Actin Depolymerization Factor/Cofilin Activation Regulates Actin Polymerization and Tension Development in Canine Tracheal Smooth Muscle*

Rong Zhao, Liping Du, Youliang Huang, Yidi Wu, and Susan J. Gunst
From the Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana 46202

The contractile activation of airway smooth muscle tissues stimulates actin polymerization, and the inhibition of actin polymerization inhibits tension development. Actin-depolymerizing factor (ADF) and cofilin are members of a family of actin-binding proteins that mediate the severing of F-actin when activated by dephosphorylation at serine 3. The role of ADF/cofilin activation in the regulation of actin dynamics and tension development during the contractile activation of smooth muscle was evaluated in intact canine tracheal smooth muscle tissues. Two-dimensional gel electrophoresis revealed that ADF and cofilin exist in similar proportions in the muscle tissues, and that ~40% of the total ADF/cofilin in unstimulated tissues is phosphorylated. Phospho-ADF/cofilin decreased concurrently with tension development in response to stimulation with acetylcholine (ACh) or potassium depolarization indicating the activation of ADF/cofilin. Expression of an inactive phospho-cofilin mimetic (cofilin S3E) but not wild type cofilin in the smooth muscle tissues inhibited endogenous ADF/cofilin dephosphorylation and ACh-induced actin polymerization. Expression of cofilin S3E in the tissues depressed tension development in response to ACh, but it did not affect myosin light chain phosphorylation. The ACh-induced dephosphorylation of ADF/cofilin required the Ca2+-dependent activation of calcineurin (PP2B). The results indicate that the activation of ADF/cofilin is regulated by contractile stimulation in tracheal smooth muscle and that cofilin activation is required for actin polymerization and tension development in response to contractile stimulation.

Cofilin, a 19-kDa protein, and the closely related protein actin depolymerization factor (ADF) are members of a family of “actin-dynamizing proteins.” These proteins play a critical role in the rapid adaptation of the actin cytoskeleton to localized cellular functions (1–3). The activation of ADF/cofilin is essential for cell motility and polarized cell migration.

The cytoskeletal organization of differentiated smooth muscle cells and tissues is dynamic, and it is regulated during contractile stimulation (4–6). Dynamic changes in cytoskeletal organization may enable smooth muscle cells to modulate their structure and contractility in response to changes in their external environment (6, 7). Actin polymerization can be triggered by contractile stimuli in many smooth muscle tissues, and tension development can be dramatically depressed by short term exposure to inhibitors of actin polymerization (5, 8–16). In airway smooth muscle, the inhibition of actin polymerization can inhibit tension development in the absence of an effect on myosin light chain phosphorylation, suggesting that actin polymerization regulates tension development by processes independent of cross-bridge cycling (5, 12, 13, 15).

Actin is present in both unassembled (globular, G) and filamentous (F) form in all cells. Actin monomers (G-actin) add preferentially to the fast growing (barbed) ends of the actin filaments; the availability of barbed ends is critical for the addition of G-actin monomers to existing actin filaments (3, 17). Cofilin activation enhances F-actin dynamics by increasing the dissociation of actin monomers from the pointed ends of actin filaments, which enhances the pool of available actin monomers (18), and by binding to F-actin and severing it to make new barbed ends available for polymerization and depolymerization. High concentrations of active cofilin can nucleate filament assembly (19, 20). The activity of ADF/cofilin is regulated by phosphorylation at a single site on the amino terminus, serine 3, which inhibits its activity. Phosphorylation at this site abolishes the ability of ADF/cofilin to bind to F-actin and thus inhibits its severing function (1, 21, 22).

We hypothesized that ADF/cofilin might play an important role in the regulation of actin dynamics in smooth muscle during contractile activation. In this study, we analyzed the effect of contractile activation on the phosphorylation of ADF/cofilin at Ser-3. To evaluate the function of ADF/cofilin in regulating actin dynamics and tension generation during contraction of smooth muscle tissues, we expressed an inactive cofilin phosphomimetic (cofilin S3E) in the tissues, which has minimal actin severing activity (23). Our results demonstrate that ADF/cofilin undergoes dephosphorylation in response to contractile stimulation in smooth muscle tissues and that ADF/cofilin dephosphorylation is necessary for both actin polymerization...
and active tension generation. We conclude that the activation of ADF/cofilin is a necessary step for the dynamic reorganization of actin that occurs during the contraction of smooth muscle tissues.

