The Small GTPase Cdc42 Regulates Actin Polymerization and Tension Development during Contractile Stimulation of Smooth Muscle*

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Contractile stimulation induces actin polymerization in smooth muscle tissues and cells, and the inhibition of actin polymerization depresses smooth muscle force development. In the present study, the role of Cdc42 in the regulation of actin polymerization and tension development in smooth muscle was evaluated. Acetylcholine stimulation of tracheal smooth muscle tissues increased the activation of Cdc42. Plasmids encoding wild type Cdc42 or a dominant negative Cdc42 mutant, Asn-17 Cdc42, were introduced into tracheal smooth muscle strips by reversible permeabilization, and tissues were incubated for 2 days to allow for protein expression. Expression of recombinant proteins was confirmed by immunoblot analysis. The expression of the dominant negative Cdc42 mutant inhibited contractile force and the increase in actin polymerization in response to acetylcholine stimulation but did not inhibit the increase in myosin light chain phosphorylation. The expression of wild type Cdc42 had no significant effect on force, actin polymerization, or myosin light chain phosphorylation. Contractile stimulation increased the association of neuronal Wiskott-Aldrich syndrome protein with Cdc42 and the Arp2/3 (actin-related protein) complex in smooth muscle tissues expressing wild type Cdc42. The agonist-induced increase in these protein interactions was inhibited in tissues expressing the inactive Cdc42 mutant. We conclude that Cdc42 activation regulates active tension development and actin polymerization during contractile stimulation. Cdc42 may regulate the activation of neuronal Wiskott-Aldrich syndrome protein and the actin-related protein complex, which in turn regulate actin filament polymerization initiated by the contractile stimulation of smooth muscle.

Actin polymerization is stimulated by contractile agonists in vascular and airway smooth muscle tissues and smooth muscle cells in culture (1–7). Inhibition of actin polymerization by pharmacologic inhibitors dramatically depresses active tension development in smooth muscle tissues (2, 7–10). The depression of tension development caused by the inhibition of actin polymerization occurs even though myosin light chain phosphorylation and cross-bridge cycling are fully activated, indicating that the polymerization of actin and the activation of cross-bridge cycling by myosin light chain phosphorylation are independently regulated events (2, 7).

Although actin polymerization plays an important role in regulating smooth muscle contraction, the mechanisms that regulate actin dynamics in smooth muscle tissues remain to be elucidated. The small GTPases Cdc42 and Rac have been shown to regulate the formation and organization of actin filaments associated with membrane ruffles, filapodium and lamellipodium, in cultured non-muscle cells such as fibroblasts and macrophages (11, 12), whereas Rho-mediated pathways have been shown to mediate stress fiber formation in cultured smooth muscle cells, macrophages, and fibroblasts (11, 13, 14). The role of Cdc42 in the regulation of actin polymerization in smooth muscle has not been investigated.

In vitro biochemical studies have shown that a seven-component protein complex, the Arp2/3 complex, promotes the nucleation of new actin filaments (15). In response to external stimulation, the Arp2/3 complex can be activated by N-WASp, which is a multidomain member of the WASp family of proteins that interacts with the Arp2/3 complex and globular (G) actin through its carboxyl-terminal domain to stimulate actin polymerization (15–18). Cdc42 can regulate the activation of N-WASp and its coupling to the Arp2/3 complex in vitro (17, 18). Once in GTP-bound form, Cdc42 interacts with N-WASp and initiates actin filament formation mediated by the Arp2/3 complex (16–18). There is evidence that N-WASp-mediated Arp2/3 complex activation regulates actin polymerization in tracheal smooth muscle (19).

The objective of this study was to evaluate the role of Cdc42 in the regulation of actin polymerization and force generation in smooth muscle tissues. Our results demonstrate that the activation of Cdc42 is necessary for actin polymerization and tension development in tracheal smooth muscle tissues in response to stimulation with acetylcholine and that it does not affect myosin light chain phosphorylation.

