*Arch Pharm Res Vol 23, No 6, 535-547, 2000*

Review

# Mechanosensitive Modulation of Receptor-Mediated Crossbridge Activation and Cytoskeletal Organization in Airway Smooth Muscle

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*(Received October 17, 2000)*

Recent findings indicate that mechanical strain (deformation) exerted by the extracellular matrix modulates activation of airway smooth muscle cells. Furthermore, cytoskeletal organization in airway smooth muscle appears to be dynamic, and subject to modulation by receptor activation and mechanical strain. Mechanosensitive modulation of crossbridge activation and cytoskeletal organization may represent intracellular feedback mechanisms that limit the shortening of airway smooth muscle during bronchoconstriction. Recent findings suggest that receptor-mediated signal transduction is the primary target of mechanosensitive modulation. Mechanical strain appears to regulate the number of functional G-proteins and/or phospholipase C enzymes in the cell membrane possibly by membrane trafficking and/or protein translocation. Dense plaques, membrane structures analogous to focal adhesions, appear to be the primary target of cytoskeletal regulation. Mechanical strain and receptor-binding appear to regulate the assembly and phosphorylation of dense plaque proteins in airway smooth muscle cells. Understanding these mechanisms may reveal new pharmacological targets for controlling airway resistance in airway diseases.

**Key words:** Asthma, Bronchodilation, Cytoskeleton, Mechanotransduction

## INTRODUCTION

Airway smooth muscle is the contractile component of airways. In static equilibrium, airway diameter is determined by a balance of force generated by airway smooth muscle cells against the elastic load exerted by the extracellular matrix. Therefore, excessive contractions of airway smooth muscle can lead to bronchoconstriction in airway diseases such as asthma. Force generated by an airway smooth muscle cell is determined by crossbridge activation, contractile and cytoskeletal organization, and the amount of contractile proteins. Recent findings indicate that mechanical strain (deformation) exerted by the extracellular matrix modulates activation of airway smooth muscle cells. Furthermore, cytoskeletal organization in airway smooth muscle appears to be dynamic, and subject to regulation by receptor activation and mechanical strain. Mechanosensitive modulation of crossbridge activation and cytoskeletal organization may represent intracellular feedback mechanisms that limits the shortening of airway smooth muscle during bronchoconstriction. Understanding the molecular mechanisms of these mechanisms may reveal new pharmacological targets for controlling airway resistance in airway diseases.

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### Mechanosensitive modulation of airway smooth muscle activation

*Mechanosensitive Modulation of Signal Transduction.* Many neurotransmitters, hormones, and cytokines bind to membrane receptors, and activate signal transduction enzymes via heterotrimeric G-proteins (Hakonarson and Grunstein, 1998). Phosphatidylinositide-specific phospholipase C is a major signal transduction enzyme in airway smooth muscle cells. Phospholipase C catalyzes the break-down of phosphatidylinositol (4,5) bisphosphate with the production of two important second messengers, namely inositol (1,4,5) trisphosphate ($IP_3$) and diacylglycerol (DAG), although phospholipase D also catalyzes DAG production (Lee and Severson, 1994). Muscarinic receptor activation has been shown to activate phosphatidylinositol (PI) turnover in airway smooth muscle (Chilvers et *al*., 1989; Grandordy et *al*., 1986). Furthermore, muscarinic receptor-coupled PI turnover is sustained during force maintenance (Chilvers et

*al.*, 1989; Chilvers and Nahorski, 1990; Yoo *et al.*, 1994). Mechanical stretch per se has been found to activate phospholipase C activity in cultured vascular smooth muscle cells (Kulik *et al.*, 1991) and intact vascular smooth muscle (Matsumoto *et al.*, 1995; Tanaka *et al.*, 1994). Furthermore, mechanical strain also modulates muscarinic receptor-coupled PI turnover in airway smooth muscle. Yoo *et al.* (1994) found that carbachol-induced PI turnover was statistically significant at optimal length ($L_o$) for contraction, but became statistically insignificant at $10\%L_o$. Yoo *et al.* (1994) also observed similar length-dependent changes in intracellular [$Ca^{2+}$] in parallel with PI turnover. Since some isoforms of phospholipase C are known to be regulated by $Ca^{2+}$ (Rhee and Bae, 1997), this observation raised the possibility that mechanosensitive modulation of PI turnover may reflect $Ca^{2+}$-dependence of phospholipase C. However, Grandordy *et al.* (1986) have measured PI turnover in airway smooth muscle activated by $K^+$-depolarization but did not observe significant increases in PI turnover. Therefore, this finding suggests that the muscarinic receptor-coupled phospholipase C isoform in airway smooth muscle is not $Ca^{2+}$-dependent within the physiological range of cytosolic [$Ca^{2+}$]. Together, these findings suggest that mechanical strain modulates signal transduction between muscarinic receptor occupation and phospholipase C activation.