MATERIALS AND METHODS

Preparation of Smooth Muscle Tissues and Measurement of Force—Mongrel dogs (20–25 kg) were euthanized with pentobarbital sodium (30 mg/kg intravenously) and quickly exsanguinated. All 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 physiological saline solution (PSS) at 22 °C containing (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 a pH of 7.4. Smooth muscle was dissected free of connective tissue and epithelium and cut into strips (1 mm wide × 0.2–0.5 mm thick × 15 mm long). 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 (Lo) for muscle contraction was determined by progressively increasing the length of the muscle until the active isometric force elicited by ACh reached a maximum. All tissues were then maintained at Lo for 30–60 min without stimulation. For experiments involving the introduction of plasmids encoding cofilin proteins, muscle strips were then subjected to the reversible permeabilization procedure described below. Two days were then allowed for expression of the recombinant proteins, at which time the active isometric force in response to ACh at Lo was determined again.

Reagents—The following antibodies were used in these studies: mouse monoclonal anti-cofilin (BIOSOURCE), rabbit polyclonal anti-phospho-ADF/cofilin at serine-3 antibody (23), and rabbit polyclonal anti-ADF/cofilin antibody (reacts with both ADF and cofilin) (24) provided by Dr. James Bamberg, Colorado State University; mouse monoclonal anti-actin (Clone AC-40, Sigma); mouse monoclonal GAPDH (RDI, Concord, MA). Polyclonal myosin light chain antibody was custom-made by BABCO (Richmond, CA). pcDNA3.1 vectors (human cytomegalovirus as promoter) encoding human wild type cofilin and inactive mutant cofilin S3E were provided by Dr. J. R. Bamberg (Colorado State University, Fort Collins).

Transfection of Smooth Muscle Tissues with Plasmids—Plasmids were introduced into tracheal smooth muscle strips by the method of reversible permeabilization as described previously (13, 15, 25). After initial equilibration and contraction to 10⁻⁵ M ACh to obtain maximal force, muscle strips were attached to metal mounts at Lo. The strips were incubated successively in each of the following solutions: Solution 1 (at 4 °C for 120 min) containing (mM): 10 EGTA, 5 Na₂ATP, 120 KCl, 2 MgCl₂, and 20 TES; solution 2 (at 4 °C overnight) containing (mM): 0.1 EGTA, 5 Na₂ATP, 120 KCl, 2 MgCl₂, 20 TES, and 10 μg/ml plasmids; Solution 3 (at 4 °C for 30 min) containing (mM): 0.1 EGTA, 5 Na₂ATP, 120 KCl, 10 MgCl₂, 20 TES; and solution 4 (at 22 °C for 60 min) containing (mM): 110 NaCl, 3.4 KCl, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 dextrose. Solutions 1–3 were maintained at pH 7.1 and aerated with 100% O₂. Solution 4 was maintained at pH 7.4 and aerated with 95% O₂, 5% CO₂. After 30 min in Solution 4, CaCl₂ was added gradually to reach a final concentration of 2.4 mM. The strips were then incubated in a CO₂ incubator at 37 °C for 2 days in serum-free Dulbecco’s modified Eagle’s medium containing 5 mM Na₂ATP, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml plasmids encoding human wild type cofilin or the inactive mutant cofilin S3E.

Analysis of ADF/Cofilin Phosphorylation—ADF/cofilin isoforms and phosphorylation were analyzed by one-dimensional and by two-dimensional electrophoresis. Muscle tissues were rapidly frozen using liquid nitrogen-cooled tongs and pulverized using a mortar and pestle. Pulverized muscle tissues were mixed with extraction buffer containing the following: 20 mM Tris-HCl, pH 7.4, 2% Triton X-100, 0.4% SDS, 2 mM EDTA, 2 mM EGTA, phosphatase inhibitors (2 mM sodium orthovanadate, 2 mM molybdate, and 2 mM sodium pyrophosphate, 50 mM sodium fluoride) and protease inhibitors (2 mM benzamidine, 0.5 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride).