EXPERIMENTAL PROCEDURES

Preparation of Smooth Muscle Tissues—Mongrel dogs (20–25 kg) were anesthetized with pentobarbital sodium (30 mg/kg, intravenously) and quickly exsanguinated. A 12–15-cm segment of extra-thoracic trachea was immediately removed and immersed in physiologic saline solution (PSS) at 22 °C (composition: 110 mM NaCl, 3.4

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† The abbreviations used are: Arp, actin-related protein; N-WASp, neuronal Wiskott-Aldrich syndrome protein; G, globular; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; GST, glutathione S-transferase; PAK, p21-activated kinase; PBD, p21-activated kinase binding domain; MLC, myosin light chain; PIPES, 1,4-piperazineethanesulfonic acid; ACh, acetylcholine; PKC, protein kinase C.
mm KCl, 2.4 mM CaCl₂, 0.8 mM MgSO₄, 25.8 mM NaHCO₃, 1.2 mM KH₂PO₄, and 5.6 mM glucose). The solution was aerated with 95% O₂, 5% CO₂ to maintain a pH of 7.4. Rectangular strips of tracheal muscle 0.6–0.8 mm in diameter and 8–10 mm in length were dissected from the trachea after removal of the epithelium and connective tissue layer. The use of an appropriately sized strip was critical for maintaining muscle contractility during the incubation period and for the successful introduction of plasmids throughout the muscle strip. Each muscle strip was placed in PSS at 37 °C in a 25-ml organ bath and attached to a Grass force transducer. At the beginning of each experiment, maximal force development was determined by increasing muscle length progressively during successive contractions until the force of active contraction in response to a contractile stimulus reached a maximum and remained stable.

Plasmids encoding recombinant Cdc42 proteins were introduced into muscle strips according to experimental procedures described below. Muscle strips were then incubated for 2 days with plasmids in serum-free Dulbecco’s modified Eagle’s medium. The strips were then returned to PSS at 37 °C in 25-ml organ baths and attached to Grass force transducers for the measurement of isometric force. For biochemical analysis, muscle strips were frozen using liquid N₂-cooled tongs and then purified under liquid N₂ using a mortar and pestle.

**Introduction of Plasmids Encoding Recombinant Cdc42 into Tracheal Tissue—** The effects of human recombinant Cdc42 and the Asn-17 Cdc42 dominant negative mutants have been previously described (11, 20). The cDNAs encoding wild type Cdc42 and Asn-17 mutant were subcloned into the mammalian expression vector pcDNA 3.1 (Invitrogen). Escherichia coli (Bluescript) transformed with these plasmids were grown in LB medium, and plasmids were purified by alkaline lysis with SDS (maxipreparation) or by a kit from Invitrogen (N.A.P., 5). Then were incubated for 2 days with plasmids in serum-free Dulbecco’s modified Eagle’s medium. The strips were then returned to PSS at 37 °C in 25-ml organ baths and attached to Grass force transducers for the measurement of isometric force. For biochemical analysis, muscle strips were frozen using liquid N₂-cooled tongs and then purified under liquid N₂ using a mortar and pestle.

**Analysis of Protein Expression—** 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 leupeptin). The tissue was homogenized by sonication at pH 7.4, and after centrifugation at 10,000 rpm for 5 min at 4 °C, the supernatant was collected and boiled in sample buffer containing 2% SDS, 0.01% bromphenol blue, and 4% 2-thioglycerol. The proteins were size fractionated by SDS-PAGE and transferred to nitrocellulose, after which the blots were blocked with 5% milk and incubated with polyclonal affinity-purified rabbit myosin light chain 20 antibody. The primary antibody was reacted with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences). The membranes were washed with 5% milk and incubated with a secondary antibody conjugated to horseradish peroxidase. The blots were visualized by ECL and quantified by scanning densitometry.

