

ADF/COFILIN ACTIVATION REGULATES ACTIN POLYMERIZATION AND
TENSION DEVELOPMENT IN CANINE TRACHEAL SMOOTH MUSCLE

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DEDICATION

I dedicate this thesis dissertation to my amazing family and those who have so selflessly taught me along the way.

To my husband: Your understanding, motivation and support go throughout the course of this thesis.

To my daughter: You are the best gift from the god.

To my parents and parents-in-law: You offer me unconditional love and support.

To all of the mentors and teachers I have been blessed to have in my life. You have each been an invaluable teacher to me at different points along my journey.

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ABSTRACT

Rong Zhao

ADF/COFILIN ACTIVATION REGULATES ACTIN POLYMERIZATION AND TENSION DEVELOPMENT IN CANINE TRACHEAL SMOOTH MUSCLE

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 airway smooth 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 approximately 40% of the total ADF/cofilin in unstimulated tissues is phosphorylated (inactivated). 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 WT 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). Expression of Slingshot (SSH) inactive phosphatase (C393S) decreased force development and cofilin dephosphorylation. Activation of ADF/cofilin was also required for the relaxation of tracheal muscle tissues induced by forskolin and isoproterenol. Cofilin activation in response to forskolin was not Ca²⁺ - dependent and was not inhibited by calcineurin inhibitors, suggesting it was regulated by a different mechanism. Cofilin activation is required for actin dynamics and tension development in response to the contractile stimulation of tracheal smooth muscle and is regulated by both contractile and relaxing stimuli. These concepts are critical to understanding the mechanisms of smooth muscle contraction and relaxation, which may provide novel targets for therapeutic intervention in the treatment of abnormal airway responsiveness.

Susan J. Gunst, Ph. D., Chair

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ABBREVIATIONS

ACh	Acetylcholine
ADF	Actin depolymerizing factor
cAMP	adenosine 3', 5' -cyclic monophosphate
EGF	epidermal growth factor
F-actin	Filamentous actin
G-actin	Globular actin
IP ₃	inositol 1,4,5,-trisphosphate
LIMK	Lim Kinases
MLC	myosin light chain
N-WASp	neuronal Wiskott-Aldrich syndrome protein
PAK	p21-activated kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PI3K	PtdIns 3-kinase
ROCK	Rho kinase
SSH	Slingshot
TESK	testicular protein kinases
WT	wild type

CHAPTER I

Introduction

Asthma is defined in the Global Initiative for Asthma guidelines as a chronic inflammatory disorder characterized by reversible airways obstruction and airway hyperresponsiveness (21). The burden from asthma in the United States has increased by 74% over the past 2 decades (109). In 2005, an estimated 7.7% of people (22.2 million) currently had asthma. Rates decreased with age; 8.9% of children (6.5 million) had asthma compared to 7.2% of adults (15.7 million). Therefore, to study the mechanisms of asthma for the development of therapy is important, both medically and economically.

Airway hyperresponsiveness is defined as exaggerated airway narrowing due to nonspecific irritants or pharmacological agonists, which is reversible by bronchodilators that relax airway smooth muscles implying that airway smooth muscle is the cause (203). Historically, inflammation has been regarded as the causal pathophysiological mechanism underlying airway hyperresponsiveness, but recent studies demonstrated dissociation between airway hyperresponsiveness and airway inflammation (9). Anti-inflammatory therapy does not “cure” asthma, and airway hyperresponsiveness can persist in asthmatics even in the absence of airway inflammation, suggesting that airway hyperresponsiveness may be the fundamental cause of asthma (9). Airway smooth muscle cells are thought to be the major effector cells of airway

narrowing, although swelling of airway wall compartments and mucus plugging may amplify the narrowing (26; 48).

Studies using isolated bronchial rings and cultures of isolated airway smooth muscle cells have shown airway smooth muscle from asthmatics can generate more force and, therefore, contract to a greater extent, or to have increased maximum shortening velocity and capacity (87; 105). Remodeling the cytoskeleton to facilitate the transmission or maintenance of force developed by actomyosin interactions (9) and reorganization of actin filament network (67; 114) may be implicated in asthma.

Although the mechanism of force development from actomyosin interaction is well established, the mechanisms underlying actin dynamics in smooth muscle are not clear. Studies on cultured cell lines have demonstrated that members of a family of “actin–dynamizing proteins”, actin depolymerization factor and cofilin (ADF/cofilin), mediate actin dynamics that are required for cell motility (14; 15; 28; 29; 35; 45). My thesis work focused on the role of ADF/cofilin in regulating actin dynamics and smooth muscle contractility.

I. Basic concepts of smooth muscle contraction

1. The ultrastructure of smooth muscle and the crossbridge mechanism for tension development

In the past several decades, growing evidence regarding the structure and regulation of the contractile apparatus of smooth muscle has accumulated. The studies on the molecular organization of the cytoskeleton and contractile

apparatus of smooth muscle have shed light on the mechanisms for the plastic properties of smooth muscle.

(1) The plastic properties of airway smooth muscle

Airway smooth muscle tissues are subjected to large changes in shape and volume during breathing under physiologic conditions *in vivo*. A great amount of data obtained from both *in vivo* and *in vitro* studies has established that the mechanical forces are important during breathing in the regulation of normal airway responsiveness. The airways are dilated by periodic deep inspirations and their responsiveness is reduced by bronchoconstrictors, and tidal breathing is necessary to maintain a normal low level of airway reactivity (90; 154; 156; 188). The isolated airway smooth muscle tissues have similar properties: mechanical oscillation or stretch reduces their stiffness and decrease responsiveness to contractile stimuli (52; 63; 69; 72; 155; 184). Thus, the effects of breathing maneuvers on airway responsiveness *in vivo* are likely to result from the intrinsic properties of the airway smooth muscle tissue itself.

Airway smooth muscle can rapidly adapt its compliance and contractility to accommodate to changes in mechanical forces in the external environment. Studies on both airway smooth muscle cells and tissues have established that the same contractile stimulus may elicit different responses from the muscle depending on its mechanical history—how it has been stretched or shortened before receiving the stimulus (64; 67; 68; 72; 73; 184). Furthermore, changes in

muscle length or mechanical strain also modulate the mechanical stiffness of the smooth muscle tissue.

(2) Ultrastructure of smooth muscle (Figure 1)

The contractile apparatus in smooth muscle cells includes thin filaments and thick filaments. The thin filaments are ~7 nm in diameter, and actin is the primary component. The thick filaments are 12–15 nm in diameter, and myosin is the primary component. The thick filaments in smooth muscle are assembled in a side–polar way, in which all myosin heads have the same polarity along one edge of the filament, and the opposite polarity on the other edge (39; 41; 79). The side–polar filaments consist of polymerized myosin monomers with the head regions extending in opposing directions on each side of the filament. The number of the thick filaments is relatively fewer than the thin filaments which surround the thick filaments. The ratio of actin to myosin filaments varies in different smooth muscle tissues. The lowest ratio is 8:1 in chicken gizzard; the middle ratio is approximately 15:1 in vascular muscle; the highest ratio is 50:1 in isolated amphibian visceral muscle. Actin is the major constituent of the thin filament. Filamentous actin (F–actin) is a polymeric protein composed of asymmetric bi–lobed 42 kDa actin monomers (83; 89; 117). Actin filaments form the thin filament backbone. Tropomyosin binds along the groove of the actin filament. The binding of tropomyosin stabilizes the actin filament (42).

In smooth muscle tissues, the actin filaments connect with dense plaques on the membrane and link to dense bodies in the cytoplasm

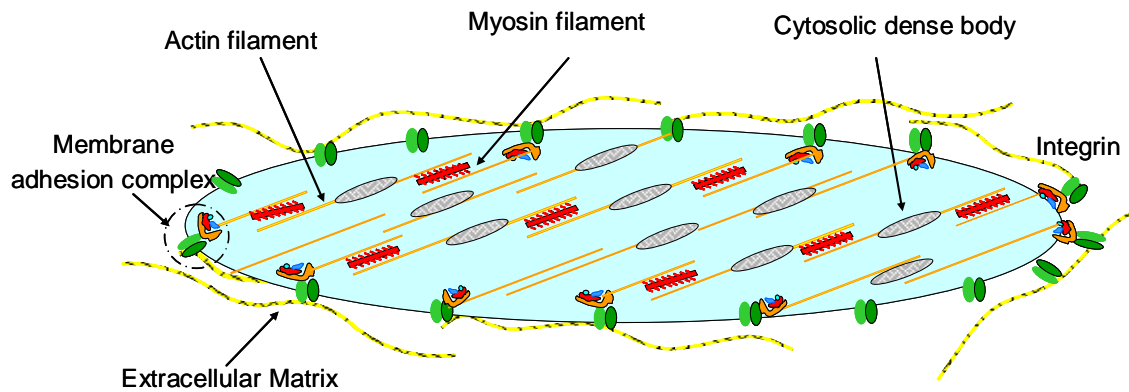


Figure 1. The ultrastructure of smooth muscle cell. Myofilaments of smooth muscle cells contain thick filaments (myosin) and thin filaments (actin). Myosin and actin filaments slide against each other to produce contraction of the cell. Actin filaments are anchored to the membrane adhesion plaques and the dense bodies in the cytoplasm. There are some actin filaments are not associated with myosin.

(70; 74; 158; 159). The membrane-associated dense bodies, also known as “macromolecular adhesion junctions,” form on the intracellular side of the plasma membrane at the junctions between F-actin and the extracellular matrix (27; 47; 157; 198), at which F-actin is connected to integrin proteins via “linker” proteins including α -actinin, talin, and filamin. These “linker” proteins can both cross-link actin filaments and bind to the β -subunit of integrin heterodimers (40; 140). Evidence that the adhesion complexes of smooth muscle are dynamic structures has been obtained in studies of airway smooth muscle, vascular smooth muscle tissues and in isolated smooth muscle cells using various methods, including coimmunoprecipitation, cell fractionation, immunofluorescence analysis, and cellular imaging (53; 92; 139; 142; 143; 199-201).

The molecular structure of the smooth muscle myosin is grossly similar to that of skeletal muscle myosin. It is a large asymmetric protein (molecular weight (MW) ~520 kDa) made up of six polypeptide chains: two ~205 kDa heavy chains that form a dimer, and two pairs of light chains, the 20 kDa “regulatory” light chains and the 17 kDa “essential” light chains (3). The myosin heavy chain dimer makes up the main body of the molecule, with each heavy chain containing a slightly elongated globular head at the amino terminus. The myosin globular heads are linked to a long α -helical coiled tail of ~120 kDa which aggregates to form the rod-like backbone of the thick filament. Each myosin globular head contains the functional motor domains which include the nucleotide and actin-binding regions. A single essential and regulatory light chain is associated with each myosin head. The light chains are localized along the α -helical segment

(“neck” region) of the heavy chain at the junction of the globular head and the rod-like backbone.

(3) The basic mechanisms of smooth muscle contraction: actomyosin crossbridge activation

Traditionally, smooth muscle contractile regulation has been attributed to mechanisms that modulate the number or cycling rate of actomyosin crossbridges, either by changing the overlap of actin and myosin filaments or by affecting crossbridge activation. Myosin generates force and/or motion by mechanical cycles when the myosin head repetitively attaches to actin and undergoes a conformational change which results in a power stroke and then detaches (189). The energy required for the mechanical power is from the enzymatic hydrolysis of adenosine triphosphate (ATP) on the globular myosin head. The “lever arm hypothesis” has been applied to explain the conversion of chemical energy into directed movement (145; 146). In this model, the binding of ATP to myosin while it is bound to actin results in a series of conformational changes in the myosin globular head which reduces its affinity for actin and allows it to hydrolyze ATP to adenosine diphosphate (ADP) and inorganic phosphate (Pi). As the myosin rebinds to actin and releases the nucleotide, the conformational changes are reversed causing movement of the myosin relative to actin. The light-chain binding domain (“the neck region”) pivots about a fulcrum near where the globular head and light chain binding domains connects to each other, resulting in the displacement of actin relative to myosin (61).

(4) The limitation of actomyosin crossbridge activation in smooth muscle contraction

The actomyosin crossbridge interaction and associated regulatory processes can explain tension development and active shortening of the smooth muscle cell; however, crossbridge interactions fail to account for the ability of the smooth muscle cell to accommodate cell mechanical responses to the physical environment, either while activated or quiescent. Other molecular interactions within the cytoskeleton are likely to be responsible for these plastic properties and consequently to be important (65).

2. Smooth muscle contraction stimulates actin polymerization independently from contractile apparatus activation

(1) Actin polymerization is documented to occur during smooth muscle contraction in various studies with diverse approaches

There is growing evidence to document that actin polymerization plays an important role in regulating active tension development in smooth muscle (71; 72). Actin polymerization can be induced by contractile agonists stimulation in smooth muscle tissues and cells in culture (33; 50; 76; 80; 81; 114; 162; 172; 199; 200; 202).

An increase in the pool of F-actin and a decrease in the pool of G-actin during smooth muscle contraction has been documented in diverse types of smooth muscle cells and smooth muscle tissues with different approaches. Since

the 1990s, the dynamics of actin assembly from G-actin to F-actin have been documented in tracheal smooth muscle tissue by several groups with various methods, such as DNase I inhibition, cellular fractionation and immunofluorescence staining (80; 88; 114; 199). A similar phenomenon has also been reported by Barany *et al.* in arterial and other smooth muscle tissues by measuring the exchange rates of actin-bound nucleotides (20). The amount of the reduction of the G-actin pool during the contractile stimulation of vascular smooth muscle was comparable to that in tracheal smooth muscle. Frequently used approaches for the measurement of actin polymerization include cell fractionation (147; 174; 199), fluorescence imaging to visualize G- and F-actin in isolated smooth muscle cells or smooth muscle tissues with G- and F-actin-specific stains (33; 50; 81; 88; 176) and electron microscopic studies to quantify actin filament density (76). All of these approaches have consistently shown that an increase in F-actin and a decrease in G-actin occurs when smooth muscle cells or tissues are activated by a contractile stimulus.

(2) Actin dynamics contributes to the plastic properties of smooth muscle

The observations of actin dynamics in smooth muscle tissues are analogous to observations in many non-muscle cells (175), but stand in marked contrast to observations in skeletal muscle, where no significant G-actin pool has been detected and the rate of actin-bound nucleotide exchange is extremely low. Thus, the dynamic state of the actin cytoskeleton may be a unique feature of smooth muscle cells, not common to cells of other muscle tissues types.

Compared with skeletal muscle tissues, smooth muscle tissues also contain a much higher ratio of F-actin to myosin and maintain a significant pool of F-actin that does not interact with myosin filaments (158). The abundance of actin in smooth muscle and its highly dynamic state may contribute to the plastic properties of smooth muscle, the unique functional characteristics of this tissue.

(3) Actin polymerization is required for smooth muscle force development

The actin filaments in smooth muscle have been classified into “contractile actin” and “cytoskeletal actin”. The evidence from our lab supports a hypothesis that the contractile actin filaments localize around the thick filaments, forming the contractile apparatus, while cytoskeletal actin filaments localize at the subcortical area and are not structurally associated with myosin (74). The dynamic nature of the cytoskeletal actin cytoskeleton in smooth muscle makes it more like that of nonmuscle motile cells than sarcomeric actin in striated muscles. A corollary of this hypothesis is that many of the same actin-modifying proteins and regulatory pathways that control actin dynamics in nonmuscle cells are important in smooth muscle contraction (55; 56; 70; 74). (Figure 2)

Tension development in many smooth muscle tissues is dramatically depressed by short term exposure to inhibitors of actin polymerization (4; 114; 132; 139; 196), which indicates that the polymerization-depolymerization of actin filaments is part of the contraction-relaxation cycle of smooth muscle. The short-term application of pharmacologic actin polymerization inhibitors, such as latrunculin, which works by sequestering actin monomers, and cytochalasin,

