

# Molecular Mechanisms for the Mechanical Modulation of Airway Responsiveness

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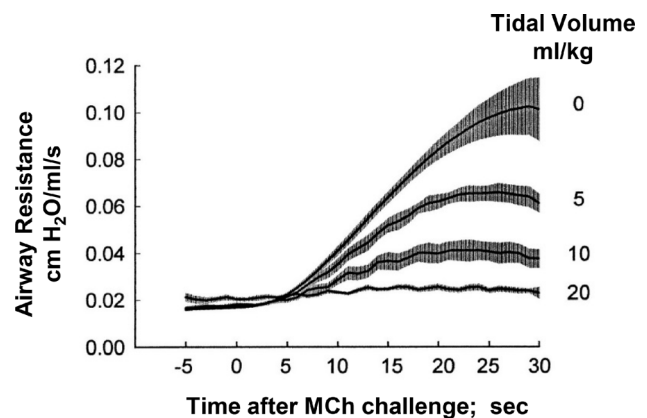
*The smooth muscle of the airways is exposed to continuously changing mechanical forces during normal breathing. The mechanical oscillations that occur during breathing have profound effects on airway tone and airway responsiveness both in experimental animals and humans in vivo and in isolated airway tissues in vitro. Experimental evidence suggests that alterations in the contractile and mechanical properties of airway smooth muscle tissues caused by mechanical perturbations result from adaptive changes in the organization of the cytoskeletal architecture of the smooth muscle cell. The cytoskeleton is a dynamic structure that undergoes rapid reorganization in response to external mechanical and pharmacologic stimuli. Contractile stimulation initiates the assembly of cytoskeletal/extracellular matrix adhesion complex proteins into large macromolecular signaling complexes (adhesomes) that undergo activation to mediate the polymerization and reorganization of a submembranous network of actin filaments at the cortex of the cell. Cortical actin polymerization is catalyzed by Neuronal-Wiskott–Aldrich syndrome protein (N-WASP) and the Arp2/3 complex, which are activated by pathways regulated by paxillin and the small GTPase, cdc42. These processes create a strong and rigid cytoskeletal framework that may serve to strengthen the membrane for the transmission of force generated by the contractile apparatus to the extracellular matrix, and to enable the adaptation of smooth muscle cells to mechanical stresses. This model for the regulation of airway smooth muscle function can provide novel perspectives to explain the normal physiologic behavior of the airways and pathophysiologic properties of the airways in asthma. [DOI: 10.1115/1.4042775]*

## Effects of Mechanical Forces on the Airways During Breathing

The smooth muscle of the airways is exposed to continuously changing mechanical forces during normal breathing. As lung volume increases and decreases with each breath, the airways are subjected to forces that cause them to expand and contract, thereby stretching and retracting the airway smooth muscle within the bronchial tree. Airway smooth muscle is also periodically subjected to larger forces of expansion caused by the intermittent deep breaths that occur during normal breathing. The mechanical oscillations that occur during breathing are well-documented to have profound effects on airway tone and airway responsiveness in both humans and experimental animals [1–12].

The lung volume oscillations that occur during tidal breathing are critical for maintaining a low level of airway tone in vivo [7]. Increasing the volume of tidal breath oscillations results in airway dilation and an increase in airway caliber, whereas a decrease in the oscillation volume leads to airway constriction [11,13] (Fig. 1). The absence of periodic deep breaths during normal breathing results in airway hyper-reactivity in experimental animals and in human subjects [8,11]. The continual stretch and retraction of airway smooth muscle during tidal breathing is also critical for maintaining normal airway reactivity in vivo [7]. Thus, the mechanical modulation of airway responsiveness during normal breathing is a fundamental physiologic property of the airways that is important for the maintenance of a low level of airway reactivity during breathing in vivo. The adaptive effects of stretch may account for the dilatory effect of tidal volume oscillations and deep inspiration on the airways.