**Assessment of Protein Interactions by Co-immunoprecipitation—** Ten small muscle strips that were pooled after identical treatment by reversible permeabilization or a large muscle strip that had not been treated by reversible permeabilization was used for each measurement of co-immunoprecipitation. Muscle extracts containing equal amounts of protein were preclarified for 30 min with 50 µl of 10% protein A-Sepharose (Sigma). The preclarified extracts were centrifuged at 4,000 rpm for 2 min. The extracts were incubated 2–3 h with goat polyclonal antibody against N-WASP (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated for 2 h with 125 µl of 10% protein G-Sepharose (Sigma) conjugated to rabbit anti-goat Ig. The immune complexes were washed four times in a buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100. All procedures of immunoprecipitation were performed at 4 °C. The immunoprecipitates were separated by SDS-PAGE followed by transfer to nitrocellulose membranes. The nitrocellulose membranes were divided into two parts; the lower part was probed with monoclonal antibody for Cdc42 (BD Biosciences), stripped and reprobed with polyclonal Arp2 antibody (Santa Cruz Biotechnology). The upper part was probed with rabbit anti-goat Ig. Proteins were quantitated by scanning densitometry of immunoblots.

**Statistical Analysis—** All statistical analysis was performed using Prism 4 software (GraphPad Software, San Diego, CA). Comparison among multiple groups was performed by one-way analysis of variance followed by Tukey’s multiple comparison test. Differences between all pairs of groups were analyzed by Student-Newman-Keuls test or Dunn’s method. Values of p refer to the number of experiments used to obtain each value. *p < 0.05 was considered to be significant.
RESULTS

Effect of ACh Stimulation on Cdc42 Activation in Smooth Muscle Strips—We assessed the effect of contractile stimulation on the activation of Cdc42 in smooth muscle tissues. Tracheal smooth muscle strips were stimulated with 10−5 M ACh for 1, 5, or 10 min, or they were unstimulated. Extracts of these muscle tissues were mixed with GST-PBD, which binds activated Cdc42. The active Cdc42/GST-PBD complex was separated using glutathione affinity beads. The amount of active Cdc42 was determined by immunoblot analysis using Cdc42 antibody. A, representative immunoblot illustrating the increased amount of activated Cdc42 in the ACh-stimulated muscle strips. B, the amount of activated Cdc42 in stimulated tissues is normalized to the amount obtained in unstimulated strips. Values represent means ± S.E. (n = 5–10). Contractile force is expressed as percent of maximal response to 10−5 M ACh (n = 5–10).

Expression of Recombinant Cdc42 Proteins in Smooth Muscle Tissues—To determine whether Cdc42 plays a role in regulating smooth muscle contraction, we introduced plasmids encoding wild type Cdc42 or the dominant negative Cdc42 mutant Asn-17 into smooth muscle strips by reversible permeabilization. These strips were then maintained in an incubator for 2 days. Expression of these untagged wild type and mutant Cdc42 in smooth muscle strips was assessed by immunoblot analysis.

Inhibition of Tension Development by Expression of the Cdc42 Mutant Asn-17—We assessed the effect of the expression of wild type or mutant Cdc42 on contractile force by evaluating ACh-induced contraction in muscle strips transfected with plasmids encoding wild type Cdc42 or the Asn-17 Cdc42 mutant. Force in response to 10−5 M ACh was compared before and after the 2-day incubation period. In muscle strips not transfected with plasmids and strips transfected with wild type Cdc42, contractile force in response to stimulation with ACh was similar before and after the 2-day incubation period (Fig. 3A). In muscle strips transfected with Asn-17 Cdc42 mutant, isometric force in response to stimulation with ACh was 10–20% of the preincubation force (Fig. 3B, n = 8–10, p < 0.05).

Effects of Asn-17 Cdc42 Mutant on the F-actin/G-actin Ratio in Smooth Muscle Tissues—We determined whether the Asn-17 Cdc42 mutant affects actin polymerization by assessing the effects of Asn-17 Cdc42 mutant on the F-actin/G-actin ratio in smooth muscle. Smooth muscle strips treated with plasmids encoding wild type Cdc42 and the Cdc42 mutant Asn-17 were stimulated with 10−5 M ACh for 5 min for the analysis of F-actin and G-actin. The ratio of F-actin/G-actin was analyzed...
by fractionation followed by Western blotting as described under "Experimental Procedures."

