Length-dependent modulation of smooth muscle activation: effects of agonist, cytochalasin, and temperature

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Youn, Thomas, Son A. Kim, and Chi-Ming Hai. Length-dependent modulation of smooth muscle activation: effects of agonist, cytochalasin, and temperature. Am. J. Physiol. Cell Physiol. 43: C1601–C1607, 1998.—We tested the hypothesis that mechanical strain modulates agonist sensitivity of smooth muscle by measuring myosin phosphorylation and contractile force in bovine tracheal smooth muscle activated by various concentrations of the muscarinic receptor agonist carbachol and at various muscle lengths. Increasing carbachol concentration by 10,000-fold did not restore myosin phosphorylation levels at shorter muscle lengths to the level at optimal length (L_{0}). Maximum levels of myosin phosphorylation induced by carbachol at 0.6, 0.8, and 1.0 L_{0} were similar but became lower at <0.6 L_{0}. Cytochalasin D significantly attenuated carbachol-induced contraction by 54%. In addition, cytochalasin D treatment induced a parallel downward shift in the length-myosin phosphorylation relation. Lowering temperature from 37 to 23°C did not significantly change the length dependencies of carbachol-induced active force and myosin phosphorylation. These results have led us to conclude that 1) agonist sensitivity and maximum level of activation (as measured by myosin phosphorylation) are targets of length-dependent modulation, 2) actin filaments involved in contraction and length-dependent modulation are distinct in sensitivity to cytochalasin D, and 3) length-dependent modulation is relatively temperature insensitive.

MECHANICAL STATE (stress and/or strain) is an important modulator of many cellular processes, including signal transduction, growth, motility, and contraction (5, 8, 13). Chen et al. (4) proposed the general hypothesis that a cell may respond to the same chemical input (integrin binding) with different functional outputs (growth vs. apoptosis), depending on the mechanical deformation of the cell. Phosphorylation of the 20,000-Da myosin light chain is the central regulatory mechanism of smooth muscle contraction (12). Similar to the findings of Chen et al., Yoo et al. (31) found that airway smooth muscle responded to the same concentration (1 μM) of a muscarinic receptor agonist (carbachol) with different levels of myosin light chain phosphorylation depending on muscle length. Yoo et al. found that carbachol-induced myosin phosphorylation was highest at optimal length (L_{0}) for contraction and became attenuated at shorter lengths. Several investigators hypothesized that mechanical strain and stress modulated the sensitivity of smooth muscle cells to contractile agonists (21, 27).

In the simplest model the sensitivity hypothesis may be described in terms of Michaelis-Menten kinetics. In this model, V = V_{max} \times \[\text{agonist}] / (K_{m} + \[\text{agonist}])

where V is cell activation induced by a given agonist concentration ([agonist]), V_{max} is maximum cell activation, and K_{m} is the dissociation constant. Agonist sensitivity may be defined as 1/K_{m}. The sensitivity hypothesis proposes that V, as measured by myosin phosphorylation, is length dependent, because K_{m} is a function of cell length. This hypothesis predicts that, at very high [agonist], when V approaches V_{max}, cell activation (myosin phosphorylation) should become length independent. The first aim of this study was to test the sensitivity hypothesis by measuring myosin light chain phosphorylation and contractile force in bovine tracheal smooth muscle stretched to various muscle lengths ranging from 10% to 100% L_{0} and activated by various concentrations of carbachol ranging from 0.1 to 100 μM.