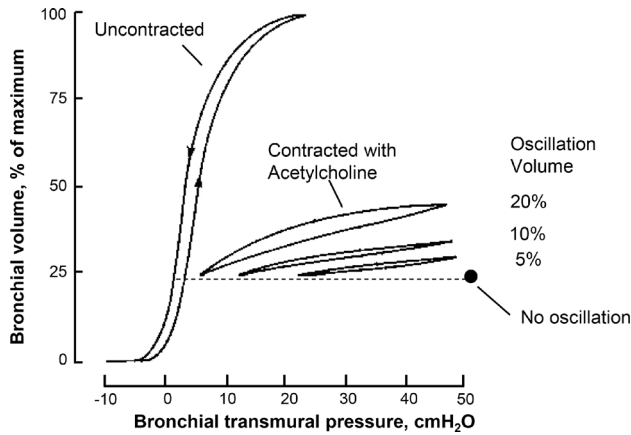
An abnormally low load on airway smooth muscle could occur in local regions of the lungs in which disease or inflammatory processes disrupt connections between small airways and the lung parenchymal tissues. Similarly, inflammatory processes in the airway wall that lead to degradation of the extracellular matrix or the disruption of connections between the extracellular matrix and transmembrane integrin proteins on the smooth muscle cells might result in local alterations in the load on the smooth muscle tissue. The unloading of airway smooth muscle tissues could potentiate their responses to inflammatory mediators, exacerbating inflammatory processes [14,15]. There is also evidence that an increase in the mechanical stress imposed on airway smooth muscle



**Fig. 1** The volume of tidal breath oscillations regulates airway dilation and airway caliber in anesthetized rabbits. Airway resistance ( $R_{aw}$ ) versus time from initiation of challenge with intravenous methacholine (MCh; 0.01 mg/kg) at different volumes of tidal ventilation. Modified from Shen et al. [11].

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**Fig. 2 Increasing the amplitude of tidal volume oscillations reduces the magnitude of the transmural pressure of isolated canine intraparenchymal bronchi contracted with acetylcholine. Modified from Gunst et al. [13].**

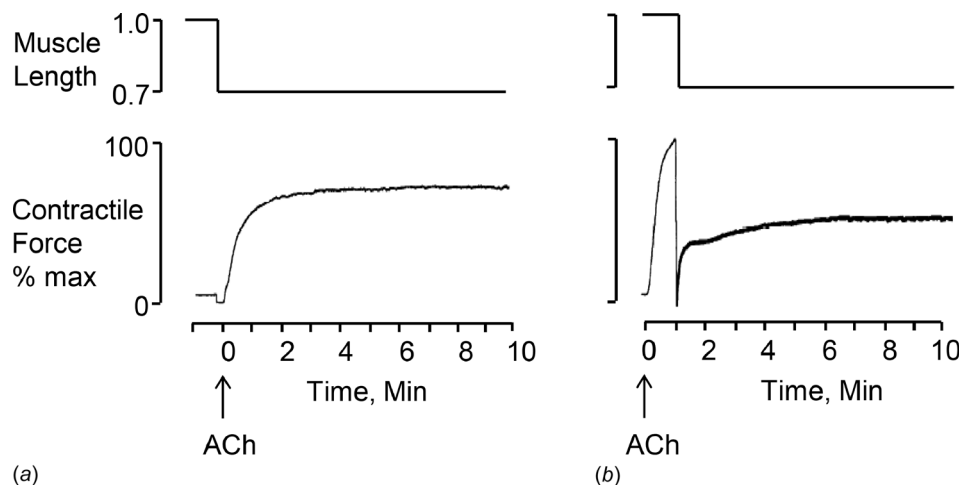
tissues, such as occurs in lungs subjected to positive end expiratory pressure, reduces the synthesis of inflammatory mediators by airway smooth muscle and helps to alleviate airway inflammation [14,16,17].

The mechanical effects of inflation and deflation on the airways in vivo can be mimicked in isolated airways and in airway smooth muscle tissues in vitro, suggesting that these properties are fundamental to airway smooth muscle [2,18–27]. Volume or pressure oscillations imposed on isolated bronchial segments in vitro after they are stimulated to contract result in dilation of the bronchi [13] (Fig. 2). Similarly, length or force oscillations imposed on isolated contracted airway smooth muscle tissues lead to relaxation [23,26]. Periodic stretches imposed on isolated airway tissues also result in their temporary relaxation, although, in the absence of further stretches, the muscle eventually redevelops contractile tone if is returned to its shorter length and maintained at a constant length [18,28]. The property by which airway smooth muscle modulates its contractile and mechanical properties to accommodate to changes in its mechanical environment has been termed “mechanical plasticity” or “length adaptation” [21,29,30]. The mechanical plasticity of airway smooth muscle may be basic to many of the observed effects of changes in lung volume on airway caliber and responsiveness in vivo.