An and Hai (1999) investigated whether mechanical strain modulates the maximal response ($V_{max}$) or the affinity of interaction ($K_m$) of the signal transduction cascade as described by Michaelis-Menten kinetics. They found that muscarinic receptor-mediated PI turnover remained linearly dependent on muscle length at maximal carbachol concentration for eliciting contraction in bovine tracheal smooth muscle. This observation suggests that affinity of interaction ($K_m$) is unlikely to be the target of mechanosensitive modulation. When the concentration-dependence of carbachol-induced PI turnover was measured at 20% and $100\%L_o$. An and Hai (1999) found that a change in maximal PI turnover alone was necessary and sufficient to explain the two concentration-response relations. These results indicate that maximal PI turnover is the target of modulation by mechanical strain in airway smooth muscle. Regarding mechanisms, this finding suggests that mechanical strain regulates the maximum capacity of the signal transduction cascade leading from muscarinic receptor to phospholipase C. Muscarinic receptors are coupled to phospholipase C via heterotrimeric G-proteins. Therefore, mechanical strain may regulate the number of functional receptors, G proteins, or phospholipase C enzymes on the cell membrane of an airway smooth muscle cell. Fluoroaluminate activates heterotrimeric G-proteins directly, thus bypassing the step of receptor activation (Bigay *et al.*, 1985; Sternweis and Gilman, 1982). Fluoroaluminate has been shown to activate PI turnover in airway smooth muscle (Hall *et al.*, 1990). An and Hai (1999) found that fluoroaluminate-induced PI turnover at maximal [fluoroaluminate] remained linearly dependent on muscle length. This finding suggests that mechanical strain regulates the number of functional G-proteins and/or phospholipase C enzymes in the cell membrane. Mechanistically, this finding suggests that mechanical strain regulates the compartmentalization of G-proteins and/or phospholipase C in airway smooth muscle cells. Possible molecular mechanisms include mechanosensitive modulation of membrane trafficking, such as the formation of caveoli where signal transduction molecules are concentrated (Anderson, 1993), and translocation of phospholipase C from the cytosol to the cell membrane (Coburn *et al.*, 1997; Cockcroft and Thomas, 1992; Liu *et al.*, 1996; Rhee and Bae, 1997).

*Mechanosensitive Modulation of Intracellular [$Ca^{2+}$].* It is known that $IP_3$ produced from receptor-mediated PI turnover induces $Ca^{2+}$ release from the sarcoplasmic reticulum (SR) by binding to $IP_3$-sensitive calcium channels (Hakonarson and Grunstein, 1998). In addition, receptor activation also stimulates $Ca^{2+}$ influx through sarcolemmal calcium channels in airway smooth muscle cells (Missiaen *et al.*, 1992; Van Breemen and Saida, 1989). Murray and Kotlikoff (1991) found that receptor-mediated $Ca^{2+}$ influx was not sensitive to organic calcium channel antagonists in airway smooth muscle. Their finding suggests that receptor agonists stimulate $Ca^{2+}$ influx through pathways that are distinct from voltage-dependent calcium channels. In contrast, other investigators have reported significant inhibition of receptor-mediated $Ca^{2+}$ influx by organic calcium channel antagonists in airway smooth muscle cells (Kajita and Yamaguchi 1993; Yang *et al.*, 1993). Kamishima *et al.* (1992) reported that carbachol increases the open probability of voltage-dependent calcium channels in airway smooth muscle cells by shifting the activation curve in the hyperpolarization direction. Tomasic *et al.* (1992) reported that bradykinin and methacholine increased the open probability of voltage-dependent calcium channels via second messengers.

Gunst (1989) studied the effect of muscle shortening on intracellular [$Ca^{2+}$] ([$Ca^{2+}$]$_i$) in electrically activated canine tracheal smooth muscle and observed increases in [$Ca^{2+}$]$_i$ at shorter muscle lengths and during isotonic shortening. In contrast, Yoo *et al.* (1994) measured carbachol-induced [$Ca^{2+}$]$_i$ at $L_o$ and $10\%L_o$, but observed lower [$Ca^{2+}$]$_i$ at shorter muscle length. Mehta et al. (1996) also observed a decrease in [$Ca^{2+}$]$_i$ when acetylcholine-activated canine tracheal smooth muscle was allowed to shorten from $L_o$ to $70\%L_o$. Therefore, [$Ca^{2+}$]$_i$ in electrically stimulated and receptor-activated airway smooth muscles appear to respond differently to muscle shortening.

In theory, mechanical stress and strain can both be the mechanical signal involved in mechanosensitive modulation of [$Ca^{2+}$]$_i$ in smooth muscle cells. External stress is transmitted to a smooth muscle cell via dense plaques on

the cell membrane (Draeger *et al.*, 1990). It has been proposed that mechanical stress-dependent conformational change of the focal adhesion complex may be sufficient to regulate a whole array of protein kinase pathways, thereby regulating cell activation (Chicurel *et al.*, 1998; Zou *et al.*, 1998). It is difficult to differentiate mechanical stress from strain during isometric contractions because the two change in parallel. However, mechanical stress and strain can be temporarily uncoupled during isotonic shortening when the external load remains constant and muscle length decreases. An and Hai (2000) investigated the mechanical signals and the role of SR $Ca^{2+}$-ATPase during shortening-induced attenuation of $[Ca^{2+}]_i$ in airway smooth muscle. An and Hai (2000) found that $[Ca^{2+}]_i$ correlated linearly with muscle length during isotonic shortening independent of external load ranging from 20% to 80% isometric force. These results indicate that mechanical strain, but not stress, is the primary mechanical signal for shortening-induced attenuation of $[Ca^{2+}]_i$ in airway smooth muscle. It is noteworthy that the opposite appears to be true for vascular smooth muscle when an increase in mechanical stress appears to be sufficient to induce myogenic vasoconstriction (VanBavel and Mulvany, 1994). Physiologically, an increase in transmural pressure leads to lung expansion during inspiration, but leads to vasoconstriction in the autoregulation of blood flow. Therefore, the different responses of airway and vascular smooth muscle cells to mechanical stress appear to be well suited for their physiological functions.