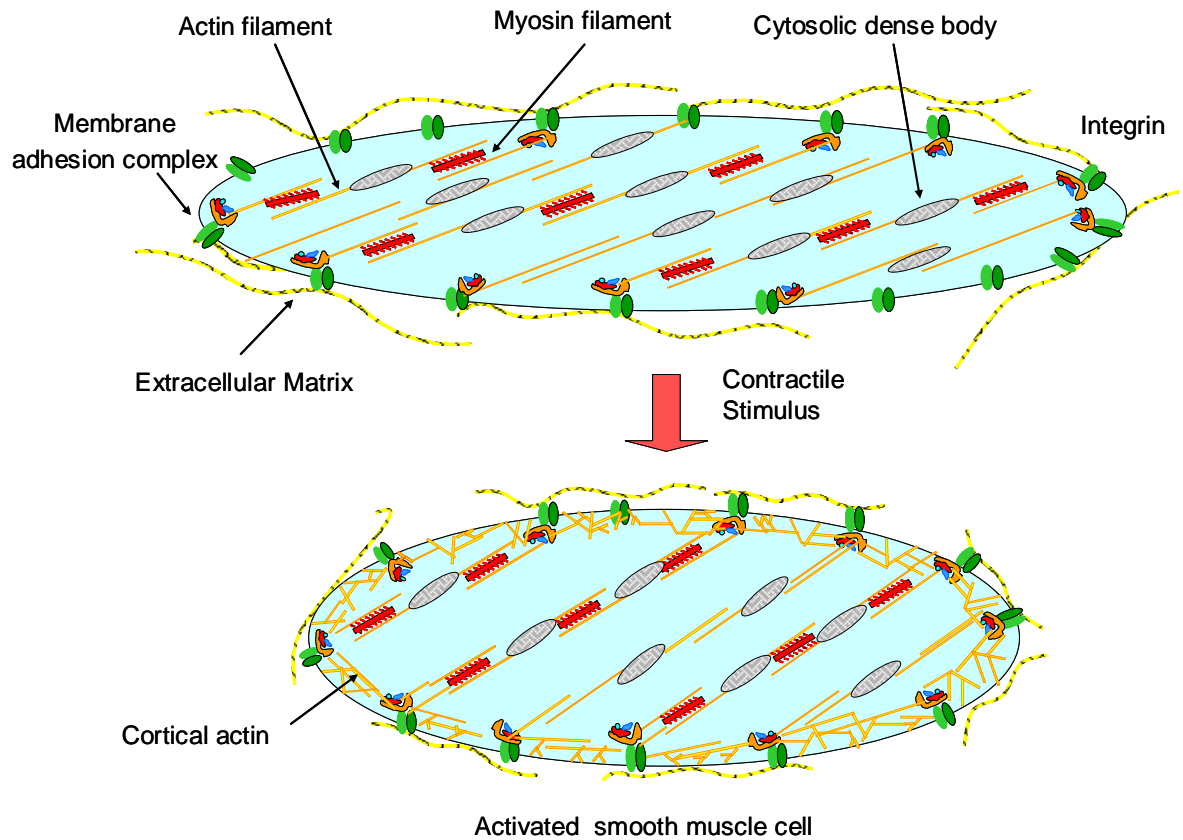


Figure 2. Hypothesized model for smooth muscle contraction (74). The cortical actin around the membrane undergoes polymerization with the contractile stimulation. The formation of a subcortical actin filament network strengthens the membrane for the transmission of force generated by the actomyosin system. Thus the dynamic character of the cortical actin cytoskeleton in smooth muscle is similar to that in several nonmuscle motile cells. A main idea of this hypothesis is that many of the actin-modifying proteins and regulatory pathways that control actin dynamics in nonmuscle cells are important in smooth muscle contraction

which works by capping actin filaments (25; 36; 38), have been reported to depress tension development in response to contractile stimulation in airway smooth muscle (10; 46; 114; 160; 180; 196), vascular smooth muscle (4; 32-34; 133; 134; 147; 149; 153; 191), uterine smooth muscle (153) and intestinal smooth muscle (110; 131). Further evidence regarding the importance of actin polymerization in the process of mechanical tension development in smooth muscle has been obtained from studies applying molecular constructs or peptides to interrupt specific steps in the actin polymerization process. These interventions also inhibit tension development in smooth muscle tissues in response to contractile stimuli (12; 169; 170; 174; 199; 200). Cellular imaging demonstrated that the depression of tension development by inhibiting actin polymerization does not result from disruption of the organization or integrity of the contractile apparatus (4; 114; 199).

(4) Actin polymerization is independent of myosin light chain (MLC) phosphorylation in smooth muscle

Actin polymerization inhibitors can dramatically reduce tension development in airway smooth muscle without significantly affecting MLC phosphorylation or myosin ATPase activity, indicating that actin polymerization induced by contractile stimulation does not regulate the processes involved in the activation of contractile protein or crossbridge cycling (4; 114; 199). These results suggest that active tension generation in smooth muscle tissues depends on two parallel cytoskeletal processes: 1) the activation of contractile proteins

leading to crossbridge cycling and the sliding of actin and myosin filaments; 2) the polymerization of actin and remodelling of cytoskeleton organization. In the absence of either of these events, tension development does not occur.

In summary, actin polymerization regulates tension development by a cellular process that is distinct from and independent of cross-bridge cycling. Thus, a new model for smooth muscle contraction has been proposed in which a contractile stimulus activates independent, but parallel, signaling pathways that regulate the processes of actin polymerization and contractile protein activation, both of which are essential to the process of shortening and tension development in smooth muscle tissues (74).

The above studies demonstrate that a relatively small amount of actin undergoes polymerization during smooth muscle contraction. It also suggests that this labile pool of actin serves a specialized function that is distinct from that of the “thin filament” actin that interacts with myosin to regulate cross-bridge cycling. However, the nature of the pool of actin that undergoes polymerization and its function during the contraction is currently an unresolved question. Much of the existing evidence supports a model in which actin polymerization occurs in a submembranous area of the smooth muscle cell (74). The formation of a network of submembranous actin in smooth muscle cells may function to enhance membrane rigidity and to connect the contractile and cytoskeletal filament lattice to the membrane to transmit the tension generated by cross-bridge cycling (66; 74; 198; 199). Submembranous actin polymerization may also enable the cell to adapt its shape and stiffness and contractility to external and

internal mechanical forces imposed on it, and it may occur in regions within smooth muscle cells in which the membrane tension is greatest (74).

II. Molecular Mechanisms for Actin Polymerization.

In the previous section (section I.2.), evidence was presented that actin polymerization and the dynamic remodeling of the actin cytoskeleton play critical roles in the regulation of contractility and the physiologic properties of airway smooth muscle, as well as in other smooth muscle tissues. However, the mechanisms of actin polymerization in smooth muscle cells are still not established.

1. The general mechanism of actin polymerization

The ability of a cell to coordinate the assembly and disassembly of its actin cytoskeleton is essential for cell integrity, motility, membrane trafficking and shape changes (116). Many of the mechanisms regulating actin filament assembly and dynamics are derived largely from studies in reconstituted *in vitro* systems, and from the evaluation of mechanisms of cell locomotion and motility via pseudopods in unicellular organisms, immune cells, migrating fibroblasts, and growth cones (144). Actin filaments are polarized *in vitro* and *in vivo*. G-actin adds preferentially to the fast-growing (barbed) end of the F-actin and this is the major site of F-actin elongation *in vivo*. The slower growing ends are called pointed ends. G-actin binds to and hydrolyzes one molecule of ATP, which provides the energy to maintain the difference in affinity for G-actin addition

between the barbed and pointed ends. The addition of ATP-G-actin is favored at the barbed end of the F-actin, while ADP-G-actin is lost from the pointed end. This, together with the fact that barbed ends are oriented towards the plasma membrane in a cell, allows the barbed end to undergo rapid growth that drives protrusion of the cell membrane and thus cell motility (116).

Actin is the most abundant protein in many eukaryotic cells, with concentrations of over 100 μM in some cell types. Pure actin requires a critical concentration of only 0.1–1 μM for polymerization at the barbed end, so the cell has evolved mechanisms to control the number of free barbed ends. Currently there are three presumed mechanisms for the free barbed end generation, and thus, the regulation of actin filament assembly, in cells (77; 116; 144).

First, cells regulate the growth of F-actin via capping proteins such as gelsolin and cap Z, which bind to the barbed ends of F-actin and prevent further elongation. Gelsolin and cap Z together are able to cap the majority of barbed ends within most animal-cell types. Caps can be removed when signals trigger actin assembly, leading to rapid F-actin elongation (116).

Second, F-actin severing by ADF (actin-depolymerizing factor)/cofilin provides a source of free barbed ends. A detailed description of the activity of ADF/cofilin is in section II.2,

Finally, *de novo* F-actin nucleation produces new F-actin seeds. Forming F-actin from G-actin alone requires the initial association of G-actin molecules to form dimers and trimers. Kinetic modeling has demonstrated that the initial formation of dimers and trimers is energetically unfavorable (78; 111).

This leads to the nucleation of F-actin from G-actin alone. It is now accepted that nucleators, such as the Arp2/3 complex, formins, and spire proteins, together with uncapping and severing proteins, nucleate actin networks in response to environmental cues (60; 111; 122; 144; 163).

2. Actin Depolymerizing Protein (ADF)/Cofilin is required for Actin polymerization

(1) Basic function of ADF/cofilin

ADF/Cofilin family is named as “actin depolymerizing factor” because it was first discovered in chicken and porcine brain based on its capacity to form “cofilamentous” structures with actin filaments and to depolymerize the actin filaments (16; 127).

(2) Isoforms of ADF/cofilin family

The ADF/cofilin family is ubiquitously present in the eukaryocytes (15; 101). The molecular weight is around 19-kDa. This small protein has a single domain, termed as ADF-homology domain (101). In mammalian systems, the ADF/cofilin family includes of three highly conserved isoforms: cofilin 1 (non-muscle cofilin) (127), cofilin 2 (muscle cofilin) (1) and ADF (actin depolymerizing factor or destrin) (121). Studies on mouse have demonstrated the various expression levels of the three isoforms in different cells or tissues (75; 183). Cofilin 1 is the predominant isoform and is expressed ubiquitously in most adult tissues. ADF is post-natally upregulated primarily in epithelial and endothelial

tissues and the amount is usually lower than cofilin 1. Cofilin 2 replaces cofilin 1 in striated muscle and is the unique isoform expressed in differentiated skeletal muscle and the major isoform in cardiac muscle at the later stages of embryogenesis (125). Cofilin 2 is expressed in low level in other adult cells or tissues, including smooth muscle.

Studies of the isoforms of ADF/cofilin suggest that different isoforms of ADF/cofilin play qualitatively similar roles in regulating actin dynamics, but the effects on actin dynamics are quantitatively different during different developmental processes (22; 23; 37; 43; 103; 127; 138; 182). Lappalainen and colleagues reported that ADF and cofilin 1 play overlapping roles in F-actin depolymerization in mouse NIH 3T3, B16F1, and Neuro 2A cells by knockdown with siRNA. However, studies on the role of ADF/cofilin during development of knockout mouse showed that cofilin 1 is critical for fetal survival (75), while ADF is more important for development after birth.

(3) The basic functions of ADF/cofilin

The studies *in vitro* demonstrate that the effects of ADF/cofilin on actin dynamics are multiple and complex. ADF/cofilin prefers to bind to ADP-actin subunits that are produced following ATP hydrolysis and the release of inorganic phosphate within the F-actin filament (15; 19; 107). ADF/cofilin inhibit nucleotide exchange on ADP-G-actin (15; 127). Direct observation of actin filaments by electron microscopy has shown that each cofilin contains two actin binding sites, which bind in the cleft between the two actin monomers in an actin filament and

weaken the lateral interactions of the F-actin (11; 24). Cofilin binding to F-actin induces a conformational twist in the actin filament structure that propagates over a long range from the actual cofilin-binding site and this is suggested to underlie their fragmenting/severing activity (54; 112; 185). ADF/cofilin accelerates spontaneous polymerization of G-actin and increases the rate of actin subunits dissociation from the pointed end (29; 30).

(4) Dephosphorylated ADF/cofilin is the active form

Phosphorylation and dephosphorylation of its NH₂-terminal Ser-3 is the critical way to regulate ADF/cofilin activity. Phosphorylation at Serine 3 abolishes the binding ability of ADF/cofilin to F-actin and thus inhibits its severing function (Figure 3) (5; 15; 18; 113; 120) and thus dephosphorylation cause reactivation of ADF/cofilin.

(5) The mechanisms of ADF/cofilin on actin dynamics

ADF/cofilin crucially contributes to actin dynamics, but how exactly ADF/cofilin regulates actin polymerization and cell motility is still under dispute. Evidence from a number of studies of different cell types (neuroblastoma, fibroblast, melanoma, mammary adenocarcinoma, glioblastoma-astrocytoma cells, T lymphoma (Jurkat) and carcinomas from the cervix epithelia (HeLa), colon (KM12), hepatocytes (HepG2), and kidney fibroblasts (COS1) *in vitro* and *in vivo* have demonstrated that the dephosphorylation and activation of ADF/cofilin is required for cell motility (6; 57; 62; 84; 126; 186; 192; 194; 195).

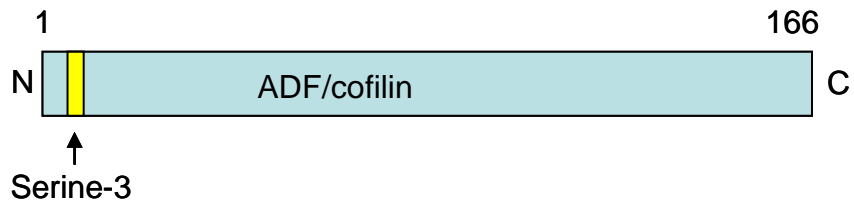


Figure 3. The molecular structure of ADF/cofilin. ADF/cofilin is a small protein. The serine 3 at the NH₂-terminal is the critical site to regulate its activation. Phosphorylation of the NH₂-terminal ser-3 on ADF/cofilin abolishes its ability to bind actin and thus its severing function is inhibited.

There are also some studies demonstrating that phosphorylation and inactivation of ADF/cofilin are necessary for motility to occur (13; 128).

The above debate results in two models of ADF/cofilin function: (1). ADF/cofilin severs the actin filaments; (2). ADF/cofilin increases the off-rate of actin subunits from the pointed end. Filament severing and off-rate enhancement not only increases turnover but can also lead to nucleated filament growth from new filament ends. Thus, whether ADF/cofilin causes filament growth or shrinkage will depend on the availability of actin subunits within the region of the cell where this process occurs (35). Studies of actin dynamics *in vitro* and in cells have shown that actin polymerization is regulated by proteins that control the availability of a G-actin pool for incorporation into actin filaments, and the availability of free “barbed” or “plus” ends of F-actin which can undergo polymerization (30; 98). The family of ADF/cofilin proteins participates in both of these processes. Thus these proteins are termed “actin-dynamizing” proteins due to their critical role in regulating the actin dynamics which enables the rapid turnover of the actin cytoskeleton (17; 28; 35).

ADF/cofilin creates free barbed ends via a severing activity that is essential in cell spreading and lamellipodia formation in response to epidermal growth factor (EGF) (31; 86; 116) and other signals. ADF/cofilin can increase the rate of depolymerization at the pointed end of the actin filament (31; 107; 167). In reconstituted *in vitro* systems, ADF/cofilin acts alone or synergistically with profilin to regulate the availability of free barbed ends of actin filaments for polymerization (35; 44; 93), with ADF/cofilin presumably increasing the

availability of barbed ends for polymerization and profilin increasing the availability of G-actin.

The important role of ADF/cofilin in cell mobility is well established (17; 28; 35). ADF/cofilin is an essential for both the actin polymerization and depolymerization processes during cell movement: It binds to actin filaments and severs them, thereby promoting actin disassembly and the formation of new barbed ends, which enables the nucleation of new actin filaments by the Arp2/3 complex. Arp2/3 complex is a cellular initiator of actin filament nucleation, (78; 106; 123). During cell migration, on one hand, new F-actin is assembled at the leading edge of the cell; on the other hand, F-actin at the rear of the actin network is disassembled (35). ADF/cofilin also contributes to F-actin assembly by replenishing the G-actin pool required for polymerization (Figure 4) (17; 28; 93; 131).

There have been very few studies of the role of ADF/cofilin in smooth muscle physiological functions. One study from Brophy and colleagues demonstrated ADF/cofilin is activated (dephosphorylated) in response to forskolin and isoproterenol in cultured human airway smooth muscle cells (95). Another study from Hellstrand and colleagues has shown that stretch induced actin polymerization and increased cofilin expression in vascular smooth muscle tissues (7). However, there is virtually no information about the role of ADF/cofilin in regulating smooth muscle contraction.

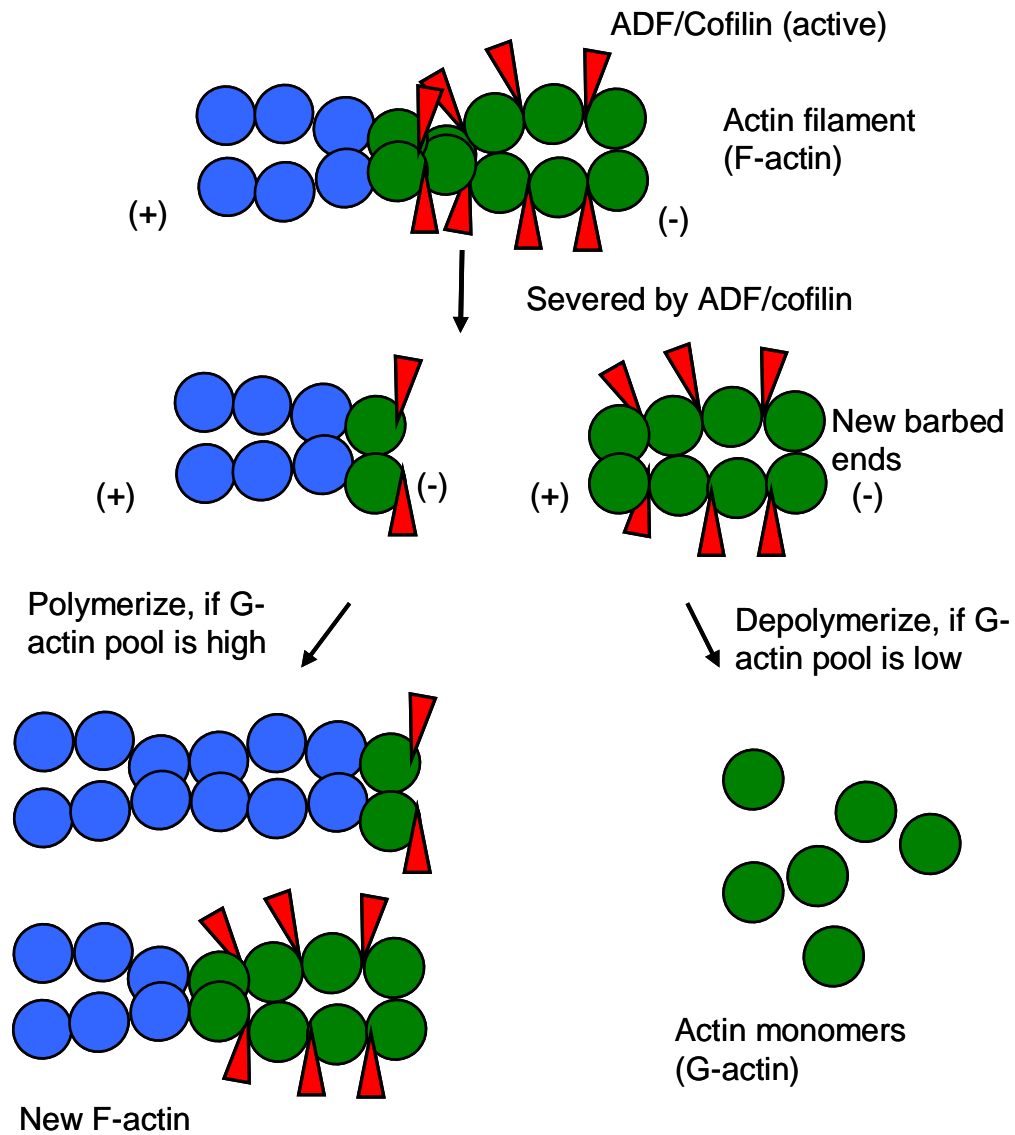


Figure 4. The role of ADF/cofilin in actin dynamics. Severing by ADF/cofilin has two possible consequences: in the presence of G-actin, severing causes nucleation of polymerization (left); in the absence of G-actin, severing causes net depolymerization (right).