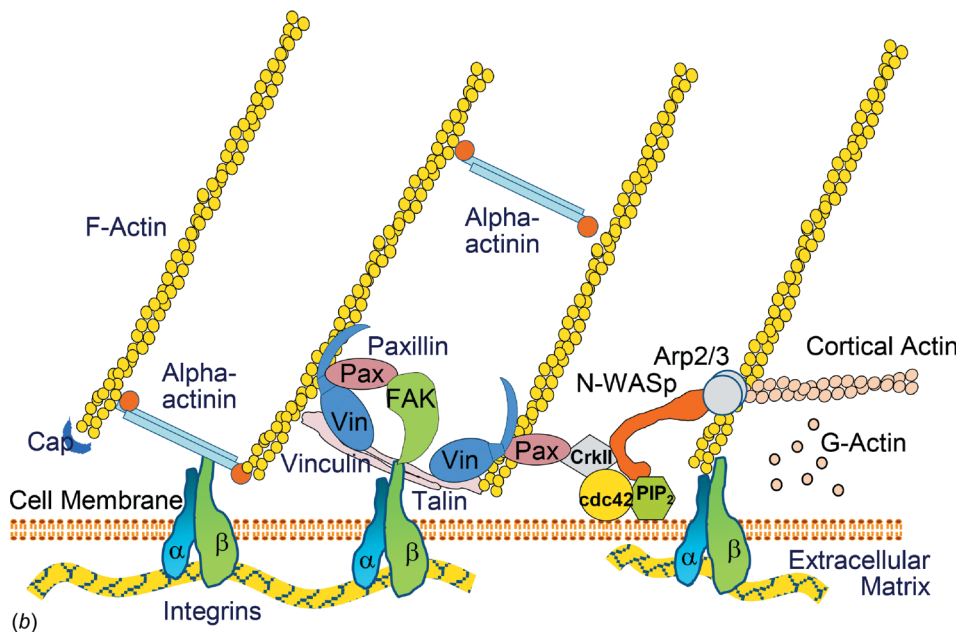
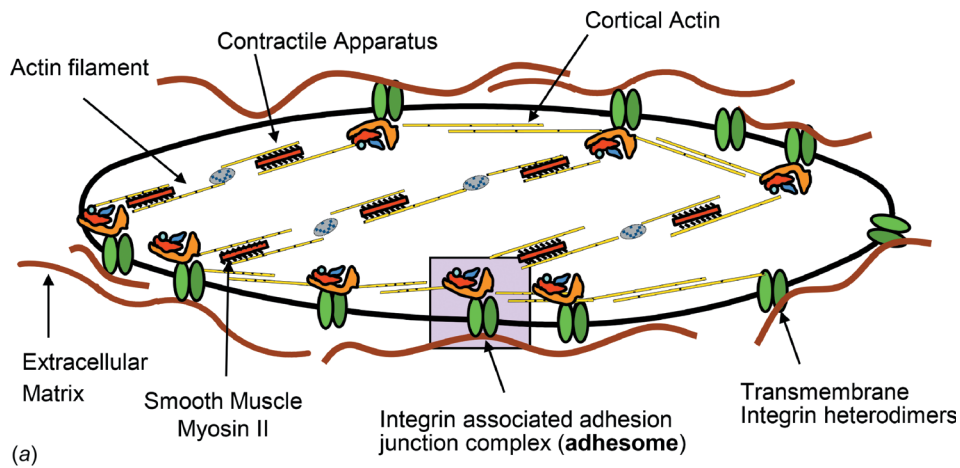
## The Mechanical Plasticity of Airway Smooth Muscle

