The effects of the small GTPase RhoA on the muscarinic contraction of airway smooth muscle result from its role in regulating actin polymerization.

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Zhang W, Du L, Gunst SJ. The effects of the small GTPase RhoA on the muscarinic contraction of airway smooth muscle result from its role in regulating actin polymerization. Am J Physiol Cell Physiol 299: C298–C306, 2010. First published May 5, 2010; doi:10.1152/ajpcell.00118.2010.—The small GTPase RhoA increases the Ca2+ sensitivity of smooth muscle contraction and myosin light chain (MLC) phosphorylation by inhibiting the activity of MLC phosphatase. RhoA is also a known regulator of cytoskeletal dynamics and actin polymerization in many cell types. In airway smooth muscle (ASM), contractile stimulation induces MLC phosphorylation and actin polymerization, which are both required for active tension generation. The objective of this study was to evaluate the primary mechanism by which RhoA regulates active tension generation in intact ASM during stimulation with acetylcholine (ACh). RhoA activity was inhibited in canine tracheal smooth muscle tissues by expressing the inactive RhoA mutant, RhoA T19N, in the intact tissues or by treating them with the cell-permeant RhoA inhibitor, exoenzyme C3 transferase. RhoA inactivation reduced ACh-induced contractile force by ~60% and completely inhibited ACh-induced actin polymerization but inhibited ACh-induced MLC phosphorylation by only ~20%. Inactivation of MLC phosphatase with calyculin A reversed the reduction in MLC phosphorylation caused by RhoA inactivation, but calyculin A did not reverse the depression of active tension and actin polymerization caused by RhoA inactivation. The MLC kinase inhibitor, ML-7, inhibited ACh-induced MLC phosphorylation by ~80% and depressed active force by ~70% but did not affect ACh-induced actin polymerization, demonstrating that ACh-stimulated actin polymerization occurs independently of MLC phosphorylation. We conclude that the RhoA-mediated regulation of ACh-induced contractile tension in ASM results from its role in mediating actin polymerization rather than from effects on MLC phosphatase or MLC phosphorylation.

cytoskeleton; calcium sensitization; myosin light chain phosphorylation; myosin light chain phosphatase; calyculin A; myosin light chain kinase

THE EFFECTS OF THE SMALL GTPase RhoA in regulating the Ca2+ sensitivity of smooth muscle contraction are well documented and have been described for many smooth muscle tissue types, including airway smooth muscle (10, 14, 15, 19, 21, 26, 34, 35, 45, 47, 53). The effects of RhoA on the Ca2+ sensitivity of contraction have been ascribed to its role in regulating the catalytic activity of smooth muscle myosin light chain (MLC) phosphatase (23, 33, 47, 53). MLC phosphatase activity can be regulated by the downstream RhoA effector, ROCK (Rho kinase), which can inhibit MLC phosphatase by phosphorylating and inhibiting activity of its regulatory subunit, myosin phosphatase subunit 1 (MYPT1), or by phosphorylating the inhibitory peptide of MLC phosphatase, CPI-17 (27, 47). The inhibition of MLC phosphatase results in the augmentation of agonist-induced MLC phosphorylation, which has been proposed as a mechanism for increasing smooth muscle contractility (19, 30, 47, 53).

RhoA is also widely recognized as a key regulator of actin cytoskeletal dynamics and assembly and can couple the activation of G protein-coupled receptors to cytoskeletal functions in a variety of cell types, including airway smooth muscle cells (5, 11, 22). Contractile agonists have been shown to stimulate actin polymerization in a number of smooth muscle cells and tissues, and the critical role of actin polymerization in regulating active tension development in smooth muscle is well documented (4, 8, 12, 16, 18, 22, 29, 38, 63, 64, 67). The inhibition of actin polymerization using either pharmacologic or molecular approaches has been shown to depress tension development in many smooth muscles with little or no effect on MLC phosphorylation or cross-bridge cycling (16, 38, 42, 43, 49, 51, 57–59, 64, 65). However, there is little information regarding the role of RhoA in the regulation of actin polymerization induced by the contractile stimulation of smooth muscle tissues, and the degree by which the effects of RhoA inhibition on contractile activation and tension development reflect its role in actin dynamics.

