Interactions of Airway Smooth Muscle Cells with Their Tissue Matrix

Implications for Contraction

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The ability of airway smooth muscle to alter its stiffness and contractility in response to mechanical oscillation and stretch is critically important for the regulation of normal airway responsiveness during breathing. The properties of mechanical adaptation in airway smooth muscle are proposed to result from dynamic cytoskeletal processes outside of the actomyosin interaction. The actomyosin interaction and crossbridge cycling are viewed as components of a complex and integrated array of cytoskeletal events that occur during cell contraction. These events are orchestrated by macromolecular protein complexes that associate with the cytoplasmic domains of integrin proteins at the adhesion junctions between muscle cells and the extracellular matrix. According to this paradigm, these concerted cytoskeletal events are essential components of the process of active tension generation in airway smooth muscle, and also serve to adapt the shape and stiffness of the smooth muscle cell to its environment. Contractile stimuli initiate actin polymerization within the submembranous cortex of the airway smooth muscle cell that may serve to determine the cells shape and strengthen the membrane. The recruitment of structural proteins such as α -actinin to adhesion junctions fortifies the strength of the connections between membrane adhesion junctions and actin filaments. These processes create a strong and rigid cytoskeletal framework for the transmission of force generated by the interaction of myosin and actin filaments. 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.

Keywords: cytoskeleton; actin polymerization; integrin

ROLE OF ADHESION JUNCTIONS IN THE REGULATION OF AIRWAY SMOOTH MUSCLE CONTRACTILITY

Airway smooth muscle tissues are subjected to large changes in shape and volume during breathing under physiologic conditions *in vivo*. A wealth of data obtained from both *in vivo* and *in vitro* studies has established the importance of mechanical forces that occur during breathing in the regulation of normal airway responsiveness. Periodic deep inspirations dilate the airways and reduce their responsiveness to bronchoconstrictors, and tidal breathing is necessary to maintain a normal low level of airway reactivity (1–4). Similar properties are exhibited by isolated airway smooth muscle tissues: mechanical oscillation or stretch reduces their stiffness and decrease its responsiveness to

contractile stimuli (5–10). 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 (8, 10–14). Furthermore, changes in muscle length or mechanical strain also modulate the mechanical stiffness of the smooth muscle tissue. These physiological effects suggest that the smooth muscle cell structure or organization is modulated by mechanical forces.

External forces that are imposed on smooth muscle tissues are transmitted from the extracellular matrix to the interior of the cells via membrane adhesion plaques, which are the junctions that connect cytoskeletal filaments within the cells with the extracellular matrix outside of the cells (Figure 1). Adhesion plaques are complex molecular assemblies of structural and signaling proteins that link actin filaments to the cytoplasmic domains of heterodimeric transmembrane integrin proteins, which are specific ligands for extracellular matrix proteins (Figures 2A and 2B). The actin filaments that connect to adhesion sites in smooth muscle are likely to include the filaments that associate with myosin filaments and activate crossbridge cycling, as well as actin filaments that are not associated with myosin. Other cytoskeletal filaments, such as intermediate filaments, may also bind to proteins in these adhesion sites (15). However, actin filaments do not bind directly to integrin proteins: other components of the molecular complexes at membrane adhesion sites provide the structural links between integrin proteins and actin filaments. Proteins that can directly link actin filaments to integrin proteins include α -actinin, talin, and filamin, all of which form homodimers that can crosslink actin filaments and also bind to the β subunit of integrin heterodimers (16–18). The macromolecular complexes at adhesion sites also contain scaffolding and signaling proteins that have been shown to regulate actin remodeling and actin dynamics in a variety of cell types, and that can modulate signaling pathways involved in the regulation of contractile protein activation. Thus, adhesion complexes are well positioned to modulate changes in smooth muscle cell mechanical properties and contractility in response to extracellular stimuli that affect the activation of integrin proteins.