The property of mechanical plasticity or length adaptation has been proposed to result from an ability of the smooth muscle cell to modulate the organization of its contractile filaments and cytoskeletal apparatus in order to accommodate to changes in cell shape imposed by mechanical forces from the external environment [18,21,23,24,31,32]. The length-history dependence of the contractile properties of airway smooth muscle provide an illustration of this property [18,20,21,23–26,28,32–37]. For example, when isolated tracheal smooth muscle tissues are maintained at a constant muscle length for a period of time and then shortened, a depression of tension redevelopment and shortening velocity at the shorter length is observed (Fig. 3). The amount of force depression is proportional to the size of the shortening step [21,28,34,35]. This depression occurs whether the shortening step is imposed on the muscle prior to or during contractile stimulation. Conversely, if the muscle is maintained at a short length for a period of time and then stimulated to contract, both tension development and shortening velocity are higher than if these parameters are measured immediately after shortening to that length [18,34,35]. Similar properties have been reported for other smooth muscle tissue types and for single smooth muscle cells [38–40], suggesting that this adaptive behavior may be a fundamental characteristic of smooth muscle cells.

The adaptive changes that are observed when airway muscles are maintained at different lengths are manifest not only as changes in their contractile dynamics but also as alterations in tissue stiffness [18,21,40]. Tracheal smooth muscle strips are significantly stiffer and less extensible after isometric contraction at a short muscle length than after isometric contraction at a long length, suggesting that the structure of the smooth muscle cell changes when it is activated at different lengths [21,34]. The increased stiffness that results from contracting a muscle at a short length can be reversed simply by stretching the muscle to a longer length, which is consistent with the hypothesis that stretch induces structural rearrangements in the muscle [18,21]. According to this hypothesis, activation of the muscle at a short length would lead the contractile filaments to organize into a short, thick filament array adapted to the shorter, thicker shape of the muscle cell, whereas activation at a long length would result in the organization of the contractile filaments into a long thin array adapted to the longer, thinner shape of the muscle cell. Stretch of the muscle to a long length after activation at a short length would result in reorganization of the cytoskeleton to accommodate to the change in shape of the muscle cell caused by the stretch. If contractile



**Fig. 3 Tracheal muscle tissues strips were subjected to length steps either before (a) or after 1 min of contractile stimulation with ACh (b). Force in response to ACh was much higher when the length step was performed prior to contractile stimulation than when the length step occurred during contractile stimulation. Modified from Gunst et al. [18].**



**Fig. 4 Smooth muscle cell cytoskeletal structure and organization. (a) Actin filaments within the contractile apparatus and at the cell cortex linked to integrin proteins at membrane adhesion junctions that connect to the extracellular matrix. (b) Molecular organization of integrin-associated adhesome complexes in smooth muscle cells.**

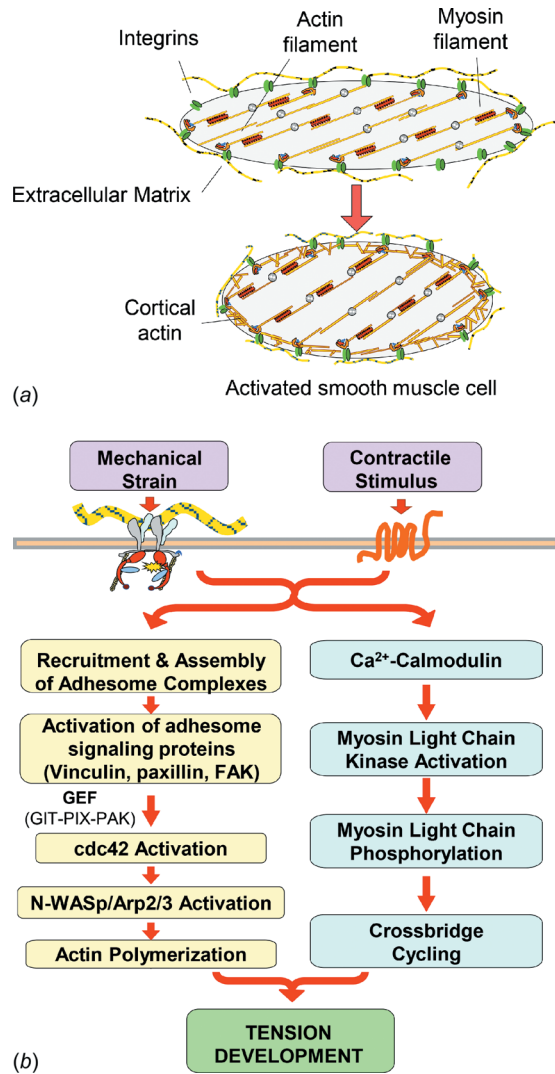
activation at different lengths results in such changes in contractile filament organization, the muscle should be stiffer and less extensible after contractile activation at a short length than after it is activated at a long length. The stiffness would also be predicted to decrease when the contracted muscle is stretched from a short length to a long length [18,21,40]. There is substantial evidence that these “plastic” or length-adaptive properties of airway smooth muscle underlie the mechanical properties of the airways that are critical for the normal regulation of airway tone [2,21,31,41].