3. Signaling pathways regulating ADF/cofilin phosphorylation (inactivation) and dephosphorylation (activation)

Phosphorylation (inactivation) of ADF/cofilin at Serine 3 is mediated by LIM kinases (LIMK) (151) and testicular protein kinases (TESK) (178). Both LIMK and TESK have two isoforms: LIMK1/2 (2; 51; 113) and TESK1/2 (113; 178). The dephosphorylation (activation) of ADF/cofilin is mediated by the ADF/cofilin-specific phosphatases slingshot (SSH) and chronophin (85) and other nonspecific phosphatases, including protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B or calcineurin) (18; 113). (Figure 5)

(1) Phosphorylation (inactivation) of ADF/cofilin by LIMK

Current evidence suggests that the signaling pathways that regulate ADF/cofilin phosphorylation are complex. ADF/cofilin is the substrate of LIM Kinase (LIMK) (119; 135; 136; 151), which can be activated through Rho-family GTPases via the activation of Rho kinase (ROCK) (8; 108; 151; 164; 165; 193), or via Cdc42/Rac-mediated activation of PAK1, 2 or 4 (13; 49; 108; 118; 128; 151; 166). Studies performed in neuroblast, kidney fibroblast, and human cervical cancer cells indicate that ADF/cofilin phosphorylation is inhibited by the inhibition of ROCK with the ROCK-specific inhibitor, Y27632 (7; 108; 164; 165). Studies *in vitro* using mutant plasmids in neuroblastoma cells and T lymphocytes encoding inactive mutants of Rac have also documented a Cdc42/Rac-PAK pathway for the regulation of LIMK activation and ADF/cofilin phosphorylation (108; 128). In

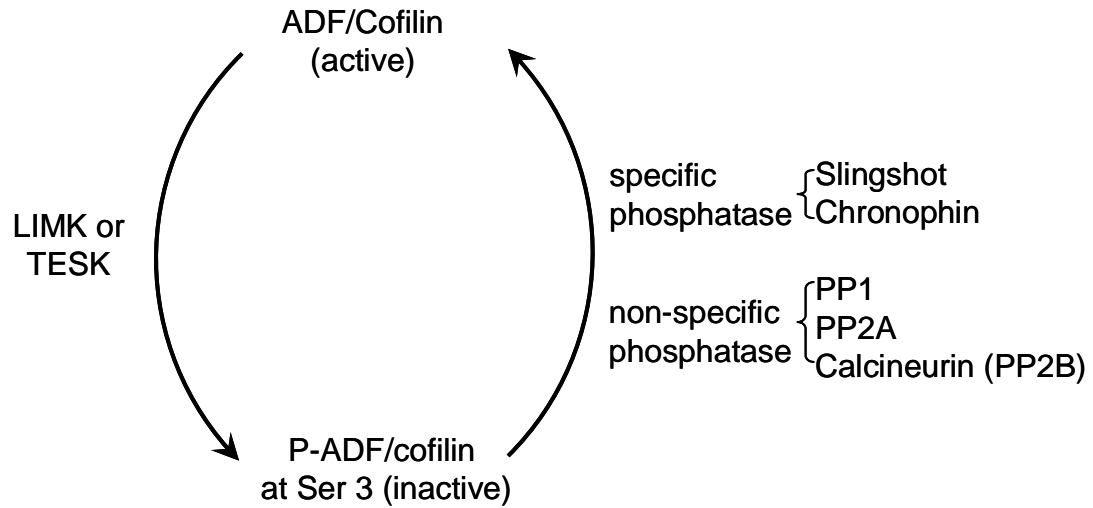


Figure 5. Signaling pathways that regulate ADF/cofilin activation and inactivation. ADF/cofilin can be phosphorylated (inactivated) at Serine 3 by LIM kinases (LIMK) and testicular protein kinases (TESK). It can also be dephosphorylated (activated) by the ADF/cofilin-specific phosphatases slingshot (SSH) and chronophin and other nonspecific phosphatases, including protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B or calcineurin).

addition, there are pathways that activate LIMK independently of GTPase. One example is calcium and integrin binding protein 1 (CIB1)–PAK1–LIMK1 pathway. It was reported by Paris and colleagues that activation of LIMK1 through CIB1–PAK1 with the application of CIB1 overexpression and RNA interference increases cofilin phosphorylation and prevent platelets migration (104). The other example is integrin–MAPKAPK–LIMK1 pathway. It was reported by Reisler and colleagues that activation of LIMK1 through integrin–MAPKAPK in endothelial cells (97).

(2) Phosphorylation (inactivation) of ADF/cofilin by TESK

TESK and LIMK appear to have different extracellular signals for ADF/cofilin phosphorylation. Activation of TESK was demonstrated to be unrelated to ROCK or PAK activation in cervical carcinoma cells by using the ROCK inhibitor (Y27632) and overexpression of Rho, Cdc42, ROCK or PAK in HeLa cells and COS7 cells (177). The activation of integrin associated signals, α -parvin (or actopaxin, a focal adhesion protein), 14–3–3 β (a member of scaffold protein family that binds phosphoserine/threonine motifs), or sprouty–4 (Spry–4, an inhibitor of receptor tyrosine kinase (RTK)–MAP kinase signaling) inhibits TESK activity in HeLa cells and COS–7 cells (100; 179; 181). The interaction between actopaxin or 14–3–3 β and TESK1 is inhibited by fibronectin (100; 179).

(3). Dephosphorylation (activation) of ADF/cofilin by phosphatases

The pathways that regulate the phosphatases that dephosphorylate ADF/cofilin are not clear. In 1998, several nonspecific phosphatases were demonstrated to dephosphorylate ADF/cofilin in rat pheochromocytoma by using pharmacological inhibitors (18; 113). In 2002, Uemura and colleagues first reported a specific phosphatase for ADF/cofilin, named “Slingshot (SSH)” (130). In 2005, Bokoch and colleagues reported another specific phosphatase for ADF/cofilin, named as “chronophin” (59).

In both human and mouse, the Slingshot phosphatases are represented by three genes (SSH-1, -2, and -3), each with long and short variants with distinct tissue expression patterns. Slingshot seems to be widely expressed in various organisms, but is notably absent in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Arabidopsis thaliana*. In mammalian cells SSH-1L, along with SSH-2L and SSH-3L, dephosphorylates both phospho-ADF and phospho-cofilin at the critical Ser3 residue. Notably, SSH-3L was less effective in dephosphorylating these substrates in comparison with the two other isoforms (85; 137).

Ca²⁺ ionophore A23187, Ca²⁺ –mobilizing agonists, such as ATP and histamine, and Ca²⁺ –calcineurin (PP2B) induced SSH-1L activation, correlating with cofilin dephosphorylation in HeLa cells (187). Dephosphorylation of Slingshot by λ-phosphatase increased its phosphatase activity, but Slingshot activity was inhibited by PAK4-mediated phosphorylation in rat hippocampal neurons (161). The PtdIns 3-kinase (PI3K) inhibitor wortmannin has been shown

to antagonize cofilin dephosphorylation induced by fMLP (Formyl–Methionyl–Leucyl–Phenylalanine), a chemotaxis, or tissue plasminogen activator (TPA) in polymorphonuclear leukocytes (15). Mizuno and colleagues (129) reported that insulin–dependent actin reorganization occurs through PI3K and SSH, since insulin stimulated human breast adenocarcinoma cells exhibit SSH activation and cofilin dephosphorylation that is abolished by PI3K inhibition (124).

In 2008, Brophy and colleagues demonstrated cofilin is activated (dephosphorylated) in response to forskolin and isoproterenol in cultured human airway smooth muscle cells, suggesting a possible role of ADF/cofilin in regulating actin dynamics during the smooth muscle relaxation (95).

There are some studies in human breast adenocarcinoma, monkey kidney fibroblasts, human embryonic kidney cells and *Xenopus* cycling extracts demonstrating that the small GTPases Rac and Rho dephosphorylate cofilin (124; 168), which suggests that small GTPase family members may regulate SSH activity. Jones and colleagues proposed a novel mechanism in which $\alpha 6\beta 4$ integrin activates cofilin via Rac1, 14–3–3 proteins, and SSH regulates cofilin activation in human epidermal keratinocytes (94).

Signal transduction pathways for chronophin activation are poorly characterized. Chronophin phosphatase activity is insensitive to classical thiol–based serine/threonine phosphatase inhibitors, which were reported for physiological ADF/cofilin phosphatase activity (15; 197). Chronophin exhibits several predicted interaction motifs regulated by both PI3K and PLC γ , both of

which were suggested to be involved in signaling to ADF/cofilin dephosphorylation in polymorphonuclear leukocytes (15).

There is also a study of the interaction between LIMK and SSH. Bernaard and colleagues proposed a reciprocal control of LIMK1 and SSH1L activity, and that SSH1L can directly dephosphorylate and regulate LIMK1 activity in mouse embryo fibroblasts (161).

In summary, the signalling pathways that regulate ADF/cofilin activation (dephosphorylation) and inactivation (phosphorylation) are complex and not established. There is currently almost no information on the mechanisms by which stimuli regulate ADF/cofilin phosphorylation during force development in smooth muscle tissues.

In conclusion, there is growing evidence that actin polymerization and dynamic remodeling of the actin cytoskeleton are important in the regulation of the physiologic properties of smooth muscle. The overall thesis work is based on the hypothesis that ADF/cofilin might play an important role in the regulation of actin dynamics in smooth muscle tension development. In Chapter II, the roles of ADF/cofilin in regulating actin polymerization in smooth muscle in response to contractile and relaxing stimuli are determined. The signal pathways that activate ADF/cofilin are evaluated.

CHAPTER II

Results

II.i.

Activation of ADF/cofilin is required for actin polymerization and contraction in canine tracheal smooth muscle

1. Summary

Recent studies have documented that the cytoskeletal organization of differentiated smooth muscle cells and tissues is dynamic, and it is regulated during contractile stimulation (66; 74; 198). Dynamic changes in cytoskeletal organization may enable smooth muscle cells to modulate their structure and contractility in response to changes in their external environment (71; 198). 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 (10; 33; 74; 80; 88; 91; 114; 169; 173; 199). 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 independently of cross-bridge cycling (74; 114; 173; 199).

ADF/cofilin play a critical role in the rapid adaptation of the actin cytoskeleton to localized cellular functions (17; 28; 35). The activation of ADF/cofilin is essential for cell motility and polarized cell migration, but the role of

ADF/cofilin on the regulation of actin dynamic and contraction in smooth muscle tissue is not known. I hypothesized that ADF/cofilin might play an important role in the regulation of actin dynamics in smooth muscle during contractile activation. In the present study, an inactive cofilin phosphomimetic (cofilin S3E), which has minimal actin severing activity (113) (Figure 6), was expressed in the airway smooth muscle tissues. 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. The results demonstrate 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.

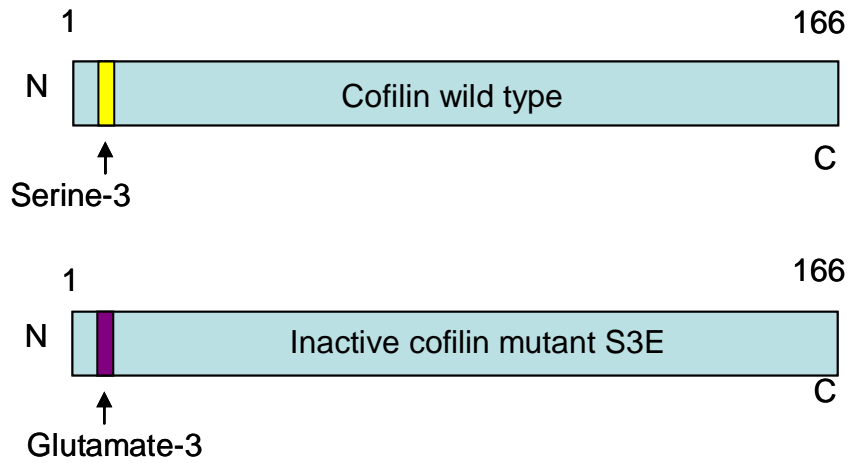


Figure 6. The structure of cofilin wild type and inactive cofilin mutant. The inactive cofilin mutant (cofilin S3E), a phospho-cofilin mimetic, is constitutively inactive, so it has no severing ability. Inactive cofilin mutant (cofilin S3E) may sequester the cofilin phosphatase, thereby preventing the activation of the endogenous cofilin.

2. Results

2.1. Contractile stimulation of tracheal smooth muscle tissues induces ADF/cofilin dephosphorylation on Ser-3 (Figure 7).

First we measured if the ADF/cofilin phosphorylation changes with contractile stimulation using acetylcholine (ACh) or KCl at different time points. 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 (Figure 7A and 7B). 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).

To determine 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

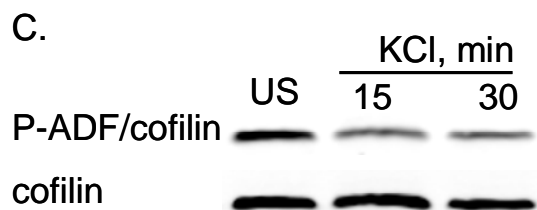
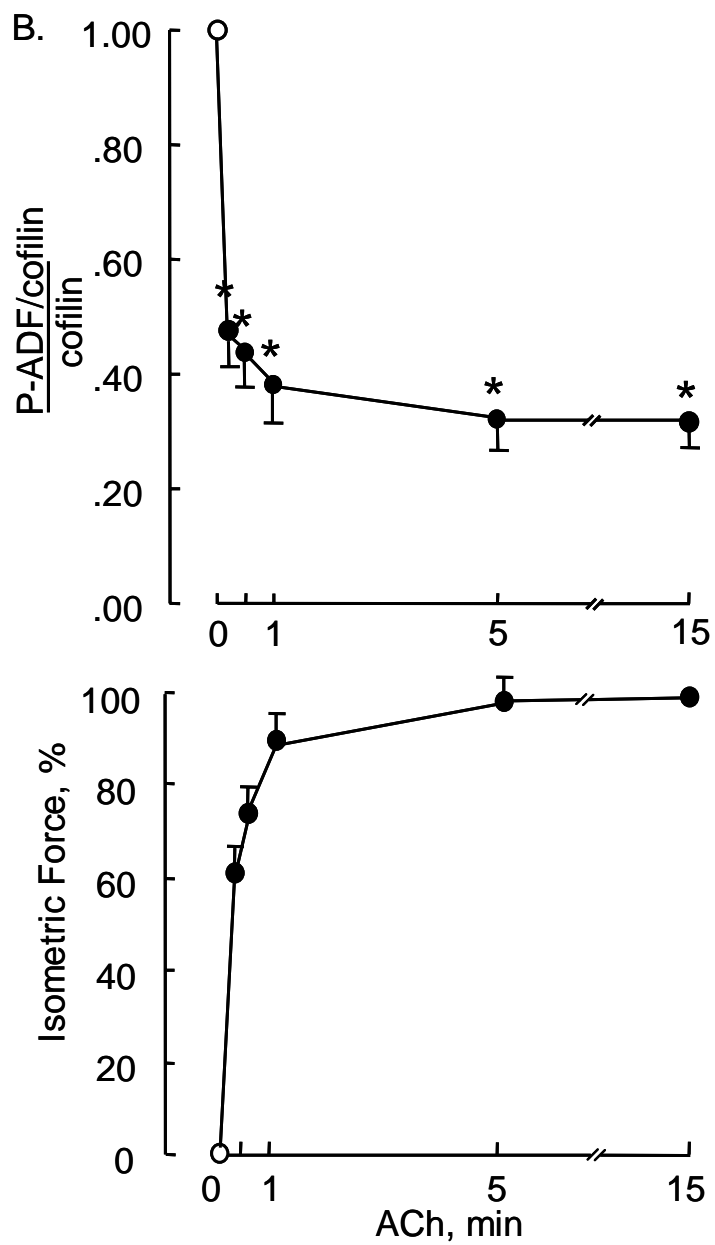
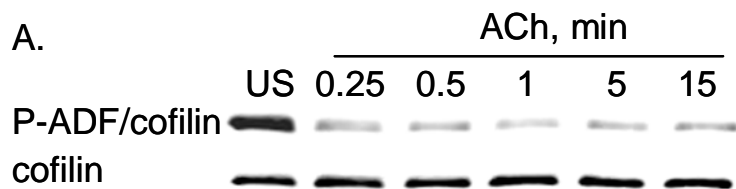


Figure 7. ADF/cofilin phosphorylation decreases during contractile stimulation with ACh or KCl in smooth muscle tissues. A and C. immunoblots of phospho-ADF/cofilin (P-ADF/cofilin) and cofilin in six muscle tissues stimulated with ACh for the indicated time periods (0.25, 0.5, 1, 5, and 15 min) or unstimulated (US), and three muscle tissues stimulated with KCl for 15 or 30 min or unstimulated. ADF/cofilin phosphorylation and total cofilin were quantitated simultaneously using a LI-COR Odyssey infrared scanner. B. Mean ratios of P-ADF/cofilin/cofilin and active tension (% of maximum) at increasing time intervals during contractile stimulation with ACh. * Significant difference in P-ADF/cofilin/cofilin between unstimulated muscles (US) and muscles stimulated with ACh ($p < 0.05$). All values are means \pm S.E. ($n = 6$).

significantly to $61 \pm 5\%$ ($n = 6$, $p < 0.05$) 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 stimulation. The results demonstrate that both receptor-mediated agonists and depolarization with KCl stimulate the dephosphorylation of ADF/cofilin and its activation in tracheal smooth muscle tissues.

