Activation of the Arp2/3 complex by N-WASp is required for actin polymerization and contraction in smooth muscle

Wenwu Zhang, Yidi Wu, Liping Du, Dale D. Tang, and Susan J. Gunst
Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana
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Zhang, Wenwu, Yidi Wu, Liping Du, Dale D. Tang, and Susan J. Gunst. Activation of the Arp2/3 complex by N-WASp is required for actin polymerization and contraction in smooth muscle. Am J Physiol Cell Physiol 288: C1145–C1160, 2005. First published December 29, 2004; doi:10.1152/ajpcell.00387.2004.—Contractile stimulation has been shown to initiate actin polymerization in smooth muscle tissues, and this actin polymerization is required for active tension development. We evaluated whether neuronal Wiskott-Aldrich syndrome protein (N-WASp)-mediated activation of the actin-related proteins 2 and 3 (Arp2/3) complex regulates actin polymerization and tension development initiated by muscarinic stimulation in canine tracheal smooth muscle tissues. In vitro, the COOH-terminal CA domain of N-WASp acts as an inhibitor of N-WASp-mediated actin polymerization; whereas the COOH-terminal CA domain of N-WASp is constitutively active and is sufficient by itself to catalyze actin polymerization. Plasmids encoding EGFP-tagged wild-type N-WASp, the N-WASp VCA domain, or enhanced green fluorescent protein (EGFP) were introduced into tracheal smooth muscle strips by reversible permeabilization, and the tissues were incubated for 2 days to allow for expression of the proteins. Expression of the CA domain inhibited actin polymerization and tension development in response to ACh, whereas expression of the wild-type N-WASp, the VCA domain, or EGFP did not. The increase in myosin light-chain (MLC) phosphorylation in response to ACh was not affected by expression of either the CA or VCA domain of N-WASp. Stimulation of the tissues with ACh increased the association of the Arp2/3 complex with N-WASp, and this association was inhibited by expression of the CA domain. The results demonstrate that 1) N-WASp-mediated activation of the Arp2/3 complex is necessary for actin polymerization and tension development in response to muscarinic stimulation in tracheal smooth muscle and 2) these effects are independent of the regulation of MLC phosphorylation.

Wiskott-Aldrich syndrome protein; actin-related protein; tracheal muscle; cytoskeleton

Recent studies have documented a critical role for actin polymerization in regulating active tension development in smooth muscle. Contractile agonists stimulate actin polymerization in smooth muscle tissues and in smooth muscle cells in culture (2, 3, 7, 14, 18, 23, 45–47). Force development in smooth muscle is dramatically depressed by short-term exposure to inhibitors of actin polymerization (1, 23, 30, 52). However, the mechanisms by which contractile stimuli initiate the polymerization of actin in smooth muscle are unknown.

Wiskott-Aldrich syndrome protein (WASP) was originally described as a protein that could induce actin polymerization in fibroblasts in connection with the small GTPase cdc42 (43). In vitro studies demonstrated that WASP family proteins bind to the actin-related proteins 2 and 3 (Arp2/3) complex and induce Arp2/3 complex activation and the nucleation of actin filaments (21, 37). The Arp2/3 complex consists of one each of seven strongly associated subunits; Arp2 and Arp3 are actin-related proteins and function as the template for actin polymerization. The Arp2/3 complex is stable and remains intact in cells; all of the subunits are associated exclusively with the complex (13, 22, 33, 50). Although proteins other than WASp may regulate activity of the Arp2/3 complex, the evidence to date suggests that WASp family proteins are central to Arp2/3 complex activation (27).

In mammalian tissues, WASp is expressed exclusively in hematopoietic cells; however, the closely related protein neuronal Wiskott-Aldrich syndrome protein (N-WASp), first discovered in the brain, is expressed ubiquitously and is conserved across eukaryotic phyla (21, 24, 33). The demonstration that the binding of active cdc42 to N-WASp in vitro enhances its ability to activate the Arp2/3 complex provided evidence for an externally activated signaling pathway that could initiate actin filament nucleation (37). The WASp family proteins have been implicated in filopodia formation in fibroblast cell lines (25) and are essential for the motility of a number of microbial pathogens within their host cells (20, 41). However, despite the ubiquitous expression of these proteins in mammalian cells, there is little information about their function in physiological processes.

WASp family proteins share a conserved domain organization that includes a module at the extreme COOH terminus responsible for binding to and activating the Arp2/3 complex. This region consists of one or two verprolin homology regions (V; alternatively referred to as W for WASp homology), followed by a central sequence (C) and an acidic region (A). The V sequence binds to G-actin and is common to many actin-binding proteins (16, 26, 32, 36, 51). The COOH-terminal acidic A domain binds the Arp2/3 complex (13, 37). Both the Arp2/3 complex and G-actin must bind to the COOH-terminal domain VCA region of N-WASp for the nucleation of an actin filament to be initiated (26, 36, 51). The VCA COOH-terminal region of N-WASp is the minimal region required to bind to and activate the Arp2/3 complex (21). It is constitutively active and sufficient by itself to stimulate actin filament assembly by the Arp2/3 complex in reconstituted systems in vitro (16).

In vitro studies suggest that N-WASp remains in an inactive conformation until activated by upstream signaling molecules (13). When N-WASp is in its inactive conformation, the VCA domain is masked, whereas in the active conformation, the

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VCA domain is exposed and able to interact with both the Arp2/3 complex and G-actin and thereby initiate the nucleation of new actin filaments. The CA region by itself binds the Arp2/3 complex but lacks the binding sites for G-actin and acts as a dominant-negative inhibitor of WASp or N-WASp protein activation (16, 36, 37, 50).

In the present study, we expressed a peptide for the CA domain of N-WASp in smooth muscle tissues to evaluate the role of N-WASp in regulating activation of the Arp2/3 complex and actin polymerization in response to contractile stimulation in smooth muscle. Our results demonstrate that the inhibition of N-WASp-mediated activation of the Arp2/3 complex in smooth muscle tissues inhibits both actin polymerization and active tension generation in response to muscarinic stimulation. We conclude that the activation of N-WASp may be an essential step in the pharmacological activation of contraction in smooth muscle.

MATERIALS AND METHODS

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. Experiments were performed in accordance with the guidelines of 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 physiologic saline solution (PSS) at 22°C (in mM: 110 NaCl, 3.4 KCl, 2.4 CaCl₂, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 glucose). PSS was aerated with 95% O₂-5% CO₂ to maintain pH 7.4. Smooth muscle strips (1 mm wide × 0.2–0.5 mm thick × 15 mm long) were dissected free of connective tissue and epithelium. Muscle strips were placed in PSS at 37°C in a 25-ml organ bath and attached to a force transducer for measurement of force. At the beginning of each experiment, the optimal length for muscle contraction was determined by progressively increasing the length of the muscle until the active isometric force elicited by ACh reached a maximum (Lmax). For experiments involving the introduction of N-WASp fusion proteins or plasmids encoding N-WASp recombinant proteins, muscle strips were then subjected to the reversible permeabilization procedure described below. After the introduction or expression of recombinant proteins, the active isometric force in response to ACh at Lmax was determined.