In the present study, we inhibited RhoA activity in airway smooth muscle tissues by expressing the inactive RhoA mutant, RhoA T19N, in the intact tissues or by treating them with the cell-permeant RhoA inhibitor, exoenzyme C3 transferase. We measured the effects of RhoA inhibition on agonist-induced tension development, actin cytoskeletal dynamics, and MLC phosphorylation and evaluated the relative importance of these RhoA-mediated processes in the regulation of the tension development. We found that the effects of RhoA on tension development in airway smooth muscle tissues activated by acetylcholine (ACh) result from its role in the regulation of actin polymerization rather than from its effects on the regulation of MLC phosphorylation.

EXPERIMENTAL PROCEDURES

Preparation of smooth muscle tissues and measurement of force.

Mongrel dogs (20–25 kg) were anesthetized with pentobarbital sodium (30 mg/kg iv) and quickly exsanguinated in accordance with procedures approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. A segment of the trachea was immediately removed and immersed in physiological saline solution (PSS) (composition in mM: 110 NaCl, 3.4 KCl, 2.4 CaCl2, 0.8 MgSO4, 25.8 NaHCO3, 1.2 KH2PO4, and 5.6 glucose). Smooth muscle strips (1.0 × 0.2–0.5 × 15 mm) were dissected free of connective and epithelial tissues. For the measurement of contractile force, muscle tissues were attached to force transducers and maintained within a tissue bath in PSS at 37°C.

Transfection of smooth muscle tissues with plasmids.

The constructs for wild-type human RhoA and the mutant human RhoA

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Asn-19 have been previously described (54). The cDNAs encoding human RhoA wild-type and hemagglutinin (HA)-RhoA Asn-19 mutant were subcloned into the mammalian expression vector pcDNA3.1. *Escherichia coli* (BlueScript) transformed with these plasmids were grown in Luria-Bertani medium, and plasmids were purified by alkaline lysis with SDS by a purification kit from Qiagen.

Plasmids were introduced into tracheal smooth muscle strips by the method of reversible permeabilization (59, 64). After equilibration of the tissues and establishment of a muscle length for the generation of maximal isometric force ($L_0$), muscle strips were attached to metal mounts to maintain them at $L_0$. The strips were incubated successively in each of the following solutions: solution 1 (at 4°C for 120 min) containing (in mM) 10 EGTA, 5 Na$_2$ATP, 120 KCl, 2 MgCl$_2$, and 20 N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES); solution 2 (at 4°C overnight) containing (in mM) 0.1 EGTA, 5 Na$_2$ATP, 120 KCl, 2 MgCl$_2$, 20 TES, and 10 µg/ml plasmids; solution 3 (at 4°C for 30 min) containing (in mM) 0.1 EGTA, 5 Na$_2$ATP, 120 KCl, 10 MgCl$_2$, 20 TES; and solution 4 (at 22°C for 60 min) containing (in mM) 100 NaCl, 3.4 KCl, 0.8 MgSO$_4$, 25.8 NaHCO$_3$, 1.2 KH$_2$PO$_4$, and 5.6 dextrose. Solutions 1–3 were maintained at pH 7.1 and were aerated with 100% O$_2$. Solution 4 was maintained at pH 7.4 and was aerated with 95% O$_2$, 5% CO$_2$. After 30 min in solution 4, CaCl$_2$ was added gradually to reach a final concentration of 2.4 mM. The strips were then incubated in a CO$_2$ incubator at 37°C for 2 days in serum-free DMEM containing 5 mM Na$_2$ATP, 100 µM penicillin, 100 µg/ml streptomycin, and 10 µg/ml plasmids.