2.2. ACh stimulation induces similar dephosphorylation at Ser-3 of ADF and cofilin in tracheal smooth muscle tissues (Figure 8). Two-dimensional gel electrophoresis was used to determine which isoforms of ADF/cofilin are present in tracheal smooth muscle and to quantitate the effects of stimulation with ACh on their phosphorylation (Figure 8A). We found that cofilin and ADF were represented in similar proportions (54 ± 2 and $46 \pm 2\%$, respectively) ($n = 4$). Cofilin2 represented less than 7% of the total ADF/cofilin detected in the muscle tissue. In the unstimulated smooth muscle tissues, the phospho-cofilin was $40 \pm 3\%$ of total cofilin, and this proportion decreased to $14 \pm 3\%$ with ACh stimulation (Figure 8B) ($n = 4$). The proportion of phospho-ADF in unstimulated smooth muscle was $38 \pm 4\%$, and this decreased to $14 \pm 1\%$ with ACh stimulation.

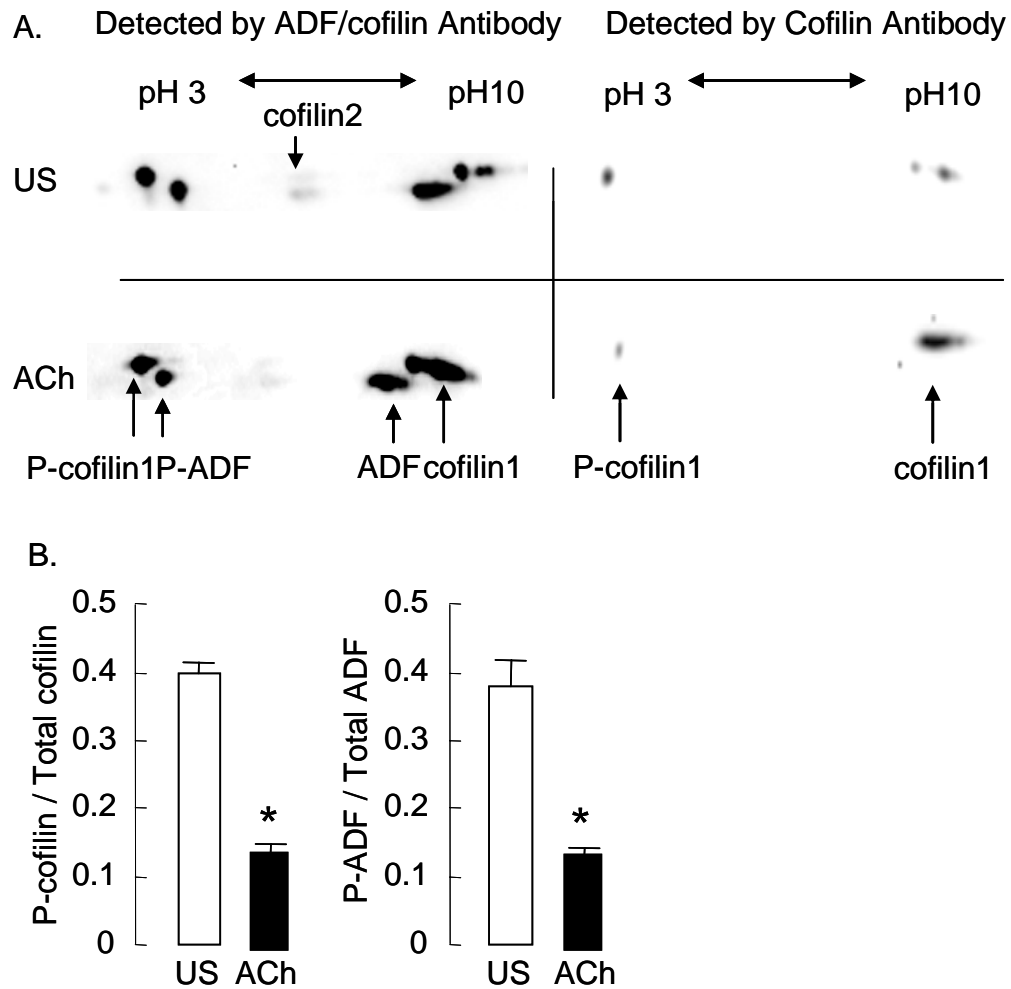


Figure 8. Phosphorylation of both cofilin and ADF decreases with ACh stimulation of smooth muscle tissues. Immunoblots ADF and cofilin isoforms obtained on extracts of stimulated and unstimulated muscles after separation of proteins by two-dimensional electrophoresis. A. representative immunoblot from extracts of one unstimulated (US) smooth muscle tissue, and one stimulated smooth muscle tissue (ACh), probed with anti-ADF/cofilin antibody (left) and stripped and reprobbed with anti-cofilin antibody (right). B. mean values of the proportions of P-cofilin/total cofilin and P-ADF/total ADF in

the muscle extracts. All values are means \pm S.E. * significantly different from unstimulated group, $p < 0.05$ ($n = 4$).

2.3. Expression of the inactive cofilin S3E mutant inhibits the dephosphorylation of endogenous ADF/Cofilin induced by ACh (Figure 9).

Plasmids encoding wild type cofilin (cofilin WT) or the inactive phosphomimetic cofilin mutant S3E (cofilin S3E) were introduced into smooth muscle strips by reversible permeabilization. Transfected tissues and control untreated tissues were then maintained in an incubator for 2 days to allow for the expression of recombinant proteins. Muscle tissues were stimulated with 10^{-5} M ACh for 5 min or left unstimulated and then frozen for the analysis of ADF/cofilin phosphorylation on Ser-3.

The amount of total cofilin was 70–80% higher in each group of muscle strips treated with cofilin WT or cofilin S3E compared with tissues without plasmid treatment ($n = 5$ or 6 , $p < 0.05$), consistent with a robust expression of the recombinant proteins in the transfected tissues (Figure 9C). There were no significant differences in the amount of cofilin in tissues expressing wild type or mutant cofilin constructs. The amount of P-ADF/cofilin in unstimulated muscle tissues treated with either cofilin WT or cofilin S3E was also significantly higher than in control tissues, reflecting the higher levels of cofilin in these tissues. When the P-ADF/cofilin values were normalized to total cofilin, the amount of phosphorylated ADF/cofilin in the cofilin WT-treated and unstimulated cofilin S3E-treated muscles was not significantly different from untreated muscles.

Stimulation with ACh caused a significant decrease in the amount of P-ADF/cofilin in untreated and cofilin WT-treated tissues; when normalized either to GAPDH or to total cofilin. In contrast, ACh did not cause a significant

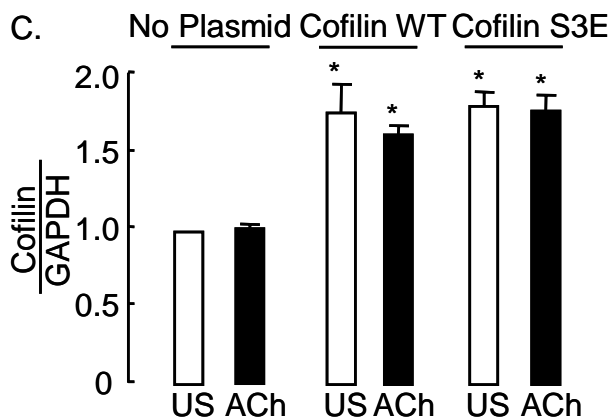
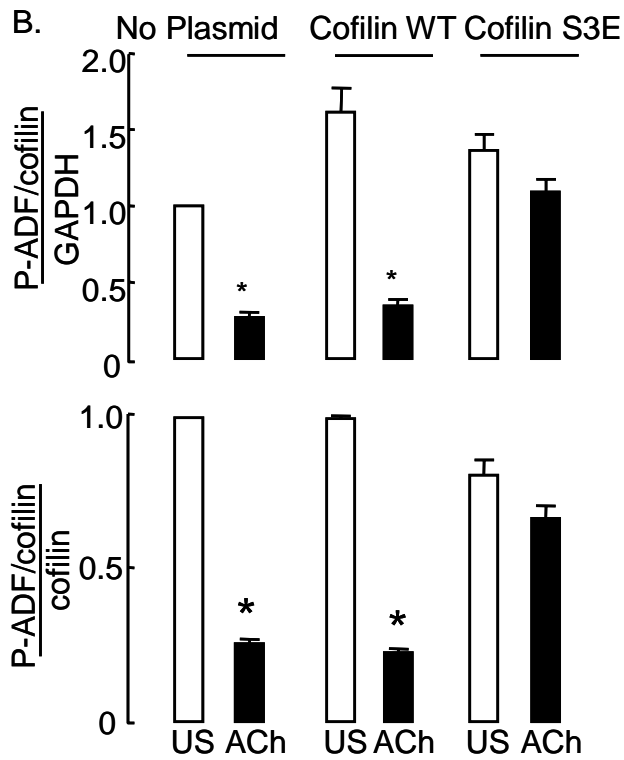
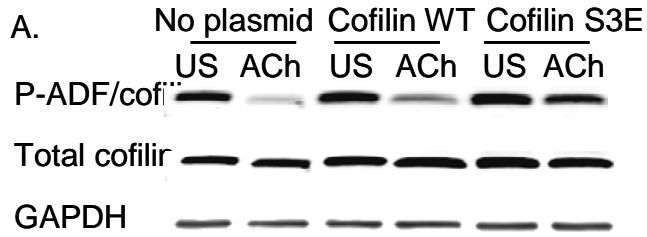


Figure 9. Expression of cofilin S3E inhibits dephosphorylation of ADF/cofilin in response to ACh. A. representative immunoblot of extracts from

six smooth muscle tissues treated with plasmids encoding wild type cofilin (cofilin 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 \pm S.E. * significantly different from the unstimulated group for the same treatment ($n = 5-6$, $p < 0.05$). 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 \pm S.E. * significantly different from the no plasmid unstimulated group ($n = 5-6$, $p < 0.05$).

decrease in the amount of phosphorylated ADF/cofilin in muscle tissues treated with cofilin S3E, whether normalized to GAPDH or total cofilin. Thus, expression of the cofilin S3E mutant in the muscle tissues markedly suppressed ADF/cofilin dephosphorylation and activation in response to stimulation with ACh (Figure 9A and 9B).

We used two-dimensional gel electrophoresis to evaluate the effects of the expression of cofilin S3E on the phosphorylation of both ADF and cofilin in response to ACh stimulation and to quantify the amount of cofilin S3E in the smooth muscle tissue (Figure 10A and 10B). Cofilin S3E represented $34 \pm 5\%$ 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.

2.4. Expression of cofilin S3E inhibits tension development in smooth muscle tissues (Figure 11). The effects of expression of cofilin S3E and cofilin WT on contractile tension was evaluated 5 min after the stimulation of muscle tissues with 10^{-5} M ACh. 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 $52 \pm 4.0\%$ 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 (Figure 11A and 11B).

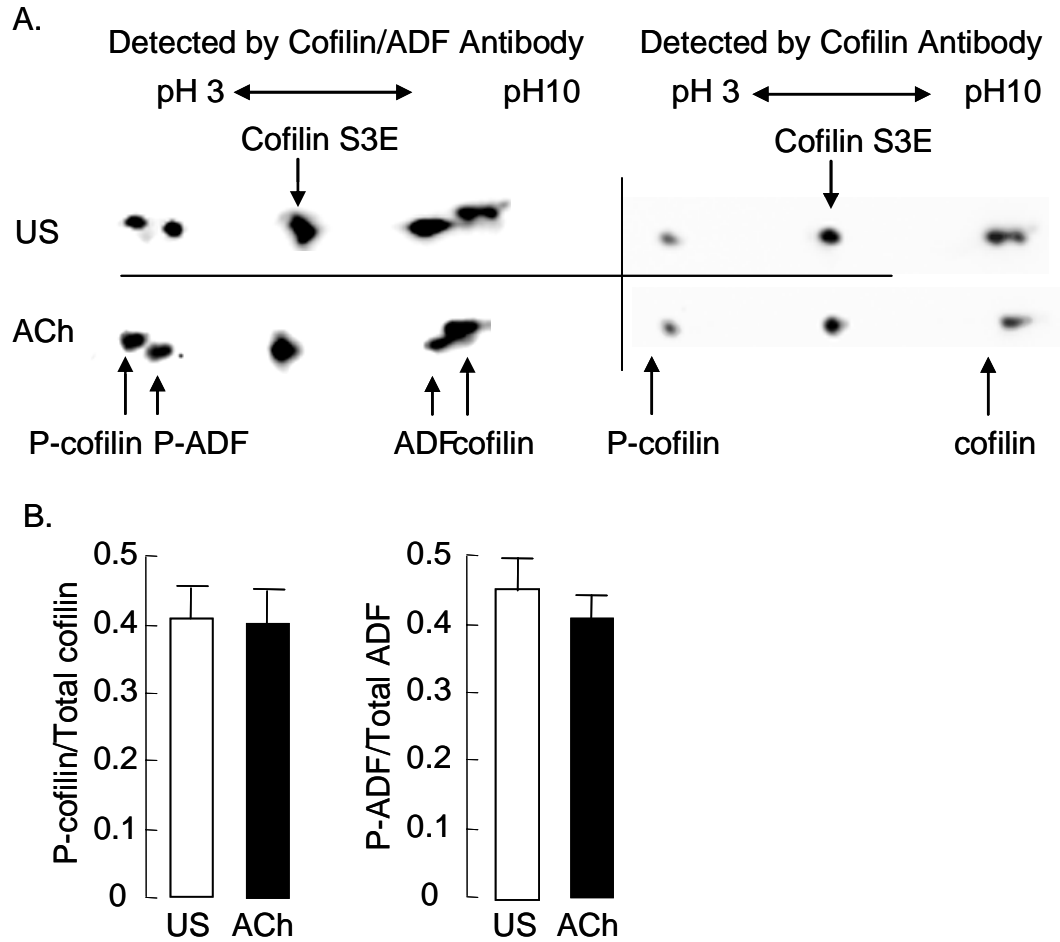


Figure 10. Dephosphorylation of both cofilin and ADF in response to ACh stimulation is inhibited by the expression of cofilin S3E in smooth muscle tissues. Immunoblots of ADF and cofilin isoforms obtained on extracts of ACh-stimulated and unstimulated (US) muscles after separation of proteins by two-dimensional electrophoresis. A. representative immunoblot from extracts of one unstimulated (US) and one stimulated (ACh) smooth muscle tissue after expression of cofilin S3E. Immunoblots were probed with anti-ADF/cofilin antibody (left), stripped, and reprobed with anti-cofilin antibody (right). B. mean values of the proportions of P-cofilin/cofilin and P-ADF/ADF in the muscle

extracts. All values are means \pm S.E. Values for ACh and unstimulated groups are not significantly different ($n = 4$).

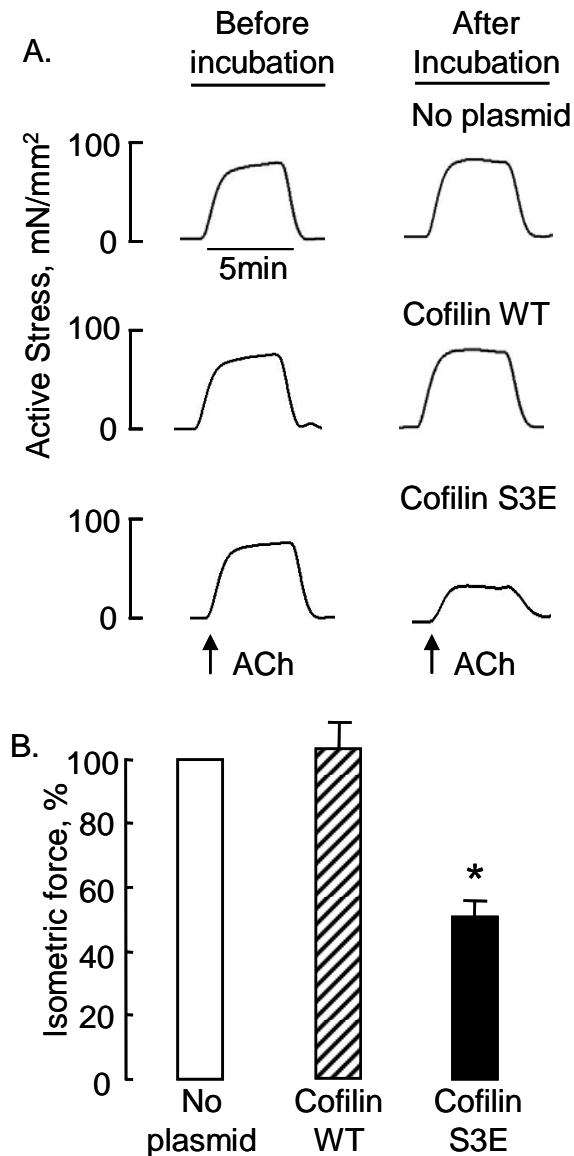


Figure 11. Expression of cofilin S3E inhibits contraction in tracheal muscle strips stimulated with 10^{-5} M ACh. A. representative isometric contractions in response to 10^{-5} M ACh obtained in three muscle strips from one experiment. Contractions were performed before and after a 2-day incubation 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 plasmids), 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 \pm S.E. * force significantly different from no plasmid group. ($n = 27$, $p < 0.05$).