We previously found that phosphatidylinositol turnover, intracellular Ca^{2+} concentration ([Ca^{2+}]), and myosin phosphorylation in cholinergically activated smooth muscle were sensitive to changes in muscle length (31). A fundamental question is how effector molecules on the cell membrane such as phospholipase C and Ca^{2+} channels sense mechanical strain and/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 effector molecules could be biochemical in nature. For example, second messengers may be produced by the focal adhesion complex at the integrin receptors. Alternatively, the communication could be mechanical in nature. For example, actin filaments may physically connect integrin receptors to the interior of a cell to regulate effector molecules by binding or sterically hinder. Several investigators have hypothesized that actin filaments may be a putative transmitter of mechanical force to intracellular target proteins and nucleus (4, 8). 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. The second aim of this study was to investigate the effect of actin filament disruption on the length dependencies of myosin phosphorylation and contraction in airway smooth muscle. Temperature is an important determinant of membrane fluidity (3, 24) and a critical determinant of myogenic contraction in vascular smooth muscle (15, 25). Laher et al. (15) found that myogenic contraction elicited by mechanical stretch per se in vascular smooth muscle occurred only at >32°C. If myogenic contraction and length-dependent modulation of receptor-mediated smooth muscle activation...
represent different manifestations of fundamentally the same cellular mechanism, then length-dependent modulation should also be highly temperature sensitive. The third aim of this study was to investigate the effect of temperature on length-dependent modulation of carbachol-induced myosin phosphorylation and contraction in bovine tracheal smooth muscle.

**METHODS**

Tissue preparation. Bovine tracheae were collected from a slaughterhouse and transported to the laboratory in cold (4°C) physiological salt solution (PSS) containing (in mM) 140.1 NaCl, 4.7 KCl, 1.2 Na2HPO4, 2.0 MOPS (pH 7.4), 0.02 Na2EDTA, 1.2 MgSO4, 1.6 CaCl2, and 5.6 d-glucose. A segment of trachea consisting of multiple rings of cartilage was used for each experiment. The smooth muscle layer, together with adventitia, mucosa, and the attached cartilage, was removed by longitudinal cuts on the cartilage. The dissected piece was placed in a petri dish containing cold PSS. The mucosal and adventitial layers were then carefully removed using microdissecting scissors and fine forceps. Smooth muscle strips were prepared by making cuts along the circumferential direction. One end of the smooth muscle strip was attached by a stainless steel clamp connected to a force transducer (Grass FT.03). The other end of the smooth muscle strip was attached by a stainless steel clamp secured on a glass rod mounted on a length manipulator.

Muscle strips were first stretched to 12.5 g and then allowed to equilibrate in PSS bubbled with air at 37°C for 1 h. After the 1st h of equilibration, muscle strips were activated briefly (3 min) by K-PSS, a solution similar to PSS in composition, except 104.95 mM NaCl was substituted by KCl. Responsive muscle strips were then allowed to relax in PSS and equilibrate for another hour in PSS. During this 2nd h of equilibration, muscle strips were restretched to 12.5 g every 15 min. At the end of the 2nd h of equilibration, muscle strips were quickly released to a passive force of 2.5 g that was found to be associated with L0 for contraction. Muscle strips were then activated by K-PSS at L0 for 10 min, and the force (F0) developed in this contraction was used to normalize force development in subsequent contractions. After this contraction, muscle strips were allowed to relax in PSS for 1 h before further experimentation.

Adjustment of muscle length. After equilibration, muscle strips were released to different muscle lengths ranging from 0.1 to 1.0 L0 by adjusting the length manipulator (0.1-mm resolution). The extent of manipulator adjustment was calculated from the measured muscle length (in mm) at L0 and the assigned fraction of L0. For example, if a muscle strip has a length of 22 mm at L0, then to release this muscle strip from L0 to 0.6 L0 requires a manipulator adjustment of 0.4 × 22 mm, or 8.8 mm. After the release, muscle strips were activated by K-PSS for 30 min to induce shortening to the assigned lengths. The rationale was that when a muscle strip has shortened to the limit set by the length manipulator, the muscle strip contracts isometrically with force production. Muscle strips at different lengths were then allowed to relax in PSS for 1 h before activation by carbachol. Muscle strips were activated by carbachol until steady-state force was reached (30 min) and then quickly frozen with an acetone-dry ice slurry for the measurement of myosin light chain phosphorylation.