The observed significant length-dependence and insignificant load-dependence of $[Ca^{2+}]_i$ as observed by An and Hai (2000) may have implications on the underlying mechanisms. Ellipsoidal geometry predicts that cell surface/volume ratio will decrease as smooth muscle cells shorten from a more elongated shape to a more spherical shape. Therefore, the excess membrane may invaginate or protrude in shortened smooth muscle cells. If some of the excess membrane and its associated signal transduction molecules become compartmentalized and inaccessible to extracellular agonists in a shortened smooth muscle cell, then the level of cell activation will be attenuated. This mechanism of membrane compartmentalization appears to be consistent with the suggestion that mechanical strain regulates the number of functional G-proteins and/or phospholipase C enzymes on the cell membrane of a smooth muscle cell (An and Hai, 1999).

An and Hai (2000) also investigated the role of SR $Ca^{2+}$-uptake during shortening-induced attenuation of $[Ca^{2+}]_i$ in airway smooth muscle. An and Hai (2000) found that cyclopiazonic acid (CPA), an inhibitor of SR $Ca^{2+}$-ATPase did not significantly change $[Ca^{2+}]_i$ during carbachol-induced isometric contractions. This observation is consistent with the findings of Amoako *et al.* (1996) and Bourreau *et al.* (1993) that CPA did not significantly inhibit muscarinic receptor-mediated isometric contractions. In contrast, Janssen *et al.* (1999) have reported CPA-dependent attenuation of acetylcholine-induced contractions and augmentation of KCl-induced contractions. However, it is not clear whether force has reached steady state in their experiments. These findings together suggest that SR-$Ca^{2+}$-uptake does not play a significant role during the steady-state phase of isometric contractions in airway smooth muscle. Maximal inhibition of SR $Ca^{2+}$-uptake by CPA also did not significantly change $[Ca^{2+}]_i$ during isotonic shrotening (An and Hai, 2000). This finding suggests that the SR does not play an important role in shortening-induced attenuation of $[Ca^{2+}]_i$ in airway smooth muscle. Since the SR has a finite capacity of storing $Ca^{2+}$, it is perhaps not surprising that the SR does not regulate $[Ca^{2+}]_i$ during the steady-state phase of isometric contraction. However, recent studies have suggested coupling between the emptying of the SR with the activation of sarcolemmal $Ca^{2+}$ influx (Putney and Bird, 1993). Therefore, the SR may indirectly regulate steady-state $[Ca^{2+}]_i$ by its coupled effect on sarcolemmal $Ca^{2+}$ influx. However, the above results suggest that this mechanism is relatively unimportant in muscarinic receptor-activation of airway smooth muscle. Therefore, mechanosensitive ion channels may be the mechanism of shortening-induced attenuation of intracellular $[Ca^{2+}]$ in muscarinic receptor-activated airway smooth muscle (Kirber *et al.*, 1988; Mehta *et al.*, 1996; Morris, 1990).

*Mechanosensitive Modulation of Myosin Light Chain Phosphorylation.* Phosphorylation of the 20,000 myosin light chain by $Ca^{2+}$, calmodulin-dependent myosin light chain kinase (MLCK) is the central activation mechanism of smooth muscle contraction (Gerthoffer, 1991; Horowitz *et al.*, 1996). Myosin light chain dephosphorylation is catalyzed by myosin light chain phosphatase (MLCP) during smooth muscle relaxation. Therefore, steady-state level of myosin phosphorylation is determined by the MLCK/MLCP ratio. Recent findings suggest that MLCK activity may be regulated by CaM kinase II (Hashimoto, Y. and T.R. Soderling, 1990; Kim *et al.*, 2000; Tansey *et al.*, 1992), protein kinase C (Stull *et al.*, 1990), and Rho-associated kinase (Amano *et al.*, 1996; Somlyo and Somlyo, 2000). Other findings indicate that MLCP activities may be regulated by Rho and protein kinase C (Kimura *et al.*, 1996; Kureishi *et al.*, 1997; Masuo *et al.*, 1994; Somlyo and Somlyo, 2000; Stull *et al.*, 1990). These mechanisms have been proposed as modulators of $Ca^{2+}$-sensitivity of myosin phosphorylation and contraction in smooth muscle (Horowitz *et al.*, 1996; Somlyo *et al.*, 1999). Bremerich *et al.* (1997) investigated the effects of PKC inhibitors (calphostin C, chelerythrine chloride, PKC peptide-19-31, and staurosporine) on acetylcholine-induced $Ca^{2+}$-sensitization in β-escin-permeabilized canine tracheal smooth muscle. However, Bremerich *et al.* (1997) found that PKC inhibitors had no effect on $Ca^{2+}$-sensitization induced by acetylcholine, suggesting that mechanisms other than

PKC may regulate $Ca^{2+}$-sensitivity in airway smooth muscle. Kai *et al.* (2000) observed similar relationships between force and myosin phosphorylation in the absence and presence of muscarinic receptor activation in α-toxin-permeabilized canine tracheal smooth muscle. These results together indicate that $Ca^{2+}$-sensitivity of myosin light chain phosphorylation is the mechanism by which receptor agonists regulate $Ca^{2+}$-sensitivity of contraction in airway smooth muscle.