Studies performed in a variety of experimental preparations from diverse cell types have shown that the activation of integrin proteins is affected by changes in the extracellular environment such as mechanical forces and changes in extracellular matrix composition (17, 19–23). Studies in smooth muscle cells and tissues suggest a similar role for integrin proteins in transducing environmental signals. Mechanical forces and changes in extracellular matrix proteins that are imposed on smooth muscle tissues can be sensed by integrin proteins and transduced to downstream pathways that can

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Figure 1. Electron micrographs of 60-nm-thick longitudinal sections of unstimulated canine tracheal smooth muscle tissues. (A) Magnification: $\times 18,500$. (B) Magnification: $\times 49,000$. White arrows point to membrane adhesion plaques. Black arrows point to actin and myosin filaments. Black triangles point to extracellular matrix. White triangles point to cytoplasmic dense bodies. Scale bar = $0.5 \mu\text{m}$.

regulate both cytoskeletal organization and contractile protein activation (24–30). Although knowledge of the molecular mechanisms for this transduction process is incomplete, considerable evidence has accumulated that the protein complexes at membrane adhesion sites play a critical role in mediating the transduction of signals from integrin proteins to downstream effectors that determine the mechanical properties of smooth muscle tissues.

CYTOSKELETAL DYNAMICS IN AIRWAY SMOOTH MUSCLE CELLS DURING CONTRACTILE ACTIVATION

Dynamic changes in cytoskeletal organization have been proposed as a mechanism by which airway smooth muscle cells may modulate their structure and contractility to accommodate to changes in their external environment (25, 31–33). Actin polymerization and remodeling form the basis for regulating the shape and stiffness of many cell types, and it is therefore plausible that smooth muscle cells might also depend on similar processes to adapt their shape and stiffness to external conditions.

A number of lines of experimental evidence support the concept that the cytoskeletal organization of smooth muscle cells is dynamic and that it is regulated by stimuli that contract and relax smooth muscle. Actin exists in both filamentous form (F actin) and in soluble monomeric form (G actin) in all cell types. The contractile stimulation of airway and other smooth muscle cell and tissue types triggers an increase in the pool of F actin and a decrease in the pool of G actin. Numerous studies have documented these transitions in the state of actin in a variety of smooth muscle tissue and cell types using diverse assays for monomeric and filamentous actin, including biochemical assays, immunofluorescence analysis, and electron microscopy (34–43). Studies of airway smooth muscle tissues suggest that the pool of G actin in resting tissues constitutes approximately 20% of the total actin in the cell, and that this pool decreases by approximately 30 to 40% in response to a contractile stimulus (37). This would mean that the percentage of F actin increases by less than 10% in activated muscle tissues over the 80% level in unactivated airway muscle tissues (37) (Figure 3). These measurements indicate that a relatively small percentage of the total pool of actin undergoes polymerization during the activation of airway smooth muscle tissues.

The transition in the polymerization state of actin clearly constitutes a critical step in the process of tension development in airway smooth muscle. An extensive literature exists in which

pharmacologic and molecular approaches have been used on various smooth muscle tissue and cell types to inhibit actin polymerization, and these studies uniformly report that the inhibition of actin polymerization markedly depresses tension development (35, 37, 43–50). Furthermore, a number of studies have shown that the actin polymerization can be inhibited with little or no effect on myosin light chain phosphorylation or crossbridge cycling (35–37). Conversely, there is also evidence that myosin light chain phosphorylation can be inhibited without inhibiting agonist-induced actin polymerization (43, 51). However, the inhibition of either process by itself markedly depresses active tension development, indicating that both actin polymerization and crossbridge cycling are essential steps in the process of tension development in airway and other smooth muscle tissues. This evidence suggests that actin polymerization must regulate tension development by a cellular process that is distinct from and independent of crossbridge cycling. The observation that only a small amount of actin undergoes polymerization during smooth muscle contraction is consistent with the concept that this actin serves a specialized function that is distinct from that of the actin that interacts with myosin to regulate crossbridge cycling. One possibility is that actin polymerization occurs predominantly in the submembranous area of the smooth muscle cell and is analogous to the cortical actin network that forms in response to the stimulation of other contractile cell types, such as endothelial cells (52–58). The formation of a network of submembranous actin in smooth muscle cells could function to enhance membrane rigidity (59), and to connect the contractile and cytoskeletal filament lattice to the membrane to transmit the tension generated by crossbridge cycling. Submembranous actin polymerization might also be localized regionally in smooth muscle cells in regions in which the membrane tension is greatest. In airway smooth muscle; the formation of a submembranous actin network could function to adapt smooth muscle cell shape and rigidity to its external environment and thereby alter the mechanical properties and contractility of the airway smooth muscle.