In cytochalasin D experiments, cytochalasin D (Sigma Chemical) was included in PSS during the last hour of relaxation and during the following 30 min of contraction. In low-temperature experiments the temperature of bathing solutions was changed to 23°C during the last hour of relaxation in PSS and during the following 30 min of carbachol-induced contraction.

Measurement of myosin light chain phosphorylation. Muscle strips were quickly frozen in a slurry of acetone and dry ice (−78°C) at 30 min after carbachol-induced contraction. The muscle strip and acetone were then slowly thawed to room temperature, resulting in the dehydration of the muscle strip. Acetone-dried tissues were homogenized in an aqueous solution containing 1% SDS, 10% glycerol, and 20 mM dithiothreitol on ice. The homogenate was then analyzed by two-dimensional PAGE, as described previously (10). Acetone-dry ice slurry has been found to be as effective as 10% TCA-90% acetone-dry ice slurry in preserving myosin phosphorylation in muscle samples (10). Tissue homogenate was first analyzed by isoelectric focusing (Pharmalyte 4-6.5, Pharmacia) in the presence of 8 M urea to separate phosphorylated and unphosphorylated myosin light chains from each other. Sodium thioglycolate (5 mM) was included in the cathodal solution to minimize protein oxidation. After isoelectric focusing, the tube gel was transferred to a slab gel for SDS-PAGE to separate myosin light chains from other proteins by molecular weight. At the end of electrophoresis the slab gel was stained by Coomassie blue and scanned in a densitometer equipped with an integrator (Helena). Unphosphorylated and phosphorylated myosin light chains appeared as two spots of different isoelectric pH but similar molecular weight. Myosin light chain phosphorylation in moles of phosphate per mole of light chain (mol Pi/mol LC) was calculated from the ratio of the amount of phosphorylated myosin light chain to the total amount of myosin light chains (sum of unphosphorylated and phosphorylated myosin light chains).

Statistics. Values are means ± SE; n represents the number of tracheal rings. Student's t-test was used for the comparison of two means; P < 0.05 was considered significant. Two-way ANOVA was used to compare two groups of length-matched data; P < 0.05 was considered significant. Correlation between two variables such as myosin phosphorylation and muscle length was analyzed by Pearson's correlation analysis; P < 0.05 was considered significant.

**RESULTS**

Length dependencies of active force and myosin phosphorylation at different carbachol concentrations. Figure 1A shows the active force and myosin phosphorylation induced by 0.1 µM carbachol at muscle lengths ranging from 0.1 to 1.0 L0. Mean active force was zero at 0.1 L0 and increased to 1.0 ± 0.07 F0 at L0, where F0 represents the active force induced by K+ depolarization at L0 at the beginning of each experiment (Fig. 1A). Correlation analysis of the data indicated significant correlation between active force and muscle length (P < 0.05). Myosin phosphorylation was 0.19 ± 0.02 mol P/mmol LC at 0.1 L0 and increased to 0.32 ± 0.03 mol P/mmol LC at L0 (Fig. 1A). Correlation analysis of the data indicated significant correlation between myosin phosphorylation and muscle length. The sensitivity hypothesis proposes that the lower level of myosin phosphorylation at shorter muscle lengths is due to a decreased agonist sensitivity. This hypothesis predicts that increasing the concentration of carbachol should restore myosin phosphorylation at shorter muscle lengths to the level at L0. This prediction was tested in the following experiments.
Figure 1, B–D, shows the active force and myosin phosphorylation induced by 1, 10, and 100 µM carbachol in bovine tracheal smooth muscle. Active force is expressed as fraction of force (F0) induced by K+ depolarization at optimal length (L0) for each muscle strip. Values are means ± SE (n = 4). Vertical bars are not shown when SE are smaller than symbol. Lines represent linear regression fits to data. LC, light chain.