Hai and Szeto (1992) measured the levels of myosin phosphorylation during isometric contraction and unloaded shortening in carbachol- and phorbol dibutyrate-activated bovine tracheal smooth muscle. Hai and Szeto (1992) found that carbachol-induced myosin phosphorylation was significantly attenuated during isotonic shortening. However, phorbol dibutyrate-induced myosin phosphorylation was insensitive to muscle shortening, suggesting that the mechanosensitive step is located upstream from diacylglycerol production. In theory, the observed length-dependence of myosin phosphorylation could also reflect changes in $Ca^{2+}$-sensitivity of myosin phosphorylation. However, this possibility was excluded by the findings of Moreland and Murphy (1988) that $Ca^{2+}$-sensitivity of myosin phosphorylation was not length-dependent. Yoo *et al.* (1994) found that myosin phosphorylation was linearly related to muscle length in both unstimulated and carbachol-activated bovine tracheal smooth muscle. However, the length-dependence of myosin phosphorylation in carbachol-activated tissues exhibited a steeper slope. As a result, suprabasal myosin phosphorylation induced by carbachol was statistically significant at $L_o$, but became insignificant at 10%$L_o$. These results indicate that mechanical strain modulates receptor-mediated myosin light chain phosphorylation in a graded manner.

Mehta *et al.* (1996) also observed a linear relationship between acetylcholine-induced myosin phosphorylation and muscle length in canine tracheal smooth muscle. In addition, Mehta *et al.* (1996) measured the concentration-response relations of acetylcholine-induced myosin phosphorylation and force at 50%, 70%, and 100%$L_o$, and observed length-dependent decreases in the half-maximal concentration ($EC_{50}$) for both force and myosin phosphorylation. This finding suggests that mechanical strain modulates sensitivity of airway smooth muscle to muscarinic receptor activation. Youn *et al.* (1998) tested the alternative hypothesis that mechanical strain modulates maximum myosin phosphorylation by measuring carbachol-induced myosin phosphorylation near the maximum end of the concentration-response curve. They found that maximum myosin phosphorylation became length-dependent at muscle lengths shorter than 50-60%$L_o$. These results indicate that agonist sensitivity may be the primary target of length-dependent modulation at muscle lengths near $L_o$, but the maximum level of activation gradually becomes attenuated at muscle lengths shorter than 60% $L_o$. Fluoroaluminate activates heterotrimeric G-proteins directly, thus bypassing the step of receptor activation (Bigay *et al.*, 1985; Sternweis and Gilman, 1982). Hai and Ma (1993) found that maximal fluoroaluminate-induced myosin phosphorylation was also linearly dependent on muscle length. The striking similarity in the dependencies of muscarinic receptor- and fluoroaluminate-mediated PI turnover and myosin phosphorylation on muscle length suggests that signal transduction may be the primary target of modulation by mechanical strain. Another possibility is that multiple steps in the activation-contraction cascade are linearly dependent on muscle length. The observed dependence of $K^+$-depolarization-induced myosin phosphorylation (Hai and Szeto, 1992) appears to support this alternative hypothesis. If this alternative hypothesis is correct, then mechanosensitive feedback must be critical to the function of airway smooth muscle cells so that redundant mechanosensitive feedback mechanisms are built into each level of the activation-contraction cascade.

*Mechanosensitive Modulation of Crossbridge Cycling.* Smooth muscle is unique in its ability to regulate crossbridge cycling rate in addition to crossbridge attachment (Murphy, 1994). Dillon *et al.* (1981) first reported that both myosin phosphorylation and crossbridge cycling rate increased transiently at the beginning of a contraction whereas isometric force increased monophasically to steady state. Therefore, myosin phosphorylation correlated linearly with crossbridge cycling rate, but nonlinearly with isometric force (Ratz *et al.*, 1989). The steady state of force maintenance with relatively low levels of myosin phosphorylation and crossbridge cycling rate has been termed the latch state (Dillon *et al.*, 1981). Thin filament-based regulatory mechanisms such as calponin and caldesmon have been investigated as alternative regulatory mechanisms during force maintenance (Horowitz *et al.*, 1996). Cooperative attachment of unphosphorylated crossbridges has also been proposed to explain high force maintenance at low levels of myosin phosphorylation (Somlyo *et al.*, 1988). Finally, Hai and Murphy (1989) have proposed a four-state crossbridge model to explain force maintenance during the latch state.