REGULATION OF ACTIN POLYMERIZATION BY ADHESION COMPLEX PROTEINS

If actin polymerization in smooth muscle tissues is a submembranous process that is regulated by external mechanical stimuli, it would be expected to be mediated by signaling proteins that can sense and respond to external mechanical

Figure 2. (A) Schematic illustration of smooth muscle cell cytoskeletal organization. Membrane adhesion junctions (box) connect the extracellular matrix surrounding the cells in smooth muscle tissues to actin filaments within the cell. Actin filaments are linked to integrin proteins at these adhesion junctions by structural proteins within macromolecular complexes that form around transmembrane integrin proteins. Actin that interacts with myosin generates tension and cell shortening. Additional actin not associated with myosin may regulate cell shape and structure. (B) Model of molecular organization of cytoskeletal/cell matrix membrane junctions in smooth muscle cells.

forces. A mechanistic model for a mechanotransduction process that regulates submembraneous actin polymerization has been developed from studies of motile cells (60, 61). During cell migration, mechanical tension at sites of membrane extension at the leading edge of the cell induces integrin clustering and

activation, the recruitment of cytoskeletal adhesion proteins, and actin polymerization, resulting in extension of the cell membrane and cell crawling (62–64). Wiskott-Aldrich Syndrome proteins (WASP) are key activators of the actin polymerization that occurs at the leading edge of the membrane in response to

Figure 3. The polymerization of actin in tracheal muscle strips in response to ACh stimulation measured by separating cellular fractions containing F (filamentous) and G (globular) actin (37). (A) Representative immunoblots of actin from soluble G actin and insoluble F actin fractions obtained from an unstimulated tracheal muscle tissue and tracheal muscle tissues stimulated with ACh. ACh stimulation significantly increases the ratio of F-actin to G-actin. (n = 16). (B) Amount of G actin in unstimulated and ACh-stimulated tracheal muscle tissues quantified as a percentage of total actin. The percentage of G-actin was approximately 40% lower in ACh-stimulated muscles. The amounts of G and F actin are also quantified as a proportion of total actin in unstimulated and stimulated tracheal muscle tissues. F-actin is more than 80% of total actin in unstimulated muscle tissues and increases to close to 90% of total actin with ACh stimulation. *Significant difference between stimulated and unstimulated tissues ($P < 0.05$).

signals from adhesion junction proteins. In motile cells, a stimulus for motility causes the activation of the small GTPase, cdc42, which catalyzes the recruitment of a WASP family proteins to the membrane and their activation, resulting in the binding of the Arp2/3 (Actin-related Protein) complex to WASp and Arp2/3 complex activation (60, 65, 66). The Arp2/3 complex, which consists of seven strongly associated protein subunits that include Arp2 and Arp3 (actin-related proteins), form a template for the formation of new actin filaments that extend toward the membrane and push the leading edge of the cell forward (61, 67).

Recent studies have demonstrated that WASp family proteins are also critical for the activation of actin polymerization in airway smooth muscle tissues in response to a contractile stimulus. In tracheal muscle tissues, a contractile stimulus such as acetylcholine initiates the activation of the small GTPase, cdc42, and its binding to the WASp family protein, N-WASp (37, 68, 69). N-WASp is recruited to the membrane of tracheal muscle cells in response to the stimulus, where it binds to and activates the Arp2/3 complex, initiating the polymerization of new actin filaments (37). The expression of a dominant-negative N-WASp C-terminal peptide in tracheal muscle tissues prevents the activation of N-WASp and inhibits tension generation and actin polymerization in response to a contractile stimulus, but does not affect myosin light chain phosphorylation.