Figure 1, B–D, shows the active force and myosin phosphorylation induced by 1, 10, and 100 µM carbachol at muscle lengths ranging from 0.1 to 1.0 L0. Correlation analysis of the data indicated a significant correlation between force and muscle length (P < 0.05) and a significant correlation between myosin phosphorylation and muscle length (P < 0.05) at all these concentrations of carbachol. These results indicated that active force and myosin phosphorylation remained significantly length dependent at 1, 10, and 100 µM carbachol.

Concentration dependencies of myosin phosphorylation and active force at different muscle lengths. To compare the concentration dependence of myosin phosphorylation at different muscle lengths, the phosphorylation data measured at the same muscle length were plotted against carbachol concentration. As shown in Fig. 2, myosin phosphorylation increased with 0.1–1 µM carbachol at all muscle lengths, but a further increase in carbachol concentration to >1 µM actually lowered myosin phosphorylation at almost all muscle lengths except 0.4 L0. Therefore, the maximum value of myosin phosphorylation was found at 1 µM carbachol for 0.1, 0.2, 0.6, 0.8, and 1.0 L0, whereas the maximum value of myosin phosphorylation was found at 10 µM carbachol at 0.4 L0. When the maximum value of myosin phosphorylation was plotted against the muscle length at which it was measured (Fig. 3), there was a significant correlation between maximum myosin phosphorylation and muscle length (P < 0.05). Maximum myosin phosphorylation appeared to be relatively length independent at muscle lengths near L0 and then progressively became length dependent at muscle lengths <0.5–0.6 L0.

Effect of cytochalasin D on the length dependencies of carbachol-induced active force and myosin phosphorylation. As shown in Fig. 4A, 1 µM cytochalasin D significantly inhibited active force induced by 1 µM carbachol at 0.4, 0.6, 0.8, and 1.0 L0 (P < 0.05). Correlation analysis of the cytochalasin D data indicated a significant correlation between force and muscle length (P < 0.05). Linear regression analysis of the cytochalasin D data yielded a slope of 0.56 ± 0.07 F0/L0, which was lower than the slope for control (1.22 ± 0.05 F0/L0) by 54.1%, indicating that 1 µM cytochalasin D inhibited active force by an average of 54.1%.

Fig. 1. Length dependencies of active force (●) and myosin phosphorylation (○) induced by 0.1 µM (A), 1 µM (B), 10 µM (C), and 100 µM (D) carbachol in bovine tracheal smooth muscle. Active force is expressed as fraction of force (F0) induced by K+ depolarization at optimal length (L0) for each muscle strip. Values are means ± SE (n = 4). Vertical bars are not shown when SE are smaller than symbol. Lines represent linear regression fits to data. LC, light chain.

Fig. 2. Concentration dependencies of myosin phosphorylation in bovine tracheal smooth muscle activated by carbachol at different muscle lengths ranging from 0.1 to 1.0 L0. Values are taken from Fig. 1 by sorting phosphorylation data by muscle length. Values are means ± SE (n = 4–7). Vertical bars are not shown when SE are smaller than symbol.
Figure 4B shows the length-myosin phosphorylation relations in control and cytochalasin D-treated smooth muscle strips activated by 1 µM carbachol. Correlation analysis of the cytochalasin D data indicated significant correlation between myosin phosphorylation and muscle length (P < 0.05). Therefore, carbachol-induced myosin phosphorylation remained length dependent in cytochalasin D-treated tissues. Cytochalasin D appeared to shift the length-myosin phosphorylation relation downward toward lower myosin phosphorylation at all muscle lengths. Two-way ANOVA of the data indicated that the control and cytochalasin D data were significantly different (P < 0.05). The slopes for cytochalasin D and control were similar (0.20 ± 0.07 and 0.22 ± 0.02 mol Pi·mol LC⁻¹·L₀⁻¹, respectively), whereas the y-intercept was lower for the cytochalasin D than for the control group by 38% (0.13 and 0.21 mol Pi/mol LC, respectively).

