internal stores. Ca^{2+} binds to calmodulin (CaM); the Ca^{2+}/CaM complex in turn binds to and activates the serine/threonine protein kinase, myosin light chain kinase (MLCK). The active MLCK complex catalyzes myosin II regulatory light chain (RLC) phosphorylation at two sites, Ser-19 and Thr-18. Phosphorylation at these sites is required for myosin II filament formation (6), myosin II interaction with F-actin (23), and an increase in myosin II ATPase activity (7). These phosphorylation driven events are essential for initiation and maintenance of myosin II-based contraction. MLCK, the enzyme responsible for initiating Ca^{2+}-dependent contraction, exists in several isoforms, resulting from splice variants with distinct intracellular localizations and tissue distributions (3,5,32,52). To date, all MLCK isoforms have been shown biochemically to be strictly dependent on Ca^{2+}/CaM for activation and are known to catalyze phosphorylation of a single substrate, myosin II RLC.

The discovery of the Rho family of proteins initiated study of possible Ca^{2+}-independent regulation of myosin II motor activity and contraction. A range of protein kinases have now been shown to activate myosin II motor activity by catalyzing phosphorylation of myosin II RLC in vitro or in vivo, including Rho-kinase (2), p21-associated protein kinase (PAK) (9, 73), and integrin-linked kinase (ILK) (10). The most intensively studied of the Ca^{2+}-independent activators of myosin II motor activity and contraction are those regulated by the Rho family of proteins. Rho is a member of the Ras superfamily of small GTPases, which functions as a molecular switch cycling between the active GTP-bound and inactive GDP-bound state. In the active GTP state, RhoA interacts with its effector molecules to initiate downstream responses. Of several possible Rho effector proteins (4,17) that bind to GTP-bound Rho, the serine/threonine kinase, Rho-kinase, is the one that has been implicated in the regulation of myosin II and contraction.

Rho-kinase exists in two isoforms, ROKα/ROCK-II (43,46) and ROKβ/ROCK-I/$p160^{ROCK}$ (29,42); both are ubiquitously expressed in various tissues and cells. Rho-kinase isoforms range in mass from 150 to 160 kDa and contain a kinase domain in their NH_2-terminal domains, a central coil-coiled domain, and a pleckstrin homology domain in the COOH-terminal domains. The coil-coiled domain contains the Rho-binding domain (RBD), which binds active GTP-Rho, activating the catalytic activity of the kinase. Rho-kinase has been proposed to regulate contraction by two distinct but functionally related processes: *1*) catalyzing phosphorylation of the myosin binding subunit of myosin phosphatase at residue Thr-695, thereby inactivating myosin phosphatase (13,28), and *2*) direct phosphorylation of myosin II RLC at Ser-19 and Thr-18 (2). Thus phosphorylation of either of these substrates results in a net increase in phosphorylation of RLC, myosin II activation, and contraction. Although phosphorylation of myosin phosphatase's myosin binding subunit by Rho-kinase inactivates the phosphatase, this phosphorylation event alone does not directly affect contraction. The Rho/Rho-kinase signaling pathway has emerged as the predominant mechanism responsible for "Ca^{2+} sensitization" in smooth muscle contraction, the process whereby contraction is stimulated when Ca^{2+} levels are held constant (62). It has been suggested that Rho-kinase regulates Ca^{2+} sensitization by combined inactivation of myosin phosphatase and direct phosphorylation of myosin II RLC. However, recent studies (14,24,37) suggest that inactivation of the myosin phosphatase and not the direct phosphorylation of RLC by Rho-kinase is responsible for Ca^{2+} sensitization. These results imply that Rho-kinase alters the balance between kinase/phosphatase activity by inactivation of the phosphatase but raise the question as to whether Rho-kinase directly phosphorylates myosin II RLC in regulating smooth muscle Ca^{2+} sensitization.

Akin to smooth muscle, nonmuscle myosin II activity and contraction are also regulated by phosphorylation and dephosphorylation of myosin II RLC. Several studies have provided evidence that nonmuscle myosin II activity and the development of isometric tension are regulated by the Ca^{2+}/CaM-dependent MLCK (23,51,69). Although numerous studies have documented the presence of Rho/Rho-kinase pathway in nonmuscle cells, little attention has focused on the mechanism by which Rho/Rho-kinase pathway regulates nonmuscle cell contraction. Pretreatment of agonist-stimulated cultures with the Rho-kinase inhibitor Y-27632 prevents *1*) phosphorylation of myosin phosphatase, *2*) inhibition of myosin phosphatase activity, and *3*) the increase in RLC phosphorylation. In addition, permeabilized cell models have established that cell contraction occurs in the presence and absence of Ca^{2+} and that Ca^{2+}-independent contraction is mediated by direct phosphorylation of myosin II RLC by Rho-kinase. Taken together, these data implicate Rho-kinase as a major player in regulating nonmuscle cell contraction. Nevertheless, these studies do not definitively determine whether Rho-kinase-catalyzed phosphorylation of myosin phosphatase and/or direct phosphorylation myosin II RLC is responsible for myosin activation and contraction in vivo. Because the basal level of cytosolic Ca^{2+} (69) is sufficient to activate MLCK, it is conceivable that the increased level of myosin II RLC phosphorylation that occurs upon Rho-activation could in part result from MLCK activation. The in vivo determination of what role each enzyme plays in regulating myosin II activation and contraction has proved extremely difficult. Most studies have relied on the availability of pharmacological inhibitors to the specific kinases to define their role(s) in the regulation of myosin II activation and contraction in nonmuscle cells. An inherent problem with this approach is that putative specific inhibitors may in fact exert their effects through inhibition of other kinases.

To further pursue the role of Rho-kinase in nonmuscle contraction, we characterized agonist-induced isometric tension development in a rat embryo fibroblast cell line (REF-52) that we have determined contains no detectable MLCK. This cell line has allowed us to define the role of Rho-kinase-dependent myosin II activation associated with isometric tension development without the potential influence by cross-activation of the Ca^{2+}/CaM-dependent MLCK pathway. We present evidence establishing that Rho-kinase directly phosphorylates myosin II RLC, upregulating myosin II activity and contraction. Experiments have established that thrombin and lysophosphatidic acid (LPA) induce a Ca^{2+}-independent activation of RhoA and Rho-kinase activity that correlates with myosin II RLC phosphorylation and isometric tension development. Inhibition of Rho-kinase with Y-27632 abolished thrombin- and LPA-stimulated contraction and RLC phosphorylation, whereas chelation of cytosolic Ca^{2+} had very little effect on isometric tension development and RLC phosphorylation.

EXPERIMENTAL PROCEDURES

Reagents

1-Oleoyl-lysophosphatidic acid (LPA) was purchased from Avanti Polar Lipids, dissolved in PBS containing 1.0% BSA, 0.5 mM $MgCl_2$, and 0.6 mM $CaCl_2$, and stored at –70°C. Bovine thrombin, BAPTA-AM, thapsigargin, and cytochalasin D were obtained from Sigma. Rho-binding domain (RBD) was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies to RhoA (catalog no. sc-179) were purchased from Santa Cruz Biotechnology, and Rho-kinase antibodies were kindly provided by K. Itoh (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan). Y-27632 was a generous gift from Yoshitomi Pharmaceutical Industries (Osaka, Japan). Fluo 4-AM and Cell-Tracker orange were purchased from Molecular Probes (Eugene, OR).

Cell culture

Rat embryo fibroblasts (REF-52 cells) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 5% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO_2. Unless otherwise indicated, experiments were carried out in HEPES-buffered saline (HBS; 10 mM HEPES, pH 7.3, 135 mM NaCl, 5 mM KCl, 0.8 mM Na_2HPO_4, 1.8 mM $CaCl_2$, and 5.5 mM glucose) containing 0.25% fatty acid-free BSA (Sigma).

Identification of MLCK and Rho-kinase in REF-52 cell extracts

REF-52 cells (2×10^6) and transfected COS-7 cells expressing $MLCK_{220}$ or $MLCK_{155}$ were lysed in extraction buffer [25 mM Tris·HCl, pH 6.8, 150 mM NaCl, 50 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 0.5% Triton X-100, 0.2 mM PMSF, 100 μg/ml benzamidine, 100 μg/ml soybean trypsin inhibitor, and 10 μg/ml each of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), aprotinin, leupeptin, and pepstatin at 4°C] and incubated on ice for 20 min. Extracts were centrifuged at 22,000 *g* for 10 min to remove cell debris, and supernatants were snap frozen in liquid nitrogen and stored at –70°C. For detection of Rho-kinase, $MLCK_{220}$, and $MLCK_{155}$, REF-52 cell lysates (35 μg) were electrophoresed in either 7.5 or 10% SDS-polyacrylamide vertical slab minigels, using the buffer system of Laemmli. Proteins were transferred to 0.2-μm polyvinylidene difluoride (PVDF) membrane and incubated with affinity-purified rabbit anti-Rho-kinase (15), anti-$MLCK_{220}$ (5), or anti-$MLCK_{155}$ (5,22) antibodies.

Northern blots

RNA was isolated from tissues and cells, and a Northern blot was prepared as described previously (19). The blot was probed with a 556-bp ^{32}P-labeled rat MLCK cRNA probe corresponding to nucleotides 3018–3574 of the rabbit uterine MLCK. This fragment of rat MLCK was generated by reverse transcriptase coupled to PCR with the use of rat uterine mRNA and oligonucleotide primers complementary to nucleotides 3018–3042 and 3551–3687 of the rabbit MLCK cDNA. Hybridization was performed at 65°C in the presence of 50% formamide, and final wash conditions were 1.25 mM sodium phosphate, pH 7.4, 30 mM NaCl, 0.2 mM EDTA, and 0.1% SDS at 68°C for 10 min.

Ca^{2+} measurements

REF-52 cells were seeded onto optical quality glass-bottomed 35-mm culture dishes (World Precision Instruments, Sarasota, FL) at a density of 1×10^5 cells and used 48 h postseeding. Cultures were incubated in HBS containing 1 μM CellTracker orange for 45 min at 37°C, media were aspirated, and cultures were immediately loaded with 2 μM fluo 4/0.04% Pluronic F-127 in HBS for an additional 45 min. For Ca^{2+} chelation experiments, cells were loaded simultaneously with fluo 4/0.04% Pluronic F-127, 10 μM BAPTA-AM, and 1 μM thapsigargin in Ca^{2+}/BSA-free HBS. Temperature was maintained at 37°C for the duration of the experiments with a Harvard Apparatus Open Perfusion Micro-incubator (model PDMI-2). Cells were viewed using a PerkinElmer UltraView laser confocal microscope, and images were captured every 500 ms with an Astro-Cam cooled charge-coupled device camera. Pixel density was calculated from whole cell averages using the UltraView software package.

RhoA activity assay

REF-52 cells were plated at a density of 1×10^6 cells per 100-mm culture dish and used 4 days postconfluence. Dishes were washed twice with HBS, pH 7.3, and incubated in either HBS or Ca^{2+}-free HBS containing 10 μM BAPTA-AM/1 μM thapsigargin for1hat room temperature, protected from light. Monolayers were stimulated with either 1 U/ml thrombin or 1 μM LPA for 1, 2.5, 5, and 10 min. Experiments were terminated by aspirating HBS and immediately

flooding cultures with 1 ml 4°C lysis buffer (50 mM Tris, pH 7.2, 0.5 M NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Cells were scraped up with a rubber policeman, cell extract was centrifuged at 22,000 *g* for 2 min at 4°C, and the supernatant was added to glutathione beads complexed with glutathione *S*-transferase (GST)-RBD (fusion protein containing the RhoA-binding domain of Rhotekin) for 1 h at 4°C as outlined previously (56). Samples were washed, analyzed on 12% SDS gels, transferred to PVDF membrane, and probed with a 1:1,000 dilution of a RhoA polyclonal antibody (Santa Cruz Biotechnology). RhoA bands complexed with GTP were visualized by enhanced chemiluminescence detection reagents (ECL; Amersham Pharmacia Biotech). X-ray films were developed at various time intervals to obtain an exposure within the linear range of the film. Films were scanned in a personal densitometer (Molecular Dynamics, Sunnyvale CA); the densitometric units (DU) for each sample (GTP-bound RhoA) were standardized on the basis of total RhoA DU and expressed as a ratio of GTP-bound RhoA to total RhoA. Results are expressed as the fold increase in GTP-bound RhoA; i.e., DU experimental to DU control (RhoA activation = $DU_{experimental}/DU_{control}$).