2.5. Expression of inactive mutant cofilin S3E inhibits actin polymerization in response to ACh (Figure 12). The effects of expression of cofilin S3E and cofilin WT on actin polymerization was evaluated. 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 (Figure 12A). 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 (Figure 12B). 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.

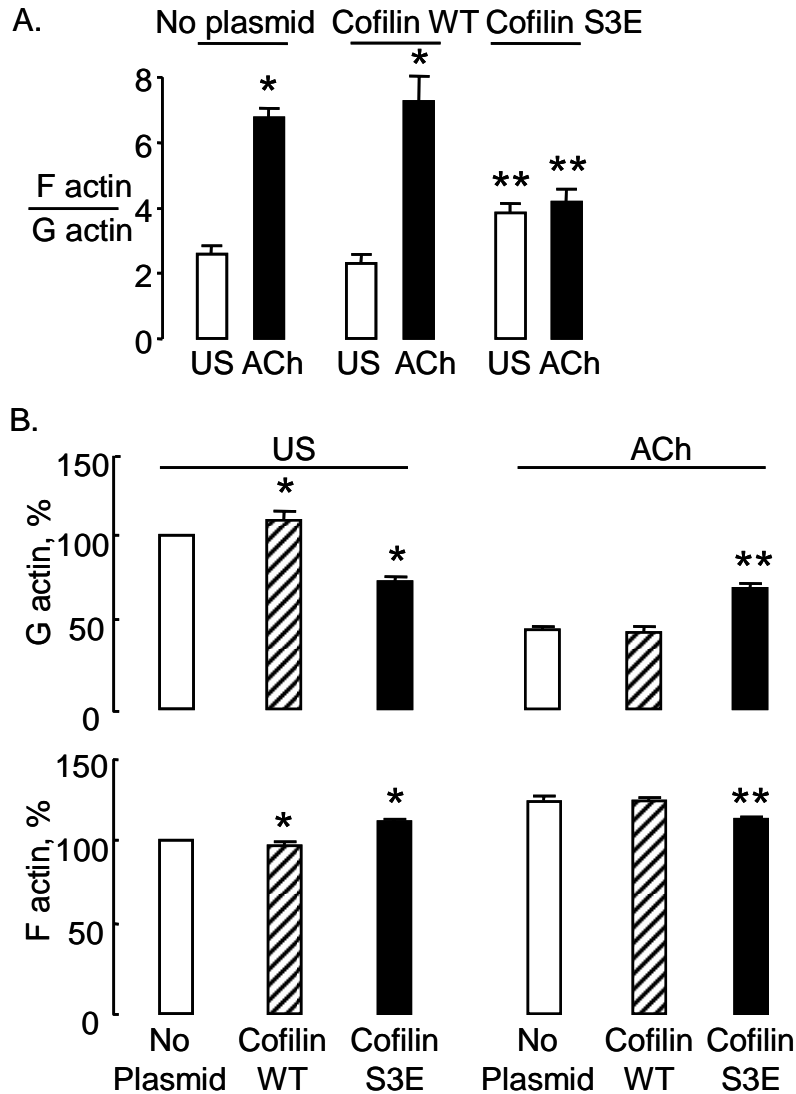


Figure 12. Expression of cofilin S3E inhibits actin polymerization in tracheal muscle strips in response to ACh stimulation. A. mean ratios of F-actin/ G-actin in muscle strips stimulated with ACh (solid bars) or unstimulated (US, open bars). ACh stimulation induced a significant increase in the ratio 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 \pm S.E. ($n = 6, p < 0.05$).

2.6. Expression of inactive cofilin S3E does not affect MLC phosphorylation in response to ACh in smooth muscle tissues (Figure 13).

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.

2.7. Depletion of intracellular Ca^{2+} from muscle tissues or treatment of the tissues with calcineurin inhibitors inhibits the dephosphorylation of ADF/cofilin induced by ACh (Figure 14 and 15).

Calcineurin, the Ca^{2+} – dependent protein phosphatase 2B (PP2B), has been shown to regulate the activation of ADF/cofilin in several non–muscle cell lines (113; 187). The Ca^{2+} dependence of ADF/cofilin activation was evaluated by depleting intracellular Ca^{2+} from muscle tissues. Tissues were incubated in Ca^{2+} –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 (171). This generally required ~60 min and resulted in a reduction of isometric force to less than 10% of the maximal force. ADF/cofilin 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

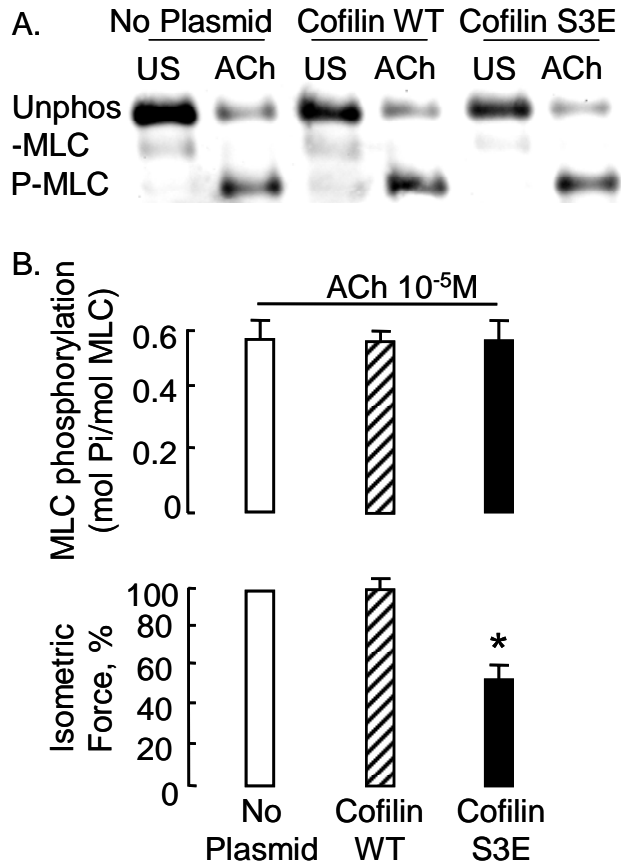


Figure 13. Expression of cofilin S3E does not inhibit the increase in MLC phosphorylation in response to ACh. Muscle tissues treated with plasmids encoding the cofilin WT or cofilin S3E, or not treated with plasmids (No Plasmid) were stimulated with 10⁻⁵ M ACh for 5 min and then frozen for the measurement 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 ($n = 4$, $p < 0.05$).

stimulated by ACh ($n = 6$, $p < 0.05$) (Figure 14). 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 ($10 \mu\text{M}$) or deltamethrin ($10 \mu\text{M}$), for 2 h to evaluate the role of calcineurin in ADF/cofilin activation during active contraction with ACh (Figure 15). ADF/cofilin dephosphorylation was inhibited significantly in ACh–stimulated muscle tissues pretreated with calcineurin inhibitors ($n = 5$, $p < 0.05$). This suggests that the dephosphorylation and activation of ADF/cofilin are mediated by calcineurin in this tissue.

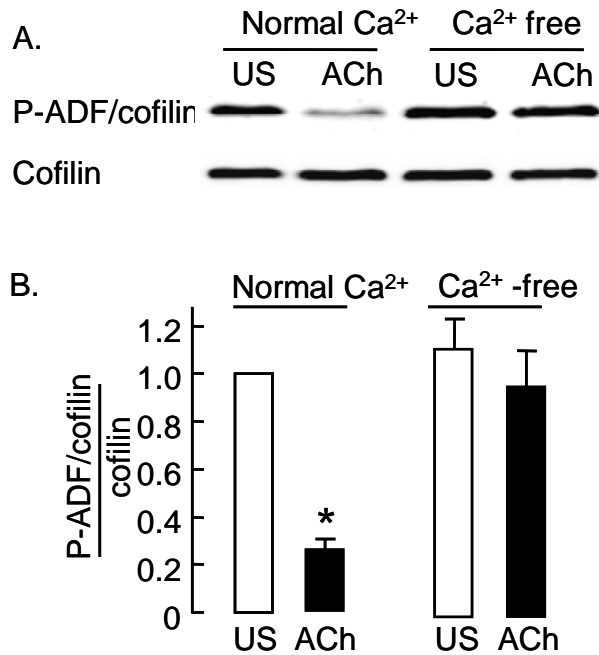


Figure 14. Depletion of intracellular Ca²⁺ inhibits cofilin dephosphorylation in response to ACh. A. immunoblots of phospho-ADF/cofilin and cofilin in four muscle tissues stimulated with ACh for 5 min in normal Ca²⁺ or Ca²⁺ -free PSS containing 0.1 mM EGTA. B. Mean ratios for P-ADF/cofilin/total cofilin 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 Ca²⁺. * significantly different from unstimulated (US) muscles ($n = 6, p < 0.05$). All values are means \pm S.E.

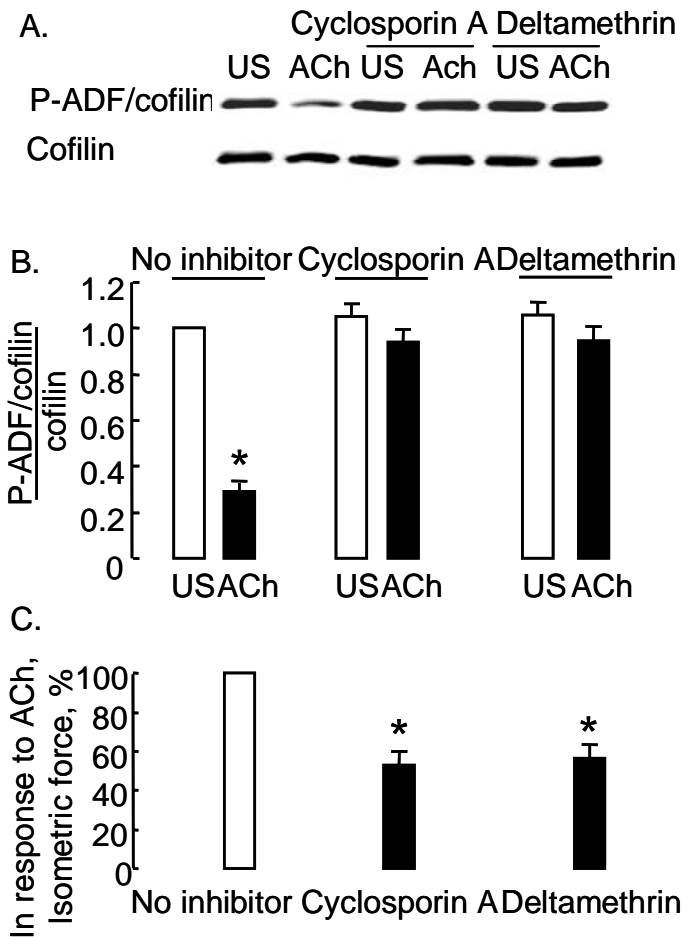


Figure 15. Calcineurin inhibitors inhibit cofilin dephosphorylation in response to ACh. A. immunoblots of phospho-ADF/cofilin and cofilin in six muscle tissues treated with calcineurin inhibitors, cyclosporin A, or deltamethrin 10 μ M for 2 h and then stimulated with ACh for 5 min or left unstimulated (US). B. Mean ratios for P-ADF/cofilin/cofilin for each treatment group. ADF/cofilin phosphorylation was significantly lower in ACh-stimulated muscle strips without calcineurin inhibitors; however, cofilin phosphorylation was not significantly different in ACh-stimulated muscle tissues with calcineurin inhibitors. C. mean force of muscle strips incubated without calcineurin inhibitors and with calcineurin inhibitors (cyclosporin A or deltamethrin). Isometric force was quantified as

percentage of the normalized force in the no plasmid group. * Significantly different from unstimulated muscles in same treatment group ($n = 5$, $p < 0.05$).

Values are means \pm S.E.

Discussion

The 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 (74; 114; 198; 199). 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 (Figure 7). 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 (15; 28; 35). 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 (85). 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 (28). 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 (86). 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 (17; 93; 141).

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 (10; 20; 33; 74; 80; 88; 91; 114; 199). In tracheal smooth muscle, contractile stimulation 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 (199). 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 (74; 198; 199; 201). 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 (74; 198; 201).

Many isoforms of tropomyosin, including muscle-specific isoforms, physiologically inhibit actin filament dynamics and the actin depolymerization and severing caused by ADF/cofilin; thus tropomyosin may serve to spatially segregate stable actin filaments from dynamic subsets of actin filaments (22; 23; 37; 43; 103; 127; 138). In smooth muscle, most of the filamentous actin that is associated with myosin is believed to be bound to tropomyosin (102); thus, it is likely that ADF/cofilin associates with a pool of actin that is not part of the contractile apparatus. This actin could reside within a dynamic submembranous pool of actin that undergoes rapid reorganization during changes in smooth muscle activation. Although there are uncertainties regarding the function of stimulus-induced actin polymerization in differentiated smooth muscle tissues, it is clear that many aspects of the molecular processes involved in the actin polymerization process are analogous to the processes that regulate actin

network assembly and disassembly at the leading edge of motile cells.

We found that the expression of inactive cofilin S3E in tracheal muscle inhibited the dephosphorylation of endogenous cofilin and endogenous ADF in response to stimulation with ACh, thus inhibiting their activation (Figure. 9 and 10). 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 (113). Our observations are consistent with those of Konakahara *et al.* (96) 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 ~70%.

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 (150). 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 (96).

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 (Figure 12). 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 polymerization during contractile activation by processes analogous to those that occur during cell migration (199).

In previous studies of smooth muscle actin dynamics, we and others have found that the inhibition of actin polymerization by latrunculin or by molecular interventions that interrupt the actin polymerization process results in a marked inhibition of active tension development without significantly affecting MLC phosphorylation (74; 114; 170; 173; 199). These 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 inactive cofilin S3E had no effect on the increase in MLC phosphorylation in response to agonist stimulation. Thus the inhibition of active force development by cofilin S3E during contractile stimulation appears to result directly from the inhibition of actin polymerization rather than from effects on MLC phosphorylation or cross-bridge cycling. This is consistent with the possibility that the dynamic pool of actin in smooth muscle is separate from the actin filaments that interact with myosin and participate in actomyosin cross-bridge cycling.

Wild type cofilin was expressed in the tissues to verify that the effects of cofilin S3E overexpression on actin dynamics and contractile function resulted from ADF/cofilin inactivation rather than the overexpression of cofilin protein. We found that the overexpression of wild type cofilin resulted in increases in both inactive phosphorylated cofilin and active cofilin, but the ratio of phospho-cofilin to cofilin was not significantly different from that in the untreated tissues (Figure 9). The dephosphorylation and activation of the ADF/cofilin in the tissues expressing wild type cofilin during contractile stimulation with ACh was also similar to that in untreated tissues. Tissues transfected with the wild type cofilin plasmid exhibited very small but significant differences in the proportions of F- and G-actin before contractile stimulation, suggesting that the increased amount of active cofilin was causing a small increase in actin depolymerization; however, we did not observe a statistically significant effect on the proportions of F- and G-actin after contractile stimulation (Figure 12). Actin polymerization and force development depend on the cooperation of actin regulatory proteins such as N-WASp and the Arp2/3 complex, which may limit the effects of cofilin overexpression on actin dynamics.

In non-muscle cells, LIM kinases and TES (testicular) protein kinases have been identified as specific mediators of ADF/cofilin phosphorylation at Ser-3 (13; 148; 193). LIM kinases are activated by extracellular stimuli and are downstream of small Rho family GTPases and their downstream protein kinases PAK and ROCK, whereas TES kinases are downstream of integrin signaling pathways. ADF/cofilin dephosphorylation is regulated by Slingshot phosphatases

via multiple signaling pathways, which may be activated by cell type and stimulus-specific signaling pathways (85).

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 (170). ACh also stimulates the activation of the small GTPase, RhoA, in this tissue (115). 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 (Figure 7). 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) (Figure 14 and 15). 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 (113; 187).

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.

CHAPTER II

II. ii

The signaling pathways that regulate activation of ADF/cofilin in canine tracheal smooth muscle tissues

1. Summary

In the previous study, the role of ADF/cofilin in the regulation of the airway smooth muscle tissue actin dynamics and contraction was evaluated. In the present study, I focused on the signaling pathways that regulate cofilin activation.