**Western blot analysis.** Pulverized muscle strips were mixed with extraction buffer containing 20 mM Tris-HCl at pH 7.4, 2% Triton X-100, 0.2% SDS, 2 mM EDTA, phosphatase inhibitors (2 mM sodium orthovanadate, 2 mM molybdate, and 2 mM sodium pyrophosphate), and protease inhibitors (2 mM benzamidine, 0.5 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Each sample was centrifuged for the collection of supernatant, and the supernatant was then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris·HCl, pH 6.8, 10% glycerol, and 0.01% bromophenol blue) for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose, after which the nitrocellulose membrane was blocked with 5% milk for 1 h and probed with primary antibodies against proteins of interest followed by horseradish peroxidase-conjugated IgG (Amersham Biosciences). Proteins were visualized by enhanced chemiluminescence.

**Assessment of RhoA activation.** The activation of RhoA was determined by using a pull-down assay for activated RhoA (Cytoskeleton, Denver, CO). Pulverized muscle tissues were mixed with lysis buffer containing 50 mM Tris·HCl at pH 7.5, 10 mM MgCl$_2$, 0.5 M NaCl, and 1% Triton X-100 for 5 min at 4°C. The extracted proteins were reacted with a peptide for the glutathione S-transferase-tagged Rho-binding domain region of Rhotekin, which has a high affinity for GTP-Rho. Activated GTP-bound RhoA was affinity-purified by glutathione beads and quantitated by Western blot.

**Measurement of MLC phosphorylation.** Frozen muscle strips were immersed in dry ice-precolded aceton containing 10% (v/vol) trichloroacetic acid and 10 mM dithiothreitol. Proteins were extracted in 8 mM urea, 20 mM Tris base, 22 mM glycine, and 10 mM dithiothreitol. Phosphorylated and unphosphorylated MLCs were separated by gelatin-urea polyacrylamide gel electrophoresis, transferred to nitrocellulose, then immunoblotted for smooth muscle MLC (64, 65). The proportions of phosphorylated and unphosphorylated MLCs were quantitated by scanning densitometry.

**Analysis of F-actin and G-actin.** The relative proportions of F-actin and G-actin in smooth muscle tissues were analyzed using an assay kit from Cytoskeleton as previously described (64–66). Briefly, each of the tracheal smooth muscle strips was homogenized in 200 µl of F-actin stabilization buffer (50 mM PIPES, pH 6.9, 50 mM NaCl, 5 mM MgCl$_2$, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween-20, 0.1% β-mercaptoethanol, 0.001% antifoam, 1 mM ATP, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 10 µg/ml benzamidine, and 500 µg/ml tosyl arginine methyl ester). Supernatants of the protein extracts were collected after centrifugation at 150,000 g for 60 min at 37°C. The pellets were resuspended in 200 µl of ice-cold water containing 10 µM cytochalasin D and then incubated on ice for 1 h to depolymerize F-actin. The resuspended pellets were gently mixed every 15 min. Four microliters of supernatant (G-actin) and pellet (F-actin) fractions were subjected to immunoblot analysis using anti-actin antibody (clone AC-40; Sigma). The relative amounts of F-actin and G-actin were determined using densitometry.

**Statistical analysis.** Comparisons between two groups were performed using paired or unpaired Student’s t-tests. Comparisons among multiple groups were performed using ANOVA. Values refer to the number of cells or tissue strips used to obtain mean values. *P* < 0.05 was considered statistically significant.

**RESULTS**

The RhoA inhibitors, exoenzyme C3 transferase and RhoA T19N, inhibit ACh-induced RhoA activation and tension development in tracheal smooth muscle tissues. The role of RhoA in ACh-induced tension development was evaluated in canine tracheal smooth muscle tissues by inhibiting RhoA activation using the cell-permeable C3 exoenzyme, which ADP-ribosylates the Asn-41 residue of RhoA to inactivate it (1, 46) (Fig. 1). Tracheal smooth muscle tissues were incubated with C3 exoenzyme at concentrations ranging from 2.5 to 20.0 µg/ml overnight in DMEM. RhoA activation was measured in treated and untreated tissues using a pull-down assay for activated RhoA. RhoA activity increased significantly in response to 10–3 M ACh stimulation and was significantly inhibited at all concentrations of C3 exoenzyme (Fig. 1, A and C). Treatment of tissues with C3 exoenzyme significantly depressed force in response to ACh at all concentrations; force was reduced to <50% of that in untreated muscles at C3 concentrations above 5 µg/ml (Fig. 1, B and C). The degree of inhibition of tension development caused by C3 treatment corresponded to the degree of inhibition of RhoA activation.