Rho-kinase activity assay

REF-52 monolayers grown in 60-mm dishes 4 days postconfluence were washed twice with HBS, pH 7.3, and treated as outlined in *RhoA activity assay*. At the appropriate time interval, cultures were flooded with 800 μl of extraction buffer (25 mM Tris·HCl, pH 6.8, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.5% Triton X-100, 0.2 mM PMSF, 100 μg/ml soybean trypsin inhibitor, 100 μg/ml benzamidine and 10 μg/ml each of TPCK, TLCK, aprotinin, leupeptin, and pepstatin at 4°C) and immediately placed on ice. Cells were scraped with a rubber policeman, and extracts were incubated on ice for 20 min. Cell extracts were centrifuged at 22,000 *g* for 10 min to remove insoluble material, and supernatants were added to protein A beads precomplexed with 15 μl of a polyclonal anti-rabbit Rho-kinase IgG fraction for 2 h at 4°C. Immune complexes bound to protein A-Sepharose 4B were collected by centrifugation at 12,000 *g* at 4°C. Pellets were washed first in extraction buffer and then twice with PBS. Protein A beads containing the immunopurified Rho-kinase were resuspended in 100 μl of phosphorylation buffer (25 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, and 1 mM DTT) containing 125 μM [γ-^{32}P]ATP and 1 μg of myosin II RLCs and then used for in vitro phosphorylation reactions as described by Goeckeler et al. (22). Phosphorylation reactions were incubated at 30°C for 10 min. Protein A beads were removed by centrifugation at 12,000 *g*, supernatants were removed and added to equal volumes of 20% TCA, and samples were processed as previously described. Phosphorylated myosin II RLCs were analyzed on 12.5% SDS gels and exposed to Phosphor-Imager plates.

Isometric tension measurements

Isometric tension measurements were performed as described in detail previously (40,67,74). Collagen gels were made by suspending 2.0×10^6 REF-52 cells/ml in a collagen/DMEM solution containing 1.0 mg/ml rat tail collagen I (Upstate Biotechnology). Collagen/cell suspension (1 ml) was poured into Teflon molds with a central mandrel and transferred to a 37°C incubator for 1 h to initiate collagen polymerization, giving rise to a collagen/cell matrix in the shape of a ring. Molds were then filled with DMEM-5% FBS and incubated for 48 h in a humidified incubator at 37°C with 5% CO_2. Collagen gels were removed from the molds, hung from the force transducers, and bathed in HBS, pH 7.3, in water-jacketed organ baths. After a stable basal tension was established, REF-52 cell-populated collagen gels were stimulated to contract by the addition of 1 U/ml thrombin or 1 μM LPA.

For Ca^{2+} chelation experiments, collagen gels were removed from molds, washed in Ca^{2+}-free HBS, hung from force transducers, and loaded with 10 μM BAPTA-AM/1 μM thapsigargin in

Ca^{2+}-free HBS for 60 min. After loading, media were replaced with Ca^{2+}-free HBS and collagen gels were allowed to establish a stable baseline tension before agonist stimulation. Tension was recorded using measurement acquisition parameters described previously (23).

MLC phosphorylation

Control and treated monolayers were flooded with 4°C 10% TCA containing 10 mM DTT and placed on ice for 1 h to precipitate REF-52 cell proteins. Cellular proteins were dissolved in urea sample buffer, and myosin II RLC phosphorylation states were separated by glycerol-urea gel electrophoresis (73). Western blots were incubated using a rabbit anti-myosin II RLC antibody.

RESULTS

Endogenous expression of Rho-kinase and MLCK in REF-52 cells

REF-52 cell extracts were analyzed for the presence of Rho-kinase and MLCK by Western blotting, using polyclonal antibodies to synthetic peptides or recombinant proteins, specifically *1*) rabbit polyclonal antibody raised against a synthetic peptide sequence (STGDSFETRFGKMDC) from the NH_2-terminal region of Rho-kinase (15); *2*) affinity-purified rabbit polyclonal antibody raised to a recombinant protein representing the first 170 amino acids of the mouse 220-kDa MLCK (5) ($MLCK_{1–170}$); *3*) affinity-purified rabbit polyclonal antibody raised to recombinant full-length rabbit 155-kDa MLCK (22); and *4*) mouse monoclonal antibody raised to smooth muscle MLCK (clone K36, catalog no. M7905; Sigma) (5). Anti-MLCK antibodies were shown by Western blots to detect <100 pg of recombinant $MLCK_{155}$ or $MLCK_{210}$. Affinitypurified rabbit polyclonal $MLCK_{155}$ and mouse monoclonal K36 recognized both $MLCK_{155}$ and $MLCK_{210}$, whereas anti-$MLCK_{170}$ detected only $MLCK_{210}$ (data not shown).

Figure 1*A* shows the presence of a 160-kDa band detected in REF-52 cell extracts with the polyclonal antibody to Rho-kinase. Immunoprecipitates with the rabbit polyclonal to Rho-kinase from REF-52 cell extracts probed with a monoclonal antibody (Santa Cruz Biotechnology) to Rho-kinase confirmed this observation (data not shown).

MLCKs expressed in smooth muscle and nonmuscle cells are products of a single MLCK gene and fall into two general classes (5), the 208- to 220-kDa MLCKs and the 125- to 155-kDa MLCKs. The variability in the masses within each class are the result of species variations in the gene. To examine the expression of MLCK isoforms in REF-52 cell extracts, Western blots were probed with either affinity-purified antibodies to $MLCK_{155}$ or affinity-purified antibodies to $MLCK_{1–170}$. Figure 1*B*, *lane 1*, shows that neither the 208- to 220-kDa or the 125-to 155-kDa MLCKs were detected in REF-52 cell extracts. Recombinant purified $MLCK_{220}$ (Fig. 1*B*, *lane 2*) and $MLCK_{155}$ (Fig. 1*B*, *lane 3*) standards were included on Western blots to confirm the reactivity of the respective antibodies. In addition, no MLCK could be detected even when an eightfold higher concentration of REF-52 extract was analyzed.

Western blots showed that no MLCK protein was expressed in REF-52 cells. To further confirm this result, Northern blots were performed to probe for the presence of MLCK mRNAs. Figure 1*C* is a representative Northern blot incubated with a cRNA probe corresponding to bp 3018–3574 of the rabbit uterine 155-kDa MLCK cDNA. This probe hybridized to mRNAs of 8.4 and 5.7 kb, encoding the 220- and 130-kDa MLCKs, respectively, in rat uterus (Fig. 1*C*, *lane 1*), rat aorta (*lane 2*), and rat embryonic thoracic aortic smooth muscle cells (A10; *lane 4*). No MLCK mRNAs were detected in REF-52 cells (Fig. 1*C*, *lane 3*). In addition, the cRNA probe detected the 2.7-kb mRNA corresponding to telokin, a protein that is identical to the carboxyl-

terminal 155 amino acids of MLCK and is expressed as an independent protein (18). No telokin mRNA was detected in REF-52 cells.

RhoA and Rho-kinase activity

To determine RhoA activation upon thrombin or LPA stimulation, an RBD assay was performed on REF-52 cells, utilizing the RBD fragment from the RhoA effector Rhotekin (54). The RBD fragment binds only active RhoA-GTP and does not bind inactive RhoA-GDP. Therefore, it is possible to determine the ratio of RhoA-GTP to total RhoA by comparing RBD-bound RhoA with total cellular RhoA. Figure 2, *A* and *B*, shows representative Western blots illustrating the effects of thrombin and LPA stimulation on RhoA activity. Cultures were treated with 1 U/ml thrombin (Fig. 2*A*) or 1 μM LPA (Fig. 2*B*) in HBS for the desired time intervals, and RhoA was immunoprecipitated as outlined in EXPERIMENTAL PROCEDURES. Quantitative data from three time-course experiments are presented in Table 1. In unstimulated controls, minimal RhoA activity was detectable as exhibited by a very low proportion of RhoA bound to GTP. Stimulation with thrombin (Fig. 2*A*) or LPA (Fig. 2*B*) for 60 s induced a rapid rise in activity as shown by a 27- and 20-fold increase in GTP-bound RhoA, respectively (Table 1). RhoA activity remained upregulated for both thrombin and LPA over the duration of the experiment. These data are consistent with previous reports (55) and suggest that agonist stimulation converts RhoA-GDP to RhoA-GTP, leading to activation of RhoA in REF-52 cells.

RhoA has been shown to affect multiple cellular functions (16) distinct from Rho-kinase activation. However, numerous studies (59) have suggested that measurement of RhoA activity is a reliable indicator of Rho-kinase activation. To confirm this assumption, we sought to directly measure Rho-kinase activity in agonist-treated REF-52 cells. To measure catalytic activity, Rho-kinase immunoprecipitated from REF-52 cells treated with either 1 U/ml thrombin or 1 μM LPA for 30 s, 2.5 min, and 5.0 min was used for in vitro phosphorylation assays on purified RLC as outlined in EXPERIMENTAL PROCEDURES. Results are shown in Fig. 2, *C* and *D*. Rho-kinase from unstimulated control cultures (Fig. 2, *C* and *D*) exhibited a low level of basal activity. Thrombin stimulation increased Rho-kinase activity 3.2-fold within 30 s, reaching maximal activity 11-fold over control cultures by 5.0 min. LPA treatment for 30 s induced a 4-fold activation of Rho-kinase activity, and by 5.0 min of continuous exposure to LPA, catalytic activity increased 8-fold over control cultures. For both LPA and thrombin, the increase in Rho-kinase activation paralleled the activation of RhoA, although maximal RhoA activity preceded maximal Rho-kinase activation. These data, taken together, indicate that agonist stimulation induces the formation of an active RhoA-GTP complex, which in turns binds to and activates the catalytic activity of Rho-kinase in REF-52 cells.

Isometric tension and myosin RLC phosphorylation

To determine the involvement of Rho/Rho-kinase in basal and agonist stimulated isometric tension, REF-52 cells were incorporated into collagen gels as outlined in EXPERIMENTAL PROCEDURES. REF-52 cells remodeled and compressed the collagen matrix within 48 h postseeding. Cell-populated collagen gels were removed from their molds and hung from an isometric force transducer as described by Wakatsuki et al. (67). After a stable baseline tension was established, collagen gels were stimulated by addition of thrombin or LPA as shown in Fig. 3, *A* and *B*, respectively. Thrombin treatment induced a steady increase in isometric tension (Fig. 3*A*), reaching a maximum tension of 65 dynes in 5–10 min. Peak tension was maintained for 15 min before slowly declining over the next 75 min. Return to baseline tension occurred within 3–4 h (data not shown). In contrast, LPA induced a rapid increase in tension, reaching a maximal tension of 75 dynes within 2–3 min (Fig. 3*B*). After peak tension was achieved, tension immediately dropped before slowly declining to establish a new baseline tension over the ensuing 60 min. Stable baseline tension as well as tension generated by REF-52 cell-populated

collagen gels in response to either thrombin or LPA was abolished by the addition of 2 μM cytochalasin D.

The increase in myosin II RLC phosphorylation in REF-52 cell monolayers in response to thrombin or LPA treatment was measured by separating cellular proteins on glycerol-urea gels, transferring to nitrocellulose membranes, and probing Western blots with an antibody to myosin II RLC. Figure 4, *A* and *B*, shows representative Western blots of REF-52 monolayers treated with thrombin or LPA, respectively. Tables 2 and 3 show the quantitative data from three time-course experiments. For thrombin experiments, buffer-treated controls showed a basal phosphorylation where 70% of the myosin RLC are unphosphorylated, 29% are monophosphorylated, and 1% are diphosphorylated. By 2.5 min, maximal RLC phosphorylation was obtained with 24% unphosphorylated, 44% monophosphorylated, and 32% diphosphorylated. After 60 min of continuous thrombin stimulation, a shift in phosphorylation was evident with unphosphorylated myosin RLC increasing to 63% while the mono- and diphosphorylated RLC increased to 33 and 4%, respectively. Although a steady decline in phosphorylation occurred with time, the level of phosphorylation was maintained above baseline control values for the 2-h duration of the experiment. The stoichiometry of myosin RLC phosphorylation (Table 2) showed that REF-52 cells exhibited a basal RLC phosphorylation of 0.31 ± 0.02 mol PO_4/mol RLC. By 2.5 min after addition of thrombin, phosphorylation rose by 348%, achieving a maximal level of 1.08 ± 0.05 mol PO_4/mol RLC (Fig. 4*A* and Table 2). Continuous thrombin stimulation sustained near-maximal levels of RLC phosphorylation for 30 min before declining to and maintaining a level of RLC phosphorylation of 0.41 ± 0.06 mol PO_4/mol. LPA-induced RLC phosphorylation (Fig. 4*B* and Table 3) was also time dependent and resulted in an increase in RLC phosphorylation similar to that which occurred in cultures treated with thrombin (Table 2). RLC phosphorylation in REF-52 populated gels was comparable to results obtained from REF-52 cell monolayers (data not shown).