In the extracts of muscle tissues not treated with plasmids, the ratio of F-actin to G-actin was 3.98 ± 0.76 in unstimulated strips and 8.79 ± 2.30 in stimulated strips 5 min after stimulation with ACh (Fig. 4, p < 0.05, n = 4). Contractile stimulation led to a significant increase in the ratio of F-actin/G-actin in smooth muscle tissues expressing wild type Cdc42; however, contractile stimulation did not significantly increase the ratio of F-actin/G-actin in strips expressing Asn-17 Cdc42 mutant (Fig. 4, p > 0.05, n = 4).

Effect of Cdc42 Mutant on Myosin Light Chain Phosphorylation in Response to Contractile Stimulation—Smooth muscle strips treated with plasmids encoding wild type Cdc42, Asn-17 Cdc42 mutants, or not treated with plasmids were frozen for the analysis of myosin light chain phosphorylation. Myosin light chain phosphorylation was determined 5 min after contractile activation with 10^{-5} M ACh. Force and myosin light chain phosphorylation in response to ACh stimulation are at a steady state by this time (23, 24). Although force production was dramatically depressed (Fig. 3), the increase in myosin light chain phosphorylation in strips expressing Asn-17 Cdc42 mutant was similar to that of the muscle strips not treated with plasmids (Fig. 5). The mean increases in myosin light chain phosphorylation 5 min after ACh stimulation in the tissues not treated with plasmids and in the muscle tissues expressing wild type Cdc42 and Asn-17 Cdc42 mutant were not significantly different (Fig. 5, n = 4–8, p > 0.05).

The Interaction of Cdc42 with N-WASp in Smooth Muscle in Response to ACh Stimulation—In vitro studies have shown that the activity of N-WASp may be regulated by binding to the small GTPase Cdc42 (17, 25). Therefore we evaluated the effect of contractile stimulation on the association of Cdc42 with N-WASp in smooth muscle tissues. Extracts of smooth muscle strips that had been treated with 10^{-5} M ACh for 5 min were immunoprecipitated with N-WASp antibody. Blots of N-WASp immunoprecipitates (Fig. 6, A and B) indicated that these two proteins do not associate with N-WASp in smooth muscle tissues.

Stimulation with ACh resulted in an increase in the association of Cdc42 with N-WASp immunocomplexes from smooth muscle tissues. As shown in Fig. 6C, the amount of Cdc42 that co-immunoprecipitated with N-WASp was significantly greater in the ACh-stimulated tissues than in unstimulated strips. The mean ratio of Cdc42/N-WASp was significantly higher in immunoprecipitates obtained from stimulated muscles as compared with unstimulated muscle strips (Fig. 6D, n = 4, p < 0.05).
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**Fig. 5.** Effects of expression of recombinant Cdc42 on myosin light chain phosphorylation in smooth muscle tissues. Myosin light chain phosphorylation was measured in smooth muscle strips (10^{-5} M ACh, 5 min) expressing wild type Cdc42 and Cdc42 mutant Asn-17 (N17 Cdc42) and in strips not treated with plasmids (No Plasmids). There were no significant differences in myosin light chain phosphorylation in strips not treated with plasmids and in strips expressing wild type Cdc42 or Asn-17 Cdc42 mutant. Values shown are mean ± S.E. (n = 4–5).

**Fig. 6.** Increases in the association of Cdc42 with N-WASp in smooth muscle in response to ACh stimulation. A, representative immunoblots (IB) show that N-WASp antibody selectively immunoprecipitates (IP) N-WASp from protein extracts of smooth muscle strips. N-WASp from smooth muscle extracts was immunoprecipitated with N-WASp antibody. Blots of the N-WASp immunoprecipitates were probed with N-WASp antibody, stripped, and reprobed with antibodies against Rho and PKCα to control for nonspecific protein associations with the immunocomplexes (n = 3). No Rho or PKCα was found in the N-WASp immunoprecipitates. B, Rho and PKCα were present in whole tissue homogenates but not in immunoprecipitates (A), demonstrating their presence in muscle extracts prior to immunoprecipitation. C, representative immunoblots illustrating the effects of ACh stimulation on the association of Cdc42 with N-WASp. Extracts of smooth muscle strips that had been treated with 10^{-5} M ACh for 5 min or untreated were immunoprecipitated with N-WASp antibody. Blots of N-WASp immunoprecipitates were detected by use of Cdc42 antibody, stripped, and reprobed with N-WASp antibody. D, the ratio of Cdc42 to N-WASp in immunoprecipitates from ACh-stimulated tissues is normalized to that obtained from unstimulated strips. Values represent means ± S.E. (n = 4). The asterisk indicates significant higher ratio of Cdc42/N-WASp in the stimulated strips relative to the ratio in unstimulated tissues (p < 0.05).