In a separate set of tracheal smooth muscle tissues, the activation of RhoA was inhibited by expressing the RhoA mutant, RhoA T19N, which acts as an inhibitor of RhoA activation (54) (Fig. 2). Plasmids encoding RhoA T19N with an HA epitope were introduced into tracheal smooth muscle tissues by reversible permeabilization. Sham-treated tissues were subjected to the reversible permeabilization procedure without plasmids. RhoA T19N expressed in muscle tissues could be detected by immunoblot (Fig. 2A). Expression of RhoA T19N significantly inhibited both RhoA activation and contractile force in response to 5-min stimulation with 10–3 M ACh (Fig. 2, B–E). The degree of inhibition of RhoA activity and contractile force caused by the expression of RhoA T19N was similar to that in tissues incubated in 5 µg or more C3 exoenzyme.

**RhoA inactivation inhibits actin polymerization in response to ACh in smooth muscle tissues.** The effect of RhoA inactivation on ACh-induced actin polymerization was determined by analyzing the proportions of G-actin and F-actin in extracts.
from unstimulated and ACh-stimulated muscle tissues expressing RhoA T19N, wild-type RhoA (RhoA WT), or subjected to sham treatment (Fig. 3). ACh induced similar increases in the ratio of F-actin to G-actin in untreated tissues and in tissues expressing RhoA T19N, wild-type RhoA (RhoA WT), or subjected to sham treatment (Fig. 3). ACh induced similar increases in the ratio of F-actin to G-actin in untreated tissues and in tissues expressing RhoA T19N, wild-type RhoA (RhoA WT), or subjected to sham treatment (Fig. 3).
expressing RhoA WT. Inhibition of RhoA activity by the expression of RhoA T19N or treatment of tissues with C3 exoenzyme completely inhibited the increase in actin polymerization stimulated by ACh.

RhoA inactivation causes a small inhibition of MLC phosphorylation in response to ACh. MLC phosphorylation in response to 10⁻⁵ M ACh was compared in muscle tissues transfected with the plasmids encoding RhoA WT or RhoA T19N, and in sham-treated tissues. The expression of RhoA T19N significantly depressed the increase in MLC phosphorylation induced by ACh; however, the amount of inhibition of MLC phosphorylation was small (Fig. 4, A and B). Treatment of muscles with 10 μg/ml C3 exoenzyme caused a similarly small but significant depression of ACh-induced MLC phosphorylation (Fig. 4C). Tension development in the same tissues was depressed to <40% of that in sham-treated or RhoA WT-treated tissues after treatment with RhoA T19N or C3 (Fig. 4D).

RhoA inactivation inhibits ACh-induced MLC phosphorylation in tracheal muscle tissues through its effect on MLC phosphatase. RhoA catalyzes the phosphorylation of regulatory subunit 1 of myosin phosphatase (MYPT1) at Thr695 and/or Thr850 by ROCK, which inhibits activity of the catalytic subunit of MLC phosphatase (53). We found that MYPT1 Thr850 phosphorylation induced by ACh was inhibited in tracheal muscle tissues treated with RhoA T19N but not in sham-treated or RhoA WT-treated tissues (Fig. 5, A and B). This suggests that the modest inhibition of MLC phosphorylation in RhoA inactivated tissues resulted from a reduction in the inhibitory effect of RhoA on myosin phosphatase activity.