Preparation of purified N-WASp VCA and CA fusion proteins and mammalian expression plasmids for wild-type N-WASp proteins and VCA and CA domains. The pGEX-2T vectors encoding glutathione S-transferase (GST) fusions of N-WASp VCA (amino acids 392–505) or CA (amino acids 450–505) peptides and pCS2+ plasmids encoding full-length bovine N-WASp were generously provided by Dr. Marc W. Kirschner (Harvard Medical School, Boston, MA). GST protein was fused to the NH₂ terminus of the N-WASp peptides. Purified N-WASp VCA and CA fusion proteins were prepared by expressing them in Escherichia coli. Proteins were affinity purified on glutathione-Sepharose beads using standard methodology.

The N-WASp VCA, N-WASp CA, and full-length wild-type N-WASp bovine cDNA were obtained by performing PCR using the pCS2+/H11001 plasmid and subcloned into the mammalian expression vector, pEGFP (Clontech, San Diego, CA). The cDNA encoding the CA (1,375–1,503) and the VCA domains of N-WASp (1,162–1,503) were obtained by performing PCR using the pCS2+ plasmid encoding full-length bovine wild-type N-WASp as the template. The primers were designed as follows: VCA upstream primer, GCTTCCAC-CAGGGAAAGCTTATGGCCCTTGCC; CA upstream primer, GCCCAGAGAAGCTTATGCGCGACCT; and the downstream primer for both VCA and CA, TCAGTCTAGATCCATCATCCTC. The PCR products containing N-WASp VCA or CA cDNA were inserted into the pEGFP vector at the HindIII and BamHI sites, in which N-WASp VCA or CA was fused to the COOH terminus of the EGFP. The full-length wild-type N-WASp cDNA (1–1,503) cDNA was obtained by cutting the pCS2+ plasmid using HindIII and SnaBI and subcloned into the pEGFP vector at the HindIII and KpnI sites by making the KpnI end blunt to produce a construct pEGFP-N-WASp(WT) in which the wild-type N-WASp protein was fused to the COOH terminus of the EGFP. Sequences of all newly made constructs were confirmed. E. coli (XL-1blue) transformed with N-WASp VCA or CA cDNA were grown in Luria-Bertani broth overnight. The bacterial cells were harvested by centrifugation at 4,000 rpm for 15 min at 4°C, and the plasmids were purified using standard methodology.

Introduction of recombinant N-WASp proteins and plasmids into smooth muscle strips. The purified N-WASp GST-tagged VCA and CA fusion proteins and mammalian expression vectors for the bovine N-WASp VCA domain, CA domain, and full-length wild-type N-WASp were introduced into tracheal smooth muscle strips using the method of reversible permeabilization (also called chemical loading) that we described previously (31, 46–48). After the optimal muscle length was determined, smooth muscle strips were placed on metal hooks under tension to maintain them at constant length. Strips were then incubated successively in each of the following solutions: solution 1 (4°C for 120 min) containing (in mM) 10 EGTA, 5 Na₂ATP, 120 KCl, 2 MgCl₂, and 20 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES); solution 2 (4°C overnight) containing (in mM) 0.1 EGTA, 5 Na₂ATP, 120 KCl, 2 MgCl₂, and 20 TES; and solution 4 (22°C for 60 min) containing (in mM) 110 NaCl, 3.4 KCl, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 dextrose. Solutions 1–3 were aerated with 100% O₂ to maintain pH 7.1, and solution 4 was aerated with 95% O₂-5% CO₂ to maintain pH 7.4. After 30 min in solution 4, CaCl₂ was added gradually to a final concentration of 2.4 mM. After proteins were introduced into the muscle strips, muscle tissues were returned to PSS at 37°C in 25-ml organ baths and attached to force transducers for the immediate measurement of isometric force. When the procedure was used to introduce plasmids into the strips, the muscle strips were then transferred to DMEM containing 5 mM Na₂ATP, 100 μM penicillin, 100 μg/ml streptomycin, and 10 μg/ml plasmids (N-WASp wild type, VCA or CA domains) and incubated for 2 days at 37°C to allow for expression of the recombinant proteins.

The efficiency of tissue transfection was evaluated by determining the percentage of cells dissociated from plasmid-treated muscle tissues that expressed GFP-labeled proteins (see method described in Dissociation of airway smooth muscle cells from tissue strips). A Zeiss LSM 510 laser scanning confocal microscope was used to view GFP fluorescence in living smooth muscle cells freshly dissociated from muscle tissues treated with plasmids encoding EGFP-tagged N-WASp VCA or CA peptides or untreated tissues. The GFP fluorescence was excited with a 488-nm argon laser light, and fluorescence emissions were collected at 500–530 nm with an Apo ×40 water-immersion lens objective (NA 1.2) (see Fig. 2).

Dissociation of airway smooth muscle cells from muscle tissue strips. After completion of the force measurements, smooth muscle cells were enzymatically dissociated from tracheal muscle strips for the analysis of cellular protein distribution using confocal microscopy (31). Tracheal muscle strips were minced and transferred into 5 ml of dissociation solution (in mM: 130 NaCl, 5 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 10 HEPES, 0.25 EDTA, 10 d-glucose, and 10 taurine, pH 7) with collagenase (type I, 400 μU/ml), papain (type IV, 30 μU/ml), bovine serum albumin (1 mg/ml), and dithiothreitol (DTT; 1 mM). All enzymes were obtained from Sigma (St. Louis, MO). The strips were then placed in a 37°C shaking water bath at 80 oscillations/min for 20–30 min, followed by washing three times with a HEPES-buffered saline solution (in mM: 130 NaCl, 5 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 20 HEPES, and 10 d-glucose, pH 7.4) and trituration with a pipette to...
liberate individual smooth muscle cells from the tissue. The solution containing the dissociated cells was poured over glass cover slips, and the cells were allowed to adhere to the coverslips for 2 h at room temperature. Cells were stimulated with ACh (10^{-3} M) for 5 min at 37 °C or left unstimulated and used as controls. Stimulated and unstimulated cells were fixed for 10 min in 4% paraformaldehyde (vol/vol) in phosphate-buffered saline (in mM: 137 NaCl, 4.3 NaHPO_4, 1.4 KH_2PO_4, and 2.7 KCl, pH 7.4).