**Fig. 7.** Stimulation with ACh increases the association of N-WASp with Arp2 in smooth muscle tissues. A, representative immunoblots illustrating the effect of ACh stimulation on the interaction of N-WASp with Arp2, a major component of the Arp2/3 complex. Tracheal smooth muscle strips were stimulated with 10^{-5} M ACh for 5 min, or they were not stimulated. Extracts of these muscle strips were immunoprecipitated with N-WASp antibody, and blots of the N-WASp immunoprecipitates (IP) were treated with Arp2 antibody, stripped, and reprobed with N-WASp antibody. B, the ratios of Arp2 to N-WASp in ACh-stimulated tissues are normalized to the values in unstimulated strips. Values represent means ± S.E. (n = 5). The asterisk indicates a significantly different ratio of Arp2/N-WASp in the stimulated strips relative to the ratio in unstimulated tissues (p < 0.05).

The Association of N-WASp with Arp2 in ACh-stimulated Smooth Muscle Strips—We evaluated the effects of contractile stimulation with ACh on the association of N-WASp with Arp2, a major component of the Arp2/3 complex. Tracheal smooth muscle strips were stimulated with 10^{-5} M ACh for 5 min or they were not stimulated. Extracts of these muscle strips were immunoprecipitated with N-WASp antibody, and N-WASp immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed using Arp2 antibody, stripped, and reprobed with N-WASp antibody.

Contractile stimulation led to an increase in the association of N-WASp with Arp2 in smooth muscle strips. More Arp2 was detected in N-WASp immunoprecipitates from tracheal muscle strips that had been stimulated with ACh (Fig. 7A). The ratio of Arp2/N-WASp in extracts from stimulated tissues was significantly higher than in extracts from unstimulated strips (Fig. 7B, n = 5, p < 0.05).

Effects of the Dominant Negative Asn-17 Cdc42 Mutant on the Association of N-WASp with Cdc42 and Arp2 in Smooth Muscle Tissues—We evaluated whether expression of the dominant negative Cdc42 mutant affects the increase in the association of Cdc42 with N-WASp and the activation of N-WASp as indicated by the association of N-WASp with the protein constituents of the Arp2/3 complex. Tracheal smooth muscle strips that were incubated for 2 days with plasmids encoding wild type Cdc42 or plasmids for Asn-17 Cdc42 mutant were stimulated with ACh for 5 min, or they were not stimulated. Untransfected muscle strips were also subjected to the same treatments. The association of N-WASp with Cdc42 and Arp2 was then determined by co-immunoprecipitation analysis.

In untransfected tissues and in tissues expressing wild type
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Fig. 8. Expression of Asn-17 Cdc42 mutant inhibited the interaction of N-WASp with Cdc42 and Arp2 in response to contractile stimulation. A, blots of N-WASp immunoprecipitates (IP) from unstimulated or stimulated muscle strips (10 inverted ACh, 5 min) expressing wild type Cdc42 (WT), the Asn-17 Cdc42 mutant (N17), or not treated with plasmids (NP) were probed with N-WASp antibody, stripped, and reprobed with antibodies against Cdc42 and Arp2. Ratios of Cdc42/N-WASp (B) and ratios of Arp2/N-WASp (C) in muscle strips expressing wild type Cdc42 or Asn-17 Cdc42 mutant are normalized to the ratios in unstimulated strips (US) not treated with plasmids (No Plasmids). The asterisk indicates significant higher ratios of Cdc42/N-WASp and ratios of Arp2/N-WASp in stimulated tissues relative to the ratios in corresponding unstimulated strips (p < 0.05).