In cultured neural cell lines, ADF/cofilin can be activated by dephosphorylation stimulated by cAMP and dephosphorylation of ADF/cofilin can be inhibited by the non-specific phosphatase, PP1 or PP2A (protein phosphatase 1 or protein phosphatase 2A) inhibitor, calyculin A, which suggest that the cAMP induced dephosphorylation of ADF/cofilin may be through cAMP-PKA-PP1/PP2A pathway (113). In human embryonic kidney (293T) cell and human cervical cancer (HeLa) cells, cofilin has been shown to be activated by the ADF/cofilin-specific protein phosphatase SSH (85), which was regulated by calcium-dependent phosphatase, calcineurin (187). Recent evidence shows that isoproterenol or forskolin led to a significant decrease in the phosphorylation of cofilin in bovine airway smooth muscle cells and bovine pulmonary artery endothelial cells (58; 95), however, the downstream pathway by which cAMP activates cofilin was not determined. However, there is no information regarding

the pathways regulating cofilin activation in smooth muscle tissues. Even at the cellular level, information on the signaling pathways that regulate ADF/cofilin activation is limited.

We hypothesized that phosphatases activate ADF/cofilin airways smooth muscle tissues in response to ACh stimulation. To evaluate this hypothesis, we used an slingshot inactive phosphatase mutant, SSH1L C393S (99), to determine whether slingshot could activate cofilin in response to ACh. We also evaluated the effects of forskolin, an activator of the enzyme adenylyl cyclase, on the regulation of ADF/cofilin activity and actin polymerization in airway smooth muscle tissues.

Our results demonstrate that ADF/cofilin dephosphorylation in response to ACh is inhibited by the inactive phosphatase slingshot mutant, SSH1L C393S, indicating that cofilin activation is regulated by slingshot and that the activity of slingshot may be regulated by Ca^{2+} -dependent calcineurin pathway (187). We also find that ADF/cofilin undergoes dephosphorylation in response to forskolin stimulation in smooth muscle tissues. In contrast to ACh, the activation of cofilin by forskolin does not appear to be regulated by calcineurin. Our results also suggest that the activation of ADF/cofilin is a required for the dynamic reorganization of actin during both the contraction and the relaxation of airway smooth muscle tissues.

2. Results

2.1. ADF/Cofilin activation (dephosphorylation) and force development were inhibited in smooth muscle tissues treated with the inactive phosphatase slingshot mutant plasmid, SSH1L C393S. pcDNA3.1 vectors (human cytomegalovirus as promoter) encoding human the inactive phosphatase slingshot1 mutant C393S (SSH1L C393S) and slingshot wild type (SSH1L WT) (Figure 16) were introduced into smooth muscle strips by reversible permeabilization. Transfected tissues and sham-treated tissues (no plasmid) were then maintained in an incubator for 2 days to allow for the expression of recombinant proteins. Muscle tissues were measured the force development in response to ACh stimulation and finally stimulated with 10^{-5} M ACh for 5 min or left unstimulated and then frozen for the analysis of ADF/cofilin phosphorylation on Ser-3.

The effect of SSH1L C393S and SSH1L WT on stimulus-induced ADF/cofilin activation in tracheal smooth muscle was determined by western blot in extracts from unstimulated and stimulated muscle tissues expressing the SSH1L C393S (Figure 17A and 17B) and SSH1L WT (Figure 17C and 17D) respectively. ACh induced a significant decrease in ADF/cofilin phosphorylation in tissues not treated with plasmids ($n = 5$, $p < 0.05$) or treated in SSH1L WT ($n = 2$, $p < 0.05$). Expression of the SSH1L C393S markedly inhibited the ADF/cofilin dephosphorylation in response to stimulation with ACh (Figure 17, $n = 5$, $p < 0.05$).

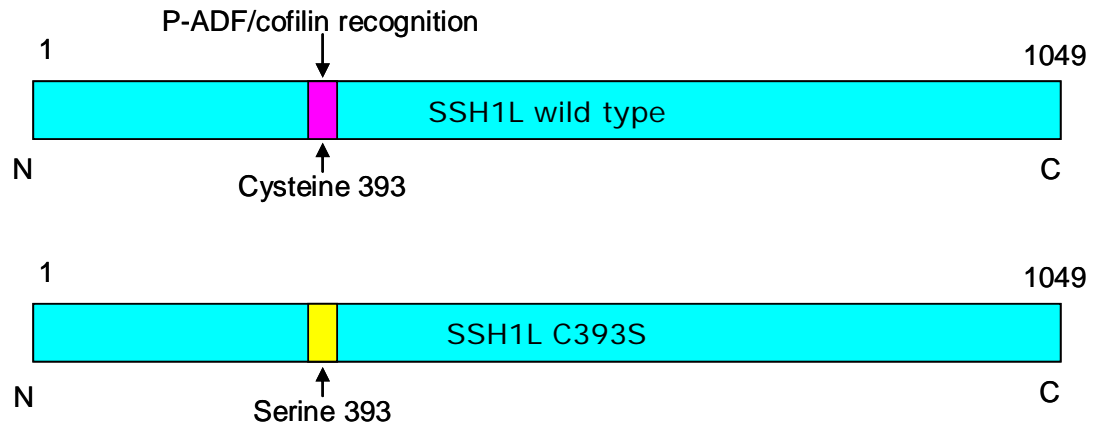


Figure 16. The structure of slingshot1L wild type and inactive phosphatase slingshot1L mutant. Cysteine 393 of slingshot is the critical site for regulating binding to the phospho-ADF/cofilin. In the inactive phosphatase slingshot1L mutant (SSH1L C393S), cysteine 393 is replaced by serine. The inactive phosphatase slingshot mutant (SSH C393S) forms an irreversible enzyme-substrate complex with phospho-ADF/cofilin and thereby inhibits the endogenous slingshot from binding to phospho-ADF/cofilin (99).

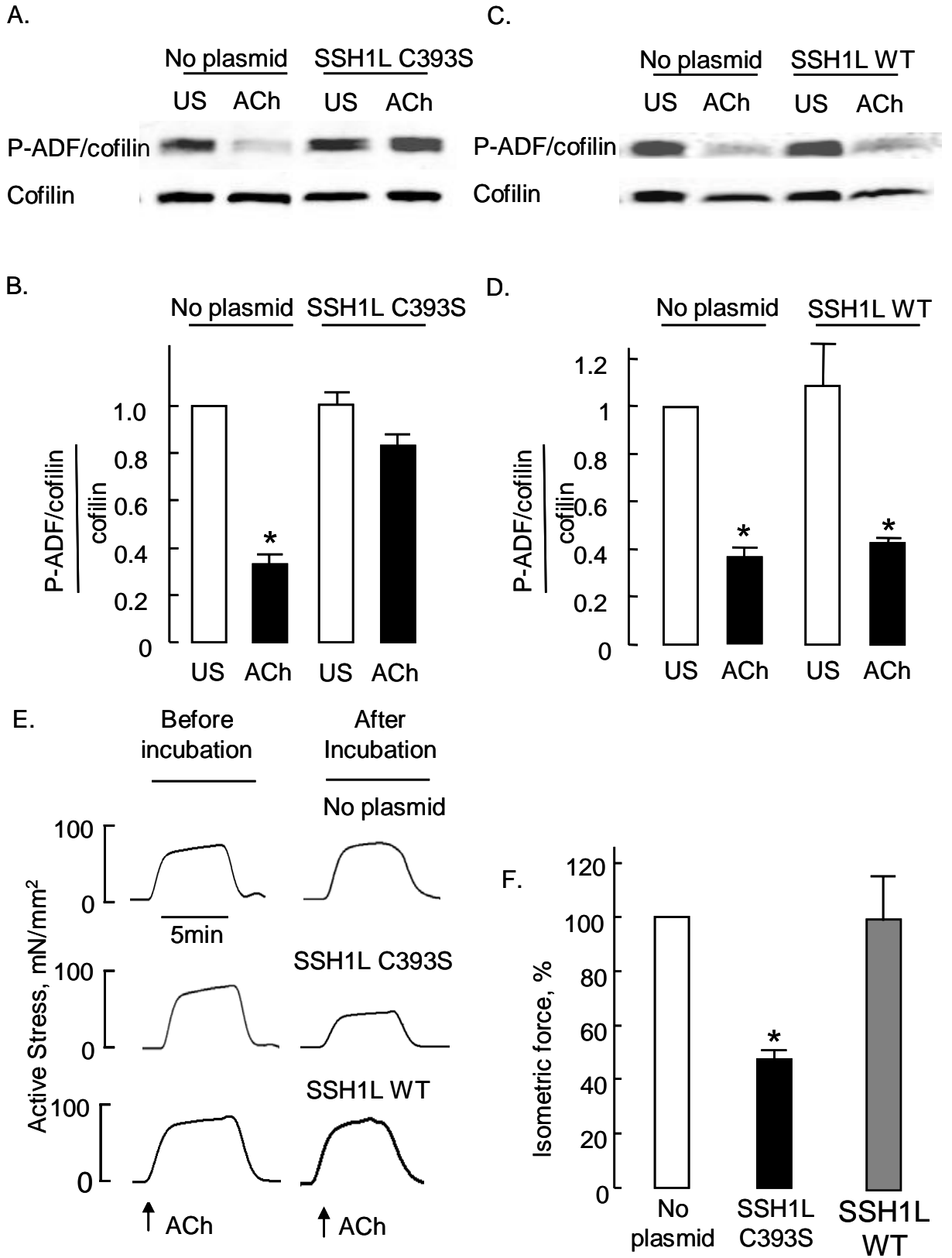


Figure 17. Expression of SSH1L C393S inhibits dephosphorylation of ADF/cofilin and force development in response to Ach in smooth muscle tissues. A. Representative immunoblot of extracts from 4 smooth muscle tissues treated with plasmids encoding inactive phosphatase slingshot mutant (SSH1L C393S), or from tissues not treated with plasmids (No Plasmid). Immunoblots were probed with antibodies to phospho ser-3 ADF/cofilin, cofilin. B. Mean values for Phospho-ADF (P-ADF/cofilin) in muscle strips without plasmid (no plasmid) or expressing SSH1L C393S stimulated with ACh or unstimulated (US). Values are normalized to the No Plasmid US. C. Representative immunoblot of extracts from 4 smooth muscle tissues treated with plasmids encoding wild type slingshot (SSH1L WT), or from tissues not treated with plasmids (No Plasmid). Immunoblots were probed with antibodies to phospho ser-3 ADF/cofilin, cofilin. D. Mean values for Phospho-ADF (P-ADF/cofilin) in muscle strips without plasmid (no plasmid) or expressing SSH1L WT stimulated with ACh or unstimulated (US). Values are normalized to the No Plasmid US. group. All values are means \pm S.E.M. * Significantly different from US group for the same treatment ($n = 2$, $p < 0.05$). E. Representative isometric contractions in response to 10^{-5} M ACh obtained in 3 muscle strips from one experiment. Contractions were performed before and after 2-day incubation with plasmids encoding inactive phosphatase slingshot mutant (SSH1L C393S) or slingshot wild type (SSH1L WT), or after incubation without plasmids (No plasmid). Contractile force in response to 10^{-5} M ACh was dramatically inhibited in tissues treated with plasmids encoding inactive phosphatase mutant SSH (SSH1L C393S), but it was not depressed in tissues

not treated with plasmids or treated with slingshot wild type (SSH1L WT) plasmids. F. Mean force of muscle strips incubated without plasmids (No plasmids), with plasmids encoding inactive phosphatase mutant SSH (SSH C393S). Isometric force was quantified as percentage of the normalized force in the No plasmid group. Values are means \pm SEM. * Force significantly different from No Plasmid group. ($n = 2-5$, $p < 0.05$).

SSH1L C393S significantly inhibited contractile force in response to 5 min stimulation with 10^{-5} M ACh relative to sham-treated smooth muscle tissues and SSH1L WT treated tissues (Figure 17E and 17F) ($n = 2-5$, $p < 0.05$).

2.2. Forskolin prevents actin polymerization induced by ACh in tracheal smooth muscle tissues (Figure 18). The effect of forskolin on actin polymerization was evaluated. Smooth muscle strips were stimulated with 10^{-5} M ACh for 5 min, and then treated with 10^{-5} M forskolin for 30 min. Control tissues were treated with ACh alone for the same time period. Then the proportions of F-actin to G-actin in muscle extracts were analyzed by cell fractionation and immunoblot. Whereas 10^{-5} M ACh stimulation significantly increased the ratio of F-actin to G-actin in smooth muscle strips not treated with forskolin ($n = 6$; $p < 0.05$), ACh stimulation did not significantly alter the ratio of F-actin to G-actin in smooth muscle tissues treated with forskolin. The results indicate that forskolin inhibits actin polymerization in muscle strips in response to ACh stimulation.

2.3. Forskolin induced ADF/cofilin dephosphorylation on Ser-3 and relaxation in tracheal smooth muscle tissues (Figure 19). The effect of forskolin on ADF/cofilin dephosphorylation on Ser-3 was evaluated. Eight canine tracheal smooth muscle strips were maintained for 30 min without contractile stimulation and then half of the muscle strips were treated with 0, 10, 30, or 100 μ M forskolin for 30 min, while the other half of the muscle strips were stimulated with 10^{-5} ACh for 5 min and then treated with 0, 10, 30, or 100 μ M forskolin for 30

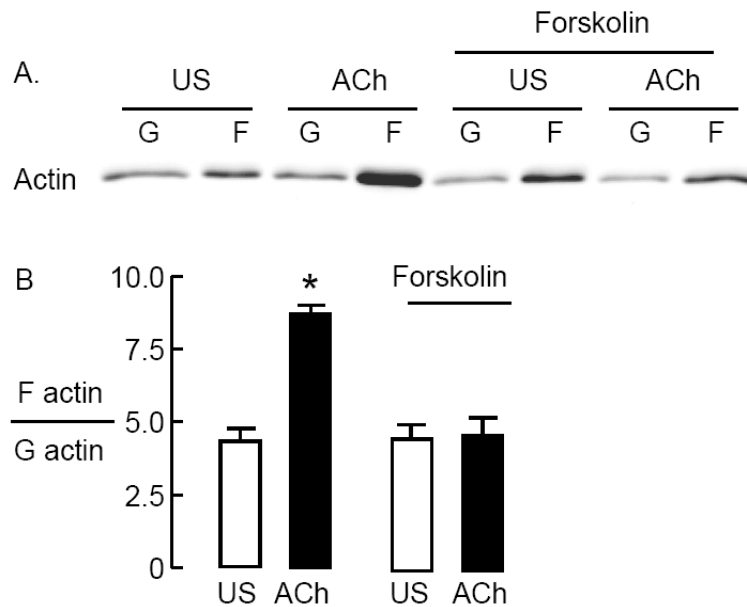


Figure 18. Forskolin reversed the actin polymerization in tracheal smooth muscle tissues. A. Representative immunoblot of extracts from 4 smooth muscle tissues unstimulated (US), stimulated with ACh, stimulated with 10^{-5} M forskolin (FSK), or stimulated with 10^{-5} M ACh for 5 min followed by 10^{-5} M forskolin (FSK) for 30 min. Immunoblots were probed with antibodies to total actin. B. Mean ratios of F-actin/G-actin for each treatment group. ACh stimulation induced a significant increase in the ratio of F-actin/G-actin in smooth muscle strips not treated with forskolin. In muscle strips treated with forskolin alone, the ratio of F-actin to G-actin was similar to that in unstimulated tissues. Stimulation by forskolin reversed the increase the ratio of F-actin to G-actin caused by ACh stimulation. * significant difference from corresponding US no FSK muscles in same treatment group. ($n = 6$, $P < 0.05$).