In tracheal smooth muscle, the activation of N-WASp can be regulated by the tyrosine phosphorylation of the adhesion complex protein, paxillin, which couples to N-WASp via the SH₂/SH₃ adaptor protein, CrkII, to catalyze its activation by cdc42 (36, 37, 68, 69). Paxillin, a scaffolding protein that binds to vinculin in adhesion complexes, can be tyrosine phosphorylated by focal adhesion kinase (FAK), which also localizes to adhesion complexes (70). Both paxillin and FAK undergo tyrosine phosphorylation during the contractile activation of tracheal smooth muscle, and the tyrosine phosphorylation of both proteins is sensitive to mechanical stimulation. When more strain is imposed on smooth muscle tissues, higher levels of phosphorylation of both paxillin and FAK are observed in response to contractile stimulation (26, 27, 71). There is also evidence that actin polymerization is mechanosensitive. The degree of tension depression caused by the inhibition of actin polymerization depends on the mechanical strain on the muscle, suggesting that actin polymerization may be important in regulating the length-sensitivity of tension development (35, 72). Modulation of the phosphorylation of paxillin in response to mechanical stimuli in airway smooth muscle tissues could provide a mechanism for the mechanosensitive regulation of N-WASp-mediated actin polymerization. This could provide a molecular pathway by which cytoskeletal organization and actin structure could be modulated to facilitate the adaptation of smooth muscle cell shape and stiffness to their environment (Figures 4, 5, and 6).

DYNAMICS OF ADHESION COMPLEX STRUCTURE IN SMOOTH MUSCLE

The macromolecular complexes at the membrane adhesion sites of smooth muscle are not static structures; proteins bound within adhesion complexes are in constant state of dynamic equilibrium with their cytoplasmic pools, where the proteins can exist in an inactive state. In cells cultured on a substrate, the process of spreading and adhesion stimulates the recruitment of adhesion junction proteins to the adhesive surface of the membrane, resulting in the enlargement of focal adhesion sites, and stimulating the formation of actin stress fibers (73–75). These adhesive complexes form around a small cluster of

ligand-bound integrins by the stepwise addition of structural and signaling proteins in temporal succession, which act to strengthen integrin–cytoskeletal connections (76, 77). The recruitment of proteins to integrin–cytoskeletal junctions during cell migration reinforces sites of adhesion so that they can withstand the force transmitted to the extracellular matrix during the generation of traction (73, 75, 78, 79). The imposition of mechanical tension or the twisting of integrin proteins bound to magnetic beads on the surface of isolated cells also stimulates the recruitment of adhesion complex proteins to sites of membrane tension (80). Mechanical force thus appears to stimulate the fortification of connections between the cytoskeletal filaments and the membrane in these cells.

Recent studies suggest that the localization of proteins within adhesion sites in differentiated smooth muscle cells and tissues is also dynamically regulated during contractile stimulation. Studies employing co-immunoprecipitation, cell fractionation, immunofluorescence analysis, and *in vivo* imaging of both differentiated and cultured smooth muscle cells have demonstrated that the pharmacologic stimulation of smooth muscle cells or tissues initiates the recruitment of a number of structural and signaling proteins to the membrane, and leads to an increase in their association with integrins and other adhesion complex proteins (37, 58, 81–84). Studies to date have documented the recruitment of talin, paxillin, vinculin, α -actinin, FAK, N-WASp, integrin-linked kinase, and its binding partner PINCH to the submembranous region of airway smooth muscle cells in response to a contractile stimulus (37, 81, 83). This recruitment may serve to catalyze the formation of submembranous actin filaments, anchor them to integrin proteins at adhesion sites, and strengthen the network of newly formed actin filaments to form a more rigid submembranous structure that is capable of transmitting the force generated by the activation of contractile proteins to the extracellular matrix filaments and across the tissue.