### Molecular Mechanisms for Mechanical Plasticity of Airway Smooth Muscle

Tension development in smooth muscle has long been attributed to interdigitating actin and myosin filaments that regulate shortening and tension development by sliding across each other through a process of cycling actomyosin crossbridges. Actin filaments associate with smooth muscle myosin II filaments to form what is commonly referred to as the contractile apparatus (Fig. 4(a)). Actin filaments within the contractile apparatus anchor to transmembrane integrins at membrane adhesion junction

complexes via a series of “linker” proteins. They also anchor within the interior of the smooth muscle cell at dense bodies that are primarily composed of the actin-binding protein alpha-actinin. Transmembrane integrin proteins connect to extracellular matrix proteins outside the cell and thereby regulate force transmission from the contractile apparatus to the extracellular matrix.

The proteins that associate with integrin proteins form large multiprotein signaling complexes termed “adhesomes” (Fig. 4(b)). Linker proteins that connect actin filaments to integrin proteins serve as a scaffold for the assembly of these integrin-associated adhesomes, which include numerous signaling modules that regulate pathways to the cytoskeleton as well as to the nucleus. In airway smooth muscle, contractile, inflammatory, and mechanical stimuli elicit the recruitment of signaling modules and linker proteins to adhesome sites, resulting in the enlargement of these complexes [41–47]. The proteins within integrin-associated adhesome complexes regulate processes of actin dynamics and cytoskeletal reorganization that enable airway smooth muscle cells to adapt to external mechanical forces by changing their shape, stiffness, and contractility [42,43,48–50]. Integrin-mediated signal transduction pathways are also important in regulating the phenotypic



**Fig. 5 (a) Model of smooth muscle shortening and tension development. Contractile and mechanical stimuli induce the recruitment of cytoskeletal signaling proteins to membrane adhesion sites and cortical actin polymerization. (b) Signals pathways regulated by integrin receptors and G protein-coupled receptors (GPCR) collaborate to regulate tension development in airway smooth muscle. Both pathways are necessary for tension development.**

properties of airway smooth muscle tissues in response to external mechanical forces: adhesome complexes initiate signaling pathways to the nucleus that initiate changes in gene expression and smooth muscle functions [14,15,51].

Submembranous actin filaments are also found at the periphery of the smooth muscle cell, where they serve functions distinct from the actin that interacts with smooth muscle myosin II (Fig. 5(a)). This peripheral actin, referred to as “cortical actin,” may interact with nonmuscle (NM) myosin II to provide a scaffold for the transport and assembly of proteins and protein complexes into adhesome complexes that are critical for the transduction of signals from extracellular stimuli to the cytoskeleton [45,46,52]. Cortical actin may also stabilize the attachment of the actin filaments within the contractile apparatus to the cell membrane, thus strengthening these sites for the transmission of force between the contractile apparatus and the extracellular matrix. The actin cytoskeleton forms a template for myosin filament binding; thus, the arrangement of actin filaments determines the organization of the contractile apparatus. As actin filaments provide the cytoskeletal