**Immunofluorescence labeling.** Stimulated and unstimulated smooth muscle cells on coverslips were washed three times in Tris-buffered saline (TBS) containing 50 mM Tris, 150 mM NaCl, and 0.1% NaN_3 and permeabilized in 0.2% Triton X-100 dissolved in TBS for 2 min. Cells were washed again in TBS and placed in a blocking solution containing 2% bovine serum albumin for 1 h at room temperature. Cells were washed repeatedly and incubated with a primary antibody against N-WASP (rabbit polyclonal, H-100; Santa Cruz Biotechnology, Santa Cruz, CA), Arp3 (goat polyclonal, G-15; Santa Cruz Biotechnology), or STAT6 (mouse monoclonal clone 23; Transduction Labs, Lexington, KY) for 1 h at 37 °C. The specificity of these antibodies for the target protein was documented using immunoblot analysis (see Figs. 7C and 8C). Cells were washed again and incubated with a secondary antibody conjugated to a fluorescent green (Alexa 488) or red (Alexa 546) fluoroprobe (Molecular Probes, Eugene, OR) for 30 min at 37 °C. In some experiments, cells were double-labeled by reacting primary antibodies with secondary antibodies conjugated to different fluorescent dyes. Cells were washed to remove excess antibodies, and coverslips were mounted onto slides with mounting medium.

**Confocal microscopy and image analysis.** The cellular localization of fluorescently labeled N-WASP, Arp3, and STAT6 were evaluated in the dissociated smooth muscle cells using a Zeiss LSM 510 laser scanning confocal microscope with an Apo ×63 oil-immersion lens objective (1.4 numerical aperture). Alexa 488-labeled (green) proteins were excited with a 488-nm argon laser light, and fluorescence emissions were collected at 500–530 nm. The fluorescence of Alexa 546-labeled (red) proteins was excited with a helium-neon laser at 543 nm, and emissions were collected at 565–615 nm. The optical pinhole was set to resolve optical sections of 1 μm in cell thickness. The plane of focus was set midway between the bottom and the top of the cell. Fluorescence intensity measurements were standardized among all cells compared within a single experiment by maintaining the same confocal settings for each fluoroprobe.

Images of smooth muscle cells were analyzed for regional differences in fluorescence intensity of labeled proteins by quantifying the pixel intensity with a series of six cross-sectional line scans along the entire length of each cell as previously described (31). The area of the nucleus was excluded from the analysis. The ratio of pixel intensity between the cell periphery and interior was determined for each line scan by calculating the ratio of the average maximum pixel intensity at the cell periphery to the minimum pixel intensity in the cell interior. The ratios of pixel intensities between the cell periphery and the cell interior for all of the six line scans performed on a given cell were averaged to obtain a single value for the ratio for each cell. The ratio of fluorescence intensity at the cell periphery to that at the cell interior was compared in cells at rest and in cells stimulated with ACh (10^{-3} M).

**Immunoblot analysis.** Pulverized smooth muscle strips were mixed with extraction buffer containing 20 mM Tris-HCl, pH 7.4, 2% Triton X-100, 0.2% SDS, 2 mM EDTA, phosphatase inhibitors (in mM: 2 sodium orthovanadate, 2 molybdate, and 2 sodium pyrophosphate), and protease inhibitors (in mM: 2 benzamidine, 0.5 aprotinin, and 1 phenylmethylsulfonyl fluoride). Each sample was centrifuged for the collection of supernatant. Muscle extracts containing equal amounts of protein were precleared for 30 min with 50 μl of 10% protein A-Sepharose. The precleared extracts were centrifuged at 14,000 rpm for 2 min. The extracts were incubated overnight with antibody against N-WASP to immunoprecipitate endogenous N-WASP and then incubated for 2 h with 125 μl of a 10% suspension of protein A-Sepharose beads conjugated to rabbit anti-goat Ig. Immunocomplexes 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 of N-WASP 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 antibody for Arp3 (G-15) or Arp2 (H-84) (Santa Cruz Biotechnology), and the upper part was probed with antibody against N-WASP (N-15; Santa Cruz Biotechnology) or monoclonal myosin heavy chain (clone hSM-V; Sigma). Proteins were quantitated using scanning densitometry.

**Analysis of MLC phosphorylation.** Analysis of myosin light-chain (MLC) phosphorylation was performed as previously described (31, 44). Muscle strips were rapidly frozen at desired time points after contractile stimulation with ACh and then immersed in acetone containing 10% (wt/vol) trichloroacetic acid (TCA) and 10 mM DTT, which was precooled using dry ice. Strips were thawed in acetone-TCA-DTT at room temperature and then washed four times with acetone-DTT. Proteins were extracted for 60 min in 8 M urea, 20 mM Tris base, 22 mM glycine, and 10 mM DTT. MLCs were separated by performing glycerol-urea-PAGE and transferred to nitrocellulose. The membranes were immunoblotted with polyclonal affinity-purified rabbit MLC 20 antibody. Unphosphorylated and phosphorylated bands of MLCs were detected using scanning densitometry. MLC phosphorylation was calculated as the ratio of phosphorylated MLCs to total MLCs.

**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 (Denver, CO). 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 distilled water containing 1 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 ratios of F-actin to G-actin were determined using densitometry.

A standard curve for actin protein was determined using immunoblot analysis with anti-total actin antibody (clone AC-40; Sigma) for actin ranging from 0.02 to 0.4 μg. The immunoblots of G-actin and F-actin fractions were compared with the standard curve to determine the amounts of G-actin and F-actin in each fraction, and then the amounts of G-actin and F-actin in the whole muscle strip were determined by adding the amounts in the soluble and insoluble.
fractions. The proportions of F-actin and G-actin in unstimulated and stimulated muscle strips were calculated on the basis of the amount of actin in each fraction relative to the total actin protein in the muscle strip.

In some experiments, the membrane was blotted with anti-β-actin antibody (clone AC-15; Sigma) and then stripped and reblotted with anti-α-actin antibody (clone 1A4; Sigma) to evaluate the polymerization of actin isoforms in smooth muscle tissues.

**Statistical analysis.** Comparisons between two groups were performed using paired *t*-tests. Comparisons among multiple groups were performed using one-way ANOVA. Values refer to the number of cells or tissue strips used to obtain mean values. *P* < 0.05 was considered statistically significant.