We evaluated the contribution of RhoA-mediated MLC phosphatase inhibition to the depression of contractile force and MLC phosphorylation in RhoA-inactivated tissues. Muscle tissues in which RhoA was inactivated were treated with calycin A, a PP1/PP2A inhibitor that inactivates the catalytic subunit of MLC phosphatase (25). Treatment with calycin A restored ACh-induced MLC phosphorylation in RhoA-inactivated tissues to the same level observed in sham-treated tissues (Fig. 5C), but the depression of ACh-induced force generation in Rho-inactivated tissues was unaffected by treatment with calycin A (Fig. 5D). Calycin A did not affect the inhibition of actin polymerization caused by RhoA inhibition (Fig. 5E). These results demonstrate that the inhibition of ACh-induced force generation caused by RhoA inactivation in canine tracheal smooth muscle does not result from the depression of MLC phosphorylation. Our results suggest that the inhibition of force caused by RhoA inactivation resulted from the inhibition of actin polymerization rather than from effects of RhoA activity on MLC phosphatase and MLC phosphorylation.

Inhibition of MLC phosphorylation does not inhibit actin polymerization. We also evaluated the possibility that MLC phosphorylation contributes to the regulation of actin polymerization. Tissues were treated with 30 μM of the MLC kinase inhibitor, ML-7, for 2 h, which resulted in a 70% inhibition of ACh-stimulated MLC phosphorylation and a 70% depression of force generation (Fig. 6, A and B). Inhibition of MLC phosphorylation had no effect on ACh-induced actin polymerization (Fig. 6C).

DISCUSSION

Our results demonstrate that activation of the small GTPase RhoA plays a critical role in the regulation of force generation in intact airway smooth muscle tissues activated by mescanin stimulation, and that RhoA activation regulates contractile tension in these tissues primarily by activating pathways that regulate actin polymerization. Previous studies have demonstrated that the polymerization of a small pool of actin during contractile activation is necessary for agonist-induced tension generation in airway smooth muscle, and this actin polymerization can be inhibited without affecting stimulus-induced MLC phosphorylation (16, 38, 64). In the present study, we found that the inhibitory effects of RhoA activation on myosin light chain phosphatase contributed to the increase in MLC phosphorylation induced by ACh; however, this effect was small in the airway smooth muscle tissues and did not contribute significantly to overall tension generation.

Studies demonstrating a role for RhoA-mediated signaling pathways in the Ca²⁺ sensitization and contraction of smooth muscle tissues have relied heavily on the use of permeabilized smooth muscle tissue preparations, in which bacterial toxins such as epidermal cell differentiation inhibitor (EDIN), exoenzyme C3 transferase, and clostridium difficile toxin B, which functionally inactivate Rho proteins, can be introduced into the muscle cells (15, 21, 26, 36, 41, 45, 47, 53, 55). In these permeabilized muscle tissue preparations, intracellular Ca²⁺ is...
maintained constant while tension is measured in response to receptor activation, so Ca\(^{2+}\)-dependent signaling pathways are not activated. These studies have shown that G protein-coupled receptor agonists activate Rho-mediated signaling pathways.

**Fig. 4.** Inactivation of RhoA in tracheal muscle tissues causes a small reduction in ACh-induced myosin light chain (MLC) phosphorylation. MLC phosphorylation and active tension were measured in muscles strips transfected with RhoA T19N, RhoA WT, or sham-treated after stimulation with ACh for 5 min or no stimulation. A: representative immunoblots of unphosphorylated and phosphorylated 20-kDa smooth muscle MLC in unstimulated and ACh-stimulated muscle strips treated with RhoA WT or RhoA T19N or sham treated. B: mean values for MLC phosphorylation in response to ACh in sham-treated tissues and RhoA WT-treated tissues. Expression of the RhoA T19N significantly inhibited MLC phosphorylation in response to ACh (n = 11). Dashed line shows basal level of MLC phosphorylation in unstimulated tissues, which was not significantly different among treatment groups. C: mean values for MLC phosphorylation in response to ACh in untreated tissues and C3-treated tissues. Treatment with C3 significantly reduced MLC phosphorylation in response to ACh (n = 9). Dashed line shows basal level of MLC phosphorylation in unstimulated tissues, which was not significantly different among treatment groups. D: treatment of tissues with RhoA T19N (n = 11) or 10 µg/ml C3 (n = 9) significantly inhibited contractile force in response to 5-min stimulation with 10\(^{-5}\) M ACh relative to sham-treated or untreated smooth muscle tissues and RhoA WT-treated tissues. Values for force are normalized to values for sham-treated muscles. *Significant difference between tissues treated with RhoA T19N or C3 and sham-treated or untreated tissues (P < 0.05). Values are means ± SE.