cdc42, the amount of Cdc42 and Arp2 that associated with N-WASp immunoprecipitates was increased in response to ACh stimulation (Fig. 8). However, in muscle strips expressing the Asn-17 Cdc42 mutant, ACh stimulation of smooth muscle did not significantly increase the amount of Cdc42 and Arp2 that co-immunoprecipitated with N-WASp. The ratios of Cdc42 to N-WASp and Arp2 to N-WASp were significantly lower in ACh-stimulated muscle tissues expressing the Asn-17 Cdc42 mutant than in untransfected tissues or muscle strips expressing wild type Cdc42 (Fig. 8, B and C, n = 4, p < 0.05). This indicates that the dominant negative Cdc42 inhibits the association of endogenous Cdc42 with N-WASp and impairs the activation of N-WASp.

DISCUSSION

The essential role of actin polymerization in the regulation of active tension development in response to stimulation with contractile agonists has been documented in a number of smooth muscle tissues (2, 4, 7–10, 26); however, the signaling pathways by which contractile stimuli activate the polymerization of actin in smooth muscle remain to be determined. The results of the present study demonstrate that Cdc42 plays a critical role in the regulation of actin polymerization and active tension development in tracheal smooth muscle. Furthermore, we find that Cdc42 regulates actin polymerization without affecting myosin light chain phosphorylation. Our data suggest that Cdc42 may trigger actin polymerization in smooth muscle by activating N-WASp and the Arp2/3 complex. These results suggest a novel role for Cdc42 in the regulation of smooth muscle contraction.

To evaluate the function of Cdc42 in smooth muscle, we introduced plasmids encoding wild type Cdc42 and the dominant negative mutant Asn-17 Cdc42 into canine tracheal smooth muscle tissues by a method of reversible permeabilization (4, 21). The expression of the recombinant proteins in the smooth muscle tissues was verified by immunoblot analysis. Whereas expression of wild type Cdc42 in smooth muscle did not inhibit contractile force, expression of the dominant negative Cdc42 mutant inhibited active tension development in response to ACh stimulation. In muscle strips expressing wild type Cdc42, ACh stimulation caused significant increases in the ratio of F-actin to G-actin during contractile stimulation. However, the expression of the inactive Cdc42 mutant inhibited actin polymerization stimulated by the contractile agonist. These results suggest that Cdc42 activation is important in the regulation of actin polymerization and tension development in smooth muscle tissues in response to contractile stimulation.

We considered possible mechanisms by which Cdc42 might regulate actin polymerization in smooth muscle. In non-muscle cells, Cdc42 has been shown to regulate the formation and organization of actin structures such as lamellipodia and filopodia (12). These structures form at the leading edge of crawling cells and function to propel the edge of the membrane forward. The formation of these actin structures is also important for other cell functions including phagocytosis and cell adhesion. Biochemical studies in vitro and studies in fibroblasts have implicated Cdc42 in the activation of N-WASp, a ubiquitously expressed WASp-family protein that can activate the Arp2/3 complex-mediated formation of new actin filaments when activated by an external stimulus (17, 18). In recent studies we have observed that the introduction of an inhibitory N-WASp carboxyl-terminal peptide into tracheal muscle strips or expression of plasmids encoding this inhibitory peptide in tracheal muscle strips inhibits tension development and actin polymerization in response to stimulation with ACh (19).

In the present study, we assessed the effect of contractile stimulation on the association of Cdc42 with N-WASp in extracts from tracheal smooth muscle tissues. The association of N-WASp with both Cdc42 and Arp2 (a major component of the Arp2/3 complex) was greater in extracts from smooth muscle tissues that had been stimulated with ACh. The expression of the dominant negative Cdc42 Asn-17 mutant inhibited the increase in association of N-WASp with Cdc42 and the Arp2/3 complex in response to agonist stimulation. These results suggest that Cdc42 may regulate actin polymerization in smooth muscle by regulating the activation of N-WASp, which in turn activates the Arp2/3 complex.