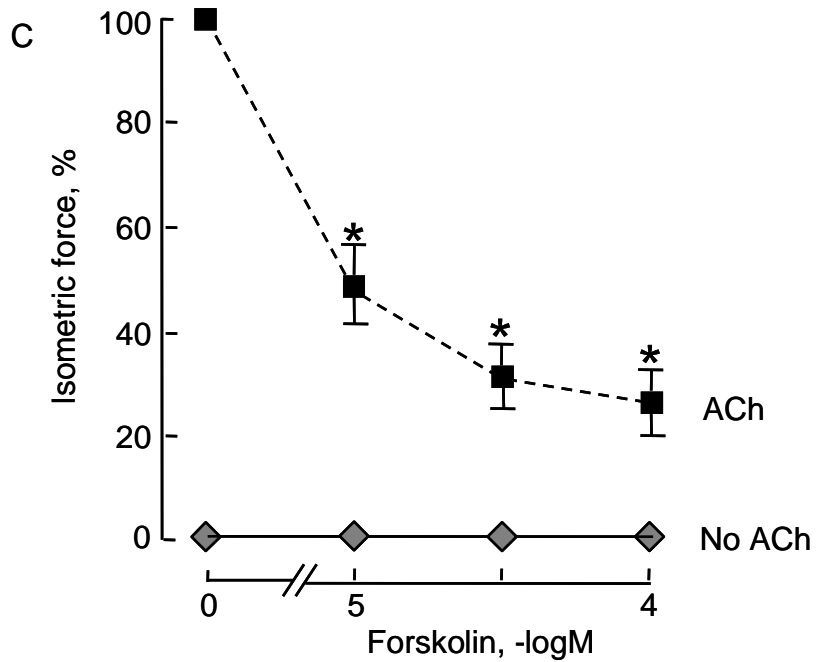
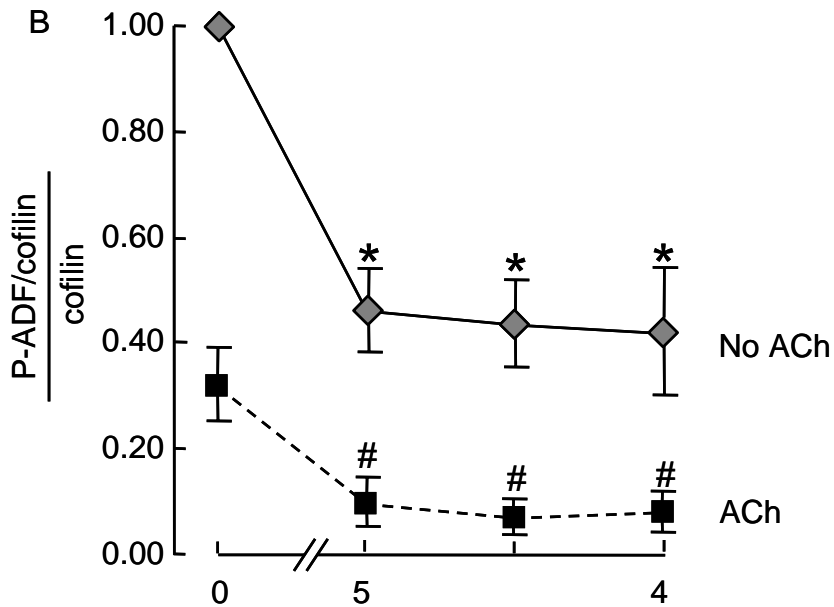
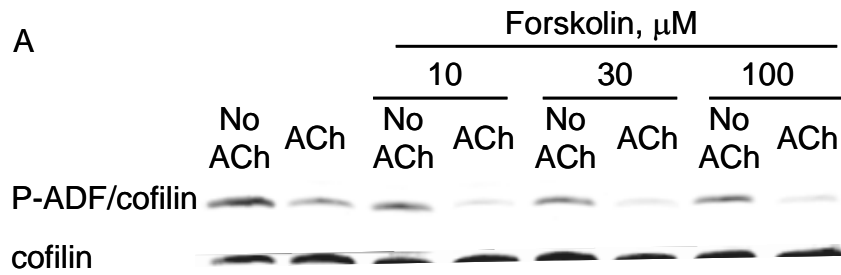


Figure 19. Forskolin induced ADF/Cofilin dephosphorylation on Ser-3 in tracheal smooth muscle tissues. A. Representative immunoblot of extracts from 8 smooth muscle tissues. Half of the muscle strips were not stimulated with ACh, but treated with 0, 10, 30, or 100 μ M forskolin for 30 min, while the rest half of the muscle strips were stimulated with 10^{-5} ACh for 5 min and then followed with 0, 10, 30, or 100 μ M forskolin for 30 min. Immunoblots were probed with antibodies to phospho ser-3 ADF/cofilin, cofilin. B. Mean values for Phospho-ADF (P-ADF)/cofilin and tension development in muscle strips treated without forskolin or forskolin (FSK) 10, 30, 100 μ M for 30 min. Values are normalized to no ACh and no forskolin treated group. All values are means \pm S.E.M. * Significantly different from no forskolin treated group ($n = 6$, $p < 0.05$).

min. ADF/cofilin phosphorylation on Ser-3 and force development were elevated in the above smooth muscle tissues. ADF/cofilin was dephosphorylated significantly in smooth muscle tissues treated with forskolin alone or treated with 10^{-5} M ACh for 5 min and then followed with forskolin (Figure 19A and 19B), and forskolin significantly decreased the force development in response to ACh stimulation (Figure 19B) ($n = 6$; $p < 0.05$).

2.4. Depletion of intracellular Ca^{2+} from muscle tissues does not inhibit the dephosphorylation of ADF/cofilin induced by forskolin (Figure 20). The Ca^{2+} –dependence of ADF/cofilin activation induced by forskolin was evaluated by depleting intracellular Ca^{2+} from muscle tissues. Tissues were incubated in Ca^{2+} –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 (171). This generally required ~60 min and resulted in a reduction of isometric force to less than 10% of the maximal force. ADF/cofilin phosphorylation was then evaluated in the Ca^{2+} –depleted tissues incubated in PSS without forskolin or with 10^{-5} M forskolin for 30 min. The depletion of Ca^{2+} did not prevent the decrease in ADF/cofilin phosphorylation stimulated by forskolin but it prevented the ADF/cofilin dephosphorylation stimulated by ACh ($n = 6$, $p < 0.05$). This suggests that the dephosphorylation and activation of ADF/cofilin induced by forskolin is mediated by Ca^{2+} – independent mechanisms in tracheal smooth muscle tissue.

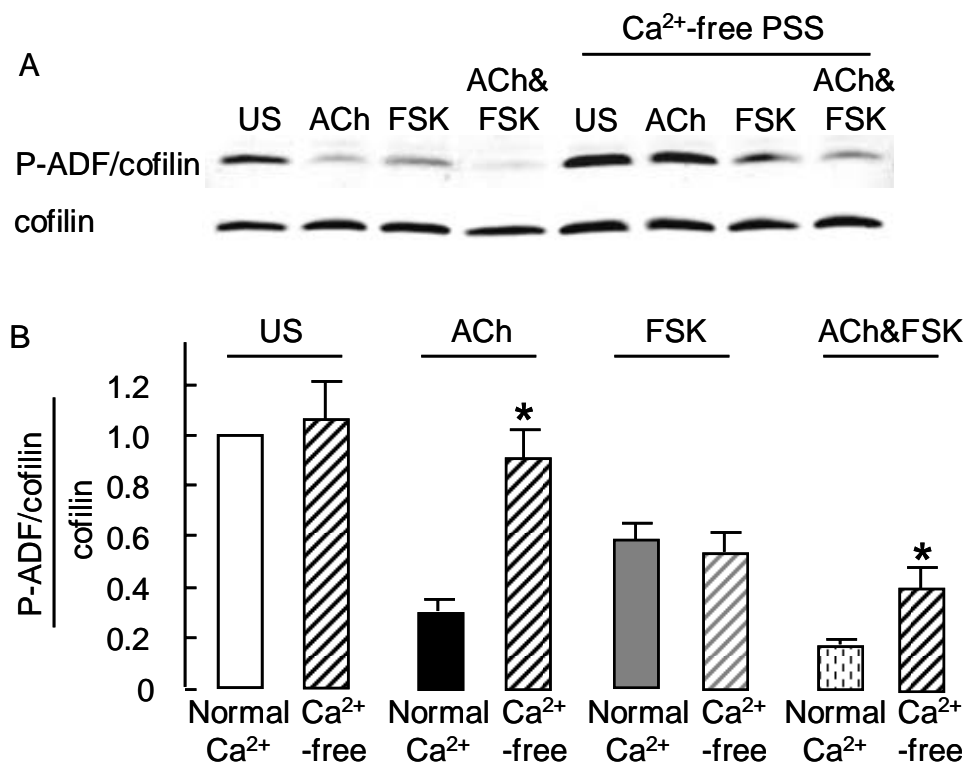


Figure 20. Forskolin induced ADF/cofilin dephosphorylation in tracheal smooth muscle tissues which is calcium insensitive. A. Representative immunoblot of extracts from 8 smooth muscle tissues of normal Ca²⁺ PSS or Ca²⁺ depletion PSS (Ca²⁺ free), The first 4 muscle strips were unstimulated (US), stimulated with 10⁻⁵ M ACh, stimulated with 10⁻⁵ M forskolin (FSK), or first simulated with 10⁻⁵ M ACh 5 min then followed by 10⁻⁵ M forskolin (ACh&FSK) for 30 min; the another 4 muscle strips are depleted with intracellular calcium (Ca²⁺ free) and then were unstimulated (US), stimulated with 10⁻⁵ M ACh, stimulated with 10⁻⁵ M forskolin (FSK), or first simulated with 10⁻⁵ M ACh 5 min then followed by 10⁻⁵ M forskolin (ACh&FSK) for 30 min. Immunoblots were probed with antibodies to phospho-ADF/cofilin at serine 3, cofilin. B. Mean ratios of phospho-ADF (P-ADF)/cofilin to cofilin for each treatment group.

Values are normalized to US group. All values are means \pm S.E.M. * Significantly different from muscles of normal Ca^{2+} PSS in the same treatment group ($n = 6$, $p < 0.05$).

2.5. ADF/cofilin activation induced by forskolin is not mediated by calcium-dependent calcineurin in the tracheal smooth muscle tissues (Figure 21). The calcineurin inhibitors, cyclosporin A (10 μ M) and deltamethrin (10 μ M) were applied to evaluate whether the ADF/cofilin dephosphorylation induced by forskolin is mediated by calcineurin. The muscle strips were treated without inhibitors (No In), with cyclosporin A (10 μ M) (CycloA), or with deltamethrin (10 μ M) (Delta) for 2 hours and then stimulated with 10⁻⁵ M forskolin (FSK) or not stimulated for 30 min. ADF/cofilin phosphorylation was then evaluated. The calcineurin inhibitors did not prevent the ADF/cofilin dephosphorylation induced by forskolin in tracheal smooth muscle ($n = 5, p < 0.05$). This suggests that forskolin did not activate ADF/cofilin through calcium-calcineurin pathway.

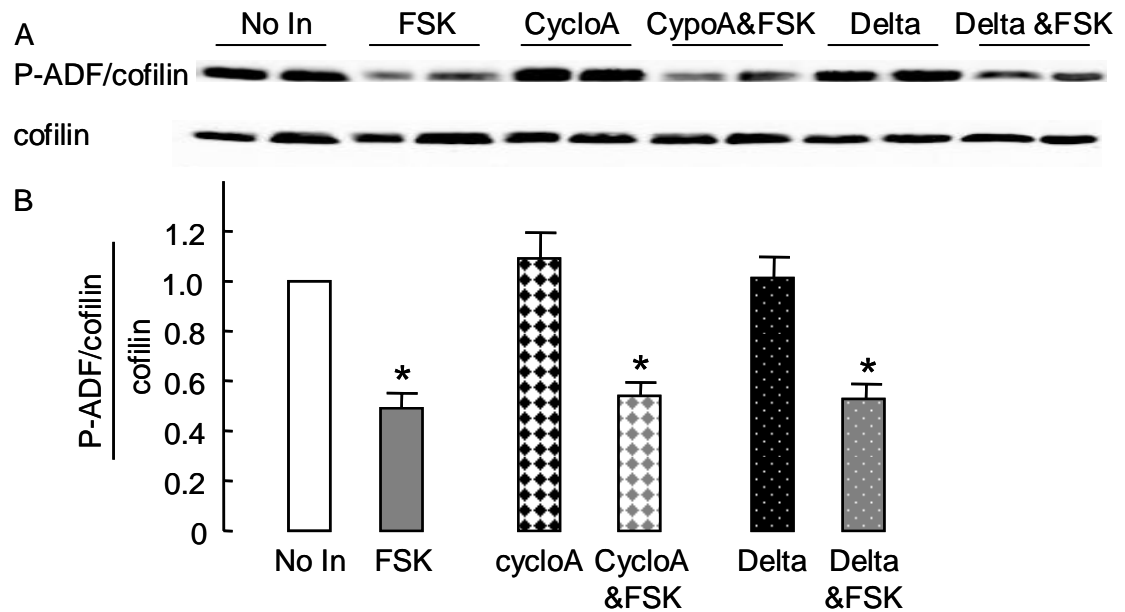


Figure 21. ADF/cofilin activation induced by forskolin is not through calcium-dependent calcineurin in the tracheal smooth muscle tissues. A. Representative immunoblot of extracts from 12 smooth muscle tissues, The first 6 muscle strips were double treated respectively with no inhibitors (No), cyclosporin A (10 μ M) (C), deltamethrin (10 μ M) (D) for 2 hours, the another 6 muscle strips were treated in the similar way and then followed by 10⁻⁵ M forskolin (FSK) for 30 min. Immunoblots were probed with antibodies to phospho-ADF/cofilin at serine 3, cofilin. B. Mean ratios of phospho-ADF (P-ADF)/cofilin to cofilin for each treatment group. Values are normalized to US group. All values are means \pm S.E.M. * Significantly different from muscles without no inhibitor (No In) and not followed by forskolin (FSK) in the same treatment group ($n = 5$, $p < 0.05$).

Discussion

The present results provide the first documentation for the regulation of the activation of ADF/cofilin is by specific phosphatases (130; 137; 168) during airway smooth muscle contraction with ACh stimulation. The inactive phosphatase slingshot1 mutant, SSH1L C393S, was designed as the dominant negative mutant (99; 168) by replacing cysteine-393 with serine. SSH1L C393S can form a stable enzyme-substrate complex with phospho-ADF/cofilin, thus it can inhibit the dephosphorylation of cofilin by endogenous phosphatases by sequestering phospho-ADF/cofilin. When the SSH1L C393S was expressed in smooth muscle tissues, force development and ADF/cofilin dephosphorylation/activation induced by acetylcholine was inhibited. This confirmed the hypothesis that phosphatases regulate ADF/cofilin activation in smooth muscle tissues in response to ACh stimulation. Combined with my previous observations (section II.i.), these results suggest that ACh-induced ADF/cofilin activation in airway smooth muscle tissues may be through a calcium-calcineurin-SSH pathway (202). Mizuno and colleagues demonstrated that in HeLa cells, ADF/cofilin is dephosphorylated/activated in response to Ca^{2+} ionophore A23187 and Ca^{2+} -mobilizing agonists, ATP and histamine, through a calcium-calcineurin-SSH pathway (187).

In results described previously (section II.i.), my data indicated that active ADF/cofilin is required for actin polymerization and force development in airway smooth muscle (202). The smooth muscle relaxant, forskolin, also dephosphorylates and activates ADF/cofilin but it prevents actin polymerization

during airway smooth muscle contraction. Similar results have been reported in cultured cells: Emala and colleagues reported that isoproterenol, a β -receptor agonist, induced actin depolymerization in cultured human airway smooth muscle cells (82). It was also reported that elevated cAMP can activate cofilin in bovine artery endothelial cells and bovine airway smooth muscle cells(58; 95). My results support the hypothesis that active ADF/cofilin is necessary for actin dynamics that occurs during both contraction and relaxation of airway smooth muscle. Whether active ADF/cofilin promotes actin polymerization or not may depend on the availability of actin subunits within the region of the cell where this process occurs, and the activation state of proteins involved in the actin polymerization process, such as N-WASp (35). My studies also suggest that the effect of ADF/cofilin activation on actin dynamics is not the same for all stimuli, as indicated by *in vitro* studies of ADF/cofilin (35). Active ADF/cofilin may generate free barbed ends on actin filaments, which will depolymerize in the presence of relaxants, such as forskolin, due to the absence of other factors that promote actin polymerization. My preliminary results suggest that active ADF/cofilin can sever the actin filaments and generate free barbed ends, but whether actin polymerization or depolymerization occurs depends on the upstream signals.

I have also evaluated the signaling pathways that regulate the activation of ADF/cofilin in response to forskolin stimulation, which induces airway smooth muscle relaxation. Previous studies on neural cells, vascular endothelial cells and bovine airway smooth muscle cells have suggested that elevated cAMP can lead to the activation of ADF/cofilin (58; 95; 113), but there are almost no studies

that demonstrate how cAMP activates ADF/cofilin. In the above studies, Meberg PJ. *et al.* (113) and Goeckeler ZM. *et al.* (58) respectively used an inhibitor (calyculin A) of the non-specific phosphatase, PP1 or PP2A (protein phosphatase 1 or protein phosphatase 2A) to prevent cofilin activation induced with by cAMP in neural cells and bovine artery endothelial cells. This evidence suggests that the dephosphorylation of ADF/cofilin induced by cAMP may be through PP1/PP2A. Our results suggest that forskolin can activate ADF/cofilin through a calcium-independent pathway. Depletion of intracellular Ca^{2+} from smooth muscle tissues does not inhibit the activation of ADF/cofilin induced by forskolin, but it does inhibit cofilin activation in response to contractile stimulation with ACh. This suggests that activation of ADF/cofilin during smooth muscle relaxation induced by forskolin is Ca^{2+} -independent.

In results described previously (section II.i.), I found that ACh stimulation induced ADF/cofilin dephosphorylation through a calcium-calcineurin dependent pathway. Thus, calcineurin inhibitors were applied to evaluate whether ADF/cofilin activation induced by forskolin is mediated by calcineurin. My results showed calcineurin inhibitors did not prevent ADF/cofilin activation induced by forskolin. This suggests that forskolin activates ADF/cofilin by a different mechanism than ACh in airway smooth muscle tissues.

CHAPTER III

Perspectives

Airway hyperresponsiveness is the fundamental mechanism of asthma and reorganization of actin filament network may be involved in this process. There is extensive evidence that actin polymerization is initiated by contractile stimulation in smooth muscle and this actin polymerization is required for active contraction (10; 20; 33; 80; 114; 173). The increase in the proportion of filamentous actin that occurs in response to the stimulation of smooth muscle cells and the essential role of stimulus-induced actin polymerization in the generation of mechanical tension have been convincingly documented in many smooth muscle tissues and cells using a wide variety of experimental approaches (55; 74). There is also extensive evidence that the functional role of actin polymerization during contraction is distinct and separately regulated from the actomyosin cross-bridge cycling process. However, much more data will be needed to establish the molecular basis for the regulation of actin polymerization and its functional roles in diverse types of smooth muscle cells and tissues.