For one-dimensional electrophoresis, 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% bromphenol blue) for 5 min. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. To measure ADF/cofilin phosphorylation, the nitrocellulose membrane was simultaneously probed with antibodies to phospho-Ser-3 ADF/cofilin and cofilin, followed by fluorophore-conjugated anti-rabbit and antimouse immunoglobulins. Fluorescence signals were detected and analyzed using an Odyssey fluorescence scanner (LI-COR Biosciences, Lincoln, NE).

For two-dimensional PAGE, protein was precipitated from smooth muscle tissue protein extracts using a methanol/chloroform/water mixture (26). The precipitated total smooth muscle protein was redissolved in ReadyPrep two-dimensional sample buffer (Bio-Rad). Isoelectric focusing was performed in a PROTEAN IEF cell with 11-cm IPG strips pH 3–10 (Bio-Rad) according to the manufacturer’s instructions. The focused proteins were then separated by means of an 18% SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane (Bio-Rad). ADF and cofilin were detected using a polyclonal anti-ADF/cofilin antibody that reacts with both ADF and cofilin (24). The ratios of phosphorylated ADF and phosphorylated cofilin to total ADF and total cofilin and the amount of recombinant cofilin expression were analyzed by scanning densitometry.

Measurement of Regulatory Myosin Light Chain Phosphorylation—Frozen muscle strips were immersed in dry ice precooled acetone containing 10% w/v trichloroacetic acid and 10 mM dithiothreitol. Proteins were extracted in 8 M urea, 20 mM Tris base, 22 mM glycine, and 10 mM dithiothreitol. Phosphorylated and unphosphorylated myosin light chains (MLCs) were separated by urea-glycerol PAGE, transferred to nitrocellulose, and then probed using antibody to the 20-kDa myosin light chain (15, 27). Proteins were visualized by enhanced chemiluminescence (ECL). The ratio of phosphorylated to unphosphorylated MLC was determined 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 a standard assay kit (Cytoskeleton, Denver, CO) as described previously (15, 27). 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 MgCl₂, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 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 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. The ratios of F-actin to G-actin were determined using densitometry.

**Statistical Analysis**—Comparisons between the two groups were performed using paired Student’s t tests. Comparisons among multiple groups were performed using repeated measures analysis of variance. Values refer to the number of tissues used to obtain mean values. *p < 0.05* was considered statistically significant.

**RESULTS**

**Contractile Stimulation of Tracheal Smooth Muscle Tissues Induces ADF/Cofilin Dephosphorylation on Ser-3 (Fig. 1)**—Canine tracheal smooth muscle strips were maintained for 30 min without contractile stimulation and then contracted isometrically with 10⁻⁵ M ACh for 15 or 30 s or 1, 5, or 15 min or left unstimulated. ADF/cofilin phosphorylation on Ser-3 was elevated in unstimulated tissues and decreased significantly in response to ACh stimulation. The decrease in ADF/cofilin phosphorylation was evident 15 s (0.25 min) after stimulation with ACh and persisted for the duration of the contraction (Fig. 1, A and B). Differences in ADF/cofilin phosphorylation in ACh-stimulated and unstimulated tissues were statistically significant at all time points (*n* = 6, *p* < 0.05). The time course of the decrease in ADF/cofilin phosphorylation was similar to the time course of the increase in force development in response to ACh stimulation. ADF/cofilin phosphorylation 30 or 60 min after stimulation with ACh was not significantly different from ADF/cofilin phosphorylation at 15 min, suggesting that the activation of ADF/cofilin was sustained for the duration of the contraction (data not shown).

We also evaluated whether the decline in ADF/cofilin phosphorylation during contractile stimulation was dependent on a receptor-mediated mechanism. Muscles were stimulated with 60 mM KCl, and ADF/cofilin phosphorylation was analyzed 15 and 30 min after stimulation. ADF/cofilin phosphorylation decreased significantly to 61 ± 5% (*p < 0.05, n = 6*) in response to KCl stimulation, and the decrease in ADF/cofilin phosphorylation persisted for the 30 min duration of the contraction. The decrease in ADF/cofilin phosphorylation was less than during ACh stimulation, but force development in response to KCl stimulation was only about 60% of that observed with ACh.
**Expression of Cofilin S3E Inhibits Tension Development in Smooth Muscle Tissues**