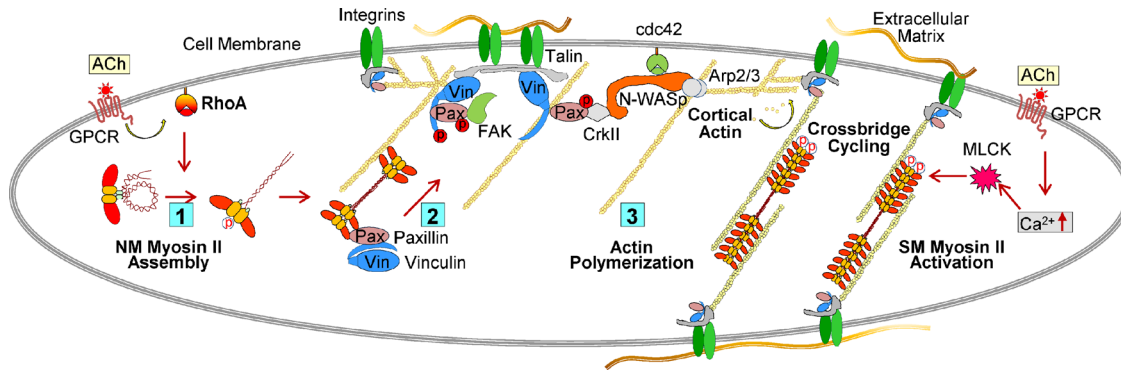
scaffolding for myosin, changes in the sites of attachment of actin filaments to the membrane or their organization would be expected to alter the organization of contractile units. This could lead to changes in smooth muscle contractility and stiffness.

In airway smooth muscle, the length and attachment sites of actin filaments may be regulated in response to environmental conditions to lead to reorganization of the actin cytoskeleton in response to changes in the shape of the smooth muscle cell. Adhesome proteins involved in regulating the organization and structure of the actin cytoskeleton are mechanosensitive in airway smooth muscle, and are regulated by contractile stimulation. Mechanical perturbations and contractile stimuli have been shown to modulate the number and organization of both actin and myosin filaments in airway smooth muscle tissues [53–58]. Thus, mechanical signals from the external environment are sensed by integrin proteins, and transduced by adhesome complexes to regulate both the structural organization and the phenotypic properties of the airway smooth muscle cell.

### The Contractile Stimulation of Airway Smooth Muscle Triggers Cytoskeletal Reorganization.

The contraction of smooth muscle cells has long been attributed to the activation of smooth muscle myosin ATPase activity and crossbridge cycling (Fig. 5(b)). This process is activated by Ca<sup>2+</sup>-calmodulin regulated myosin light chain (MLC) kinase, which regulates the phosphorylation of the 20 KD light chain of smooth muscle myosin. Myosin light chain phosphorylation promotes the actin-activated ATPase activity of the myosin head, which catalyzes crossbridge cycling and acto-myosin filament sliding [59–62]. However, studies performed in airway smooth muscle tissues have established that the activation of myosin and crossbridge cycling is not sufficient by itself to account for shortening and tension development in response to the contractile stimulation of smooth muscle [42,43,63]. The contractile stimulation of airway and other smooth muscle tissues also regulates the polymerization of a pool of actin, and the process of actin polymerization is also required for tension development to occur [46,47,58,63–75]. Mechanisms for the regulation of actin polymerization and its role in regulating activation of the contractile apparatus and tension development have been analyzed extensively in these tissues. The inhibition of either actin polymerization or of MLC phosphorylation in airway smooth muscle tissues depresses tension development in response to stimulation with ACh. However, the inhibition of actin polymerization using either molecular or pharmacologic approaches has little or no effect on the increase in MLC phosphorylation in response to agonist stimulation [63,67,71,75]. Conversely, agonist-induced actin polymerization is not suppressed when MLC phosphorylation is inhibited [71]. Thus, in airway smooth muscle, actin polymerization and smooth muscle MLC phosphorylation are distinct cytoskeletal processes that are independently regulated during contractile stimulation (Fig. 5).