ADF/cofilin is a critical factor in regulating actin dynamics. The first study (Chapter II.i.) addressed the hypothesis that the activation of ADF/cofilin is necessary in smooth muscle contraction and actin polymerization. To address this objective, the roles of ADF/cofilin in regulating actin polymerization in smooth muscle in response to extracellular stimuli were determined. The phosphomimetic mutant of cofilin, cofilin S3E, which can inhibit the endogenous

activation of the ADF/cofilin and actin polymerization *in vitro* studies, was expressed in muscle strips to inhibit the activation of the endogenous ADF/cofilin. Expression of the cofilin S3E may inhibit endogenous ADF/cofilin dephosphorylation by competitively binding to the ADF/cofilin specific phosphatase, slingshot. This inhibition of the ADF/cofilin activation caused by cofilin S3E inhibited actin polymerization and tension development in response to ACh, but it does not affect MLC phosphorylation in the smooth muscle tissues. These results suggest active ADF/cofilin is necessary for agonist-induced actin polymerization and tension development in smooth muscle.

In a separate set of studies (Chapter II.ii.), I evaluated the pathways that regulate the activation of ADF/cofilin during smooth muscle contraction. To address this objective, the SSH1 dominant negative mutant, SSH1L C393S, was introduced in muscle strips to inhibit the activation of the endogenous ADF/cofilin. The inhibition of the ADF/cofilin activation caused by SSH1L C393S inhibited tension development in response to ACh in the smooth muscle tissues. These results suggest activation of ADF/cofilin in response to contractile stimuli is regulated by phosphatases.

The role of active ADF/cofilin in the regulation of actin polymerization during smooth muscle relaxation and the pathways that activate ADF/cofilin during smooth muscle relaxation were also evaluated. The activator of adenylyl cyclase, forskolin, was used to determine the role of ADF/cofilin in smooth muscle relaxation and actin depolymerization and the pathways that activate ADF/cofilin during smooth muscle relaxation. These results suggest ADF/cofilin is

activated during smooth muscle relaxation, and that actin polymerization is inhibited. This pathway that activates ADF/cofilin on response to forskolin was not calcium-dependent.

In conclusion, actin polymerization initiated by contractile stimulation and actin depolymerization initiated by relaxing stimulation in smooth muscle tissues both require active ADF/cofilin activation (Figure 22).

However, there are some interesting questions that need to be explored further. The first question is whether nonspecific phosphatases, such as calcineurin (PP2B), regulate slingshot activation. Since the inactive phosphatase slingshot mutant can prevent the endogenous slingshot from binding with the phospho-ADF/cofilin, it can also inhibit other nonspecific phosphatases by a similar mechanism. Thus, knockdown the endogenous slingshot by introducing the siRNA of slingshot would establish whether slingshot is the only phosphatase that directly dephosphorylates cofilin. If the ADF/cofilin dephosphorylation is inhibited by knocking down slingshot, it will suggest that slingshot activity is required for the regulation of ADF/cofilin activation in airway smooth muscle, and that other phosphatases act upstream of slingshot. The measurement of slingshot activity *in vitro* with a calcineurin inhibitor or a dominant negative calcineurin mutant (187) would also provide evidence as to whether calcineurin activates slingshot. However, based on my present results it is unclear whether or not calcineurin directly dephosphorylates cofilin.

Secondly, the pathways that regulate cofilin activation induced by forskolin are not yet clear. However, in airway smooth muscle, the activation of

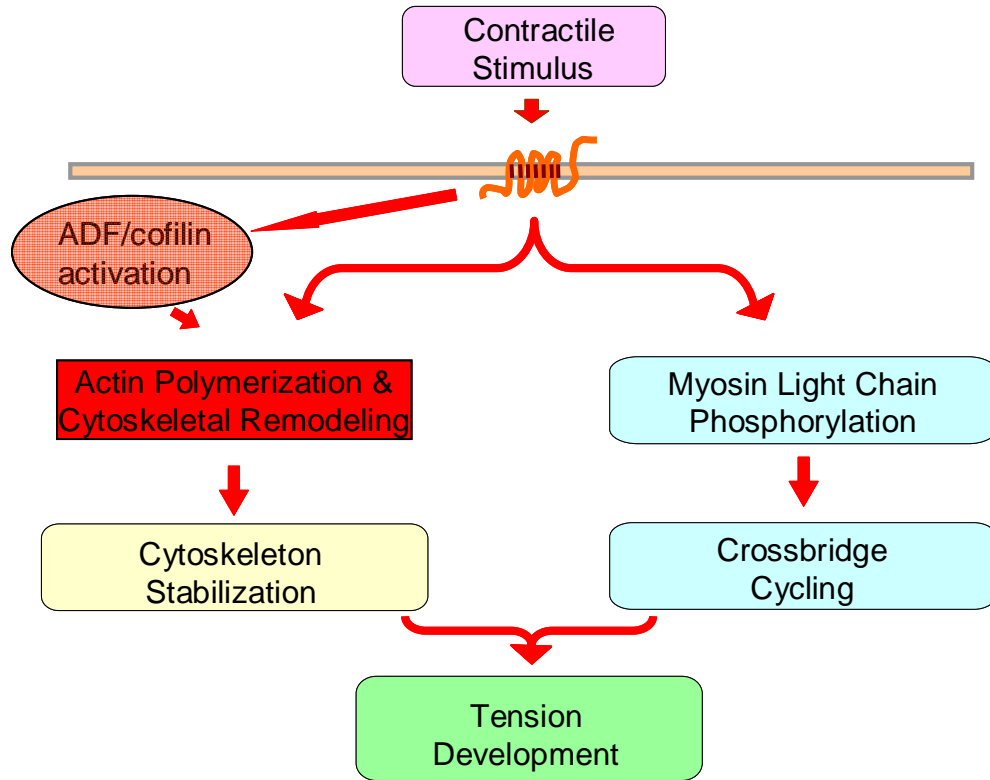


Figure 22. The role of active ADF/cofilin in smooth muscle force development. The activation of ADF/cofilin regulates actin dynamics, which is required for tension development in smooth muscle tissues.

ADF/cofilin induced by forskolin appears to be mediated by a Ca^{2+} insensitive mechanism that does not involve calcineurin. The role of slingshot in forskolin activated ADF/cofilin dephosphorylation is currently unknown. The first step is to knockdown slingshot by introducing the siRNA against slingshot. Then the dephosphorylation of ADF/cofilin can be evaluated. If expression of slingshot siRNA prevents dephosphorylation of ADF/cofilin induced by ACh or forskolin, it suggests that slingshot is the specific phosphatase that regulates ADF/cofilin activity with ACh or forskolin stimulation. If not, another phosphatase might be involved or the inactivation of LIMK might be the cause of ADF/cofilin dephosphorylation.

The research presented in this dissertation has unveiled the important role of ADF/cofilin in regulating actin polymerization and force generation in airway smooth muscle. ADF/cofilin is ubiquitously expressed across cell types and is likely to be present in all smooth muscle tissues. Thus, cofilin is likely to play a similar role in the regulation of contraction and relaxation in other smooth muscle tissues. Better understanding of the roles of ADF/cofilin activation in actin dynamics during smooth muscle tension development and its physiological functions may provide important insights into the physiological regulation of the properties of smooth muscle tissues and organs and may lead to novel targets for the development of new therapeutic agents.

Chapter IV

Experimental Procedures

Preparation of smooth muscle tissues and measurement of force.

Mongrel dogs (20–25 kg) were anesthetized with pentobarbital sodium (30 mg/kg, i.v.) and quickly exsanguinated. Experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee, Indiana University School of Medicine. A segment of the trachea was immediately removed and immersed in physiological saline solution (PSS) at 22 °C (composition 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 a pH of 7.4. Smooth muscle strips (1 mm wide x 0.2–0.5 mm thick x 15mm 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 (L_{max}). All tissues were then maintained at *L_o* 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 *L_o* was determined again. For experiments involving the

introduction of cAMP elevating agents (Forskolin 10, 30, 100 μ M), muscle strips were incubated in PSS containing cAMP elevating agents for 30 minutes or 2 hours. Then 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 (113), and rabbit polyclonal anti-ADF/cofilin antibody (reacts with both ADF and cofilin) (152) 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, inactive mutant cofilin S3E, human wild type slingshot1 and inactive phosphatase slingshot1 mutant C393S were provided by Dr. J. R. Bamberg (Colorado State University, Fort Collins). Cyclosporine A, deltamethrin and forskolin, isoproterenol (Sigma).

Transfection of smooth muscle tissues with plasmids. Plasmids were introduced into tracheal smooth muscle strips by the method of reversible permeabilization as described previously (139; 173; 199). 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, 100μg/ml penicillin, 100μg/ml streptomycin, and 10 μg/ml plasmids encoding human wild type cofilin, the inactive mutant cofilin S3E or slingshot phosphatase inactive C393S.

Dissociation of airway smooth muscle cells from 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 by confocal microscopy (139). Tracheal muscle strips were minced and transferred to 5ml of dissociation solution (composition 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 U/ml), papain (type IV, 30 U/ml), bovine serum albumin (1 mg/ml), and dithiothreitol (DTT; 1 mM). All enzymes were obtained from Sigma Co. (St. Louis, MO). The strips were then placed in a 37 °C shaking water bath for 20–30 min at 80 oscillations/min. The strips were then washed three times with a HEPES-buffered saline solution (composition in mM:

130 NaCl, 5 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 20 HEPES, and 10 D–glucose, pH 7.4) and triturated with a pipette to liberate individual smooth muscle cells from the tissue. The cells were fixed for 10 min in 4% paraformaldehyde (vol/vol) in phosphate–buffered saline (composition in mM: 137 NaCl, 4.3 Na₂HPO₄, 1.4 KH₂PO₄, and 2.7 KCl, pH 7.4).

Confocal microscopy and image analysis. The efficiency of tissue transfection (Figure 23) was evaluated by determining the percentage of cells dissociated from plasmid–treated muscle tissues that expressed RFP–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 RFP fluorescence in fixed smooth muscle cells freshly dissociated from muscle tissues treated with plasmids encoding RFP–tagged cofilin or untreated tissues. The RFP fluorescence was excited with a 543–nm argon laser light, and fluorescence emissions were collected at 565–615 nm with an Apo x40 water–immersion lens objective (NA 1.2)

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

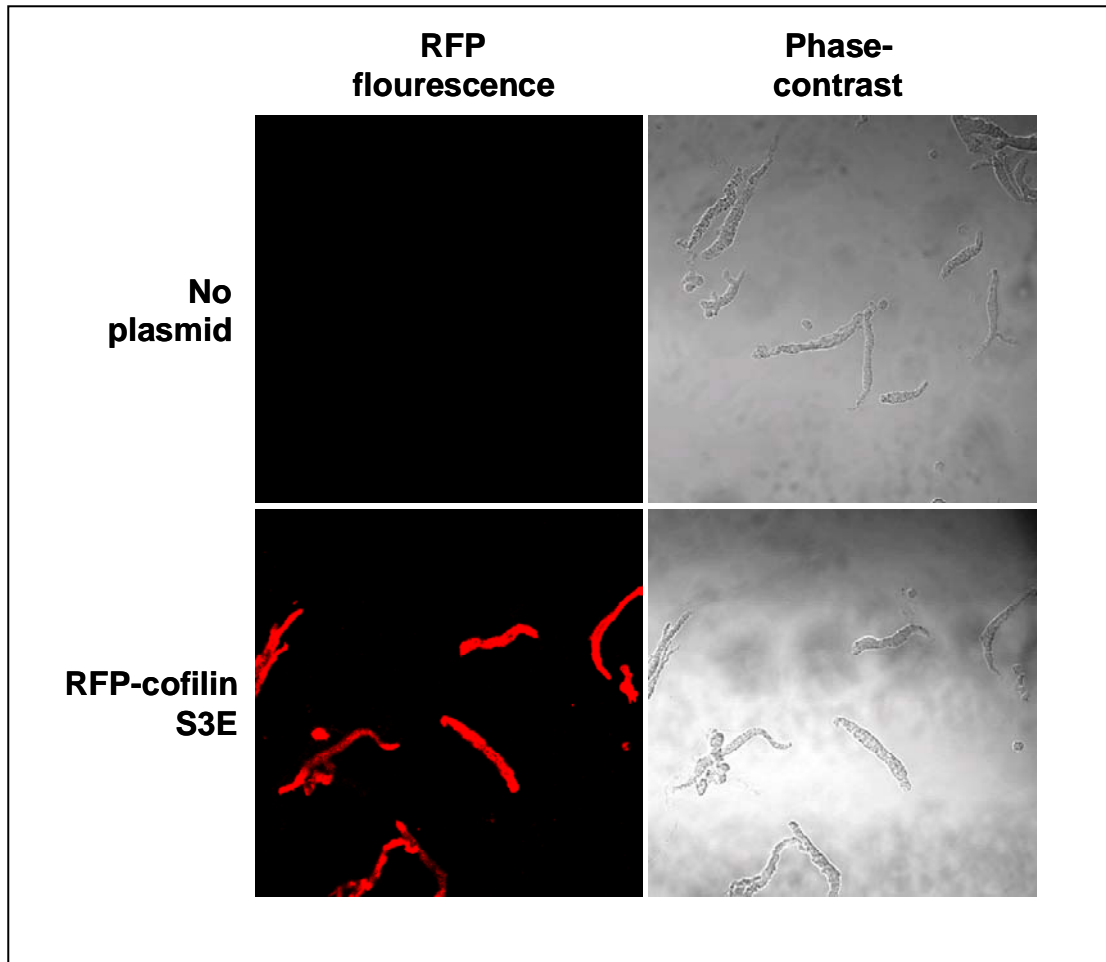


Figure 23. Expression of cofilin S3E in smooth muscle tissues. RFP fluorescence of live cells freshly dissociated from muscle strips treated with plasmids encoding the cofilin S3E or from muscle strips not treated with plasmids. Left: fluorescence images; right: phase-contrast images of the same fields. The efficiency of tissue transfection was evaluated by determining the percentage of cells dissociated from plasmid-treated and untreated muscle tissues that expressed RFP-labeled proteins. Most (80–90%) of the cells dissociated from the transfected tissues exhibited RFP fluorescence, whereas no fluorescence was observed in cells dissociated from untreated tissues.

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 (190). 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 (152). 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 (199; 200). 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 (199; 200). Briefly, each of the tracheal smooth muscle strips was homogenized in 200 μ l 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% Tween 20, 0.1% β –mercaptoethanol, 0.001% antifoam, 1 mM ATP, 1 μ g/ml pepstatin, 1 μ g/ml leupeptin, 10 μ g/ml benzamidine, and 500 μ g/ml tosyl arginine methyl ester). Supernatants of the protein extracts were collected after centrifugation at 150,000 \times g for 60 min at 37 °C. The pellets were resuspended in 200 μ l of ice–cold distilled water containing 10 μ M 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.

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Education

Indiana University, Indianapolis, IN USA
Major: Cellular & Integrative Physiology
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PhD, July 2009

Beijing Medical University, Beijing, China
Major: Medicine
MD, July 1998

Awards and Honors

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Invited speaker: American Thoracic Society International Conference.
Symposium: "Mechanisms Regulating Airway Smooth Muscle Phenotype and
Function" May 2007, San Francisco, CA.
Publication recommended by Faculty of 1000 Biology (2009)
Travel Fellowship from Indiana University (2006)
Travel Fellowship from Indiana University (2004)
University Fellowship from Indiana University (2003-2004)
Student Scholarship from Beijing Medical University (1995-1997)

Professional Organizations and Meetings

American Physiological Society
Chinese Medical Association
Doctor Society, Beijing Division
Red Cross Society, China Division
Sigma Xi, the Scientific Research Society

American Thoracic Society, International Congress, San Francisco (2007)
Experimental Biology, San Francisco (2006)
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National Cardiovascular Conference, Beijing (2001-2003)
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Working Experience

2001–2003 Cardiovascular Surgery Department, Fuwai Hospital, Peking Union
Medical College (PUMC). Beijing, China

- Resident doctor: Critical care for postoperative patients with heart and vascular diseases
- 1999–2001 People's Hospital, Beijing, China
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- 1998–1999 Cardiovascular Surgery Department, Fuwai Hospital, Peking Union Medical College (PUMC). Beijing, China
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Oral Presentations

Zhao R, Du LP, Bamburg, JR, Gunst SJ. Cofilin Phosphorylation Regulates Actin Dynamics during Contraction of Canine Tracheal Smooth muscle (TSM) tissues. Proceedings of the American Thoracic Society (PATS). 2007

Cofilin phosphorylation regulates actin dynamics during contraction of canine tracheal smooth muscle. Statewide Physiology Department Retreat, Sept 2007

Regulation of cofilin phosphorylation during airway smooth muscle contraction. Physiology Department Research in Progress Seminar, Mar 2007

Regulation of cofilin phosphorylation during airway smooth muscle contraction. Sigma Xi Society oral competition, Jun 2006

Regulation of cofilin phosphorylation during airway smooth muscle contraction. Department Research in Progress Seminar, Mar 2006

Length sensitivity of actin polymerization and cofilin regulation during smooth muscle contraction. Sigma Xi oral competition, May 2005

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Poster Presentations

American Thoracic Society International Conference. May 2007, San Francisco, CA.

Statewide Physiology Department Retreat, Sept 2007

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Experimental Biology. April 2006, San Francisco, CA.

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Publications

Zhao R, Du LP, Huang Y, Wu Y, Gunst SJ. Actin Depolymerization Factor/Cofilin Activation Regulates Actin Polymerization and Tension Development in Canine Tracheal Smooth Muscle. *J. Biol. Chem.*, Vol. 283, Issue 52, 36522-36531, 2008

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Zhao R, Zhang HT. Patient-controlled analgesia after cardiovascular surgery operations, *Surgery of Foreign Medicine*, 29 (3), 163-166, 2002

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