We used two-dimensional gel electrophoresis to evaluate the effects of expression of cofilin S3E in the smooth muscle tissues treated with either cofilin WT or cofilin S3E, whether normalized to GAPDH or total cofilin. In contrast, ACh did not cause a significant decrease in the amount of phospho-cofilin. The amount of P-ADF/cofilin in unstimulated muscle tissues marked suppression of ADF/cofilin phosphorylation in muscle tissues treated with cofilin S3E; when normalized to GAPDH or total cofilin. Thus, expression of the cofilin S3E mutant in the muscle tissues markedly suppressed ADF/cofilin phosphorylation and activation in response to stimulation with ACh (Fig. 3, A and B).

We used two-dimensional gel electrophoresis to evaluate the effects of expression of cofilin S3E on the phosphorylation of both ADF and cofilin in response to ACh, and to quantify the amount of cofilin S3E expressed in smooth muscle tissue (Fig. 4, A and B). Cofilin S3E represented 34 ± 5% of the total ADF/cofilin (n = 4). The expression of cofilin S3E caused comparable inhibition of the dephosphorylation of both cofilin and ADF in response to ACh.

**Expression of Cofilin S3E Inhibits Tension Development in Smooth Muscle Tissues (Fig. 5)**—We evaluated the effects of expression of cofilin S3E and cofilin WT on contractile tension 5 min after the stimulation of muscle tissues with $10^{-5}$ M ACh.
ADF/cofilin Activation Regulates Smooth Muscle Contraction

The contractile force generated in response to stimulation with ACh was significantly inhibited in tissues expressing cofilin S3E. The mean tension in cofilin S3E-treated tissues was 52 ± 4.0% of force in untreated or WT-treated tissues (n = 27; p < 0.01). In contrast, the contractile force in tissues expressing the cofilin WT was not significantly different from force in untreated tissues (Fig. 5, A and B).

Expression of Inactive Mutant Cofilin S3E Inhibits Actin Polymerization in Response to ACh (Fig. 6)—Smooth muscle strips incubated without plasmids or with plasmids encoding the cofilin S3E or cofilin WT were stimulated with 10⁻⁵ M ACh for 5 min, and the proportions of F-actin to G-actin in muscle extracts were analyzed by cell fractionation and immunoblot. Whereas ACh stimulation significantly increased the ratio of F-actin to G-actin in untreated and cofilin WT-treated smooth muscle strips (n = 6; p < 0.05), ACh stimulation did not significantly alter the ratio of F-actin to G-actin in smooth muscle tissues expressing inactive cofilin S3E (Fig. 6A). In tissues treated with cofilin S3E, the ratio of F/G-actin was significantly elevated in unstimulated muscles and significantly depressed in ACh-stimulated muscles relative to untreated or cofilin WT-treated muscles.

The effect of ADF/cofilin inactivation on the pools of G-actin and F-actin was evaluated in cofilin S3E, cofilin WT, and untreated muscles (Fig. 6B). Unstimulated muscles treated with cofilin S3E had significantly less G-actin and more F-actin than untreated or cofilin WT-treated muscles (n = 6, p < 0.05). Cofilin WT-treated muscles exhibited a small but statistically significant increase in G-actin and a decrease in F-actin compared with untreated muscles (n = 6, p < 0.05). The results suggest that expression of the cofilin S3E protein inhibited actin polymerization in muscle strips in response to ACh stimulation, and that cofilin S3E inhibited actin depolymerization in unstimulated muscle tissues.

Expression of Inactive Cofilin S3E Does Not Affect MLC Phosphorylation in Response to ACh in Smooth Muscle Tissues (Fig. 7)—The effects of stimulation with ACh on MLC phosphorylation were compared in muscle tissues transfected with the plasmids encoding inactive cofilin S3E and cofilin WT and in tissues incubated with no plasmids. There were no significant differences in MLC phosphorylation in unstimulated or ACh-stimulated muscles expressing the inactive cofilin S3E, cofilin WT, or muscles not treated with plasmids (n = 4, p > 0.05). Thus, the inhibition of contraction by cofilin S3E did not affect the signaling pathways that regulate MLC phosphorylation.