The sliding filament model derived from skeletal muscle research explains the length-dependence of force by the overlap of actin and myosin filaments. Recent findings suggest that this model may be incomplete for airway smooth muscle (Gunst, 1999). Sasaki and Hoppin (1979) found that activated smooth muscle exhibited significant hysteresis during cyclical length changes. Gunst (1983) and Fredberg *et al.* (1997) found that sinusoidal force oscillation of acetylcholine-activated airway smooth muscle would lead to muscle lengthening if the amplitude of force oscillation was above a threshold. Pratusevich *et al.* (1995) measured shortening velocity, compliance, and isometric force development in electrically stimulated airway smooth muscle at initial length, 75% initial length,

and 150% initial length. They found that shortening velocity and compliance exhibited strong dependencies on muscle length. In contrast, isometric force changed only initially when muscle length was changed, but eventually redeveloped to similar levels independent of muscle length. Chan *et al.* (2000) found that, when tonically activated airway smooth muscle was stretched rapidly with high stress, they exhibited memory-like behavior. Specifically, when mechanical stretch was applied to an activated muscle, post-stretch force and myosin phosphorylation remained correlated with initial length but not final length. Subsequent force development induced by $K^+$-depolarization also remained correlated with initial length but not final length. These results together suggest that multiple length-force relations may exist in airway smooth muscle depending on mechanical strain history.

Fredberg *et al.* (1999) have proposed the conditional stability hypothesis which postulates that periodic mechanical stretching places actomyosin crossbridges in a mechanically perturbed state that differs from static equilibrium. Gunst *et al.* (1995) have found that, when a tonically activated smooth muscle is allowed to shorten to a new length and redevelop force, the stiffness-force relation was steeper than that during isometric contraction at the same length. Therefore, Gunst *et al.* (1995) hypothesized that contractile and cytoskeletal structures in an activated smooth muscle cell are relatively fixed so that force development after a sudden length change is suboptimal for the new length. However, contractile and cytoskeletal structures in a resting smooth muscle cell are plastic and can reorganize to optimize force production at any muscle length. Consistent with Gunsts hypothesis, Mehta *et al.* (1996) found that rapid release of acetylcholine-activated canine tracheal smooth muscle to a shorter length resulted in a lower force than that during isometric contraction at the same final length. In contrast, the levels of myosin phosphorylation with or without prior shortening were not significantly different. Pratusevich *et al.* (1995) proposed the plasticity hypothesis that the number of contractile units in series changes with muscle length to maintain a relatively constant force independent of muscle length. These observations suggest that mechanical strain may modulate airway smooth muscle contraction by modulating the organization of cytoskeletal and contractile filaments.

## Mechanosensitive modulation of cytoskeletal organization in airway smooth muscle

Thin, intermediate, and thick filaments constitute the cytoskeletal and contractile filaments in smooth muscle cells. However, contractile units analogous to the sarcomeres in striated muscle have not been identified in smooth muscle cells. Nevertheless, the sliding filament model derived from skeletal muscle is considered to be applicable to smooth muscle. Accordingly, actomyosin interactions are often considered as the sole target of regulation, with the implicit assumption that contractile filaments and their anchoring sites at the cell membrane are stable in smooth muscle. Recent findings suggest that this assumption may be invalid for smooth muscle cells.

*Thin Filaments.* Thin filaments consist of actin and actin-binding proteins such as tropomyosin, caldesmon, and calponin. A major difference between smooth and striated muscles is the absence of the regulatory protein, troponin in smooth muscle. Much research has been done on identifying putative thin filament-based regulatory mechanisms in smooth muscle. Caldesmon and calponin are considered likely candidates (Marston *et al.*, 1998; Winder *et al.*, 1998). Caldesmon and calponin are actin-binding proteins that block the attachment of crossbridges to actin filaments. Receptor-mediated phosphorylation of caldesmon and calponin abolishes the blocking effects of caldesmon and calponin, thereby allowing crossbridge attachment to the actin filament and force production. However, it remains unclear whether caldesmon and calponin play a permissive or regulatory role in regulating smooth muscle contraction.

Actin is essential for the motility of both non-muscle and muscle cells. In non-muscle cells, dynamic actin polymerization and depolymerization are the basic mechanisms of cell motility (Chen *et al.*, 2000; Cooper and Schafer, 2000). In contrast, actin filaments are stable in skeletal muscle. Recent findings suggest that actin filaments in smooth muscle may not be as stable as those in striated muscles. First, the length-force relationship in smooth muscle appeared to be variable, depending on G protein activation and mechanical strain history (Gunst, 1986; Hai and Ma, 1993; Harris and Warshaw, 1991). Second, unlike skeletal muscle, smooth muscle contractions are inhibited by cytochalasins and latrunculin-A (Adler et al., 1983; Mehta and Gunst, 1999; Obara and Yabu, 1994; Wright and Hurn, 1994; Tseng *et al.*, 1997), agents known to be effective only on actin filaments undergoing dynamic polymerization (Cooper, 1987). These results suggest that actin filaments in smooth muscle may be dynamic, as found in non-muscle cells.

It is noteworthy that disruption of actin filaments by cytochalasins in non-muscle cells not only inhibits cell motility but also attenuates signal transduction, exocytosis, and ion channel activity (Cantiello *et al.*, 1991; Feuilloley *et al.*, 1993, 1994). These results suggest that actin cytoskeleton is not just a mechanical structure, but also a modulator of cellular processes in non-muscle cells. Obara and Yabu (1994) investigated the effect of cytochalasin B on $K^+$-depolarized intestinal smooth muscle but did not observe significant changes in intracellular $[Ca^{2+}]$ and myosin phosphorylation. Tseng *et al.* (1997) first reported that cytochalasin D induced dramatic change in morphology and organization of filamentous actin in