**Fig. 5.** RhoA T19N inhibits MLC phosphorylation through its effect on MLC phosphatase in tracheal smooth muscle tissues. A: protein extracts from sham-treated muscle tissues or muscle tissues treated with RhoA WT or RhoA T19N were probed with anti-phospho-Thr850 myosin phosphatase (MYPT) antibody. Immunoblot shows phospho-MYPT and total MYPT in 6 extracts from muscle tissues that were sham treated or treated with RhoA WT or with RhoA T19, then stimulated with 10\(^{-5}\) M ACh or not stimulated. B: ACh stimulation significantly increased MYPT1 Thr850 phosphorylation in sham-treated tissues and RhoA WT-treated tissues. Expression of the RhoA T19N significantly inhibited the ACh-induced Thr850 phosphorylation of MYPT1 (n = 7). C: in ACh-stimulated tissues, RhoA T19N depressed MLC phosphorylation significantly compared with sham-treated tissues. Treatment with calyculin A prevented the inhibition of MLC phosphorylation by RhoA T19N (n = 9). D: RhoA T19N significantly depressed contractile force, and this depression was not reversed by calyculin A (n = 6). E: RhoA T19N inhibited the increase in F-actin/G-actin ratio in response to ACh stimulation in tissues with or without treatment with calyculin A. Calyculin A did not affect the increase in the F-actin/G-actin ratio in sham-treated tissues (n = 6). *Significant difference between ACh-stimulated tissues treated with RhoA T19N and sham-treated tissues (P < 0.05). Values are means ± SE.
that regulate MLC phosphorylation and tension generation. Extensive evidence from these studies indicates that RhoA can potentiate agonist-induced MLC phosphorylation by activating pathways that inhibit the catalytic activity of smooth muscle MLC phosphatase, and thereby enhance the Ca\(^{2+}\) sensitivity of the contractile apparatus. However, the importance of RhoA-mediated signaling pathways in tension development in smooth muscle tissues contracted under physiologic conditions in which receptor-activated Ca\(^{2+}\) signaling is maintained cannot be assessed from studies of permeabilized smooth muscle tissues. In the present study, we found that in tracheal smooth muscle tissues activated with ACh under physiologic conditions, the inactivation of RhoA with a cell-permeable C3 exoenzyme or the inactive RhoA mutant RhoA T19N resulted in a profound depression of contractile tension but caused only a small depression of MLC phosphorylation.

The role of RhoA in tension development under physiologic conditions of stimulation has been investigated in a few studies of vascular and intestinal muscles using membrane-permeable RhoA toxins (14, 36, 45). In intestinal muscle tissues, Lucius et al. (36) found that the inactivation of RhoA using exoenzyme C3 transferase inhibited the second peak of a biphasic contraction induced by carbachol and completely inhibited MLC phosphorylation but did not affect the initial rise in tension or Alter intracellular Ca\(^{2+}\) transients. In rabbit portal vein smooth muscle tissues, RhoA inactivation with cell-permeant C3 inhibited the tonic phase of a phenylephrine-induced contraction but did not inhibit the initial phasic increase in tension (14). While these studies demonstrated an important role for RhoA in the receptor-mediated contractile responses of these muscle tissues, they suggested that RhoA-mediated processes contributed mainly to the tonic or secondary phase of the contractile response and not to the initial phase of contractile activation and tension development. The observations of Lucius et al. (36) also suggest that RhoA activation is critical to the regulation of carbachol-induced MLC phosphorylation in intestinal smooth muscle. Our observations of the effects of both C3 transferase and RhoA T19N suggest that in airway smooth muscle, RhoA plays a critical role in both the phasic and the tonic phases of contraction, but that it has a relatively small effect on the regulation of MLC phosphorylation. Our findings suggest that the mechanisms by which RhoA regulates contraction may differ significantly among different types of smooth muscle tissues.