There is compelling evidence that the actin cytoskeleton remains in a dynamic state in airway smooth muscle, and that the polymerization and depolymerization of actin filaments is part of the contraction-relaxation cycle. Mehta and Gunst [63] found that approximately 30–40% of the total actin in unstimulated tracheal smooth muscle tissues exists in the form of monomeric globular (G) actin, and that the amount of G-actin decreases by approximately 30% during contractile stimulation, consistent with its incorporation into actin filaments. This represents the polymerization of approximately 10–15% of the total actin in the smooth muscle cell. A transition from G to F actin during contractile activation has also been documented in other smooth muscle tissues [66,76–79]. Studies of airway smooth muscle have also demonstrated that the molecular processes that catalyze actin polymerization occur at the cell cortex, suggesting that the actin filaments polymerized in response to stimulation with contractile agonists are localized to a cortical network of filaments [43,44,46,47,52,72,75,80,81].



**Fig. 6 Molecular mechanism for the assembly of adheresome complexes in airway smooth muscle: (1) ACh stimulation activates RhoA, which regulates regulatory light chain phosphorylation of NM myosin and the assembly and activation of NM myosin II. (2) Activated NM actomyosin mediates the recruitment of inactive proteins to membrane adheresome complexes, where they undergo activation to regulate cytoskeletal signaling pathways. (3) Cdc42 activates N-WASP and the Arp2/3 complex, which catalyzes cortical actin polymerization. Signals from G-protein coupled receptors (GPCR) activate MLC kinase which regulates SM myosin regulatory light chain phosphorylation and the activation of SM myosin crossbridge cycling.**

The molecular mechanisms for actin polymerization have been elucidated in detail in airway smooth muscle [42,45,80] (Figs. 5(b) and 6). Agonist-induced actin polymerization is mediated by the actin nucleation promoting protein, neuronal Wiskott–Aldrich syndrome protein (N-WASP) in airway smooth muscle tissues [75]. N-WASP undergoes a change in conformation during its activation that enables it to bind to the actin-related protein complex (Arp2/3 complex). The Arp2/3 complex creates a template for actin polymerization that facilitates the addition of monomeric actin (G-actin) to existing F-actin filaments [82–84]. N-WASP activation is directly and specifically regulated by the binding of the small GTPase cdc42 to its CRIB (Cdc42- and Rac-interactive binding) domain [82,84–86]. In airway smooth muscle tissues, cdc42 activation is necessary for N-WASP activation, actin polymerization, and active tension development [67].

Transmembrane integrins are uniquely situated to mediate the transduction of mechanical signals to intracellular signaling pathways that regulate the cytoskeletal structure and contractility of cells. Integrins are membrane-spanning proteins that ligate extracellular matrix proteins on the outside of the cells and connect to the actin cytoskeleton on the inside [87–89]. Mechanical strain or tension applied directly to the extracellular domain of integrins regulates the activity of adheresome signaling proteins, cytoskeletal stiffening, and the activation of downstream signaling pathways, indicating that integrins can function as mechanotransducers [48–50,90].

The activation of airway smooth muscle results in the rapid recruitment and assembly of multiple proteins into adheresome complexes [44,46,47,52,72,73,75,81]. The time course of adheresome protein recruitment is rapid and can be visualized in dissociated cells and intact airway smooth muscle tissues by immunofluorescence techniques and proximity ligation assays, and in living or fixed dissociated cells expressing green fluorescent protein constructs of adheresome proteins [44,47,73,75,91]. Paxillin, focal adhesion kinase (FAK), vinculin, and multiple other components of integrin-associated signaling complexes are recruited to adheresome complexes in response to external stimulation where they undergo phosphorylation and activation [14,15,44,47,81,92]. The recruitment and activation of vinculin, paxillin, and other adheresome proteins is required for agonist-stimulated actin polymerization and contraction in airway smooth muscle [44,46,47,52,69,72,81,93]. Many of these proteins, including paxillin, vinculin, and FAK, are sensitive to mechanical stimulation: their activation in the airway smooth muscle tissues is regulated by external mechanical forces imposed on the tissues as well as by contractile stimulation [14,15,47,94]. Paxillin and vinculin exist in the cytoplasm in a stable inactive complex and are recruited to adheresome complexes in response to external

