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

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Emmert, Daniel A., Judy A. Fee, Zoe M. Goeckeler, Jeremy M. Grojean, Tetsuro Wakatsuki, Elliot L. Elson, B. Paul Herring, Patricia J. Gallagher, and Robert B. Wysolmerski. Rho-kinase-mediated Ca\(^{2+}\)-independent contraction in rat embryo fibroblasts. Am J Physiol Cell Physiol 286:C8–C21, 2004. First published September 10, 2003; 10.1152/ajpcell.00428.2002—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.

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 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 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, ROK\(\alpha\)/ROCK-II (43, 46) and ROK\(\beta\)/ROCK-I/p160ROCK (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

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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\textsuperscript{2+} sensitization” in smooth muscle contraction, the process whereby contraction is stimulated when Ca\textsuperscript{2+} levels are held constant (62). It has been suggested that Rho-kinase regulates Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} and that Ca\textsuperscript{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 of myosin II RLC is responsible for myosin activation and contraction in vivo. Because the basal level of cytosolic Ca\textsuperscript{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\textsuperscript{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 thomboin and lysophosphatidic acid (LPA) induce a Ca\textsuperscript{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\textsuperscript{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\textsubscript{2}, and 0.6 mM CaCl\textsubscript{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). Fluoro-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\textsubscript{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\textsubscript{2}HPO\textsubscript{4}, 1.8 mM CaCl\textsubscript{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\textsuperscript{5}) and transfected COS-7 cells expressing MLCK\textsubscript{220} or MLCK\textsubscript{155} were lysed in extraction buffer [25 mM Tris-HCl, pH 6.8, 150 mM NaCl, 50 mM MgCl\textsubscript{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\textsubscript{220}, and MLCK\textsubscript{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-mesh polyvinylidene difluoride (PVDF) membrane and incubated with affinity-purified rabbit anti-Rho-kinase (15), anti-MLCK\textsubscript{220} (5), or anti-MLCK\textsubscript{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 32P-labeled rat MLCK cDNA probe corresponding to nucleotides 3018–3574 of the rabbit uterine MLCK. This fragment of rat MLCK was generated by reverse transcriptase-coupled 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 M sodium phosphate, pH 7.4, 30 mM NaCl, 0.2 mM EDTA, and 0.1% SDS at 68°C for 10 min.
Ca²⁺ 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 x 10⁵ cells and used for 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% Fluronic F-127 in HBS for an additional 45 min. For Ca²⁺ chelation experiments, cells were loaded simultaneously with fluo 4/0.04% Fluronic F-127, 10 μM BAPTA-AM, and 1 μM thapsigargin in Ca²⁺/BSA-free HBS. Temperature was maintained at 37°C for the duration of the experiment. (RhoA) were standardized on the basis of total RhoA DU and expression was expressed as the fold increase in GTP-bound RhoA; i.e., DU experimental /DU control).

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 (STGDSFETRFGKMD) from the NH₂-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) (MLCK170); 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 MLCK155 or MLCK210. Affinity-purified rabbit polyclonal MLCK155 and mouse monoclonal K36 recognized both MLCK155 and MLCK210, whereas anti-MLCK170 detected only MLCK210 (data not shown).

Figure 1A 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 to synthetic peptides or recombinant proteins, specifically 1) rabbit polyclonal antibody raised against a synthetic peptide sequence (STGDSFETRFGKMD) from the NH₂-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) (MLCK170); 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 MLCK155 or MLCK210. Affinity-purified rabbit polyclonal MLCK155 and mouse monoclonal K36 recognized both MLCK155 and MLCK210, whereas anti-MLCK170 detected only MLCK210 (data not shown).
fied antibodies to MLCK$_{155}$ or affinity-purified antibodies to MLCK$_{1-170}$. Figure 1B, 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. 1B, lane 2) and MLCK$_{155}$ (Fig. 1B, 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 1C 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. 1C, 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. 1C, 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. 2A) or 1 μM LPA (Fig. 2B) 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. 2A) or LPA (Fig. 2B) 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 turn 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. 3A), 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. 3B). 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.