Smooth muscle MLC phosphorylation can be regulated by the RhoA effector, ROCK, which phosphorylates the regulatory subunit of MLC phosphatase, MYPT1, at Thr851, at Thr852, and Thr853, resulting in an augmentation of agonist-induced MLC phosphorylation. RhoA-mediated ROCK activation and regulation of MLC phosphatase have been demonstrated during the cholinergic activation of airway smooth muscles (24, 34, 50). We found that stimulation with ACh increased the phosphorylation of the myosin regulatory subunit (MYPT1) of MLC phosphatase at Thr850, and that ACh-induced MYPT1 phosphorylation was completely inhibited by the inactivation of RhoA. These observations are consistent with the premise that the activation of RhoA/ROCK pathway by ACh stimulation in the tracheal tissues augments MLC phosphorylation by inhibiting MLC phosphatase activity.

The deficit in MLC phosphorylation in RhoA-inactivated tissues was small relative to the profound depression of contractile tension caused by RhoA inactivation, which raised the question as to whether the Rho-mediated MLC phosphorylation contributes significantly to the regulation of contractile tension in airway smooth muscle. We evaluated the contribution of the RhoA-mediated MLC phosphorylation to active force development by treating the tissues with calyculin A to inhibit the activity of the catalytic subunit of MLC phosphatase (53). Calyculin A reversed the depression of ACh-induced MLC phosphorylation in RhoA-inactivated tissues, but it had no effect on the depression of contractile force caused by RhoA inactivation. We therefore concluded that RhoA activity does

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**Fig. 6.** Inhibition of MLC kinase prevents ACh-stimulated MLC phosphorylation and force development. The F-actin/G-actin ratios and MLC phosphorylation were determined in muscle strips that were treated with 30 μM ML-7 or untreated. A: treatment with ML-7 dramatically depressed ACh-induced MLC phosphorylation (n = 4). B: ML-7 significantly inhibited force development in response to ACh stimulation (n = 8). C: incubation of ML-7 did not affect the increase in F-actin/G-actin ratio in response to ACh stimulation (n = 6). *Significant difference between ML-7-treated tissues and untreated tissues (P < 0.05). Values are means ± SE.
not regulate contractile tension through its effects on MLC phosphorylation.

Rho GTPases are well known to regulate the formation of actin structures in motile cells and to regulate the assembly of focal adhesions and stress fibers in adherent cells in culture (5, 11, 17). Rho activation can regulate stress fiber formation in response to carbachol and other contractile agonists in primary cultures of airway smooth muscle cells (22, 52, 60). We therefore evaluated the possibility that RhoA might regulate the contractile tension in airway smooth muscle tissues primarily by regulating stimulus-induced actin polymerization. ACh-induced actin polymerization was completely suppressed in tracheal muscle tissues in which RhoA activation was inhibited by C3 exoenzyme or RhoA T19N, leading us to conclude that RhoA regulates ACh-induced tension development in airway smooth muscle primarily by regulating pathways that activate actin polymerization.