In vivo imaging of the recruitment of these proteins in freshly dissociated smooth muscle cells indicates that the translocation to the membrane can occur very rapidly, within seconds of the administration of a contractile agonist (81, 85). In freshly dissociated tracheal smooth muscle cells, α -actinin, an actin cross-linking protein that binds to the cytoplasmic tail of the β integrin subunit, is recruited to the periphery cells within a few seconds of stimulation with acetylcholine (81) (see Figure 4; see also the real-time video file of GFP- α -actinin in the online supplement). Co-immunoprecipitation analysis of airway muscle tissue extracts shows that contractile stimulation increases the association of α -actinin with integrin proteins at these sites. The recruitment of α -actinin can be blocked in the tissues by expressing an α -actinin fragment that consists only of the integrin-binding rod domain, which competes with endogenous α -actinin for binding to integrin proteins (81). Inhibition of the recruitment of α -actinin by the α -actinin rod domain peptide causes a marked inhibition of the contraction of the tracheal muscle tissues, without inhibiting actin polymerization or myosin light chain phosphorylation. This suggests that the recruitment of α -actinin does not contribute to the activation or regulation of either actin polymerization or myosin light chain phosphorylation, but might instead be involved in strengthening and anchoring a newly formed actin network.

The mechanism by which proteins are recruited to adhesion sites has not been established. In migrating cells, new adhesive complexes that form at the leading edge of the cell have been proposed to form around a small cluster of ligand-bound integrins by the stepwise addition of structural and signaling proteins in temporal succession, which act to strengthen integrin–cytoskeletal connections (76, 77). In migrating fibroblasts, the scaffolding

Figure 4. Fluorescence images obtained on live tracheal smooth muscle cells freshly dissociated from tracheal muscle tissues that were transfected with plasmids encoding EGFP α -actinin (81). EGFP α -actinin was visualized in live cells during stimulation with ACh. Cells were scanned once per second for 60 seconds using

confocal microscopy. Panels show the same cell before stimulation and 1, 2, and 10 seconds after stimulation. α -actinin is rapidly recruited to the cell membrane immediately after ACh is administered. Images are excerpted from real-time video images (see video file in the online supplement).

Figure 5. Proposed cytoskeletal processes that occur during shortening and tension development in airway smooth muscle.

Figure 6. 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. Cytoskeletal proteins such as α -actinin are recruited to stabilize membrane/cytoskeletal junctions and the cortical actin filament lattice and adapt the shape of the cell to its surrounding environment. The cortical actin/cytoskeletal network provides a rigid structure for the transmission of the tension generated by crossbridge cycling to the outside of the cell.

protein paxillin is recruited to adhesion sites early in the process, whereas α -actinin subsequently colocalizes with paxillin (76).

In airway muscle cells, paxillin and vinculin are recruited to the membrane of dissociated smooth muscle cells in response to contractile stimulation, and the recruitment of vinculin requires the presence of paxillin (83). However, talin appears to be recruited independently of paxillin or vinculin. When the recruitment of α -actinin is inhibited by expression of the α -actinin rod domain, it does not prevent the recruitment of paxillin, talin, or vinculin (81). This sequence of events is consistent with the hypothesis that paxillin and vinculin are necessary for the initiation of actin polymerization, but that α -actinin is recruited later in the process to stabilize actin filaments after they form. The sequence of protein recruitment at adhesion complex proteins in tracheal muscle cells appears to be generally analogous to that reported to occur during the formation of focal adhesions in cells cultured on a substrate, although the time course is much faster.

Integrin-linked kinase (ILK) is a multidomain protein that binds directly to the cytoplasmic domain of β 1 integrins and serves as a scaffolding protein for the organization of cytoskeletal signaling proteins at adhesion complexes (86) (Figure 2). ILK may play a critical role in the assembly of macromolecular complexes at smooth muscle adhesion sites during contractile stimulation. ILK forms a heterotrimeric complex with PINCH, an adaptor protein that consists of a tandem array of five LIM domains, and α -parvin, which binds to actin filaments (87–93). ILK also binds to paxillin (36, 68). Through its interactions with paxillin and PINCH, ILK may transduce signals from integrin receptors to regulate the initiation of actin polymerization. Paxillin may couple to N-WASp via the adaptor protein CrkII (94), and PINCH may couple to N-WASp via the adaptor protein Nck. ILK and its binding partners are thus positioned to coordinate signaling pathways that regulate cytoskeletal remodeling and to orchestrate the formation of structural links between integrin proteins and the actin cytoskeleton.