### RESULTS

**Expression of the N-WASp CA domain in muscle strips inhibits force development.** We expressed EGFP-labeled N-WASp VCA or CA domains in tracheal muscle strips by introducing plasmids into the tissue by a reversible permeabilization procedure. Smooth muscle strips were also treated with plasmids encoding EGFP, full-length wild-type N-WASp, or no plasmids (control). After introduction of the plasmids, tracheal muscle strips were incubated for 2 days to allow for expression of the recombinant proteins. We evaluated contractile responses of tissues treated with plasmids or no plasmids to 10⁻⁵ M ACh (Fig. 1). There were no significant differences in force measured before incubation among the treated and untreated tissues (Fig. 1A). After 2 days of incubation, the contractile force generated in response to 5-min ACh stimulation was significantly inhibited in tissues expressing the N-WASp CA domain (Fig. 1), with a mean tension of 45.5 ± 2.8% of preincubation force (Fig. 1B) (*n* = 44; *P* < 0.01). In contrast, the contractile force in response to 5-min ACh stimulation was slightly but significantly increased to 112.4 ± 3.4% of preincubation force in tissues expressing the N-WASp VCA domain (Fig. 1B) (*n* = 44; *P* < 0.05). In muscle strips treated with EGFP plasmids, wild-type N-WASp plasmids, or no plasmids, there was no inhibition or potentiation of tension development in response to 5-min ACh stimulation.

The efficiency of tissue transfection was evaluated by determining the percentage of cells dissociated from plasmid-treated and untreated muscle tissues that expressed EGFP-labeled proteins (Fig. 2A). EGFP fluorescence was observed in 88.6 ± 4.2% of the cells dissociated from the N-WASp VCA plasmid-transfected tissues and in 89.2 ± 3.9% of the cells dissociated from tissues transfected with the N-WASp CA plasmid (a total of 180 cells in 4 separate experiments). No fluorescence was observed in cells dissociated from untreated tissues. Western blot analysis also was performed to confirm the expression of recombinant N-WASp domains in smooth muscle tissues (Fig. 2B). Because the N-WASp antibody did not react with the COOH-terminal domain peptides of N-WASp, the presence of the N-WASp peptides was confirmed using an antibody to the EGFP tag (polyclonal GFP antibody; Abcam, Cambridge, MA). The EGFP-tagged recombinant N-WASp VCA (∼37 kDa) and CA (∼32 kDa) peptides were detected in smooth muscle tissues treated with plasmids encoding N-WASp VCA or CA peptides, but not in untreated muscle strips or muscle strips treated with EGFP plasmids.

**Introduction of N-WASp CA peptides into muscle strips inhibits force development in tracheal smooth muscle tissue.** Purified GST fusion proteins for the VCA and CA domains of N-WASp were introduced into tracheal smooth muscle tissue strips by performing reversible permeabilization. Force in response to 10⁻⁵ M ACh was measured 1–2 h after the introduction of the N-WASp fusion proteins and compared with force measured before incubation (Fig. 3). In smooth muscle strips treated with the GST fusion proteins for the N-WASp CA peptide, isometric contractile force in response to 5-min stimulation with ACh was significantly inhibited (45.5 ± 2.8% of preincubation force; *P* < 0.05). Contractile force in response to ACh was significantly increased (112.4 ± 3.4% of preincubation force) in tissues treated with plasmids encoding the N-WASp CA domain, but it was not depressed in tissues treated with plasmids encoding the VCA domain, N-WASp(WT), EGFP alone, or tissues not treated with plasmids. *B* mean force of muscle strips incubated without plasmids (No plasmids), with plasmids encoding EGFP, N-WASp(VCA), N-WASp(WT), or the CA domain of N-WASp, or after incubation without plasmids. Contractile force in response to ACh was dramatically inhibited in tissues treated with plasmids encoding the N-WASp CA domain, but it was not depressed in tissues treated with plasmids encoding the VCA domain, N-WASp(WT), EGFP alone, or tissues not treated with plasmids. *B* mean force of muscle strips incubated without plasmids (No plasmids), with plasmids encoding EGFP, N-WASp(VCA), N-WASp(WT), or the CA domain of N-WASp, or after incubation without plasmids. Contractile force in response to ACh was significantly inhibited (45.5 ± 2.8% of preincubation force; *P* < 0.05). Contractile force in response to ACh was significantly increased (112.4 ± 3.4% of preincubation force) in tissues treated with plasmids encoding the VCA domain, N-WASp(WT), or the CA domain of N-WASp. There were no significant differences in the mean contractile responses of tissues treated with plasmids encoding EGFP, N-WASp(WT), and tissues not treated with plasmids. *Significantly different from mean force of tissues incubated without plasmids.*
reduced to 69.8 ± 3.8% of preintroduction force ($n = 14$; $P < 0.05$). In contrast, in strips treated with GST fusion proteins for the N-WASp VCA domain or with no proteins, isometric force in response to 5-min stimulation with ACh was not significantly different from the preintroduction force (Fig. 3B) ($n = 14$; $P > 0.05$). Protein extracts from muscle strips were examined using Western blot analysis to confirm that the N-WASp peptides were present in smooth muscle strips (Fig. 3C). The depression of force caused by the introduction of the GST fusion proteins for the N-WASp CA domain was somewhat less than that obtained when the N-WASp CA peptide was expressed in the tissue by introducing plasmids, possibly because the latter method resulted in higher levels of the CA domain peptides in the smooth muscle tissues. These results demonstrate that isometric contraction in response to ACh in smooth muscle strips also can be inhibited by short-term (~1 h) exposure to N-WASp CA peptides.

Fig. 2. Expression of recombinant N-WASp domains in smooth muscle tissues. A: GFP fluorescence of live cells freshly dissociated from muscle strips treated with plasmids encoding the VCA domain of N-WASp or the CA domain of N-WASp or from muscle strips not treated with plasmids. Left: immunofluorescence images; right: phase-contrast images of the same fields. The efficiency of tissue transfection was evaluated by determining the percentage of cells dissociated from plasmid-treated and untreated muscle tissues that expressed GFP-labeled proteins. Approximately 90% of the cells dissociated from the transfected tissues exhibited GFP fluorescence, whereas no fluorescence was observed in cells dissociated from untreated tissues. Red arrowheads (middle, right) point to two cells not expressing EGFP-tagged recombinant domains. B: immunoblots against GFP detected a 32-kDa protein in tissues treated with plasmids encoding the CA domain of N-WASp and a 37-kDa protein in tissues incubated with plasmids encoding the EGFP-tagged N-WASp VCA domain. These proteins were not detected in strips expressing GFP alone or in untreated muscle strips (No plasmids). The N-WASp antibody does not react with the COOH-terminal domains of N-WASp.
Expression of the N-WASp CA domain in muscle strips inhibits actin polymerization in tracheal smooth muscle tissues in response to contractile stimulation. Smooth muscle strips incubated without plasmids or with plasmids encoding the N-WASp VCA or CA domains were stimulated with $10^{-5}$ M ACh for 5 min and then fractionated to analyze changes in the proportion of F-actin to G-actin in muscle extracts. Figure 4A shows representative immunoblots of actin in the soluble and insoluble fractions of muscle extracts from a single set of muscle strips from one experiment; Fig. 4B shows mean data for 10 muscle strips treated under each condition. ACh stimulation significantly increased the ratio of F-actin to G-actin from $3.8 \pm 0.4$ to $7.8 \pm 0.5$ in smooth muscle strips not treated with plasmids ($n = 10; P < 0.05$). In muscle strips treated with plasmids encoding the N-WASp VCA domain, the ratio of F-actin to G-actin in response to ACh stimulation increased to a level similar to that in untreated muscle strips, from 4.5 $\pm$ 1.0 to 7.6 $\pm$ 0.7 ($n = 10; P < 0.05$). In contrast, ACh stimulation with plasmids ($n = 10$).
did not significantly increase the ratio of F-actin to G-actin in smooth muscle tissues expressing the N-WASp CA domain (Fig. 4B) \( (n = 10; P > 0.05) \).