### Table 1. Effect of thrombin and LPA on RhoA activation in REF-52 cells

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<td>1</td>
<td>27±2</td>
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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 lysosphatidic 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.
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 PO₄/mol RLC. By 2.5 min after addition of thrombin, phosphorylation rose by 348%, achieving a maximal level of 1.08 ± 0.05 mol PO₄/mol RLC (Fig. 4A 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 PO₄/mol. LPA-induced RLC phosphorylation (Fig. 4B 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).

**Ca²⁺ requirement for RhoA and Rho-kinase activity.** We next sought to determine whether Ca²⁺ was required for agonist activation of the Rho/Rho-kinase pathway. Both thrombin and LPA induce a rise in cytosolic Ca²⁺ by stimulating direct entry of extracellular Ca²⁺ and coupling via a G protein receptor-dependent pathway to stimulate inositol 1,4,5-trisphosphate production, which releases Ca²⁺ from internal stores (53). To ascertain the involvement of Ca²⁺ 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 Ca²⁺ as outlined under EXPERIMENTAL PROCEDURES. To confirm that the BAPTA-AM/thapsigargin effectively blocked LPA- and thrombin-induced increases in Ca²⁺, cells were loaded with fluo 4, and the increase in intracellular Ca²⁺ was monitored by confocal microscopy as outlined in EXPERIMENTAL PROCEDURES. Figure 5 demonstrates representative Ca²⁺ tracings of agonist-stimulated REF-52 cells in the presence and absence of Ca²⁺ chelation. Fluo 4-loaded monolayers treated with the Ca²⁺ ionophore ionomycin showed an immediate influx of Ca²⁺ that rapidly declined within 2 min (Fig. 5A). In contrast, cultures loaded with BAPTA-AM/thapsigargin showed no rise in cytosolic Ca²⁺ upon ionomycin treatment (Fig. 5B), confirming that the Ca²⁺ chelation protocol effectively quenched intracellular Ca²⁺. Both thrombin (Fig. 5C) and LPA (Fig. 5E) induced an immediate rise in intracellular Ca²⁺ that peaked within 1 min and rapidly returned to basal levels. As with ionomycin, BAPTA-AM/thapsigargin blocked the rise in cytosolic Ca²⁺ induced by both agonists (Fig. 5, D and F). To determine whether the Ca²⁺ 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. 5G) or 1 μM LPA (Fig. 5I). Upon thrombin and LPA treatment, the magnitude and duration of Ca²⁺ response in REF-52 cells embedded within collagen gels were similar to the response elicited in monolayer cultures. Both thrombin and LPA caused an immediate rise in intracellular Ca²⁺, 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 Ca²⁺ induced by both agonists (Fig. 5, H and J).
Table 2. Distribution and stoichiometry of thrombin induced RLC phosphorylation in REF-52 cells

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<td>4.0±1.2</td>
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<td>RLC phosphorylation, mol PO₄/mol RLC</td>
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<td>0.41±0.05</td>
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Values are means ± SE of 3 separate experiments. Monolayers were stimulated with 1 μM 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 PO₄/mol RLC) is calculated with the formula M/M₀ = 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

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<td>36±2.6</td>
<td>42±4.0</td>
<td>46±3.2</td>
<td>45±3.1</td>
<td>50±3.6</td>
<td>49±0.4</td>
<td>44±4.8</td>
</tr>
<tr>
<td>Monophosphorylated</td>
<td>31±2.9</td>
<td>46±0.8</td>
<td>43±4.3</td>
<td>43±1.0</td>
<td>43±0.6</td>
<td>43±1.8</td>
<td>41±2.4</td>
<td>47±2.3</td>
</tr>
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<td>Diphasphorylated</td>
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<td>18±2.2</td>
<td>15±1.1</td>
<td>11±2.2</td>
<td>12±3.0</td>
<td>7.0±1.7</td>
<td>10±2.9</td>
<td>9±2.8</td>
</tr>
<tr>
<td>RLC phosphorylation, mol PO₄/mol RLC</td>
<td>0.33±0.03</td>
<td>0.82±0.05</td>
<td>0.73±0.04</td>
<td>0.65±0.06</td>
<td>0.67±0.06</td>
<td>0.57±0.06</td>
<td>0.61±0.04</td>
<td>0.65±0.08</td>
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</table>