Ca^{2+} requirement for RhoA and Rho-kinase activity

We next sought to determine whether Ca^{2+} was required for agonist activation of the Rho/Rho-kinase pathway. Both thrombin and LPA induce a rise in cytosolic Ca^{2+} by stimulating direct entry of extracellular Ca^{2+} and coupling via a G protein receptor-dependent pathway to stimulate inositol 1,4,5-trisphosphate production, which releases Ca^{2+} from internal stores (53). To ascertain the involvement of Ca^{2+} in Rho/Rho-kinase activation, REF-52 cell cultures and collagen gels were pretreated with 10 μM BAPTA-AM/1 μM thapsigargin in media free of extracellular Ca^{2+} as outlined under EXPERIMENTAL PROCEDURES. To confirm that the BAPTA-AM/thapsigargin effectively blocked LPA- and thrombin-induced increases in Ca^{2+}, cells were loaded with fluo 4, and the increase in intracellular Ca^{2+} was monitored by confocal microscopy as outlined in EXPERIMENTAL PROCEDURES. Figure 5 demonstrates representative Ca^{2+} tracings of agonist-stimulated REF-52 cells in the presence and absence of Ca^{2+} chelation. Fluo 4-loaded monolayers treated with the Ca^{2+} ionophore ionomycin showed an immediate influx of Ca^{2+} that rapidly declined within 2 min (Fig. 5*A*). In contrast, cultures loaded with BAPTA-AM/thapsigargin showed no rise in cytosolic Ca^{2+} upon ionomycin treatment (Fig. 5*B*), confirming that the Ca^{2+} chelation protocol effectively quenched intracellular Ca^{2+}. Both thrombin (Fig. 5*C*) and LPA (Fig. 5*E*) induced an immediate rise in intracellular Ca^{2+} that peaked within 1 min and rapidly returned to basal levels. As with ionomycin, BAPTA-AM/thapsigargin blocked the rise in cytosolic Ca^{2+} induced by both agonists (Fig. 5, *D* and *F*). To determine whether the Ca^{2+} response from REF-52 cell-populated collagen gels was similar to that of monolayer cultures, collagen gels were loaded with fluo 4 and stimulated with 1 U/ml thrombin (Fig. 5*G*) or 1 μM LPA (Fig. 5*I*). Upon thrombin and LPA treatment, the magnitude and duration of Ca^{2+} response in REF-52 cells embedded within collagen gels were similar to the response elicited in monolayer cultures. Both thrombin and LPA caused an influx in

intracellular Ca^{2+}, reaching peak concentrations within 1–2 min and then slowly returning to baseline levels within 3–4 min. As shown in monolayer cultures (Fig. 5, *D* and *F*), BAPTA-AM/thapsigargin blocked the rise in cytosolic Ca^{2+} induced by both agonists (Fig. 5, *H* and *J*).

To assess the state of RhoA activity in the absence of Ca^{2+}, BAPTA-AM/thapsigargin-pretreated cultures were stimulated with thrombin or LPA, and RhoA activity was determined. Figure 6, *A* and *B*, shows representative Western blots demonstrating that cytosolic Ca^{2+} chelation had minimal effects on RhoA activity. In control cultures, active RhoA was marginally detectable. By 60 s, thrombin induced a 47-fold increase in GTP-bound RhoA (Table 4). In addition, LPA stimulation of RhoA activity in Ca^{2+}-depleted REF-52 cultures elicited a 9-fold increase in GTP-bound RhoA (Fig. 6*B* and Table 4).

To further assess Ca^{2+} requirements for activation of the Rho/Rho-kinase pathway, Rho-kinase catalytic activity was assessed from REF-52 cultures pretreated with BAPTA-AM/thapsigargin. Rho-kinase was immunoprecipitated and used for in vitro phosphorylation assay. Figure 6, *C* and *D*, shows that unstimulated control cells contained little active Rho-kinase. In contrast, thrombin stimulation produced a time-dependent increased in Rho-kinase catalytic activity, reaching maximal activity 12-fold over control cultures by 5 min as evident by the increase in RLC phosphorylation (Fig. 6*C*). LPA treatment increased Rho-kinase activity 3.4-fold within 30 s, reaching a 10-fold increase in activity over control cultures by 5 min (Fig. 6*D*). These data confirm that in vivo RhoA activation and upregulation of Rho-kinase catalytic activity can occur in the complete absence of increases in cytosolic Ca^{2+}.

Effect of Ca^{2+} on isometric tension and MLC phosphorylation

To determine the role of Ca^{2+} in LPA- and thrombin-stimulated isometric tension generation, REF-52 cell-populated collagen gels were pretreated in Ca^{2+}-free HBS containing 10 μM BAPTA-AM/ 1 μM thapsigargin as outlined in EXPERIMENTAL PROCEDURES. Figure 7 depicts representative tension tracings illustrating the effects of Ca^{2+} chelation on thrombin- or LPA-induced isometric tension development. Removal of Ca^{2+} had minimal effects on basal tension. Ca^{2+} chelation had no significant effect on the kinetics or the peak tension developed in response to 1 U/ml thrombin compared with gels stimulated in Ca^{2+}-complete media (Fig. 7*A*). However, under Ca^{2+}-free conditions, the duration of peak force generation was diminished, being maintained for only 5 min before tension declined to baseline levels over the ensuing 60 min. In contrast, REF-52 gels in Ca^{2+}-complete HBS maintained peak tension for ~30–40 min followed by a slow, steady decline in tension (Fig. 7*A*), returning to baseline levels within 3–4 h. No significant difference in LPA-induced tension generation was detected in the presence or absence of Ca^{2+} (Fig. 7*B*). Maximal tension generated was 80.3 ± 6.4 dynes (n = 4) for samples without Ca^{2+} compared with 80.0 ± 8.8 dynes (n = 4) for LPA-stimulated gels in the presence of Ca^{2+} (Fig. 7*B*).

To assess whether elevation of intracellular Ca^{2+} was sufficient to initiate REF-52 cell contraction, collagen gels were treated with ionomycin and its effects on isometric tension development were assessed. Raising intracellular Ca^{2+} levels by the addition of 1 μM ionomycin (Fig. 7*C*) in the presence and absence of extracellular Ca^{2+} caused no detectable increase in REF-52 cell isometric tension. Addition of thrombin elicited an increase in tension, indicating that there was no toxicity from elevated intracellular Ca^{2+}. These results clearly demonstrated that an increase in intracellular Ca^{2+} ion concentration is neither necessary nor sufficient for isometric tension production in REF-52 cells.

To determine whether Ca^{2+} chelation prevented an increase in RLC phosphorylation in response to thrombin and LPA stimulation, REF-52 cultures were pretreated with BAPTA-AM/thapsigargin for 60 min and RLC phosphorylation was assessed after the addition of 1 U/

ml thrombin or 1 μM LPA. Figure 8 demonstrates typical phosphorylation experiments in the absence of Ca^{2+}, showing the time-dependent rise in RLC phosphorylation upon thrombin (*A*) or LPA treatment (*B*). Quantitative analysis is presented in Table 5. Chelation of cytosolic Ca^{2+} had little effect on baseline phosphorylation. Control cultures had a baseline level of RLC phosphorylation of 0.23 ± 0.02 to 0.26 ± 0.05 mol PO_4/mol RLC (Table 5), whereas cells in Ca^{2+}-complete media exhibited a basal phosphorylation of 0.31 ± 0.02 to 0.33 ± 0.03 mol PO_4/mol RLC (see Tables 2 and 3). Upon thrombin stimulation, RLC phosphorylation increased by 408% to 0.94 ± 0.02 mol PO_4/mol RLC by 5 min and returned to baseline levels by 2 h post-stimulation (Table 5), in contrast to thrombin treatment in the presence of Ca^{2+} where RLC phosphorylation remained elevated for up to 2 h poststimulation (Table 2). The decline in RLC phosphorylation correlates well with the decline in thrombin generated tension observed upon Ca^{2+} chelation (Fig. 7*A*). Figure 8*B* shows a typical Western blot of LPA-induced RLC phosphorylation in the BAPTA-AM/thapsigargin-treated cultures. The rate and extent of RLC phosphorylation were marginally reduced (Table 5). RLC phosphorylation increased 253% over baseline to 0.66 ± 0.03 mol PO_4/mol RLC by 5 min, whereas peak phosphorylation in the presence of Ca^{2+} occurred at 2.5 min, achieving levels of 0.82 ± 0.05 mol PO_4/mol RLC (Table 3).

Effects of Y-27632 on isometric tension and RLC phosphorylation

The data have thus far established that *1*) REF-52 cells contain Rho-kinase but no detectable isoforms of MLCK; *2*) both thrombin and LPA upregulate a Ca^{2+}-independent increase in RhoA and Rho-kinase activity; and *3*) thrombin and LPA elicit a Ca^{2+}-independent increase in isometric tension and RLC phosphorylation. In the next set of experiments, the effects of direct inhibition of Rho-kinase on REF-52 myosin II RLC phosphorylation and basal and agonist-stimulated isometric tension were determined. Figure 9 shows typical isometric tension tracings of REF-52 cell-populated collagen gels incubated in 10 μM Y-27632, a specific inhibitor of Rho-kinase (30,47,65). Isometric tension declined rapidly within the first 3–4 min, decreased steadily over the next 5 min, and established a new baseline of 14–18 dynes, a 90% reduction, within 12 min. Isometric tension reproducibly declined to 85–90% of pretreatment levels ($n = 6$) and remained at a new baseline level for the duration of the experiment. Addition of 1 μM LPA (Fig. 9*A*) or 1 U/ml thrombin (Fig. 9*B*) in the continuous presence of Y-27632 showed a modest 8- and 12-dyne increase in tension, respectively. The small rise in tension elicited by thrombin (Fig. 9*B*) was maintained for the duration of the experiment, whereas LPA-induced tension returned to baseline within 10 min (Fig. 9*A*).

To determine whether the inhibitory effects of Y-27632 could be overcome, organ baths were drained and collagen gels were bathed in two changes of medium without inhibitor. Figure 9*A*, *inset*, illustrates the successful removal of Y-27632 from REF-52 cell-populated collagen gels. Upon removal of Y-27632, tension steadily increased, establishing a steady baseline within 3 h. Addition of 1 μM LPA to washout gels elicited a typical rapid rise in isometric tension, peak tension development within 5 min, followed by an immediate but partial decline in tension toward baseline values.

The effects of Y-27632 on myosin II RLC phosphorylation are shown in Fig. 10. Quantitative analysis from three experiments is presented in Table 6. REF-52 monolayers incubated with 10 μM Y-27632 alone showed a decrease in the extent of RLC phosphorylation for thrombin controls from 0.15 ± 0.02 to 0.09 ± 0.01 mol PO_4/mol RLC (Fig. 10*A*, *lanes 1* and *3*) and for LPA from 0.21 ± 0.08 to 0.07 ± 0.01 mol PO_4/mol RLC (Fig. 10*B*, *lanes 1* and *3*) by 15 min, which represents a 40 and 66% decrease from control values, respectively (Table 6). Cultures pretreated with Y-27632 for 15 min and then stimulated with thrombin (Fig. 10*A*, *lane 4*) or LPA (Fig. 10*B*, *lane 4*) in the presence of Y-27632 for 5 min exhibited a small increase in RLC phosphorylation to 0.15 ± 0.03 and 0.12 ± 0.03 mol PO_4/mol RLC, respectively (Table 6). In

contrast, monolayers stimulated with either thrombin (Fig. 10*A*, *lane 2*) or LPA alone (Fig. 10*B*, *lane 2*) showed a marked increase in RLC phosphorylation (Table 6). These data suggest that in REF-52 cells, Rho-kinase serves as the principle regulator of RLC phosphorylation and isometric tension development.

DISCUSSION

We have shown that our REF-52 cells are a unique model system for the analysis of the regulation and activity of the Rho/Rho-kinase signaling pathway. The surprising discovery that a cell line that lacks any endogenous MLCK and its mRNA is viable, proliferates, and contracts indicates that other regulatory pathways play a significant role in cytoskeletal dynamics. Two previous reports utilizing MLCK cDNA knockouts have investigated changes in cell morphology (60) and RLC phosphorylation (38) and indicated that MLCK suppression by antisense RNA altered morphology but not RLC phosphorylation. Our results extend these previous findings by taking advantage of a cell line naturally devoid of endogenous MLCK, without resorting to overexpression systems or genetic manipulations. The findings presented here indicate that even though these REF-52 cells lack detectable levels of endogenous MLCK mRNA and protein, they can be stimulated by thrombin or LPA to contract, as shown by measuring the tension exerted by collagen gels made from these fibroblasts. Contraction correlates with increased phosphorylation of myosin RLC. Ca^{2+}-independent RLC phosphorylation and contraction are nearly identical to RLC phosphorylation and contraction at physiological Ca^{2+} levels. We have also shown that the agonist-stimulated activations of both Rho and Rho-kinase are correlated with RLC phosphorylation and contraction with or without the presence of cytosolic Ca^{2+}. When Rho/Rho-kinase signaling is blocked by the specific inhibitor Y-27632, RLC phosphorylation levels and contraction of REF-52 collagen gels are severely decreased.

These studies expand on previous ones that have shown the involvement of the Rho/Rho-kinase signaling pathway in the contraction of nonmuscle cells in collagen gels. C3 transferase, which causes ADP-ribosylation and inactivation of Rho, inhibited agonist-induced contraction of gels from human (25,51) or chicken fibroblasts (72). Likewise, our result of Y-27632 inhibition of contraction in REF-52 cells is supported by similar Y-27632 effects in fibroblast or hepatic stellate cell gels (35,51,71,72). Furthermore, efforts to stimulate contraction with Ca^{2+} ionophores or to inhibit cytosolic Ca^{2+} ion concentration had little or no effect on contractile force (41,51). For collagen gels containing NIH/3T3 cells, Nobe and coworkers (50) concluded that internal Ca^{2+} stores, but not external Ca^{2+}, was important for serum-stimulated isometric contraction. However, with cells containing a Ca^{2+}-activated myosin kinase, it is difficult to ascribe contraction solely to a Ca^{2+}-independent pathway. The signaling leading to contraction is easier to analyze in our REF-52 collagen gels because these cells lack MLCK.