stimulation, where paxillin is activated by FAK [47,81]. After recruitment, vinculin undergoes phosphorylation on its tail domain at tyrosine 1065: the phosphorylation of vinculin at this site regulates its conversion to an open ligand binding conformation that enables it to bind to talin and F-actin as well as to other proteins involved in cytoskeletal dynamics [47,81]. Higher levels of mechanical strain increase the phosphorylation of FAK, paxillin and vinculin, and provide a mechanism for the mechanosensitive regulation of signaling pathways mediated by these proteins [14,15,95]. In airway smooth muscle, the assembly and activation of adheresome complexes is a necessary prerequisite to the process of cortical actin polymerization; thus the process of actin polymerization can be regulated in a mechanosensitive fashion.

There is evidence that smooth muscle myosin filaments may also be dynamic and regulated by external contractile and mechanical stimuli in airway smooth muscle. Electron micrographic studies have shown that cytosolic myosin filaments increase in response to the contractile stimulation of airway smooth muscle cells [53,55–57,96,97].

#### **Molecular Mechanisms for the Assembly of Adheresome Complexes in Response to External Stimulation in Airway Smooth Muscle.**

The process of adheresome complex assembly in response to external stimulation of airway smooth muscle is a regulated process involving the stepwise recruitment and activation of multiple scaffolding proteins and signaling modules that interact within these macromolecular membrane complexes (Fig. 6). The recruitment of proteins from the cytosol to membrane sites is catalyzed by NM myosin II. Nonmuscle myosin II is ubiquitously expressed in all cell types and constitutes the primary motor for motility, migration and adhesion in nonmuscle cells. Airway smooth muscle contains a pool of nonmuscle myosin II, which localizes primarily to the cortical region of the airway smooth muscle cell [52]. NM myosin II is present in the airway smooth muscle in both monomeric and filamentous form [52]. Monomeric myosin II exists in an inactive folded conformation that is unable to assemble into filaments [52,98,99]. The contractile stimulation of airway smooth muscle tissues with ACh regulates the assembly of nonmuscle myosin II filaments as well as their activation by catalyzing a conformational change in the myosin II monomer that enables it to assemble into filaments. This process is required for the recruitment of vinculin, paxillin, and FAK to membrane adhesion complexes in response to a contractile stimulus [52].

RhoA is a critical regulator of nonmuscle myosin II assembly and activation in airway smooth muscle, and thus regulates the recruitment and activation of adheresome proteins and actin polymerization [46,52,71]. In airway smooth muscle tissues, RhoA

has no significant effect on the regulation of the SM myosin light chain phosphorylation during contractile stimulation, but it is a critical regulator of NM myosin regulatory light chain phosphorylation [52]. Nonmuscle myosin II light chain phosphorylation mediates the conversion of NM myosin II inactive monomers to an open extended conformation that is capable of assembling into NM myosin II filaments [52,100]. The ability of RhoA to selectively regulate the assembly of NM myosin II in airway smooth muscle provides a mechanism by which external stimulation can activate adhesome complex assembly and regulate actin polymerization independently of the activation of smooth muscle myosin and crossbridge cycling.

## Summary and Conclusions

**Mechanism for Adaptation of the Airway Smooth Muscle Cell to External Mechanical Forces.** In summary, the plastic properties of airway smooth muscle may underlie many of the physiologic effects of lung volume changes on airway caliber and airway responsiveness in vivo in normal humans and experimental animals. These properties may have their basis in dynamic cytoskeletal processes that can be defined at the molecular level. The chronic mechanical and pharmacologic stressors present in asthma may affect actin filament dynamics and cytoskeletal signaling pathways resulting in actin filament remodeling and cell stiffening. Actin filament dynamics in response to mechanical or contractile stimuli is regulated by signaling pathways mediated by adhesome complexes that associate with transmembrane integrins. RhoA GTPase and nonmuscle myosin regulate the assembly and activation of integrin adhesome complexes, independently of the processes that mediate the activation of smooth muscle myosin and crossbridge cycling. Further definition of the mechanisms that regulate actin and myosin polymerization and cytoskeletal dynamics may lead to new insights in the mechanisms underlying the plasticity of airway smooth muscle mechanical properties.

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