We also compared the proportions of G-actin and F-actin in the treated and untreated muscle strips (Fig. 4C). In unstimulated smooth muscle tissues not treated with plasmids, G-actin content was 16.3 ± 1.5% of total cellular actin and F-actin content was 83.7 ± 1.5% of total actin. The amounts of G-actin or F-actin were similar in unstimulated VCA-treated and CA-treated muscle strips. In muscles not treated with plasmids, ACh stimulation decreased G-actin to 9.3 ± 1.5% and increased F-actin to 90.7 ± 1.5% of total actin. This represents polymerization of ~7% of the total cellular actin in response to contractile stimulation. ACh stimulation caused similar changes in the amounts of G-actin and F-actin in smooth muscle tissues treated with plasmids encoding the N-WASp VCA domain \( (n = 10; P < 0.05) \). However, in smooth muscle strips expressing the N-WASp CA domain, stimulation with ACh did not cause significant changes in the amounts G-actin or F-actin. In CA-treated muscle strips stimulated with ACh, the G-actin content was significantly higher, and the F-actin content was significantly lower, than that in the unstimulated or VCA-treated muscle strips. These results indicate that expression of the N-WASp CA domain inhibited actin polymerization in muscle strips in response to ACh stimulation.

In separate experiments, the G-actin and F-actin fractions were blotted using anti-α-actin antibody and anti-β-actin antibody to evaluate which isoforms of actin were undergoing polymerization in response to contractile stimulation. Our results showed that both α-actin and β-actin polymerized in response to ACh stimulation in tracheal smooth muscle tissues. In unstimulated muscle strips, the ratios of F-actin to G-actin were 3.0 ± 0.6 for β-actin and 2.8 ± 0.3 in α-actin. ACh stimulation increased the ratio of F-actin to G-actin to 6.3 ± 2.2 for β-actin and to 6.0 ± 0.6 for α-actin. There were no significant differences in the proportions of α-actin and β-actin isoforms of either G- or F-actin (Fig. 5) \( (n = 4; P > 0.05) \).

Expression of the N-WASp CA domain in muscle strips does not affect MLC phosphorylation in response to contractile stimulation in tracheal smooth muscle tissues. MLC phosphorylation was evaluated in muscle strips incubated either with plasmids encoding the N-WASp VCA or N-WASp CA domain or with no plasmids. Muscle strips were stimulated with \( 10^{-4} \) M ACh for 5 min and then frozen for the evaluation of MLC phosphorylation. Tension development was inhibited in tissues expressing the CA domain by 49.8 ± 3.7% of preincubation force; however, the increase in MLC phosphorylation caused by ACh was not inhibited. There were no significant differences in MLC phosphorylation in response to stimulation with ACh in muscles expressing the CA domain or in肌肉组织 not treated with plasmids (Fig. 6) \( (n = 8; P > 0.05) \).

N-WASP and Arp3 redistribute to the cell periphery in response to ACh in freshly dissociated smooth muscle cells. Figure 7A illustrates freshly dissociated smooth muscle cells in which the localization of endogenous N-WASp and Arp3 proteins were visualized using immunofluorescence microscopic imaging. Cells were fixed after 5-min stimulation with ACh \( (10^{-3} \) M) and double-stained using antibodies against N-WASp and Arp3. The specificity of the antibodies was confirmed by immunoblots of extracts of whole tissue strips obtained with antibodies to N-WASp and Arp3 (Fig. 7C). The effect of stimulation with ACh on the cellular localization of each of the fluorescently labeled proteins was assessed by quantifying the pixel intensity of fluorescence using multiple
line scans across the cell as previously described (31). The fluorescence intensity profile recorded from a single representative line scan for each cell is shown as an inset in Fig. 7A. In unstimulated cells, the fluorescence intensity profiles were relatively flat, indicating that the N-WASp and Arp3 distributions were uniform throughout the cell, with a somewhat higher concentration at the cell periphery. In contrast, in cells stimulated with ACh, fluorescence intensity was markedly higher at the periphery of each cell relative to the interior of the cell for both proteins. The cellular distribution of Arp3 was similar to that of N-WASp in both unstimulated and stimulated muscle cells.
Ratios of fluorescence intensity between the cell periphery and the cell interior for N-WASp and Arp3 were calculated for unstimulated cells and for cells stimulated with ACh (Fig. 7B). A total of 35 unstimulated and 38 stimulated smooth muscle cells obtained from six experiments were analyzed for each protein. Fluorescence intensities for both N-WASp and Arp3 were ~2.5 times higher at the cell periphery than in the cell interior after stimulation with ACh (P < 0.05). In contrast, in the unstimulated cells, the concentrations of N-WASp and Arp3 at the cell periphery were only slightly elevated relative to the cell interior.

Fig. 8. Effect of ACh stimulation on the localization of STAT6 and Arp3 in freshly dissociated smooth muscle cells. Smooth muscle cells were freshly dissociated from tracheal muscle tissue strips. Cells were stimulated with ACh for 5 min or left unstimulated. The distribution of STAT6 and Arp3 proteins was determined using immunofluorescence microscopy after fixation of the cells. Cells were double-immunostained with anti-STAT6 antibody and anti-Arp3 antibody. In unstimulated cells, both STAT6 and Arp3 were distributed throughout the cytoplasm of the cells. In cells stimulated with ACh, there was marked redistribution of Arp3 to the membrane, but STAT6 remained distributed throughout the cytoplasm. A: representative results showing one unstimulated and one ACh-stimulated cell. The cell nucleus was not stained and is represented by the dark area near the cell center. B: mean ratios of pixel intensity for STAT6 and Arp3 in unstimulated and stimulated smooth muscle cells. Protein distribution is expressed as the ratio of fluorescence intensity in the cell periphery to the cell interior. After ACh stimulation, there was a marked increase in the distribution of Arp3 at the membrane relative to the cell interior but no change in the distribution of STAT6 (unstimulated cells, n = 13 experiments; stimulated cells, n = 20 experiments). *Significant difference between the fluorescence intensity ratio for stimulated and unstimulated cells. C: immunoblot of extract from whole tissue strip showing the specificity of the STAT6 antibody.
N-WASP regulates actin polymerization and tension in smooth muscle
to those in the cell interior (~1.2 times higher for both N-WASp and Arp3). These results indicate that stimulation with a contractile agonist promotes the recruitment of both N-WASp and the Arp2/3 complex to the smooth muscle cell membrane.