Previous investigators have also described Ca^{2+}-independent RLC phosphorylation and contraction (41); however, it is difficult to compare our results with those because of technical differences between sample preparation. The use of the Ca^{2+}-chelating agent BAPTA must be done with care to ensure its chemical stability during experimentation because amino acids or amines in the media may extracellularly cleave the AM ester group and inhibit BAPTA-AM from crossing the cell membrane (26).

Recent work by other laboratories has investigated the role of Rho/Rho-kinase in isolated stress fibers. Katoh and coworkers (34) developed a permeabilized cell system to identify that Rho/Rho-kinase mediates Ca^{2+}-independent stress fiber contraction. However, the authors suggested that in a two-pathway system, containing both MLCK and Rho/Rho-kinase activity, Rho/Rho-kinase predominantly regulates myosin phosphatase activity to perform sustained contraction versus the fast contractions mediated by Ca^{2+}-dependent MLCK. We have

expanded upon their work to demonstrate that in an intact cell system, Rho/Rho-kinase can regulate cell contraction independent of MLCK activity. Because our model system does not contain MLCK, we cannot ascertain the relative rates of each pathway. Yet, we have demonstrated that the Rho/Rho-kinase mediates endogenous agonist-induced contraction, a phenomenon more similar to physiological signaling than permeabilized cell systems.

Despite the fact that our studies, along with these others cited, have shown that Rho/Rho kinase signaling is important in the regulation of MLC phosphorylation and cytoskeletal changes leading to contraction, this does not rule out the existence of other signaling pathways regulating actomyosin interactions. In endothelial cells, the p21-activated kinase PAK2, which is activated by the GTP-binding proteins Cdc42 and Rac, can phosphorylate Ser-19 of the RLC and cause cell retraction (9,73). Furthermore, contraction can be affected by the binding of other proteins to microfilaments. For example, nonmuscle caldesmon has been shown to inhibit stress fiber formation and contractility in fibroblasts (27). Caldesmon binding, in turn, can be regulated by phosphorylation by PAK (66).

The putative pathway for Rho-kinase-regulated cytoskeletal rearrangements has focused on myosin phosphatase inhibition (13,36). Yet, in vitro data have suggested a role for Rho-kinase in direct phosphorylation of RLC, but this activity has yet to be shown in intact cells (2). Many investigators have attributed Rho-kinase-mediated RLC phosphorylation mainly due to inhibition of myosin phosphatase via phosphorylation at Thr-695 and/or Thr-850, and the direct phosphorylation activity upon RLC is attributed to MLCK (12,14,17,37). However, our unique model system lacks MLCK and therefore demonstrates the new finding that Rho-kinase can mediate agonist-stimulated contraction independent of MLCK activity. In every other system, RLC phosphorylation results are confounded by the presence of competing phosphorylation pathways of MLCK and Rho/Rho-kinase. Cell lines that contain both kinase pathways require the use of multiple kinase inhibitors, recombinant knockouts, or overly active constitutively active proteins, leading to potentially aggravated cellular aberration. We suggest that in light of our results, Rho-kinase (or an undiscovered kinase) can directly phosphorylate RLC and generate subsequent cellular contraction. Additional data supporting direct phosphorylation of RLC by Rho-kinase have been obtained from preliminary experiments employing both the myosin phosphatase inhibitor calyculin A and Rho-kinase inhibitor Y-27632. Inhibition of both Rho-kinase and myosin phosphatase followed by agonist stimulation results in only marginal increases in RLC phosphorylation. Tryptic peptide mapping has shown that this limited RLC phosphorylation is not associated with either Ser-19 or Thr-18, the myosin II activation sites phosphorylated by Rho-kinase (unpublished observation). These data further indicated that Rho-kinase directly phosphorylates RLC in response to agonist stimulation in REF-52 cells. Zip kinase and ILK have been suggested as additional mediators of Rho-kinase phosphorylation of RLC, and whether these agents are involved in Rho-kinase-mediated RLC phosphorylation in our system is not known (10,45,49).

A noteworthy caveat to consider is that the contribution of the Rho/Rho-kinase pathway to contraction in these cells may be exaggerated compared with cell lines that also contain MLCK. Potentially, the absence of MLCK in these cells has upregulated Rho-kinase activity, therefore demonstrating an enhancement of the role of Rho-kinase in cell contraction. Nonetheless, the absence of MLCK does not seem to effect cell viability or phenotype.

Our results demonstrate that cytosolic calcium is not necessary for RLC phosphorylation and subsequent contraction. Chelation of Ca^{2+} mildly attenuated RLC phosphorylation and the magnitude of contraction. Although Ca^{2+} may serve a minor regulatory role, it is not paramount to Rho-kinase-mediated agonist-stimulated contraction. Because MLCK is dependent on Ca^{2+}/CaM activation, its role in agonist-stimulated contraction would be severely ablated by Ca^{2+}-free conditions. The negligible difference between Ca^{2+}-containing and Ca^{2+}-free RLC

phosphorylation and contraction indicates that a Ca^{2+}-free pathway is sufficient for basal tone and agonist-induced contraction mediated by RLC phosphorylation.

We have described a model system that allows for analysis of the regulation and activity of the Rho/Rho-kinase for cell contraction. We have shown that our REF-52 cells lack detectable levels of endogenous MLCK protein and its mRNA. Even in the absence of MLCK, we have shown that REF-52 cells can be stimulated by agonists thrombin or LPA to contract. This agonist-stimulated contraction is mediated by RLC phosphorylation. Additionally, we have demonstrated that Ca^{2+}-independent RLC phosphorylation and contraction are nearly identical to RLC phosphorylation and contraction performed in conditions containing Ca^{2+}. Furthermore, the specific Rho-kinase inhibitor Y-27632 severely inhibits both cell contraction and RLC phosphorylation.

Acknowledgments

This work was supported by National Heart, Lung, and Blood Institute Grants HL-45788 (to R. B. Wysolmerski), HL-54118 (to P. J. Gallagher), HL-58571 (to P. Herring), and National Institute of General Medical Sciences Grant GM-38838 (to E. L. Elson). Z. M. Goeckeler was supported by a American Heart Association (AHA) Grant in Aid, and D. A. Emmert was supported by an AHA Predoctoral Fellowship.

REFERENCES

1. Amano M, Chihara K, Nakamura N, Fukata Y, Yano T, Shibata M, Ikebe M, Kaibuchi K. Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. Genes Cells 1998;3:177–188. [PubMed: 9619630]
2. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 1996;271:20246–20249. [PubMed: 8702756]
3. Birukov KG, Schavocky JP, Shirinsky VP, Chibalina MV, Van Eldik LJ, Watterson DM. Organization of the genetic locus for chicken myosin light chain kinase is complex: multiple proteins are encoded and exhibit differential expression and localization. J Cell Biochem 1998;70:402–413. [PubMed: 9706877]
4. Bishop AL, Hall A. Rho GTPases and their effector proteins. Biochem J 2000;348:241–255. [PubMed: 10816416]
5. Blue EK, Goeckeler ZM, Jin Y, Hou L, Dixon SA, Herring BP, Wysolmerski RB, Gallagher PJ. 220- and 130-kDa MLCKs have distinct tissue distributions and intracellular localization patterns. Am J Physiol Cell Physiol 2002;282:C451–C460. [PubMed: 11832329]
6. Bresnick AR. Molecular mechanisms of nonmuscle myosin-II regulation. Curr Opin Cell Biol 1999;11:26–33. [PubMed: 10047526]
7. Bresnick AR, Wolff-Long VL, Baumann O, Pollard TD. Phosphorylation on threonine-18 of the regulatory light chain dissociates the ATPase and motor properties of smooth muscle myosin II. Biochemistry 1995;34:12576–12583. [PubMed: 7548006]
8. Bridgman PC, Dave S, Asnes CF, Tullio AN, Adelstein RS. Myosin IIB is required for growth cone motility. J Neurosci 2001;21:6159–6169. [PubMed: 11487639]
9. Chew TL, Masaracchia RA, Goeckeler ZM, Wysolmerski RB. Phosphorylation of non-muscle myosin II regulatory light chain by p21-activated kinase (gamma-PAK). J Muscle Res Cell Motil 1998;19:839–854. [PubMed: 10047984]
10. Deng JT, Van Lierop JE, Sutherland C, Walsh MP. Ca^{2+}-independent smooth muscle contraction: a novel function for integrin-linked kinase. J Biol Chem 2001;276:16365–16373. [PubMed: 11278951]
11. Elson EL, Pasternak C, Liu ZY, Young JI, d B. Schwab Worthen GS, Downey G, Michaels R, McConnaughey WB, McDaniel M, Wolf B, Zahalak GI. Activation of mechanical responses in leukocytes. Biorheology 1990;27:849–858. [PubMed: 2093393]
12. Essler M, Amano M, Kruse HJ, Kaibuchi K, Weber PC, Aepfelbacher M. Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells. J Biol Chem 1998;273:21867–21874. [PubMed: 9705325]

13. Feng J, Ito M, Ichikawa K, Isaka N, Nishikawa M, Hartshorne DJ, Nakano T. Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. J Biol Chem 1999;274:37385–37390. [PubMed: 10601309]
14. Feng J, Ito M, Kureishi Y, Ichikawa K, Amano M, Isaka N, Okawa K, Iwamatsu A, Kaibuchi K, Hartshorne DJ, Nakano T. Rho-associated kinase of chicken gizzard smooth muscle. J Biol Chem 1999;274:3744–3752. [PubMed: 9920927]
15. Fujita A, Saito Y, Ishizaki T, Maekawa M, Fujisawa K, Ushikubi F, Narumiya S. Integrin-dependent translocation of p160^{ROCK} to cytoskeletal complex in thrombin-stimulated human platelets. Biochem J 1997;328:769–775. [PubMed: 9396719]
16. Fujisawa K, Madaule P, Ishizaki T, Watanabe G, Bito H, Saito Y, Hall A, Narumiya S. Different regions of Rho determine Rho-selective binding of different classes of Rho target molecules. J Biol Chem 1998;273:18943–18949. [PubMed: 9668072]
17. Fukata Y, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. Trends Pharmacol Sci 2001;22:32–39. [PubMed: 11165670]
18. Gallagher PJ, Herring BP. The carboxyl terminus of the smooth muscle myosin light chain kinase is expressed as an independent protein, telokin. J Biol Chem 1991;266:23945–23952. [PubMed: 1748667]
19. Gallagher PJ, Herring BP, Griffin SA, Stull JT. Molecular characterization of a mammalian smooth muscle myosin light chain kinase. J Biol Chem 1991;266:23936–23944. [Corrigendum. *J Biol Chem* 267: May 1992, p. 9450.]. [PubMed: 1748666]
20. Giuliano KA, Kolega J, DeBiasio RL, Taylor DL. Myosin II phosphorylation and the dynamics of stress fibers in serum-deprived and stimulated fibroblasts. Mol Biol Cell 1992;3:1037–1048. [PubMed: 1421576]
21. Giuliano KA, Taylor DL. Formation, transport, contraction, and disassembly of stress fibers in fibroblasts. Cell Motil Cytoskeleton 1990;16:14–21. [PubMed: 2354525]
22. Goeckeler ZM, Masaracchia RA, Zeng Q, Chew TL, Gallagher P, Wysolmerski RB. Phosphorylation of myosin light chain kinase by p21-activated kinase PAK2. J Biol Chem 2000;275:18366–18374. [PubMed: 10748018]
23. Goeckeler ZM, Wysolmerski RB. Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. J Cell Biol 1995;130:613–627. [PubMed: 7622562]
24. Gong MC, Cohen P, Kitazawa T, Ikebe M, Masuo M, Somlyo AP, Somlyo AV. Myosin light chain phosphatase activities and the effects of phosphatase inhibitors in tonic and phasic smooth muscle. J Biol Chem 1992;267:14662–14668. [PubMed: 1321813]
25. Grinnell F, Ho CH, Lin YC, Skuta G. Differences in the regulation of fibroblast contraction of floating versus stressed collagen matrices. J Biol Chem 1999;274:918–923. [PubMed: 9873032]
26. Haugland, RP. Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes; Eugene, OR: 1996. p. 549
27. Helfman DM, Levy ET, Berthier C, Shtutman M, Riveline D, Grosheva I, Lachish-Zalait A, Elbaum M, Bershadsky AD. Caldesmon inhibits nonmuscle cell contractility and interferes with the formation of focal adhesions. Mol Biol Cell 1999;10:3097–3112. [PubMed: 10512853]
28. Ichikawa K, Ito M, Hartshorne DJ. Phosphorylation of the large subunit of myosin phosphatase and inhibition of phosphatase activity. J Biol Chem 1996;271:4733–4740. [PubMed: 8617739]
29. Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 1996;15:1885–1893. [PubMed: 8617235]
30. Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M, Narumiya S. Pharmacological properties of Y-27632, a specific inhibitor of Rho-associated kinases. Mol Pharmacol 2000;57:976–983. [PubMed: 10779382]
31. Jay PY, Pham PA, Wong SA, Elson EL. A mechanical function of myosin II in cell motility. J Cell Sci 1995;108:387–393. [PubMed: 7738114]