Depletion of Intracellular Ca²⁺ from Muscle Tissues or Treatment of the Tissues with Calcineurin Inhibitors Inhibits the Dephosphorylation of ADF/Cofilin Induced by ACh (Fig. 8)—Calcineurin, the Ca²⁺-dependent protein phosphatase 2B (PP2B), has been shown to regulate the activation of ADF/cofilin in several non-muscle cell lines (23, 28). We evaluated the Ca²⁺ dependence of ADF/cofilin activation by depleting intracellular Ca²⁺ from muscle tissues. Tissues were incubated in Ca²⁺-free PSS containing 0.1 mM EGTA. The tissues were then repeatedly stimulated for 5-min time periods with ACh until the contractile response decreased to a minimum (29). This generally required ~60 min and resulted in a reduction of isometric force to less than 10% of the maximal force. ADF/cofilin

| FIGURE 3. Expression of cofilin S3E inhibits dephosphorylation of both cofilin and ADF in response to ACh. A, representative immunoblot of extracts from six smooth muscle tissues treated with plasmids encoding wild type cofilin (Coiln WT), cofilin S3E, or from tissues not treated with plasmids (No plasmid). Immunoblots were probed with antibodies to phospho-ser-3 ADF/cofilin, cofilin, and GAPDH. B, mean values for P-ADF/cofilin/GAPDH and P-ADF/cofilin/total cofilin in muscle strips expressing wild type cofilin or cofilin S3E and stimulated with ACh or unstimulated (US). Values are normalized to the no plasmid unstimulated group. All values are means ± S.E., * significantly different from the unstimulated group for the same treatment (p < 0.05, n = 5–6). C, mean values for cofilin/GAPDH in muscle strips expressing wild type cofilin or cofilin S3E and stimulated with ACh or unstimulated (US). Values are normalized to the no plasmid unstimulated group. All values are means ± S.E., * significantly different from the no plasmid unstimulated group (p < 0.05, n = 5–6). |
phosphorylation was then evaluated after ACh stimulation of the Ca\(^{2+}\)-depleted tissues for 5 min. The depletion of Ca\(^{2+}\) markedly inhibited the decrease in ADF/cofilin phosphorylation stimulated by ACh (p < 0.05, n = 6). This suggests that the dephosphorylation and activation of ADF/cofilin are mediated by Ca\(^{2+}\)-dependent mechanisms in this tissue.

Muscle tissues were incubated with the calcineurin inhibitors, cyclosporin A (10 \(\mu\)M) or dexamethasone (10 \(\mu\)M), for 2 h to evaluate the role of calcineurin in ADF/cofilin activation during active contraction with ACh (Fig. 9). ADF/cofilin dephosphorylation was inhibited significantly in ACh-stimulated muscle tissues pretreated with calcineurin inhibitors (p < 0.05, n = 5). This suggests that the dephosphorylation and activation of ADF/cofilin are mediated by calcineurin in this tissue.

**DISCUSSION**

Our results provide the first documentation that the activation of ADF/cofilin is necessary for the development of active contractile tension in smooth muscle. Previous studies have shown that active tension development in tracheal smooth muscle depends on the polymerization of a small pool of actin, and that this actin polymerization is catalyzed by activation of the Arp2/3 complex (5, 6, 12, 15). In this study, we found that endogenous cofilin and ADF are both significantly (~40%) phosphorylated on serine 3 in unstimulated tracheal muscle tissues. Stimulation of the tissues with either ACh or KCl results in a 70% reduction in the phosphorylation of both cofilin and ADF, indicating they are undergoing activation. The time course of ADF/cofilin dephosphorylation in response to contractile stimulation was well correlated with the time course of the smooth muscle tension development (Fig. 1). Both force development and actin polymerization induced by stimulation with acetylcholine were inhibited in tracheal muscles by the expression of an inactive cofilin phosphomimetic, cofilin S3E. Cofilin S3E inhibited the dephosphorylation of endogenous cofilin and ADF in muscle tissues, thereby preventing their activation. The expression of cofilin S3E did not affect myosin light chain phosphorylation, indicating that the inhibition of tension development caused by the expression of cofilin S3E did not result from an inhibitory effect of ADF/cofilin inactivation on pathways that regulate the activation of myosin. These observations suggest that the regulation of ADF/cofilin activation through its dephosphorylation may provide an important mechanism for modulating tension development in smooth muscle tissues that is independent of the pathways that regulate actomyosin activation and cross-bridge cycling.