Contractile stimulation induces both actin polymerization and MLC phosphorylation in many types of smooth muscle. Both processes are necessary for tension development in tracheal smooth muscle as well as in a number of other types of smooth muscle tissues (3, 16, 29, 61, 63). Studies of airway smooth muscle and some other types of smooth muscle have shown that the treatment of smooth muscle tissues with pharmacologic or molecular inhibitors of actin polymerization have little or no effect on the increase in MLC phosphorylation induced by contractile stimulation, which suggests that the actin polymerization does not regulate contraction by contributing to processes that modulate MLC phosphorylation and cross-bridge cycling (16, 38, 42, 43, 49, 51, 57–59, 64, 65). However, the activation of MLC phosphorylation and actomyosin activity has been shown to be necessary for stimulus-induced actin polymerization in some non-muscle cultured cell lines (7). We assessed the possibility that MLC phosphorylation contributes to the regulation of actin polymerization by treating the tissues with ML-7, an inhibitor of MLC kinase. ML-7 almost completely inhibited ACh-induced MLC phosphorylation and dramatically suppressed contraction, but it had no effect on ACh-induced actin polymerization. These observations support the premise that actin polymerization and MLC phosphorylation induced by contractile stimulation of airway smooth muscle are regulated independently.

There are a number of possible molecular mechanisms by which RhoA activation might regulate actin polymerization and cytoskeletal dynamics in airway smooth muscle. RhoA has been shown to regulate phosphorylation of the adhesion junction protein paxillin and its kinase, focal adhesion kinase, in a number of non-muscle cell lines (13, 20, 39, 40, 48, 62). These proteins contribute to the regulation of stimulus-induced actin polymerization in airway smooth muscle (16, 56–58, 63). RhoA has also been shown to modulate the activity of integrin-linked kinase (28, 31), which binds to both β-integrins and paxillin at cell adhesion junctions, and can regulate airway muscle contractility and actin polymerization through its scaffolding activity (65). There is also evidence that RhoA can regulate activity of the actin dynamizing protein coflin (2, 37, 44), which is involved in regulating stimulus-induced actin filament polymerization and reorganization during the contraction of airway smooth muscle (67). RhoA activation may be involved in catalyzing multiple cytoskeletal processes that regulate actin dynamics and cytoskeletal organization during the contractile activation of airway smooth muscle.

While our study indicates that RhoA-mediated regulation of MLC phosphatase is relatively unimportant in regulating the contraction of airway smooth muscle, there is compelling evidence that it plays a significant role in the regulation of MLC phosphorylation and activation of the contractile apparatus in other smooth muscle tissue types, such as vascular smooth muscle (47, 53). Our observations may reflect differences in RhoA-mediated mechanisms that regulate smooth muscle contraction in different smooth muscle tissue types. Alternatively, given the paucity of studies in which RhoA inactivation has been studied in intact smooth muscle preparations, it may also reflect differences in the predominant effects of RhoA-mediated processes in permeabilized versus intact smooth muscle tissues. For example, in a study of intact rat cerebral arteries, Corteling and colleagues (9) found that the depletion of RhoA protein by small interfering RNA knockdown abolished UTP-induced vasoconstriction and inhibited actin polymerization but had little effect on MLC phosphorylation. They also concluded that the primary mechanism by which RhoA regulated tension development in these arteries was through its effects on actin polymerization.

There is evidence that the inflammatory mediator IL-13 can upregulate the expression of RhoA proteins in airway smooth muscle, and this has been proposed as a mechanism for airway smooth muscle hyperresponsiveness during airway inflammation (6). On the basis of its role in the regulation of airway smooth muscle contractility, RhoA has also been proposed as a potential target for asthma therapy (32). Our results suggest that downstream effectors in the RhoA pathway that regulate cytoskeletal dynamics may provide very effective targets for the mitigation of airway hyperresponsiveness with airway inflammation and asthma.

In summary, our results indicate that RhoA-mediated signaling pathways play an important role in the regulation of contractile tension in airway smooth muscle but that the major function of RhoA activation in active contraction is to regulate the polymerization of cytoskeletal actin that occurs with contractile activation. This effect appears to be independent of its effects on MLC phosphorylation, which are quite modest in this tissue and do not contribute significantly to contractile tension development or maintenance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


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RhoA AFFECTS MUSCLE CONTRACTION BY REGULATING ACTIN