32. Kamm KE, Stull JT. Dedicated myosin light chain kinases with diverse cellular functions. J Biol Chem 2001;276:4527–4530. [PubMed: 11096123]
33. Katoh K, Kano Y, Amano M, Kaibuchi K, Fujiwara K. Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. Am J Physiol Cell Physiol 2001;280:C1669–C1679. [PubMed: 11350763]
34. Katoh K, Kano Y, Amano M, Onishi H, Kaibuchi K, Fujiwara K. Rho-kinase-mediated contraction of isolated stress fibers. J Cell Biol 2001;153:569–584. [PubMed: 11331307]
35. Kawada N, Seki S, Kuroki T, Kaneda K. ROCK inhibitor Y-27632 attenuates stellate cell contraction and portal pressure increase induced by endothelin-1. Biochem Biophys Res Commun 1999;266:296–300. [PubMed: 10600496]
36. Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T, Ito M, Matsumura F, Inagaki M, Kaibuchi K. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. J Cell Biol 1999;147:1023–1038. [PubMed: 10579722]
37. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 1996;273:245–248. [PubMed: 8662509]
38. Kishi H, Mikawa T, Seto M, Sasaki Y, Kanayasu-Toyoda T, Yamaguchi T, Imamura M, Ito M, Karaki H, Bao J, Nakamura A, Ishikawa R, Kohama K. Stable transfectants of smooth muscle cell line lacking the expression of myosin light chain kinase and their characterization with respect to the actomyosin system. J Biol Chem 2000;275:1414–1420. [PubMed: 10625693]
39. Kitazawa T, Gaylinn BD, Denney GH, Somlyo AP. G-protein-mediated Ca^{2+} sensitization of smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem 1991;266:1708–1715. [PubMed: 1671041]
40. Kolodney MS, Elson EL. Correlation of myosin light chain phosphorylation with isometric contraction of fibroblasts. J Biol Chem 1993;268:23850–23855. [PubMed: 8226923]
41. Kolodney MS, Thimgan MS, Honda HM, Tsai G, Yee HF Jr. Ca^{2+}-independent myosin II phosphorylation and contraction in chicken embryo fibroblasts. J Physiol 1999;515:87–92. [PubMed: 9925880]
42. Leung T, Chen XQ, Manser E, Lim L. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol 1996;16:5313–5327. [PubMed: 8816443]
43. Leung T, Manser E, Tan L, Lim L. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem 1995;270:29051–29054. [PubMed: 7493923]
44. Manning TJ Jr, Parker JC, Sontheimer H. Role of lysophosphatidic acid and rho in glioma cell motility. Cell Motil Cytoskeleton 2000;45:185–199. [PubMed: 10706774]
45. Murata-Hori M, Suizu F, Iwasaki T, Kikuchi A, Hosoya H. ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. FEBS Lett 1999;451:81–84. [PubMed: 10356987]
46. Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S. ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. FEBS Lett 1996;392:189–193. [PubMed: 8772201]
47. Narumiya S, Ishizaki T, Uehata M. Use and properties of ROCK-specific inhibitor Y-27632. Methods Enzymol 2000;325:273–284. [PubMed: 11036610]
48. Niggli V. Rho-kinase in human neutrophils: a role in signalling for myosin light chain phosphorylation and cell migration. FEBS Lett 1999;445:69–72. [PubMed: 10069376]
49. Niiro N, Ikebe M. Zipper-interacting protein kinase induces Ca^{2+}-free smooth muscle contraction via myosin light chain phosphorylation. J Biol Chem 2001;276:29567–29574. [PubMed: 11384979]
50. Nobe K, Nobe H, Obara K, Paul RJ. Preferential role of intracellular Ca^{2+} stores in regulation of isometric force in NIH 3T3 fibroblast fibres. J Physiol 2000;529:669–679. [PubMed: 11118497]
51. Parizi M, Howard EW, Tomasek JJ. Regulation of LPA-promoted myofibroblast contraction: role of Rho, myosin light chain kinase, and myosin light chain phosphatase. Exp Cell Res 2000;254:210–220. [PubMed: 10640419]
52. Poperechnaya A, Varlamova O, Lin P, Stull JT, Bresnick AR. Localization and activity of myosin light chain kinase isoforms during the cell cycle. J Cell Biol 2000;151:697–708. [PubMed: 11062269]

53. Rayan GM, Parizi M, Tomasek JJ. Pharmacologic regulation of Dupuytren's fibroblast contraction in vitro. J Hand Surg [Am] 1996;21:1065–1070.
54. Reid T, Furuyashiki T, Ishizaki T, Watanabe G, Watanabe N, Fujisawa K, Morii N, Madaule P, Narumiya S. Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. J Biol Chem 1996;271:13556–13560. [PubMed: 8662891]
55. Ren XD, Kiosses WB, Schwartz MA. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J 1999;18:578–585. [PubMed: 9927417]
56. Ren XD, Schwartz MA. Determination of GTP loading on Rho. Methods Enzymol 2000;325:264–272. [PubMed: 11036609]
57. Rochlin MW, Itoh K, Adelstein RS, Bridgman PC. Localization of myosin II A and B isoforms in cultured neurons. J Cell Sci 1995;108:3661–3670. [PubMed: 8719872]
58. Sanders LC, Matsumura F, Bokoch GM, de Lanerolle P. Inhibition of myosin light chain kinase by p21-activated kinase. Science 1999;283:2083–2085. [PubMed: 10092231]
59. Sato M, Tani E, Fujikawa H, Kaibuchi K. Involvement of Rho-kinase-mediated phosphorylation of myosin light chain in enhancement of cerebral vasospasm. Circ Res 2000;87:195–200. [PubMed: 10926869]
60. Shoemaker MO, Lau W, Shattuck RL, Kwiatkowski AP, Matrisian PE, Guerra-Santos L, Wilson E, Lukas TJ, Van Eldik LJ, Watterson DM. Use of DNA sequence and mutant analyses and antisense oligodeoxynucleotides to examine the molecular basis of nonmuscle myosin light chain kinase autoinhibition, calmodulin recognition, and activity. J Cell Biol 1990;111:1107–1125. [PubMed: 2202734]
61. Skuta G, Ho CH, Grinnell F. Increased myosin light chain phosphorylation is not required for growth factor stimulation of collagen matrix contraction. J Biol Chem 1999;274:30163–30168. [PubMed: 10514506]
62. Somlyo AP, Somlyo AV. Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J Physiol 2000;522:177–185. [PubMed: 10639096]
63. Sward K, Dreja K, Susnjar M, Hellstrand P, Hartshorne DJ, Walsh MP. Inhibition of Rho-associated kinase blocks agonist-induced Ca^{2+} sensitization of myosin phosphorylation and force in guinea-pig ileum. J Physiol 2000;522:33–49. [PubMed: 10618150]
64. Totsukawa G, Yamakita Y, Yamashiro S, Hartshorne DJ, Sasaki Y, Matsumura F. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. J Cell Biol 2000;150:797–806. [PubMed: 10953004]
65. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 1997;389:990–994. [PubMed: 9353125]
66. Van Eyk JE, Arrell DK, Foster DB, Strauss JD, Heinonen TY, Furmaniak-Kazmierczak E, Cote GP, Mak AS. Different molecular mechanisms for Rho family GTPase-dependent, Ca^{2+}-independent contraction of smooth muscle. J Biol Chem 1998;273:23433–23439. [PubMed: 9722579]
67. Wakatsuki T, Kolodney MS, Zahalak GI, Elson EL. Cell mechanics studied by a reconstituted model tissue. Biophys J 2000;79:2353–2368. [PubMed: 11053115]
68. Weber LP, Van Lierop JE, Walsh MP. Ca^{2+}-independent phosphorylation of myosin in rat caudal artery and chicken gizzard myofilaments. J Physiol 1999;516:805–824. [PubMed: 10200427]
69. Wysolmerski RB, Lagunoff D. Involvement of myosin light-chain kinase in endothelial cell retraction. Proc Natl Acad Sci USA 1990;87:16–20. [PubMed: 2296576]
70. Wysolmerski RB, Lagunoff D. Regulation of permeabilized endothelial cell retraction by myosin phosphorylation. Am J Physiol Cell Physiol 1991;261:C32–C40.
71. Yanase M, Ikeda H, Matsui A, Maekawa H, Noiri E, Tomiya T, Arai M, Yano T, Shibata M, Ikebe M, Fujiwara K, Rojkind M, Ogata I. Lysophosphatidic acid enhances collagen gel contraction by hepatic stellate cells: association with Rho-kinase. Biochem Biophys Res Commun 2000;277:72–78. [PubMed: 11027642]
72. Yee HF Jr, Melton AC, Tran BN. RhoA/rho-associated kinase mediates fibroblast contractile force generation. Biochem Biophys Res Commun 2001;280:1340–1345. [PubMed: 11162676]

73. Zeng Q, Lagunoff D, Masaracchia R, Goeckeler Z, Cote G, Wysolmerski R. Endothelial cell retraction is induced by PAK2 mono-phosphorylation of myosin II. J Cell Sci 2000;113:471–482. [PubMed: 10639334]

74. Zutter MM, Santoro SA, Wu JE, Wakatsuki T, Dickeson SK, Elson EL. Collagen receptor control of epithelial morphogenesis and cell cycle progression. Am J Pathol 1999;155:927–940. [PubMed: 10487850]

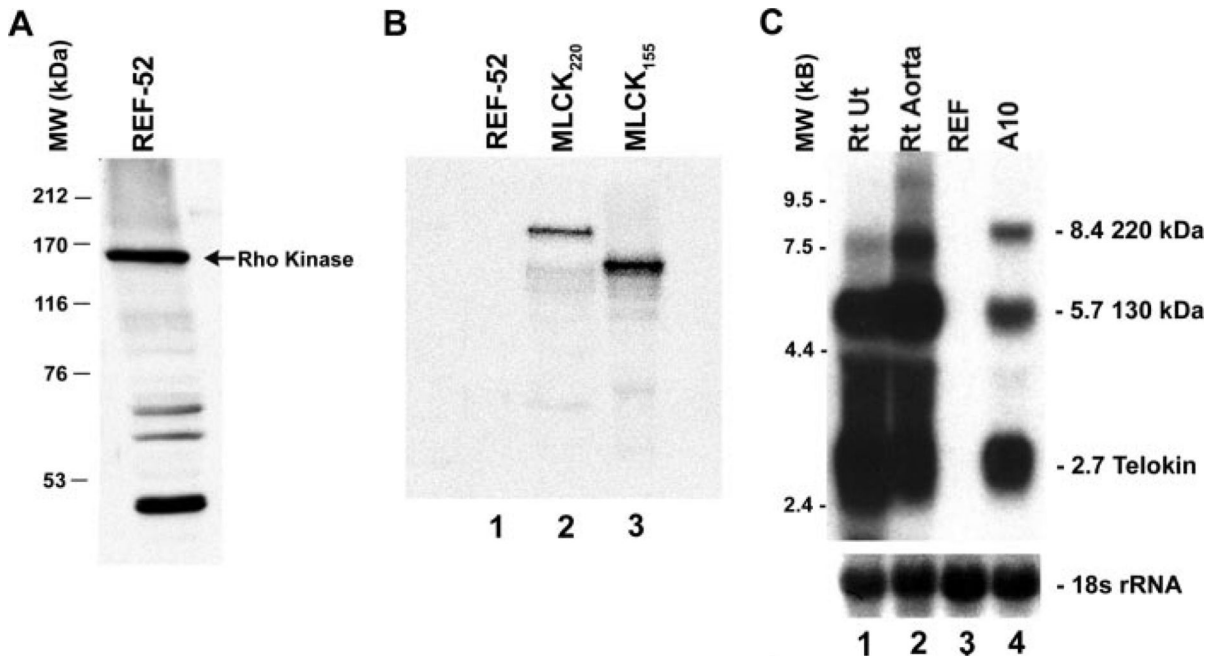

Fig. 1.

Expression of Rho-kinase and myosin light chain kinase (MLCK) in rat embryo fibroblast (REF)-52 cells. REF-52 cell pellets (2×10^6 cells) were lysed and extracted as outlined in EXPERTMENTAL PROCEDURES, electrophoresed on 10% SDS-PAGE, transferred to 0.45-μm polyvinylidene difluoride (PVDF) membrane, and probed with rabbit polyclonal antibody to Rho-kinase. *A*: REF-52 cell extracts probed with a rabbit polyclonal antibody to Rho-kinase. Rho-kinase was found to have a molecular mass of 160 kDa. *B*: REF-52 cell extracts probed with an affinity-purified rabbit polyclonal antibody to $MLCK_{155}$, which recognizes both isoforms of MLCK. No endogenous MLCK was detected in REF-52 cells (*lane 1*). *Lanes 2* and *3* are recombinant $MLCK_{220}$ and $MLCK_{155}$ standards, respectively. *C*: Northern analysis of MLCK mRNAs in rat cells and tissues. RNA (15 μg total RNA/lane) was isolated from rat uterus (Rt Ut; *lane 1*), rat aorta (*lane 2*), rat embryo fibroblasts (REF; *lane 3*), and rat embryonic thoracic aorta smooth muscle cells (A10; *lane 4*) and probed as outlined in EXPERIMENTAL PROCEDURES. The positions of the RNA molecular weight standards (kB) are shown at *left*. *Bottom*: photograph documenting the amounts of 18S rRNA present in each lane.