bovine tracheal smooth muscle cells. Furthermore, Tseng *et al.* (1997) found that disruption of actin filament organization by cytochalasin B significantly attenuated carbachol-induced intracellular [$Ca^{2+}$], and the effect was most dramatic on the initial peak intracellular [$Ca^{2+}$], suggesting that integrity of actin filament organization may be important for muscarinic receptor-mediated $Ca^{2+}$ release. This finding is novel for smooth muscle research because signal transduction in smooth muscle is often depicted as a unidirectional flow of information from the cell membrane to contractile proteins without feedback control from the contractile filaments (Somlyo and Somlyo, 1994). Results from Tseng *et al.* (1997) suggest that actin filaments may be an integral component of signal transduction and/or $Ca^{2+}$ regulation in smooth muscle cells. The concept of actin cytoskeleton-dependent modulation of signal transduction has been investigated extensively in non-muscle cells, and published results suggest several mechanisms. Feuilloley *et al.* (1993) have reported that cytochalasin B inhibited phosphatidylinositol turnover in adrenocortical cells, and Bourguignon et al. (1993) have reported that cytochalasin D inhibited inositol 1,4,5-trisphosphate ($IP_3$)-induced intracellular $Ca^{2+}$ release in blood platelets. Phosphatidylinositol turnover and $IP_3$-induced $Ca^{2+}$ release are both integral components of muscarinic and other receptor-coupled signal transduction in smooth muscle (Hakonarson and Grunstein, 1998). It is conceivable that actin filaments may also modulate these processes in smooth muscle cells. Cytochalasin B also attenuated carbachol-induced myosin phosphorylation in airway smooth muscle (Tseng *et al.*, 1997), but the [$Ca^{2+}$]-myosin phosphorylation relations in control and cytochalasin B-treated smooth muscle appeared to be similar, suggesting that the effect of cytochalasin B on carbachol-induced myosin phosphorylation could be explained by its effect on intracellular [$Ca^{2+}$]. Actin cytoskeleton has been proposed as an important transducer of mechanosensitive modulation (Ingber, 1991; Jacobson *et al.*, 1995). These results of Tseng *et al.* (1997) are consistent with this proposal.

A fundamental question is how signal transduction molecules such as phospholipase C and calcium channels sense mechanical strain an/or stress. When mechanical stress is applied to smooth muscle, it is transmitted to the cell surface via the extracellular matrix bound to integrin receptors. The communication between integrin receptors and signal transduction molecules could be biochemical in nature. For example, integrin-associated focal adhesion kinase may initiate a cascade of protein phosphorylation at focal adhesions. Alternatively, the communication could be mechanical in nature. For example, actin filaments may physically connect integrin receptors to signal transduction molecules (Chen *et al.*, 1997; Davis, 1995). This mechanical model predicts that disruption of actin filaments by cytochalasin D should disrupt the connection between mechanical stress on the cell surface and the interior of a cell, thereby inhibiting length-dependent modulation. Youn *et al.* (1998) investigated the effect of actin filament disruption by cytochalasin D on the length-dependencies of myosin phosphorylation and contraction in airway smooth muscle. Youn *et al* (1998) found that cytochalasin D significantly attenuated carbachol-induced active force, suggesting that cytochalasin D disrupted actin filaments involved in contraction. Furthermore, cytochalasin D significantly shifted the length-myosin phosphorylation relation downward to lower levels of myosin phosphorylation. However, as a measure of length sensitivity, the slopes of the length-myosin phosphorylation relation in control and cytochalasin D-treated tissues were not significantly different. This effect of cytochalsin D was also confirmed by Mehta and Gunst (1999) in canine tracheal smooth muscle. Furthermore, Mehta and Gunst (1999) used latrunculin-A to inhibit actin polymerization, and found that latrunculin-A significantly inhibited force development without significantly changing the length-dependence of myosin phosphorylation. These findings suggest that actin filaments involved in contraction and length-dependent modulation may be distinguished by their different sensitivities to cytochalasin D. A potential implication of these results is that actin filaments bound by different actin-binding proteins (Cooper, 1987) may be differentially involved in contraction and mechanosensitive modulation.

Mehta and Gunst (1999) compared the effects of cytochalasin D and latrunculin-A on acetylcholine-induced force and myosin phosphorylation in canine tracheal smooth muscle. Cytochalasin D is expected to cap the barbed end of actin filaments, thereby disrupting actin filaments. Latrunculin-A is expected to bind to actin monomers, thereby preventing actin polymerization. Mehta and Gunst (1999) found that cytochalasin D inhibited force development by the same proportion independent of muscle length, whereas the inhibition of force by latrunculin-A was greater at longer muscle lengths. Furthermore, cytochalasin D significantly inhibited myosin phosphorylation whereas latrunculin-A did not significantly inhibit myosin phosphorylation. Mehta and Gunst (1999) suggested that capping of existing actin filaments by cytochalasin D could not prevent remodeling of the uncapped actin filaments in response to mechanical strain. In contrast, prevention of new actin filament formation by latrunculin-A would prevent remodeling of actin filaments in response to mechanical strain. Since latrunculin-A inhibited force without inhibiting myosin phosphorylation, Mehta and Gunst (1999) concluded that inhibition of actin polymerization inhibited force development without disrupting signal transduction mechanisms in airway smooth muscle cells. This finding together with the finding of Tseng *et al.* (1998) suggest that two distinct classes of actin filaments possibly associated with different

actin-binding proteins are involved in contraction and signal transduction in airway smooth muscle cells.