The contractile activation of tracheal smooth muscle stimulates the recruitment of the ILK/PINCH complex to membrane adhesion complexes and increases its interaction with β integrins, paxillin and vinculin (85). The assembly of the ILK protein complex at membrane adhesion sites in tracheal smooth muscle is necessary for tension development and for N-WASp-mediated actin polymerization during contractile activation. This suggests that ILK may be an important coordinator of signals from integrin receptors to multiple downstream pathways that regulate actin polymerization and cytoskeletal organization in smooth muscle during contractile stimulation.

A NEW PARADIGM FOR SMOOTH MUSCLE CONTRACTION

The actomyosin cross-bridge interaction and associated regulatory processes provide a mechanism that can account for tension development and active shortening of the smooth muscle cell, but crossbridge interactions cannot account for the malleability of airway smooth muscle and its ability to adapt to environmental influences. The ability of airway smooth muscle to alter its stiffness and contractility in response to mechanical oscillation and stretch has been widely observed and described, and is considered to be critically important for the regulation of normal airway responsiveness (2, 9, 32). Such adaptive properties are likely to result from cytoskeletal processes outside of the actomyosin interaction (24, 25, 32, 95). Thus, it is important to consider the actomyosin interaction and crossbridge cycling as a component of a complex and integrated series of cytoskeletal events that occur during the contraction of the airway smooth

muscle cell. These concerted events not only result in tension generation and shortening of the smooth muscle cell, but also modulate the cell's shape and stiffness, and can thereby affect airway compliance and responsiveness. The signals that regulate dynamic cytoskeletal rearrangements and actin polymerization originate within the macromolecular protein complexes that reside at adhesion junctions where the cytoskeleton links to the extracellular matrix via transmembrane integrin proteins.

The current mechanistic paradigm for cytoskeletal processes that transpire during airway muscle contraction entails a dynamic process in which the response of the smooth muscle cell to changes in shape imposed by external physiologic forces is mediated by local mechanotransduction events that serve to catalyze actin polymerization and cytoskeletal remodeling (8, 13, 24, 25, 32) (Figures 5 and 6). Signaling events triggered by the pharmacologic activation of smooth muscle cells elicit coordinated responses throughout the cell that modulate the structure and conformation of the actin filament lattice, fix its structure, and increase its rigidity, and activate crossbridge cycling and the sliding of the thick and thin filaments. The remodeling of the actin cytoskeleton serves to set the shape of the airway smooth muscle cell and may occur concurrently with the remodeling of other cytoskeletal filament systems, including myosin and intermediate filaments (96–98). Additional cytoskeletal processes within the cell fortify the strength of the connections between membrane adhesion junctions and actin filaments within the contractile apparatus and cytoskeletal network, thus providing a strong and rigid framework for the transmission of force generated by the interaction of myosin and actin filaments to the outside of the cell (74, 75, 81, 83). According to this paradigm, mechanotransduction events at integrin adhesion sites modulate the cytoskeletal events that enable the cell to adapt and remodel its cytoskeletal structure and organization to conform to forces generated by external and internal physiologic processes (13, 25). Actin polymerization and remodeling may occur preferentially at points of tension or mechanical strain in the cell membrane, and the strengthening of points of tension transmission to the exterior of the cell may occur at discrete points of membrane stress or strain (37, 64, 75, 81, 99).

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. Pathophysiologic processes that disturb normal cytoskeletal dynamics might result in abnormalities in the ability of the airways to regulate their compliance and adapt to mechanical forces that are imposed on them during breathing. Airway hyperresponsiveness could be a by-product of such disturbances. However, much remains to be determined regarding the mechanistic basis for the properties of mechanical adaptation of airway smooth muscle, and the role of dynamic cytoskeletal processes in the regulation of the compliance and contractility of airway smooth muscle. A better understanding of these processes could provide novel insights into the basis for the normal physiologic properties of the airways and in disturbances in their function that occur during disease.

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