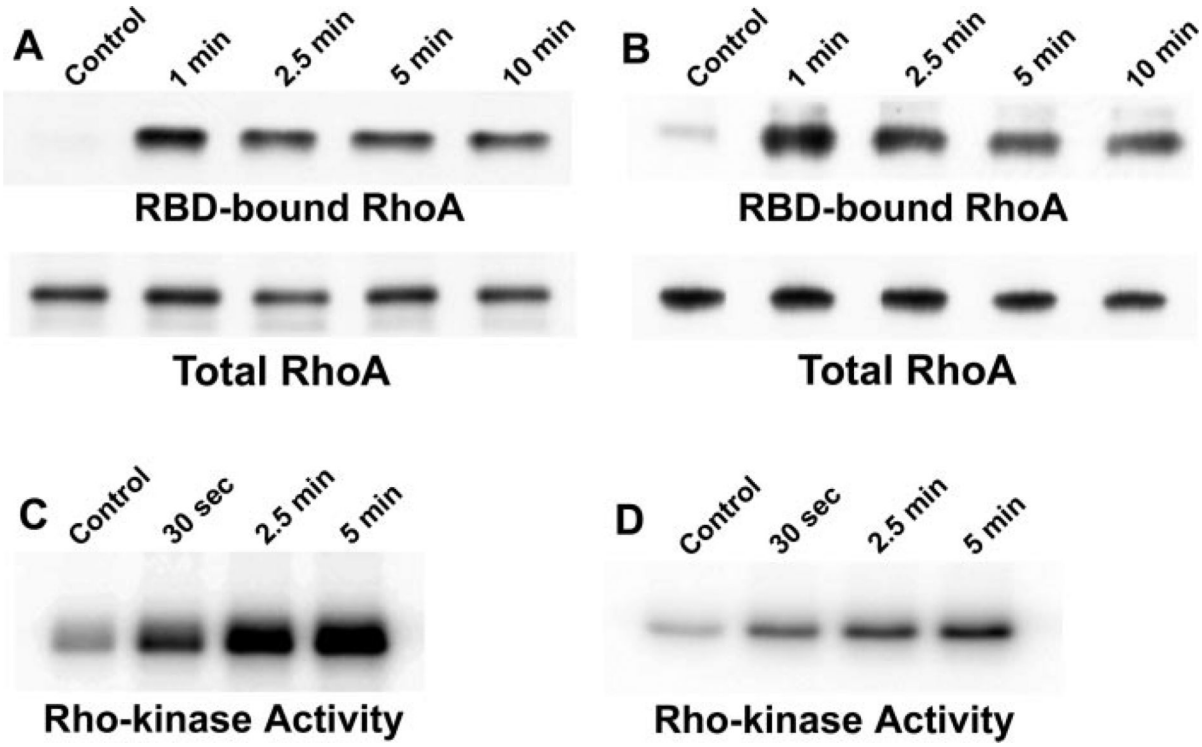

Fig. 2.

RhoA and Rho-kinase activity. *A* and *B*: representative Western blots demonstrate RhoA activation. REF-52 cells 4 days postconfluence were stimulated with 1 U/ml thrombin (*A*) or 1 μM lysophosphatidic acid (LPA; *B*) for 0, 1, 2.5, 5, or 10 min. Cell lysates were incubated with glutathione *S*-transferase-Rho-binding domain (GST-RBD) fusion protein for 1 h at 4°C, electrophoresed, transferred to PVDF, and probed with RhoA antibody. RhoA activity was determined as the amount of RBD-bound active RhoA (GTP-bound RhoA) normalized to the total amount of RhoA in the cell lysate. Below the blot is shown a whole cell lysate for total RhoA. *C* and *D*: thrombin and LPA induced Rho-kinase activation. REF-52 monolayers were stimulated with 1 U/ml thrombin (*C*) or 1 μM LPA (*D*) for the indicated time intervals, cells were lysed, and Rho-kinase was immunoprecipitated. Immunopurified Rho-kinase was used for in vitro phosphorylation reactions using purified myosin II regulatory light chain (RLC) as substrate as described in EXPERIMENTAL PROCEDURES. Phosphorylated myosin II RLCs were analyzed on 12.5% SDS gels and exposed to a PhosphorImager plate. Thrombin and LPA treatment resulted in an 11- and 8-fold increase in Rho-kinase catalytic activity, respectively.

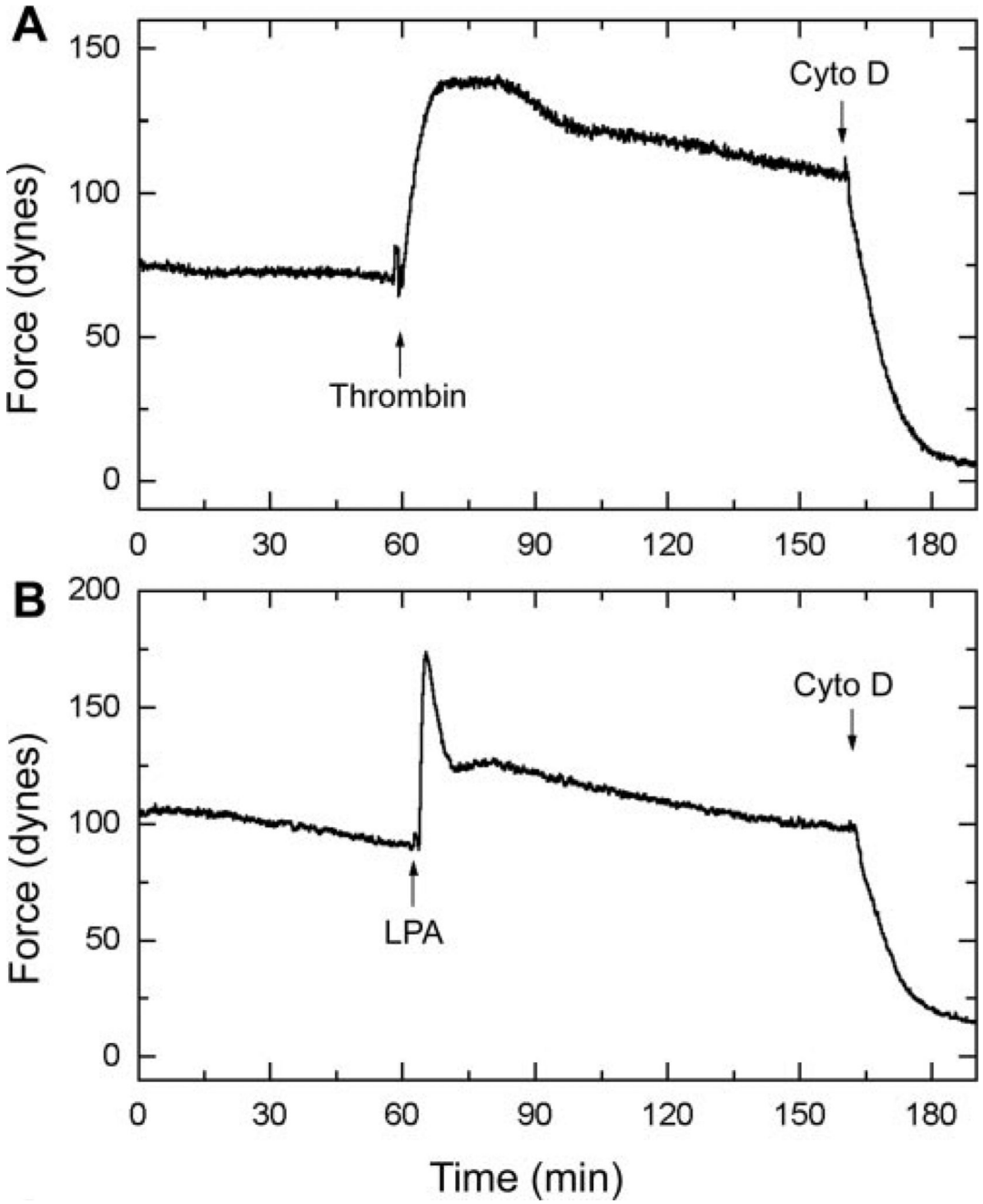

Fig. 3.

Agonist-induced isometric contraction in REF-52 collagen gels. Representative tension tracings were produced by REF-52 cells treated with thrombin or LPA. Collagen gels were mounted on force transducers in HEPES-buffered saline (HBS) and allowed 60 min to establish a stable baseline force before being stimulated with either 1 U/ml thrombin (*A*) or 1 μM LPA (*B*). Both agonists rapidly increased isometric tension, which remained elevated for over 2 h. Addition of cytochalasin D (cyto D) ablated cellular tension.

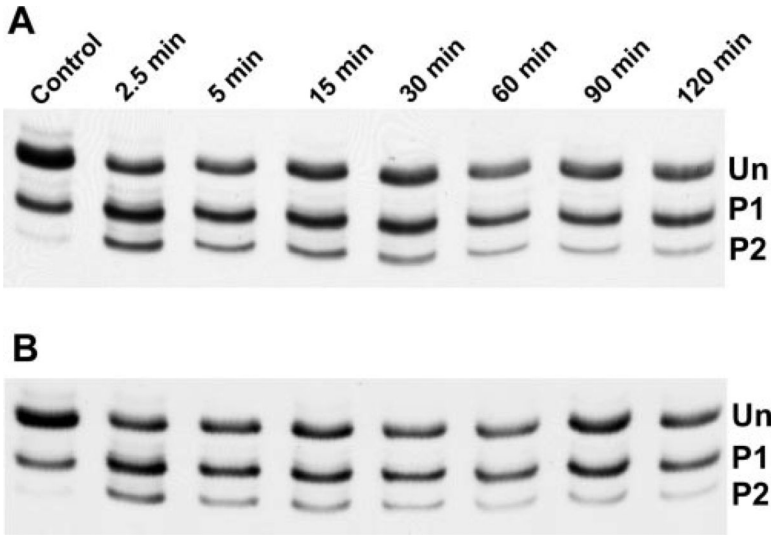

Fig. 4.

Agonist-induced MLC phosphorylation. REF-52 monolayers were preincubated for 1 h at room temperature in HBS. Cultures were stimulated with 1 U/ml thrombin (*A*)or1 μM LPA (*B*) for 0, 2.5, 5, 15, 30, 60, 90, or 120 min. Samples were separated by glycerol-urea gel electrophoresis, transferred to nitrocellulose, and probed with anti-myosin RLC antibody. The unphosphorylated (Un), monophosphorylated (P1), and diphosphorylated (P2) states of myosin II RLC are represented.

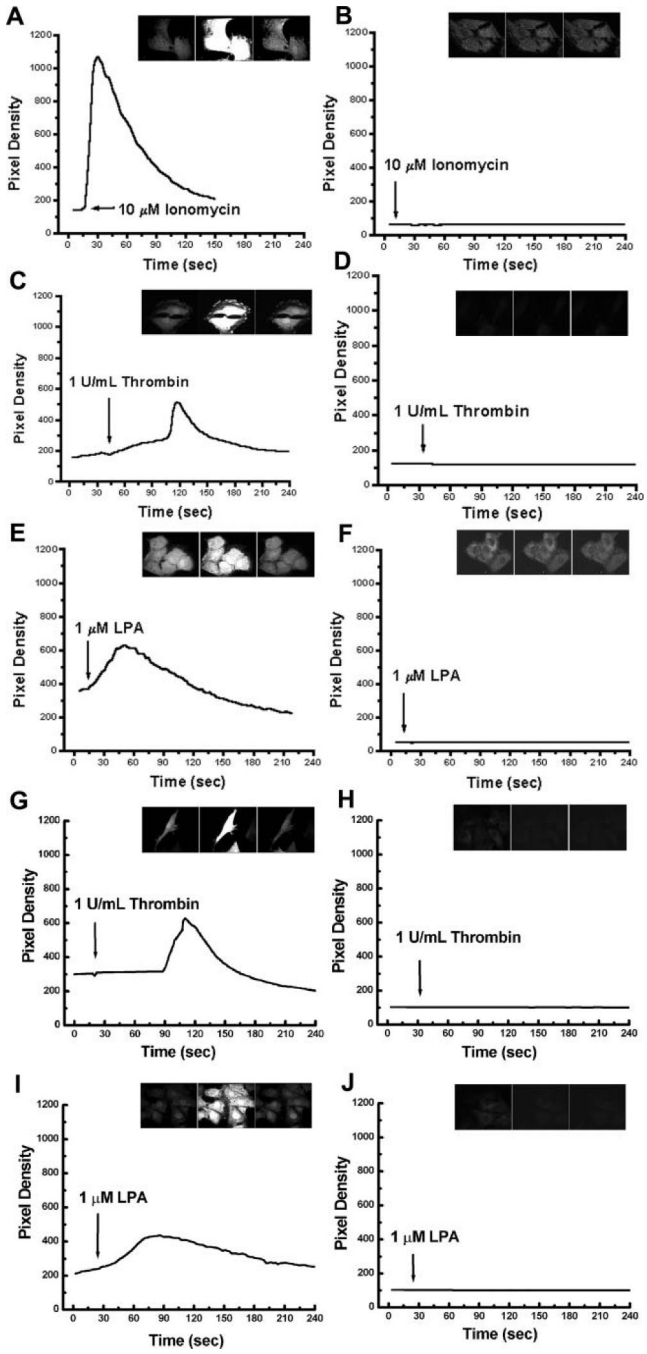

Fig. 5.