We found that the F-actin/G-actin ratio in unstimulated smooth muscle tissues was ~4, and that contractile stimulation increased the ratio of F-actin/G-actin to ~8. This result suggests that the F-actin pool constitutes ~80% of the total actin in unstimulated tracheal smooth muscle tissues and that contractile stimulation increases F-actin to nearly 90% of the total actin. These results are similar to our previous findings in this tissue (2, 4, 26). The mechanism by which a relatively small change in the amount of actin might regulate tension development remains speculative. We have previously reported that the treatment of freshly dissociated tracheal smooth cells with
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Inhibitors that cause substantial inhibition of actin polymerization does not result alterations in the organization of actin filaments or cellular structure that are evident on electron micrographs (2). However, detailed quantitative morphometric analysis of electron micrographs of tracheal muscle tissues has provided evidence for an increase in the number of actin filaments in tracheal muscle after contractile stimulation (27).

In motile cells, WASp family proteins bind to the membrane upon activation where they activate the Arp2/3 complex to catalyze the nucleation of actin filaments that branch from existing actin filaments at a 70° angle (15, 17, 18). Thus, it is possible that the actin polymerization mediated by Cdc42 during the contractile stimulation of smooth muscle tissues occurs in local pools of submembranous actin. This actin might serve to regulate the connections of actin filaments to proteins at sites of cell adhesion and thereby regulate tension development and cytoskeletal organization.

We also considered the possibility that the inhibition of tension development in smooth muscle by dominant negative Cdc42 was caused by an inhibition of myosin light chain phosphorylation because myosin light chain phosphorylation is recognized as the primary mechanism for the activation of cross-bridge cycling and tension development during contractile stimulation (28, 29). However, we found that the expression of Asn-17 Cdc42 mutant protein had no effect on myosin light chain phosphorylation in response to ACh stimulation, indicating that this was unlikely to be the mechanism for its inhibition of active tension development in these tissues.

Several previous studies of permeabilized smooth muscle tissues and cultured cells have provided evidence that Cdc42 activation enhances the activity of PAK, which can phosphorylate myosin light chain kinase and inhibit its activity (30–33). These observations suggest that the activation of Cdc42 might depress myosin light chain phosphorylation because of the inhibition of myosin light chain kinase activity. Based on these observations the dominant negative Asn-17 Cdc42 would be expected to prevent PAK activation and myosin light chain kinase inhibition and thereby prevent the inhibition of myosin light chain phosphorylation. However, we found no effect of the overexpression of wild type Cdc42 on myosin light chain phosphorylation or active tension, suggesting that Cdc42 does not significantly affect the activity of myosin light chain kinase in intact tracheal smooth muscle tissues during muscarinic stimulation. This difference may reflect the dominance of Ca2+-mediated regulation of myosin light chain kinase in the tracheal tissues in which Ca2+-signaling is intact, or it could reflect differences in the experimental preparations used for these studies.

In additional studies, the introduction of a constitutively active Cdc42 mutant into β-escin-permeabilized rabbit portal vein smooth muscle tissues has been reported to inhibit the Ca2+-sensitization of tension development induced by the administration of a constitutively active RhoA mutant (34). This may also reflect an effect of Cdc42 on signaling processes present in permeabilized tissues stimulated by the introduction of an activated protein that are not as prominent in intact tissues subjected to pharmacological stimulation.

In summary, contractile stimulation with acetylcholine increases the activation of Cdc42 in tracheal smooth muscle tissues. The expression of a dominant negative Cdc42 mutant in smooth muscle tissues dramatically inhibits tension generation and prevents actin polymerization in response to contractile stimulation without significantly inhibiting myosin light chain phosphorylation. These results suggest that Cdc42 activation plays a critical role in the regulation of actin polymerization and active tension generation in tracheal smooth muscle. Cdc42 may regulate actin polymerization and tension in smooth muscle by regulating the activation of N-WASp and the Arp2/3 complex. These results suggest a novel signaling pathway for the regulation of agonist-induced contraction in smooth muscle.

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