We considered the possibility that the localization of Arp3 and N-WASp at the membrane of ACh-stimulated smooth muscle cells might result from a nonspecific effect of contraction on the distribution of cytoplasmic proteins in the isolated smooth muscle cells. To evaluate this possibility, we used immunofluorescence microscopy to compare the localization of Arp3 and the signal transduction protein, STAT6, in freshly isolated smooth muscle cells that were stimulated with ACh or left unstimulated (Fig. 8). The cells were fixed and double-immunostained for Arp3 and STAT6 and then visualized using confocal microscopy. STAT6 can be activated in airway smooth muscle by stimulating the interleukin-4 receptors (19), which causes STAT6 to redistribute to the nucleus. STAT6 would not be expected to undergo activation or translocation within the cell in response to stimulation with ACh.

Figure 8 illustrates the response of freshly dissociated smooth muscle cells that were fixed and double-stained for Arp3 and STAT6 unstimulated cells and cells stimulated with ACh. The results shown are typical of observations obtained using 13 unstimulated cells and 20 stimulated cells from smooth muscle tissues obtained from two separate experiments. The mean results for all cells are shown in Fig. 8B. Arp3 was distributed throughout the cytoplasm of unstimulated cells and relocalized to the cell membrane in response to stimulation with ACh. STAT6 was also distributed throughout the cytoplasm of unstimulated cells. However, in contrast to Arp3, the cellular localization of STAT6 did not change in response to stimulation with ACh.

Expression of the N-WASp CA domain inhibits the recruitment of Arp3 to the cell periphery in response to stimulation with ACh. Smooth muscle cells were freshly dissociated from muscle strips and double-stained with antibodies to N-WASp and Arp3 to evaluate the effect of the expression of the N-WASp VCA and CA domain peptides on the redistribution of endogenous N-WASp and Arp2/3 complex in response to contractile stimulation. In freshly dissociated smooth muscle cells expressing either wild-type N-WASp or the N-WASp VCA domain peptides, the fluorescence of both endogenous N-WASp and Arp3 was significantly increased at the cell periphery relative to the cell interior in cells stimulated with ACh (Fig. 9A). In these groups of cells, the fluorescence intensity ratios between the cell periphery and the cell interior for endogenous N-WASp and Arp3 ranged from 2.5 to 2.7 (Fig. 9B). In smooth muscle cells dissociated from muscle strips expressing the N-WASp CA domain peptides, endogenous N-WASp redistributed to the cell periphery in response to ACh stimulation (ratio, 2.7 ± 0.4; n = 32), but there was very little redistribution of Arp3 to the cell periphery (ratio, 1.4 ± 0.3; n = 32) (Fig. 9). Thus expression of N-WASp CA domain peptides in tracheal smooth muscle strips inhibited the recruitment of endogenous Arp3 to the cell periphery in response to ACh stimulation. In unstimulated cells expressing the VCA or CA domain peptides or wild-type N-WASp, Arp3 and N-WASp were distributed throughout the cytoplasm. The distribution of the endogenous N-WASp and Arp3 in these cells was not different from that in cells from untreated tissues.

The localization of the recombinant N-WASp, CA, and VCA proteins was compared with that of endogenous Arp3 in separate sets of dissociated cells by evaluating the distribution of GFP fluorescence and endogenous Arp3 (Fig. 10). In cells expressing recombinant wild-type N-WASp, both Arp3 and recombinant wild-type N-WASp redistributed to the cell membrane in response to ACh stimulation. In smooth muscle cells expressing the N-WASp VCA domain peptides, ACh stimulation elicited the redistribution of endogenous Arp3 to the cell membrane, but the VCA peptides remained distributed throughout the cytoplasm. In cells expressing the recombinant N-WASp CA domain, neither Arp3 nor the CA domain peptides localized at the cell membrane in response to stimulation with ACh. These results confirm that the expression of the CA domain of N-WASp in smooth muscle tissues inhibits the redistribution of the Arp2/3 complex to the cell membrane. They also confirm that the N-WASp VCA or CA domain peptides are not recruited to the cell membrane in response to ACh stimulation.

Contractile stimulation increases the amount of Arp2/3 proteins that coprecipitate with N-WASp in smooth muscle extracts. The effect of contractile stimulation of smooth muscle tissues on the association of the Arp2/3 complex with N-WASp was evaluated by immunoprecipitating N-WASp from extracts of smooth muscle strips and bloting them for Arp2 or Arp3. In extracts from tissue strips not treated with plasmids, ACh...
stimulation increased the amount of both Arp2 and Arp3 that coprecipitated with N-WASp by 1.99 ± 0.14 times and 1.86 ± 0.14 times, respectively, compared with unstimulated tissues (Fig. 11) \((n = 4 \text{ and } 5; \ P < 0.05)\). This indicates that contractile stimulation in smooth muscle tissues increases the association of the Arp2/3 complex with N-WASp.