Muscarinic receptor activation has been found to increase filamentous/monomeric actin ratio and induce actin stress fiber formation in cultured airway smooth muscle cells (Togashi *et al.*, 1998). This response appears to be coupled by the Gαi2 protein and mediated by Rho and tyrosine phosphorylation (Hirshman *et al.*, 1998). This response has also been observed in differentiated airway smooth muscle activated by carbachol (Mehta and Gunst, 1999). Smith *et al.* (1997) have reported that mechanical strain is sufficient to stimulate actin stress fiber formation without receptor activation in cultured airway smooth muscle cells. These results provide direct evidence that the actin cytoskeleton is dynamic in airway smooth muscle cells. Furthermore, muscarinic receptor activation and mechanical strain appear to converge on a common pathway, leading to cytoskeletal remodeling in airway smooth muscle cells.

*Thick Filaments.* The tails of myosin molecules form the thick filaments whereas the head portion form the cross-bridges as in all muscle cells. However, myosin filaments in smooth muscle cells appear to be side-polar instead of bipolar as found in skeletal muscle cells (Craig and Megerman, 1977; Small, 1977). This side-polar structure may provide a longer distance for the sliding of actin filaments, thereby increasing the range of muscle length for force production in smooth muscle. Another unique property of smooth muscle filaments is their instability in vitro. At physiological [MgATP], ionic strength, and pH, smooth muscle myosin filaments disassemble in vitro, whereas skeletal muscle myosin filaments remain in the filamentous state (Onishi *et al.*, 1978). Furthermore, phosphorylation of the 20,000 dalton myosin light chain by $Ca^{2+}$, calmodulin-dependent myosin light chain kinase promote the assembly of smooth muscle myosin filaments (Suzuki *et al.*, 1978; Craig *et al.*, 1983). This observation suggested that myosin filaments might assemble and disassemble during contraction and relaxation cycles in smooth muscle cells (Cande *et al.*, 1983). However, Somlyo *et al.* (1981) and Tsukita *et al.* (1982) found that myosin filaments contained unphosphorylated light chains in relaxed smooth muscle cells. This observation indicates that myosin filaments do not totally disassemble in relaxed smooth muscle cells, but does not exclude quantitative changes in myosin filaments during cycles of relaxation and contraction. In fact, Gillis *et al.* (1988) have reported an increase in myosin filament density during smooth muscle contraction in anococcygeus smooth muscle. Watanabe *et al.* (1993) have reported an increase in the number of myosin filaments during contraction in rat anococcygeus smooth muscle, but not in guinea pig taenia coli. This observation was later confirmed by Xu *et al.* (1997) using low temperature electron microscopic methods for preserving the in vivo state of myosin filaments in rat anococcygeus smooth muscle and guinea taenia coli. Horowitz *et al.* (1994) labeled filamentous and monomeric myosin molecules using monoclonal antibodies, but did not observe significant changes in monomeric myosin during contraction and relaxation in avian gizzard smooth muscle. Therefore, contraction-induced assembly of myosin filaments appears to be a property of some but not all smooth muscle types. It remains to be shown whether myosin filaments undergo dynamic assembly and disassembly during relaxation-contraction cycles in airway smooth muscle. Pratusevich *et al.* (1995) studied the length-dependencies of isometric force, compliance, and shortening velocity in canine tracheal smooth muscle. They found that compliance and velocity exhibited strong dependence on muscle length, whereas isometric force was relatively insensitive to changes in muscle length. Based on these observations, Pratusevich *et al.* (1995) have hypothesized that the number of contractile units in series can change as a function of muscle length to optimize force development in airway smooth muscle. This hypothesis implies that the number of myosin filaments can undergo dynamic organization in response to length changes in airway smooth muscle. This hypothesis remains to be proven experimentally.

*Dense Bodies, Dense Plaques and Focal Adhesions.* Dense bodies are cytoplasmic sites, and dense plaques are membrane-associated sites for the anchoring of actin filaments in smooth muscle cells. The sliding filament paradigm derived from skeletal muscle research is often considered applicable to smooth muscle. Therefore, earlier studies on dense bodies and dense plaques were focused on revealing the structure of contractile units in smooth muscle cells. Focal adhesions are membrane structures at which cultured cells are attached to the extracellular matrix. Therefore, dense plaques and focal adhesions are analogous structures and possibly made up of similar proteins. Focal adhesions in cultured cells are dynamic structures. Assembly and disassembly of focal adhesions represent the basic mechanism of cell motility. Recent studies suggest that dense plaques in smooth muscle cells may also be dynamic structures, subject to regulation by mechanical strain and receptor activation.