Although a role for ADF/cofilin in muscle contraction has not been previously described, the importance of ADF/cofilin for cell motility is well established (2, 3, 30). Movement of a cell involves the generation of branched actin filaments at the leading edge of the cell and the disassembly of actin at the rear of the actin network through concerted processes mediated by actin-polymerizing and actin-depolymerizing factors (31). ADF/cofilin plays an essential role in both the actin polymerization and depolymerization processes during cell movement. It promotes actin disassembly by severing actin filaments and by accelerating the off-rate of actin monomers from the pointed ends of actin filaments (2). The severing of actin filaments increases the number of free barbed filament ends, which promotes the dendritic nucleation of new actin filaments by the Arp2/3 complex (32). By enhancing the disassembly of actin filaments from the pointed end of the filament, ADF/cofilin also contributes to actin filament assembly by replenishing the actin monomer pool required for polymerization (1, 18, 33).

Evidence from studies of a variety of types of smooth muscle tissues and cells has shown that contractile stimulation catalyzes the formation of additional F-actin, and that the polymerization of actin is a necessary step in tension generation (5, 8–12, 15, 16, 34). In tracheal smooth muscle, contractile stim-
ulation causes activation of the Arp2/3 complex mediated by the actin-nucleating protein, N-WASp (neuronal Wiskott-Aldrich syndrome protein). The inhibition of N-WASp-induced Arp2/3 complex activation inhibits stimulus-induced actin polymerization and tension development, without affecting myosin light chain phosphorylation (15). Although the role of the newly polymerized actin in regulating the contractile function of intact smooth muscle tissues is currently unclear, there is evidence that actin polymerization occurs submembranously in the cortex of the cell (5, 6, 15, 27). It is possible that a network of submembranous actin forms to increase the rigidity of the membrane during tension development and to strengthen membrane adhesion complexes that are involved in transmitting force between the contractile apparatus and the outside of the cell (5, 6, 27).

**FIGURE 5.** Expression of cofilin S3E inhibits contraction in tracheal muscle strips in response to ACh stimulation. **A,** representative isometric contractions in response to 10^-5 M ACh obtained in three muscle strips from one experiment. Contractions were performed before and after a 2-day incubation with plasmids encoding cofilin wild type (Cofilin WT), inactive mutant cofilin (Cofilin S3E), or after incubation without plasmids (No plasmid). Contractile force in response to ACh was dramatically inhibited in tissues treated with plasmids encoding inactive mutant cofilin S3E, but it was not depressed in tissues treated with plasmids encoding cofilin WT or tissues not treated with plasmids. **B,** mean force of muscle strips incubated without plasmids (No plasmid), with plasmids encoding cofilin WT, and plasmids encoding inactive mutant cofilin S3E. Isometric force was quantified as percentage of the normalized force in the no plasmid group. Values are means ± S.E. *, force significantly different from no plasmid group. (n = 27, p < 0.05).

**FIGURE 6.** Expression of cofilin S3E inhibits actin polymerization in tracheal muscle strips in response to ACh stimulation. **A,** mean ratios of F-actin/G-actin in muscle strips stimulated with ACh (solid bars) or unstimulated (US, open bars). ACh stimulation induced a significant increase in the ratio of F-actin/G-actin in muscle strips treated with cofilin WT and in strips that did not undergo plasmid treatment (No plasmid). In muscle strips treated with the cofilin S3E plasmid, ACh stimulation did not significantly increase the ratio of F-actin/G-actin. The F-actin/G-actin ratio was also increased significantly in unstimulated muscle strips treated with cofilin S3E plasmid compared with no plasmid and cofilin WT-treated tissues. *, significant difference from unstimulated tissues for each treatment group (n = 6, p < 0.05). **, significant difference from corresponding no plasmid group for unstimulated or ACh-stimulated tissues. **B,** relative changes in the percent of G-actin and F-actin to total actin for each treatment group for unstimulated (US) and stimulated (ACh) muscles. Data are normalized to values for unstimulated muscles in the no plasmid treatment group. In the unstimulated muscles, the G-actin was lower and F-actin was higher in tissues treated with cofilin S3E. In muscles treated with cofilin WT, G-actin was higher, and F-actin was lower. *, significant difference from no plasmid, unstimulated group. In ACh-stimulated muscles treated with cofilin S3E, G-actin was higher, and F-actin was lower than in the muscle strips without plasmids or strips treated with cofilin WT. **, significant difference from no plasmid ACh-stimulated group. Values are means ± S.E. (n = 6, p < 0.05).
ADF/Cofilin Activation Regulates Smooth Muscle Contraction

A.