Ca^{2+} fluorescence signal in REF-52 cells. REF-52 cells were incubated in 37°C HBS containing 1 μM Cell-Tracker orange for 45 min, followed by an additional 45-min incubation at room temperature in HBS/2 μM fluo 4-AM/0.04% (wt/vol) Pluronic F-127 supplemented with (*A*, *C*, and *E*) or without 1.8 mM Ca^{2+} (*B*, *D*, and *F*). For chelation of Ca^{2+}, BAPTA-AM (10 μM) and thapsigargin (1 μM) were added to monolayers in a Ca^{2+}-free medium (*B*, *D*, and *F*). Cells were visualized, and images were captured using a Wallac UltraView spinning disk confocal laser microscope fitted onto an Olympus IX70 inverted fluorescence microscope. Cells were maintained at 37°C with a heated stage, and images were collected every 500 ms with an AstroCam cooled charge-coupled device camera. Pixel density was calculated from

whole cell averages, less background intensity. Cells were localized by Cell-Tracker orange cytoplasmic dye (at 568 nm), and intracellular Ca^{2+} signal was measured with fluo 4 (at 488 nm). *A* and *B* demonstrate the effects of 10 μM ionomycin to stimulate Ca^{2+} influx in 1.8 mM Ca^{2+} and Ca^{2+}-free conditions, respectively. *C* and *D* demonstrate the effects of 1 U/ml thrombin to stimulate Ca^{2+} influx in 1.8 mM Ca^{2+} and Ca^{2+}-free conditions, respectively. *E* and *F* demonstrate the results for 1 μM LPA. The 3 *insets* in each panel, from *left* to *right*, display Ca^{2+} fluorescence images before stimulation, at peak Ca^{2+} influx, and after cell recovery. *G–J* show representative measurements of REF-52 cell-populated collagen gels loaded with fluo 4 in 1.8 mM Ca^{2+} (*G* and *I*) or under Ca^{2+} chelation conditions (*H* and *J*) as outlined in EXPERIMENTAL PROCEDURES. Collagen gels stimulated with 1 U/ml thrombin (*G*) or 1 μM LPA (*I*) evoked an influx of extracellular Ca^{2+} similar to the response shown in monolayer cultures (*C* and *E*). As with monolayer cultures, BAPTA-AM/thapsigargin blocked the rise in cytosolic Ca^{2+} shown with thrombin (*H*) and LPA (*J*).

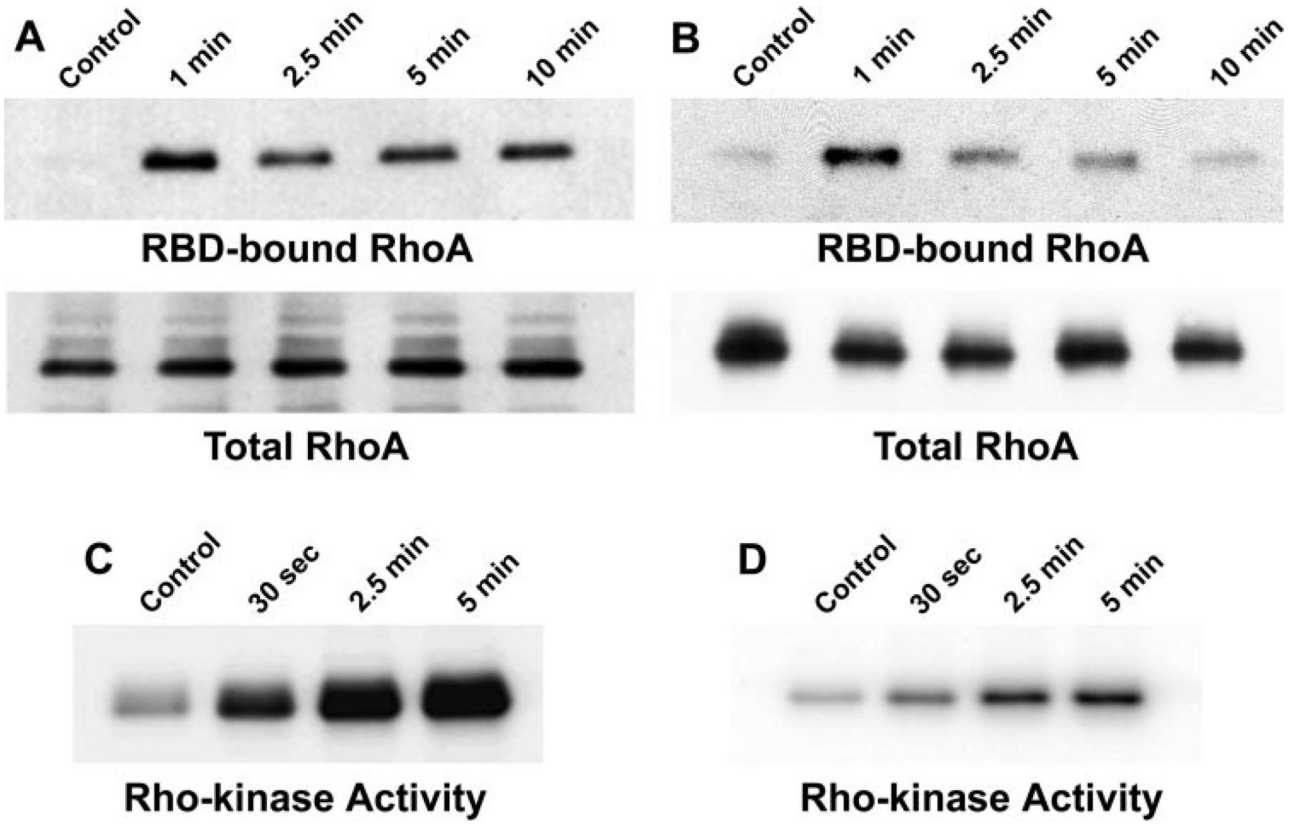

Fig. 6.

Ca^{2+}-independent RhoA and Rho-kinase activity. *A* and *B*: representative Western blots of Ca^{2+}-independent RhoA activity. REF-52 cultures were preincubated for 1 h at room temperature in Ca^{2+}-free HBS containing 10 μM BAPTA-AM/1 μM thapsigargin. Cells were stimulated with 1 U/ml thrombin (*A*) or 1 μM LPA (*B*) for the indicated time intervals, monolayers were lysed, and cell lysates were incubated with GST-RBD for 1 h. Samples were electrophoresed, transferred to PVDF, and probed with RhoA antibody. Below the blot is shown a whole cell lysate for total RhoA to indicate equal loading. *C* and *D*: thrombin and LPA induced Ca^{2+}-independent Rho-kinase activity. REF-52 monolayers were preincubated for 1 h at room temperature in Ca^{2+}-free HBS containing 10 μM BAPTA-AM/1 μM thapsigargin. Cells were stimulated with 1 U/ml thrombin (*C*) or 1 μM LPA (*D*) for the indicated time intervals, cells were lysed, and Rho-kinase was immunoprecipitated. Immunopurified Rho-kinase was used for in vitro phosphorylation reactions with purified myosin II RLC as substrate as described in EXPERIMENTAL PROCEDURES. Phosphorylated myosin II RLCs were analyzed on 12.5% SDS gels and exposed to a PhosphorImager plate. Both thrombin and LPA upregulated Ca^{2+}-independent Rho-kinase catalytic activity.

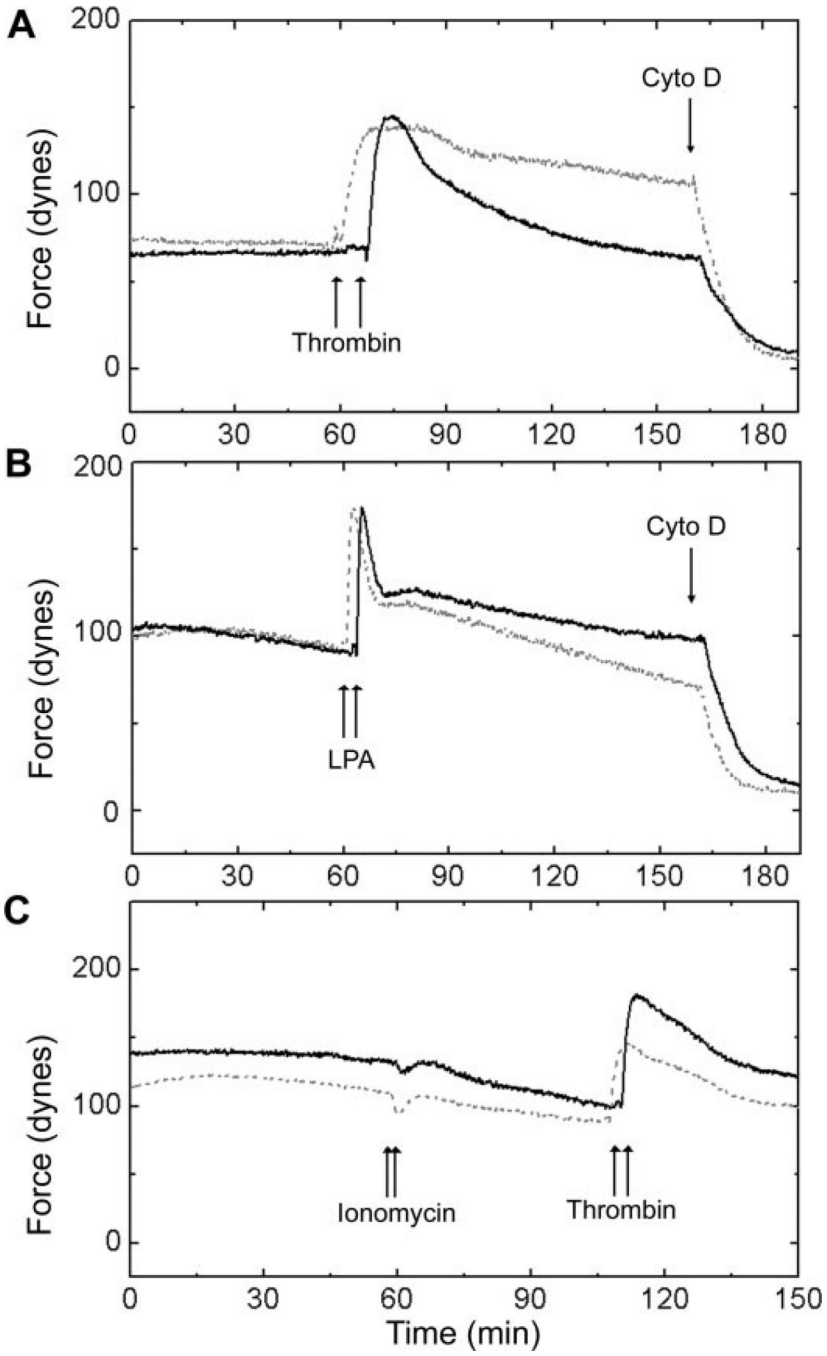

Fig. 7.

Ca^{2+}-independent agonist-induced isometric contraction in REF-52 collagen gels. REF-52 cell-populated collagen gels were mounted on isometric force transducers and incubated for 1 h in either HBS (dashed lines) or Ca^{2+}-free HBS supplemented with 10 μM BAPTA-AM and 1 μM thapsigargin (solid lines). After 1 h, the medium was replaced with fresh, inhibitor-free 37°C HBS or Ca^{2+}-free HBS. Basal tension was monitored for 1 h before the addition of 1 U/ml thrombin (*A*), 1 μM LPA (*B*), or 1 μM ionomycin (*C*).