In smooth muscle tissues expressing the N-WASp VCA domains, contractile stimulation also significantly increased the amount of Arp2 and Arp3 that coprecipitated with N-WASp by 1.66 ± 0.14 and 1.61 ± 0.15 times, respectively, compared with that of the tissues not treated with plasmids (Fig. 11) \((n = 4 \text{ and } 5; \ P < 0.05)\). The amounts of Arp2/3 complex proteins that coprecipitated with N-WASp in response to ACh stimulation in tissue strips expressing the N-WASp VCA domain and the tissues not treated with plasmids were not statistically significant \((P > 0.05)\). In extracts from smooth muscle strips expressing the N-WASp CA domain, ACh stimulation did not significantly increase the amounts of Arp2 or Arp3 in the N-WASp immunoprecipitates (Fig. 11) \((n = 4 \text{ and } 5; \ P > 0.05)\). These results suggest that the expression of the
Fig. 10. The redistribution of Arp3 and EGFP-tagged recombinant N-WASp or N-WASp peptides in ACh-stimulated freshly dissociated smooth muscle cells. Smooth muscle cells were freshly dissociated from muscle tissues expressing wild-type N-WASp or VCA or CA domain peptides of N-WASp and stimulated with ACh or left unstimulated. Arp3 immunofluorescence and GFP-labeled recombinant proteins were visualized in each cell. A: fluorescence images of endogenous Arp3, recombinant EGFP-N-WASp, EGFP-VCA peptides, or EGFP-CA peptides, and colocalization of Arp3 and EGFP recombinant proteins. In cells expressing WT N-WASp, there was a marked increase in fluorescence intensity at the cell periphery relative to the cell interior for both wild-type EGFP N-WASp and endogenous Arp3. In cells expressing the EGFP VCA peptides, the fluorescence intensity of Arp3 was increased at the cell periphery but the EGFP-VCA domain peptides were distributed throughout the cytoplasm. In cells expressing the EGFP N-WASp CA domain peptides, both endogenous Arp3 and the EGFP-CA peptides were distributed throughout the cytoplasm. B: mean ratios of fluorescence intensity between the cell periphery and the cell interior of endogenous Arp3 and EGFP recombinant proteins in ACh-stimulated and unstimulated cells. In unstimulated cells, Arp3 and EGFP recombinant proteins were distributed throughout the cytoplasm with protein distribution ratios slightly >1.0 (open bars). In cells expressing WT N-WASp (n = 10) or EGFP VCA peptides (n = 16), protein distribution ratios for Arp3 and EGFP-N-WASp were significantly higher for ACh-stimulated than for unstimulated cells. In ACh-stimulated cells expressing N-WASp CA domain peptides (n = 18), the protein distribution ratios for both Arp3 and EGFP were close to 1 and were not significantly higher than the ratios obtained in unstimulated cells. Cells from each treatment group were dissociated from tissues from three separate experiments.
population induces the polymerization of F-actin from a pool of extracts of smooth muscle strips expressing the N-WASp CA domain.

The amount of Arp3 or Arp2 that coprecipitates with endogenous N-WASp in blotted for N-WASp and Arp3 or Arp2. ACh stimulation did not increase the representative immunoblots of N-WASp immunoprecipitates from six muscles. DISCUSSION

WASp. The expression of the CA domain of N-WASp in muscle tissues inhibited active tension development stimulated by the administration of exogenous ACh by ~50%. Expression of the CA domain also inhibited ACh-induced actin polymerization as assessed by measuring the relative contents of G-actin and F-actin in stimulated and unstimulated muscle strips using a fractionation assay. In contrast, expression of the VCA domain of N-WASp, which is constitutively active in vitro, did not inhibit tension development or actin polymerization in these smooth muscle tissues.

The activation of N-WASp is thought to require its translocation and anchoring at the membrane by activator molecules such as cdc42 or phosphatidylinositol 4,5-bisphosphate (PIP2) (5, 6, 12, 24, 50). Upon activation, N-WASp undergoes a conformational change that allows it to bind to the Arp2/3 complex (16, 37). Binding of the Arp2/3 complex to N-WASp results in activation of the complex, enabling it to catalyze the polymerization of actin. To obtain further evidence that N-WASp and the Arp2/3 complex are activated during the contractile stimulation of smooth muscle, we assessed the effect of ACh on the association of Arp2/3 complex proteins with N-WASp in extracts from smooth muscle tissues. The amount of Arp2 or Arp3 that coprecipitated with N-WASp increased significantly in extracts from tissues that had been stimulated with ACh compared with extracts from unstimulated tissues, indicating that the association of the Arp2/3 complex with endogenous N-WASp is required for actin polymerization and tension development in smooth muscle tissue.

The expression of the CA domain of N-WASp in tracheal smooth muscle tissues prevented the increase in the association of endogenous N-WASp with Arp2 or Arp3 in ACh-stimulated tissues. Thus expression of the CA domain of N-WASp in these tissues is effective in inhibiting the activation of the Arp2/3 complex by N-WASp in response to stimulation with ACh. Further evidence for the activation of N-WASp and the Arp2/3 complex by ACh was obtained by evaluating changes in the localization of these proteins in muscle cells in response to contractile stimulation. Smooth muscle cells were enzymatically dissociated from muscle tissues, and the localization of N-WASp CA domain in smooth muscle tissues inhibits the association of the Arp2/3 complex with endogenous N-WASp.

DISCUSSION

Recent studies have provided evidence that contractile stimulation induces the polymerization of F-actin from a pool of G-actin in smooth muscle tissues (2, 3, 7, 18, 23, 46) and in cultured smooth muscle cells (2, 14, 17), as well as that the inhibition of actin polymerization inhibits active tension development in a variety of smooth muscle tissue types (2, 3, 14, 18, 23, 52). In the present study, we have demonstrated that activation of the Arp2/3 complex by the WASp family protein N-WASp is required for actin polymerization and tension development in smooth muscle tissue in response to stimulation with the contractile agonist ACh. These results demonstrate the importance of activation of the Arp2/3 complex by a WASp family protein in the physiological regulation of active tension development in response to receptor stimulation. The N-WASp-mediated activation of the Arp2/3 complex thus represents a novel signaling pathway for the regulation of tension generation in smooth muscle.

We evaluated the involvement of N-WASp in the regulation of contraction in smooth muscle by expressing a fusion protein encoding the GFP-tagged COOH-terminal domain of N-WASp, the CA domain, in tracheal smooth muscle tissues. In reconstituted actin polymerization assay systems in vitro, the CA domain of the WASp family proteins acts as a dominant-negative inhibitor for activation of the Arp2/3 complex by competing with full-length WASp or N-WASp proteins for binding to the Arp2/3 complex (16, 37). In tracheal smooth muscle tissues, expression of the CA domain of N-WASp in muscle tissues inhibited active tension development stimulated by the administration of exogenous ACh by approximately 50%. Expression of the CA domain also inhibited ACh-induced actin polymerization as assessed by measuring the relative contents of G-actin and F-actin in stimulated and unstimulated muscle strips using a fractionation assay. In contrast, expression of the VCA domain of N-WASp, which is constitutively active in vitro, did not inhibit tension development or actin polymerization in these smooth muscle tissues.

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Further evidence for the activation of N-WASp and the Arp2/3 complex by ACh was obtained by evaluating changes in the localization of these proteins in muscle cells in response to contractile stimulation. Smooth muscle cells were enzymatically dissociated from muscle tissues, and the localization of N-WASp CA domain in smooth muscle tissues inhibits the association of the Arp2/3 complex with endogenous N-WASp.
N-WASp and Arp proteins was visualized using immunofluorescence and confocal microscopy. After stimulation with ACh, the concentration of both N-WASp and Arp3 increased at the membrane, whereas both proteins were distributed more evenly throughout the cytoplasm in unstimulated cells.