<table>
<thead>
<tr>
<th></th>
<th>No Plasmid</th>
<th>Cofilin WT</th>
<th>Cofilin S3E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unphos MLC</td>
<td>US</td>
<td>ACh</td>
<td>US</td>
</tr>
<tr>
<td>P-MLC</td>
<td>US</td>
<td>ACh</td>
<td>US</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>ACh $10^{-5}$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLC phosphorylation (mol P/mol MLC)</td>
<td>0.6</td>
</tr>
<tr>
<td>Isometric Force, %</td>
<td>100</td>
</tr>
</tbody>
</table>

FIGURE 7. Expression of cofilin S3E does not inhibit the increase in MLC phosphorylation in response to ACh. Muscle tissues treated with plasmids encoding the cofilin WT or cofilin S3E, or not treated with plasmids (No Plasmid) were stimulated with $10^{-5}$ M ACh for 5 min and then frozen for the measurement of MLC phosphorylation. A, representative immunoblot showing unphosphorylated and phosphorylated MLC obtained from extracts of each of six muscle tissues with or without ACh from a single experiment. B, mean values for MLC phosphorylation and force in ACh-stimulated no plasmid, cofilin WT, and cofilin S3E-treated muscles. No significant differences in MLC phosphorylation among the groups were detected, whereas force was significantly depressed in cofilin S3E-treated tissues. Values shown are means $\pm$ S.E. *, indicates significant difference from the no plasmid group ($p < 0.05$, $n = 4$).

FIGURE 8. Depletion of intracellular Ca$^{2+}$ inhibits cofilin dephosphorylation in response to ACh. A, immunoblots of phospho-ADF/cofilin and cofilin in four muscle tissues stimulated with ACh for 5 min in normal Ca$^{2+}$ or Ca$^{2+}$-free PSS containing 0.1 mM EGTA. B, mean ratios for P-ADF/cofilin/total cofilin for each treatment group. ADF/cofilin phosphorylation was significantly lower in ACh-stimulated muscle strips in normal calcium PSS; however, ADF/cofilin phosphorylation was not significantly different in ACh-stimulated muscle tissues after depletion of intracellular Ca$^{2+}$. *, significantly different from unstimulated (US) muscles ($p < 0.05$, $n = 6$). All values are means $\pm$ S.E.

Expression of inactive cofilin SE3 in tracheal muscle inhibited the dephosphorylation of endogenous cofilin and endogenous ADF in response to stimulation with ACh, thus inhibiting their activation (Figs. 3 and 4). The cofilin S3E mutant is a phosphomimetic, with glutamate substituted at the serine 3 site. Its actin severing ability is negligible because it has minimal capability for binding to F-actin (23). Our observations are consistent with those of Konakahara et al. (43), who found that the expression of the cofilin S3E mutant in a hematopoietic progenitor cell line caused the inactivation of endogenous cofilin and inhibition of the rapid turnover of actin filaments needed for cell migration. The introduction of plasmids encoding either wild type cofilin or cofilin S3E into tracheal smooth muscle tissues increased the amount of total cofilin in the tissues by $\sim70%$.

There are several possible mechanisms by which cofilin S3E might inhibit endogenous ADF/cofilin dephosphorylation and activation. Cofilin S3E competes with phosphorylated cofilin and ADF for localization to sites where they are activated (44). Cofilin S3E may also compete with endogenous ADF/cofilin for binding to the ADF/cofilin-specific phosphatase, Slingshot, thereby preventing the activation of endogenous ADF/cofilin (43).