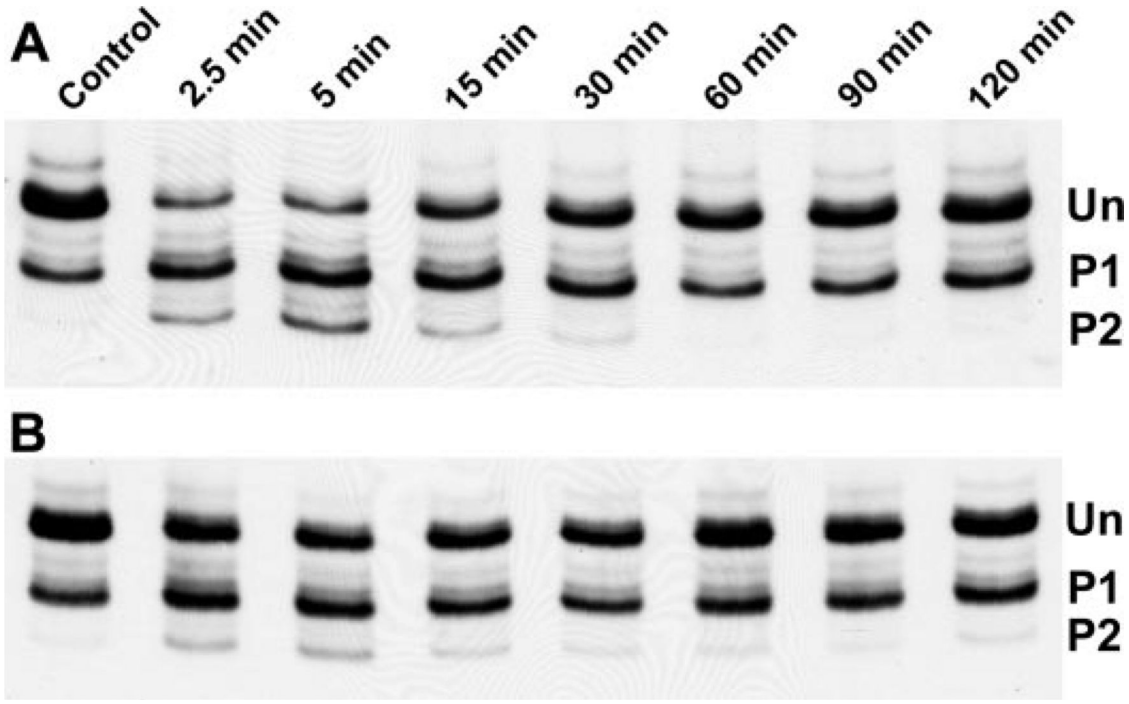

Fig. 8.

Ca^{2+}-independent agonist-induced MLC phosphorylation. Monolayers were preincubated for1hat room temperature in Ca^{2+}-free HBS containing 10 μM BAPTA-AM/1 μM thapsigargin. Cells were stimulated with 1 U/ml thrombin (*A*) or 1 μM LPA (*B*) for the indicated time intervals. Samples were separated by glycerol-urea gel electrophoresis, transferred to nitrocellulose, and probed with anti-myosin RLC antibody. Unphosphorylated (Un), monophosphorylated (P1), and diphosphorylated (P2) states of myosin II RLC are represented.

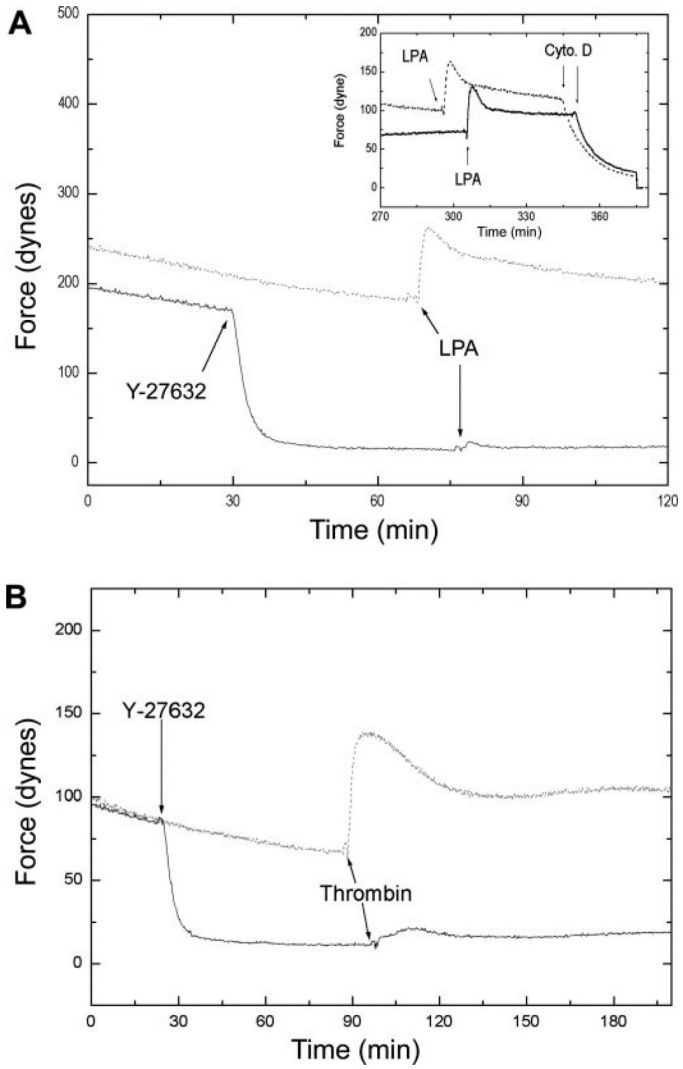

Fig. 9.

Effects of Rho-kinase inhibitor Y-27632 on basal and agonist-induced isometric tension. REF-52 cell-populated collagen gels were incubated with 10 μM Y-27632 for 1 h before the addition of agonists in the presence of Y-27632 (solid lines). *A*: effects of Y-27632 on LPA-induced isometric tension development. Y-27632 caused a rapid drop in basal tension, establishing a new baseline tension within 10 min. Pretreatment with Y-27632 inhibited the LPA-induced isometric tension development. *Inset*: reversibility of Y-27632. Organ baths were drained and replaced with media without Y-27632. Tension steadily increased, establishing a steady baseline tension within 3 h. Addition of a second dose of LPA resulted in a rapid rise in tension (*inset*). *B*: Y-27632 also inhibited thrombin-induced isometric tension. Results are representative of 3 experiments.

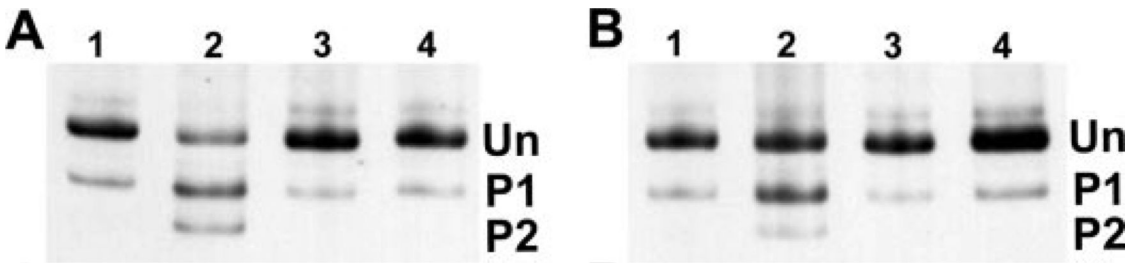

Fig. 10.

Effects of Rho-kinase inhibitor Y-27632 on basal and agonist-stimulated MLC phosphorylation. REF-52 cultures were pretreated with 10 μM Y-27632 for 15 min (*A* and *B*, *lanes 3* and *4*) or without Y-27632 (*A* and *B*, *lanes 1* and *2*). Monolayers were stimulated with 1 U/ml thrombin (*A*, *lanes 2* and *4*) or 1 μM LPA (*B*, *lanes 2* and *4*) for 5 min. Samples were separated by glycerol-urea gel electrophoresis, transferred to nitrocellulose, and probed with anti-myosin RLC antibody. Unphosphorylated (Un), monophosphorylated (P1), and diphosphorylated (P2) states of myosin II RLC are represented. *A* and *B*, *lane 1*, represent unstimulated control cells.

Table 1

Effect of thrombin and LPA on RhoA activation in REF-52 cells

	Control	1 min	2.5 min	5 min	10 min
Thrombin	1	27±4	22±3	25±2	20±3
LPA	1	20±4	21±5	16±4	14±5

Values are means ± SE of 3 separate experiments on the time course of RhoA activation. Rat embryo fibroblast (REF)-52 cultures were exposed to thrombin or lysophosphatidic acid (LPA) for the indicated time periods, extracted in 1 ml of lysis buffer and processed as outlined in EXPERIMENTAL PROCEDURES. RhoA-GTP content was assessed by Western blotting, using a rabbit polyclonal antibody to RhoA. Active RhoA-GTP content in unstimulated control cultures was designated as 1, and the increase in RhoA-GTP from normalized cell extracts was used as a measure of RhoA activation. All data were normalized to total cellular RhoA and expressed as the fold increase in GTP-bound RhoA as described in EXPERIMENTAL PROCEDURES.

Am J Physiol Cell Physiol. Author manuscript; available in PMC 2010 February 17.

Table 2

Distribution and stoichiometry of thrombin induced RLC phosphorylation in REF-52 cells

	Control	2.5 min	5 min	15 min	30 min	60 min	90 min	120 min
Unphosphorylated	70±1.3	24±2.7	30±3.6	24±5.2	35±1.7	63±4.2	63±5.2	62±5.2
Monophosphorylated	29±1.1	44±1.1	40±0.9	43±3.7	42±1.8	33±3.6	34±4.0	35±4.3
Diphosphorylated	1±0.2	32±1.9	30±2.7	33±3.7	23±0.2	4.0±1.2	3.0±1.2	3.0±1.4
RLC phosphorylation, mol PO_4/mol RLC	0.31±0.02	1.08±0.05	1.00±0.06	1.09±0.09	0.88±0.02	0.41±0.05	0.40±0.06	0.41±0.06

Values are means ± SE of 3 separate experiments. Monolayers were stimulated with 1 U/ml thrombin for the indicated time intervals. Samples were separated by glycerol-urea gel electrophoresis, transferred to nitrocellulose, probed with anti-myosin regulatory light chain (RLC) antibody, and X-ray film digitized by laser scanning densitometry. The stoichiometry of RLC phosphorylation (mol PO_4/mol RLC) is calculated with the formula M/M = P1 + (2×P2)/U + P1 + P2, where U = %unphosphorylated RLC, P1 = %monophosphorylated RLC, and P2 = %diphosphorylated RLC.

Table 3

Distribution and stoichiometry of LPA-induced RLC phosphorylation in REF-52 cells

	Control	2.5 min	5 min	15 min	30 min	60 min	90 min	120 min
Unphosphorylated	68±2.8	36±2.6	42±4.0	46±3.2	45±3.1	50±3.6	49±0.4	44±4.8
Monophosphorylated	31±2.9	46±0.8	43±4.3	43±1.0	43±0.6	43±1.8	41±2.4	47±2.3
Diphosphorylated	1.0±0.3	18±2.2	15±1.1	11±2.2	12±3.0	7±1.7	10±2.9	9±2.8
RLC phosphorylation, mol PO_4/mol RLC	0.33±0.03	0.82±0.05	0.73 ±0.04	0.65±0.06	0.67±0.06	0.57±0.06	0.61 ±0.04	0.65±0.08

Values are means ± SE of 3 separate experiments. Cultures were stimulated with 1 μM LPA for the indicated time intervals. Samples were TCA precipitated and electrophoresed through a glycerol-urea gel, transferred to nitrocellulose, probed with anti-myosin RLC antibody, and exposed to X-ray films, and the stoichiometry of RLC phosphorylation was calculated as described by Goeckeler and Wysolmerski (23).

Table 4

Effect of Ca^{2+} depletion on thrombin- and LPA-induced RhoA activation in REF-52 cells

	Control	1 min	2.5 min	5 min	10 min
Thrombin	1	47±18	30±8	40±12	29±10
LPA	1	9±0	9±0	2±0	2±1

Values are means ± SE of 3 separate experiments. REF-52 monolayers were incubated in Ca^{2+}-free HBS containing 10 μM BAPTA-AM/1 μM thapsigargin for1h at room temperature, stimulated with thrombin or LPA for the indicated time periods, extracted in 1 ml of lysis buffer, and processed as outlined in EXPERIMENTAL PROCEDURES. Active RhoA-GTP content in unstimulated control cultures was designated as 1, and the increase in RhoA-GTP from normalized cell extracts was used as a measure of RhoA activation. All data were normalized to total cellular RhoA and expressed as the fold increase in GTP-bound RhoA as described in EXPERIMENTAL PROCEDURES.

Table 5

Stoichiometry of Ca^{2+}-independent agonist-stimulated REF-52 RLC phosphorylation

	Control	2.5 min	5 min	15 min	30 min	60 min	90 min	120 min
Thrombin	0.23±0.02	0.81±0.04	0.94±0.02	0.80±0.09	0.57±0.04	0.35±0.01	0.26±0.02	0.23±0.06
LPA	0.26±0.05	0.58±0.01	0.66±0.03	0.57±0.03	0.42±0.04	0.33±0.04	0.30±0.08	0.27±0.08

Values are means ± SE of 3 separate experiments. Monolayers were stimulated with 1 U/ml thrombin or 1 μM LPA for the indicated time intervals. Samples were analyzed as described by Goeckeler and Wysolmerski (23).

Table 6

Effects of Y-27632 on basal and agonist-stimulated RLC phosphorylation

	Control	Agonist	Y-27632	Y-27632+Agonist
Thrombin	0.15±0.02	0.85±0.04	0.09±0.01	0.15±0.03
LPA	0.21±0.08	0.56±0.06	0.07±0.01	0.12±0.03

Values are means ± SE of 3 separate experiments. Stoichiometry of RLC phosphorylation was calculated as described by Goeckeler and Wysolmerski (23).