Dense bodies and dense plaques in smooth muscle cells are first identified by electron microscopy (Bond and Somlyo, 1982; Davis and Shivers, 1992; McGuffee and Little, 1992; McGuffee *et al.*, 1991; Tsukita *et al.*, 1983). These studies focused on the composition and ultrastructural organization of dense bodies and dense plaques. Fay *et al.* (1983) labeled dense bodies with α-actinin antibodies and observed string-like array of cytosolic dense bodies and more irregularly shaped dense plaques at the cell membrane. Small (1985) reported that α-actinin was associated with cytoplasmic dense bodies whereas vinculin was associated with membrane-associated dense plaques. Draeger *et al.* (1989) identified transverse bands of talin or vinculin-containing actin-anchorage sites on the mem-

brane of smooth muscle cells. Kargacin *et al.* (1989) observed specific patterns of dense body movements during shortening of single smooth muscle cells. Their findings suggest well-ordered structure of dense bodies in smooth muscle cells. Using confocal microscopy, Draeger *et al.* (1990) and North *et al.* (1994) showed that both desmin-containing intermediate filaments and actin-containing thin filaments bind to cytoplasmic dense bodies. This convergence of cytoskeletal and contractile filaments onto the cytoplasmic dense bodies have led to the hypothesis that the two classes of filaments may interact mechanically in force generation by smooth muscle cells (Small, 1995).

Focal adhesions are highly dynamic membrane structures that undergo assembly and disassembly during cell spreading and migration (Craig and Johnson, 1996; Gilmore and Burridge, 1996; Goldman *et al.*, 1996). Integrins are integral proteins that connect the cytoskeleton to the extracellular matrix. The extracellular domain of integrins bind to the extracellular matrix, whereas the cytoplasmic domain of integrins bind to an array of focal adhesion proteins and actin filaments (Burridge and Fath, 1989; Horwitz *et al.*, 1986). Therefore, focal adhesions serve as a mechanical connection between cytoskeleton and the extracellular matrix. In addition to structural proteins, signaling enzymes are also concentrated at focal adhesions (Clark and Brugge, 1995; Guan, 1997). Therefore, focal adhesions are also signal transduction sites where chemical and mechanical signals are integrated and transduced into enzyme cascades. Many focal adhesion proteins have been identified, but how individual proteins are recruited to focal adhesions is not fully understood.

Alpha-actinin and talin are focal adhesion proteins that can cross-link integrins with actin filaments (Muguruma *et al.*, 1990; Otey *et al.*, 1990). Therefore, α-actinin and talin are major markers of focal adhesions, and recruitment of these proteins are important for the assembly of focal adhesions. However, there is no evidence for direct interaction between α-actinin and talin (Muguruma *et al.*, 1992). Alpha-actinin can also cross-link actin filaments (Winkler *et al.*, 1997, and is therefore important for stress fiber formation. Vinculin binds to both α-actinin and talin (Burridge and Mangeat, 1984). Therefore, vinculin can cross-link α-actinin and talin, thereby strengthening the mechanical integrity of focal adhesions. Vinculin is also an actin-binding protein. Therefore, vinculin can also anchor actin filaments to focal adhesions. Regulation of the assembly of focal adhesions is highly complex and not fully understood. The small GTP-binding protein, Rho is known to elevate phosphatidylinositol-4,5-bisphosphate ($PIP_2$) levels, and induce focal adhesion assembly (Burridge and Chrzanowska-Wodnicka, 1996). Gilmore and Burridge (1996) have proposed that the elevated $PIP_2$ could then dissociate the head-tail folding of vinculin as described by Johnson and Craig (1994, 1995), thereby allowing vinculin to cross-link actin filaments to talin at the focal adhesions. Consistent with this hypothesis, Hai *et al.* (2000) recently reported mechanical strain-dependent cytoskeletal recruitment of vinculin in airway smooth muscle cells.

Paxillin is an integrin- and vinculin-binding protein (Liu *et al.*, 1999; Tanaka *et al.*, 1996; Turner *et al.*, 1990). In addition, paxillin also binds to focal adhesion kinase (Tachibana *et al.*, 1995), a major integrin-associated signaling molecule (Schaller and Parsons, 1994). Therefore, paxillin and focal adhesion kinase are considered key proteins involved in integrin-associated signaling at the focal adhesions. Phosphorylation of focal adhesion proteins is recognized to be an important mechanism in the assembly of focal adhesions (Clark and Brugge, 1995; Guan, 1997). Rho has been shown to stimulate tyrosine phosphorylation of focal adhesion kinase and paxillin in cultured cells (Flinn and Ridley, 1996). Muscarinic receptor activation has been found to stimulate phosphorylation of talin and paxillin phosphorylation in airway smooth muscle (Pavalko *et al.*, 1995). However, paxillin phosphorylation was also observed during KCl-induced contractions (Wang *et al.*, 1996), suggesting that receptor activation was not necessary for paxillin phosphorylation. Furthermore, paxillin phosphorylation was also observed in the absence of force when airway smooth muscle was stimulated by acetylcholine in the absence of extracellular $Ca^{2+}$ (Mehta *et al.*, 1998). This finding suggests that contraction is also not necessary for paxillin phosphorylation. Mechanical strain has also be found to stimulate phosphorylation of focal adhesion kinase and paxillin in airway smooth muscle cells (Smith *et al.*, 1998; Tang *et al.*, 1999). Many other molecules are associated with focal adhesions, but cannot be discussed in this brief review.

In summary, airway smooth muscle cells function in a mechanically active environment. Recent findings indicate that chemical and mechanical signals are integrated to regulate crossbridge activation and cytoskeletal organization in airway smooth muscle cells. Understanding these highly complex mechanisms is likely to reveal new targets for pharmacological control of airway smooth muscle contraction in airway diseases.

## ACKNOWLEDGEMENTS

This work is supported by National Heart, Lung, and Blood Institute Grant HL-52714.

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