In unstimulated muscle tissues, the expression of cofilin S3E significantly decreased the pool of G-actin and increased the pool of F-actin, suggesting that ADF/cofilin inactivation may inhibit actin depolymerization (Fig. 6). Expression of cofilin S3E in the tissues also inhibited the increase in F-actin that occurs when the tissues are stimulated with acetylcholine. The inhibitory effect of cofilin S3E on stimulus-induced actin polymerization may reflect the absence of an adequate pool of G-actin, an inadequate number of actin filament barbed ends available for interaction with the Arp2/3 complex, or both. In the tracheal muscle tissues, activated ADF/cofilin may act collaboratively with the Arp2/3 complex to regulate actin polym-
The dynamic pool of actin in smooth muscle is separate from the cross-bridge cycling. This is consistent with the possibility that actomyosin ATPase activity and cross-bridge cycling. Our previous studies of tracheal smooth muscle tissues demonstrated that contractile stimulation with ACh increases the activation of the small GTPases, Cdc42, and that Cdc42 activation is necessary for tension generation and actin polymerization (14). ACh also stimulates the activation of the small GTPase, RhoA, in this tissue (48). As ADF/cofilin dephosphorylation (activation) occurs concurrently with the ACh-induced activation of these small GTPases in tracheal muscle, the phosphorylation of ADF/cofilin in response to ACh in this tissue might not be stimulated by kinases activated downstream of these GTPases.

In this study, we showed that the activation of ADF/cofilin by dephosphorylation is required for actin polymerization and tension development in tracheal smooth muscle tissues in response to stimulation with the contractile agonist ACh. Previous studies of tracheal smooth muscle tissues demonstrated that contractile stimulation with ACh increases the activation of the small GTPase, Cdc42, and that Cdc42 activation is necessary for tension generation and actin polymerization (14). ACh also stimulates the activation of the small GTPase, RhoA, in this tissue (48). As ADF/cofilin dephosphorylation (activation) occurs concurrently with the ACh-induced activation of these small GTPases in tracheal muscle, the phosphorylation of ADF/cofilin in response to ACh in this tissue might not be stimulated by kinases activated downstream of these GTPases.

Membrane-depolarizing concentrations of KCl elicited a decrease in ADF/cofilin phosphorylation in tracheal muscle tissues (Fig. 1). We also found that ACh-induced ADF/cofilin dephosphorylation was inhibited in muscles that were depleted of intracellular Ca\(^{2+}\) or that were treated with inhibitors of the Ca\(^{2+}\)-dependent phosphatase, calcineurin (PP2B) (Figs. 8 and 9). These observations indicate that the pathways regulating the activation of ADF/cofilin in tracheal smooth muscle in response to stimulation with ACh are mediated by a pathway requiring the Ca\(^{2+}\)-dependent activation of calcineurin. These
results are consistent with studies in several cultured non-muscle cell lines demonstrating that the Ca$^{2+}$-dependent dephosphorylation of cofilin by the Slingshot phosphatases requires activation of calcineurin (23, 28).

In conclusion, this study demonstrates that ADF/cofilin is dephosphorylated and activated by the contractile stimulation of smooth muscle tissues, and that the activation of ADF/cofilin is required for actin polymerization and tension development during contractile stimulation. In tracheal smooth muscle tissues, ADF/cofilin may regulate the dynamics of a pool of actin that is distinct from tropomyosin-bound actin that interacts with myosin and participates in cross-bridge cycling. Although the function of actin polymerization in smooth muscle is uncertain, our results suggest that the regulation of ADF/cofilin phosphorylation provides a distinct process for modulating tension generation in smooth muscle that is independent of the pathways that regulate myosin light chain phosphorylation and cross-bridge cycling.

Acknowledgments—We are grateful for the generous assistance and donation of plasmids and antibodies from Dr. J. R. Bamburg, Colorado State University, Fort Collins, CO.

REFERENCES
Actin Depolymerization Factor/Cofilin Activation Regulates Actin Polymerization and Tension Development in Canine Tracheal Smooth Muscle
Rong Zhao, Liping Du, Youliang Huang, Yidi Wu and Susan J. Gunst

doi: 10.1074/jbc.M805294200 originally published online October 27, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M805294200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 47 references, 13 of which can be accessed free at http://www.jbc.org/content/283/52/36522.full.html#ref-list-1