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 an anti-myosin RLC antibody, and exposed to X-ray films, and the stoichiometry of RLC phosphorylation was calculated as described by Goeckeler and Wyssolmerski (23).
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 Cell-Tracker orange for 45 min, followed by an additional 45-min incubation 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).
phorylation increased by 408% to 0.94 (see Tables 2 and 3). Upon thrombin stimulation, RLC phosphorylation was measured at 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 PhosphoImager plate. Both thrombin and LPA upregulated Ca2+-independent Rho-kinase catalytic activity.

cytosolic Ca2+ 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 PO4/mol RLC (Table 5), whereas cells in Ca2+-complete media exhibited a basal phosphorylation of 0.31 ± 0.02 to 0.33 ± 0.03 mol PO4/mol RLC (see Tables 2 and 3). Upon thrombin stimulation, RLC phosphorylation increased by 408% to 0.94 ± 0.02 mol PO4/mol RLC by 5 min and returned to baseline levels by 2 h poststimulation (Table 5), in contrast to thombin treatment in the presence of Ca2+ 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 Ca2+ chelation (Fig. 7A).

Figure 8A 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 PO4/mol RLC by 5 min, whereas peak phosphorylation in the presence of Ca2+ occurred at 2.5 min, achieving levels of 0.82 ± 0.05 mol PO4/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 isofoms of MLCK; 2) both thrombin and LPA upregulate a Ca2+-independent increase in RhoA and Rho-kinase activity; and 3) thrombin and LPA elicit a Ca2+-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. 9A) or 1 U/ml thrombin (Fig. 9B) in the continuous

<table>
<thead>
<tr>
<th>Control</th>
<th>1 min</th>
<th>2.5 min</th>
<th>5 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>1</td>
<td>47±18</td>
<td>30±8</td>
<td>40±12</td>
</tr>
<tr>
<td>LPA</td>
<td>1</td>
<td>9±0</td>
<td>9±0</td>
<td>2±0</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 separate experiments. REF-52 monolayers were incubated in Ca2+-free HBS containing 10 μM BAPTA-AM/1 μM thapsigargin for 1 h 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.
The presence of Y-27632 showed a modest 8- and 12-dyne increase in tension, respectively. The small rise in tension elicited by thrombin (Fig. 9B) was maintained for the duration of the experiment, whereas LPA-induced tension returned to baseline within 10 min (Fig. 9A).

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 9A, 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. 10A, lanes 1 and 3) and for LPA from 0.21 ± 0.08 to 0.07 ± 0.01 mol PO_4/mol RLC (Fig. 10B, 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. 10A, lane 4) or LPA (Fig. 10B, 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. 10A, lane 2) or LPA alone (Fig. 10B, 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...
Table 5. Stoichiometry of Ca$^{2+}$-independent agonist-stimulated REF-52 RLC phosphorylation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2.5 min</th>
<th>5 min</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>0.23±0.02</td>
<td>0.81±0.04</td>
<td>0.94±0.02</td>
<td>0.80±0.09</td>
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<td>0.35±0.01</td>
<td>0.26±0.02</td>
<td>0.23±0.06</td>
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</tbody>
</table>
| LPA had an effect inhibiting thrombin-induced isometric tension. Results are representative

inset). A second dose of LPA resulted in a rapid rise in tension (control). 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.

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).

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 results 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

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Agonist</th>
<th>Y-27632</th>
<th>Y-27632+Agonist</th>
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<tr>
<td>Thrombin</td>
<td>0.15±0.02</td>
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<td>LPA</td>
<td>0.21±0.08</td>
<td>0.56±0.06</td>
<td>0.07±0.01</td>
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</table>

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

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, recombiant 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.

GRANTS

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