In cells dissociated from tissues expressing the CA domain peptide of N-WASp, the redistribution of Arp3 to the membrane in response to ACh was inhibited. In these cells, stimulation with ACh resulted in the redistribution of endogenous N-WASp to the membrane, but Arp3 and the recombinant CA peptide remained distributed throughout the cytoplasm. This observation is consistent with in vitro studies that have suggested that the CA fragment of N-WASp binds to and sequesters the Arp2/3 complex and thereby prevents its activation (16). In contrast, the N-WASp VCA domain peptides did not inhibit the redistribution of Arp3 to the membrane in response to stimulation with ACh. Neither VCA nor CA N-WASp COOH-terminal peptides contain the centrally located N-WASp sequences required for N-WASp to bind to cdc42 and PIP2 and anchor at the membrane (12, 36, 37). Thus membrane localization of these COOH-terminal peptides would not be expected. Although the VCA domain of N-WASp can bind to the Arp2/3 complex, there is evidence that this interaction is transient and that the VCA domain of N-WASp is released from the Arp2/3 complex when actin polymerization is initiated (9, 49). Thus the VCA domain peptide would not be expected to sequester the Arp2/3 complex or to inhibit the activation of endogenous N-WASp.

Our immunofluorescence data provide further evidence that the N-WASp CA domain peptide inhibits the activation of the Arp2/3 complex by endogenous N-WASp in smooth muscle tissues in response to stimulation with ACh.

In tracheal smooth muscle, the inhibition of actin polymerization by cytochalasin or latrunculin results in a marked inhibition of active tension development without significantly affecting MLC phosphorylation (23). Moreover, actin polymerization also can be inhibited by preventing the phosphorylation of the scaffolding protein paxillin, which inhibits active contraction without affecting either MLC phosphorylation or myosin ATPase activity (47). These previous studies suggested that actin polymerization functions in the regulation of tension development separately from and independently of the activation of myosin ATPase activity and cross-bridge cycling. Our present results are consistent with our previous observations in that the inhibition of actin polymerization by the CA domain of N-WASp had no effect on the increase in MLC phosphorylation in response to agonist stimulation. Thus the effect of the CA domain in inhibiting active force development during contractile stimulation appears to result directly from the inhibition of actin polymerization rather than from effects on MLC phosphorylation or cross-bridge cycling.

The functional role of actin polymerization in the regulation of tension development in smooth muscle is not known. Estimates from our present study suggest that the pool of G-actin constitutes <20% of the total actin in unstimulated tracheal smooth muscle tissues. This estimate is slightly lower than that obtained in a previous study in which we estimated the G-actin pool to be ~30% of total actin (23), which may reflect better separation of soluble and polymerized actin in extracts of smooth muscle tissues using our current methods. Contractile stimulation decreased the amount of G-actin by ~40%, resulting in a twofold increase in the ratio of F-actin to G-actin. These values are consistent with our previous observations regarding tracheal muscle tissues (23, 46, 47) and with previous reports of smooth muscle cells in which contractile stimulation increased the ratio of F-actin (estimated by FITC-phalloidin staining) to G-actin (estimated by Texas Red-labeled DNase I staining) approximately twofold (15).

A critical question is how a 7–10% increase in the amount of F-actin might regulate tension development during contractile stimulation. The molar ratio of actin to myosin in smooth muscle is quite high: It has been reported to be as low as 8:1 in chicken gizzard and ~15:1 in vascular muscle and as high as 50:1 in isolated amphibian visceral muscle (8, 29, 42). Thus it seems unlikely that the polymerization of new actin would be necessary to form actin filaments to interact with myosin for the process of cross-bridge cycling. Our observation that the inhibition of actin polymerization does not disrupt myosin ATPase activity or MLC phosphorylation also supports the idea that the newly polymerized actin is not involved in signaling processes that regulate cross-bridge cycling or the formation of filaments that interact with myosin (23, 47). Furthermore, the inhibition of actin polymerization in smooth muscle tissues by either of the inhibitors cytochalasin or latrunculin does not result in evident alterations in cytoskeletal structure when the tissues are examined using electron microscopy (23).

Smooth muscle cells contain both α- and β-isoforms of actin (39). It has been proposed that myosin may interact preferentially with α-actin, while β-actin may sustain a structural function by cross-linking the contractile apparatus to dense bodies and dense plaques in smooth muscle (39). Using specific antibodies for actin isoforms in the present study, we found that both α-actin and β-actin isoforms undergo polymerization in response to contractile stimulation; thus the polymerization of actin during contractile activation is not selective for a specific actin isoform.

N-WASp-mediated actin polymerization may be involved in regulating smooth muscle cell structure and in forming connections to the cell membrane that are necessary to transmit tension from the contractile apparatus to the extracellular matrix. The Arp2/3 complex is a critical player in the initiation of actin polymerization that drives lamellipodium and filopodium protrusion in motile nonmuscle cells (40). In the motile cells, a local change of signal catalyzes the assembly of meshwork actin filaments at the cell membrane that propel the protrusion of the edge of the membrane (34, 40). The assembly of these actin-based structures is regulated by the small GTPases Rac and cdc42 (11, 28, 35), which can be activated by growth factors and integrin receptors. N-WASp and WASp are the only known effectors of cdc42 that can directly activate actin assembly through activation of the nucleating activity of the Arp2/3 complex. There is evidence that the activation of cdc42 is required for N-WASp-mediated actin polymerization in tracheal smooth muscle (45).

Smooth muscle lines hollow organs that undergo large changes in shape and volume under physiological conditions in vivo, and the muscle must rapidly adapt its compliance and contractility to accommodate external mechanical forces (10). Actin filaments attach to adhesion plaques at the membrane, where transmembrane integrins connect to the extracellular matrix, enabling the transmission of tension between the inside
and the outside of the cell (4, 38). The ability of smooth muscle to adapt its properties to changes in its length or shape may result from the reorganization of the actin filament network in a manner that is analogous to lamellipodia formation during cell spreading and cell migration in nonmuscle cells. The polymerization of new actin filaments and their attachment to adhesion sites along the membrane may provide a mechanism for the cell to adapt its structure to shape changes imposed by external forces. The connections formed by the new actin filaments may be necessary for the transmission of tension from the contractile apparatus to the extracellular matrix during active contraction.

In conclusion, our present study demonstrates that actin polymerization in smooth muscle requires N-WASP-mediated activation of the Arp2/3 complex and that this process plays an essential role in tension generation in response to a contractile stimulus. Only a small percentage (<10%) of total actin polymerizes in response to contractile stimulation. This actin may be critical for the transmission of tension from the contractile apparatus to adhesion sites on the membrane during contractile stimulation. Newly formed actin filaments may localize beneath the cell membrane and regulate changes in smooth muscle cell shape and stiffness in response to external mechanical forces.

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