Effect of lowering temperature on length dependencies of carbachol-induced active force and myosin phosphorylation. As shown in Fig. 5A, lowering temperature from 37 to 23°C had a small effect on carbachol-induced active force. Correlation analysis of the data indicated a significant correlation between active force and muscle length (P < 0.05) and a significant correlation between myosin phosphorylation and muscle length at 23°C (P < 0.05). Two-way ANOVA of the data indicated that the 37 and 23°C data were not significantly different. As shown in Fig. 5B, lowering temperature from 37 to 23°C had a small effect on the length dependence of carbachol-induced myosin phosphorylation. Two-way analysis of the data indicated that the 37 and 23°C data were not significantly different. These results indicated that lowering temperature from 37 to 23°C did not significantly alter the length dependencies of carbachol-induced active force and myosin phosphorylation in airway smooth muscle.

DISCUSSION

Length-dependent modulation of receptor-mediated myosin phosphorylation has been observed in airway, arterial, and gastrointestinal smooth muscles (1, 9, 11, 16, 23, 28, 31), suggesting that this phenomenon may be a basic property of all smooth muscle types. Several investigators have hypothesized that mechanical strain modulates smooth muscle reactivity by modulating agonist sensitivity of smooth muscle cells (21, 27). The possibility that muscle length may modulate a maxi-
The maximum level of smooth muscle activation has not been seriously questioned. The first aim of this study was to test the hypothesis that agonist sensitivity is the primary target of length-dependent modulation. One difficulty in testing this hypothesis is that measurement of contractile force alone cannot distinguish the effects of actomyosin filament overlap and smooth muscle activation. An independent indicator of smooth muscle activation must be measured. In this study we chose myosin light chain phosphorylation as a measure of smooth muscle activation, because 

\[ \text{myosin phosphorylation} \]

is the central regulatory mechanism of smooth muscle activation (6, 12). In theory, myoplasmic 

\[ \text{[Ca}^{2+}] \]

and myosin phosphorylation could change independently if 

\[ \text{Ca}^{2+} \]

sensitivity of myosin phosphorylation is modulated by muscle length. However, Moreland and Murphy (18) found that 

\[ \text{[Ca}^{2+}] \]

sensitivity of myosin phosphorylation was not significantly length dependent in skinned swine carotid media. In agreement with the finding of Moreland and Murphy, we previously found that myoplasmic 

\[ \text{[Ca}^{2+}] \]

and myosin phosphorylation changed in parallel as a function of muscle length (31). Furthermore, Zou et al. (32) found that myosin phosphorylation was the primary mechanism by which 

\[ \text{Ca}^{2+} \]

regulated myogenic contractions of arterioles. We previously found that myosin phosphorylation induced by 1 µM carbachol was length dependent, such that suprabasal myosin phosphorylation was highest at 

\[ L_o \]

and became statistically insignificant at 0.1 

\[ L_o \]

(31). According to the “sensitivity” hypothesis, the lower levels of myosin phosphorylation at shorter muscle lengths were due to lower sensitivity to receptor agonists. This hypothesis predicts that the lower sensitivity at shorter muscle lengths can be overcome by increasing the concentration of carbachol. Accordingly, we tested this hypothesis by choosing concentrations of carbachol near the maximum end of the concentration-response curve of carbachol-induced contractions (29). As shown in Fig. 1, carbachol-induced myosin phosphorylation remained significantly correlated with muscle length at 0.1, 1, 10, and 100 µM carbachol. Therefore, contrary to this prediction, we found that increasing carbachol concentration by up to 10,000-fold did not increase myosin phosphorylation levels at shorter muscle lengths to the level at 

\[ L_o \] (Fig. 2), suggesting that length-dependent modulation of myosin phosphorylation could not be explained by length-dependent modulation of agonist sensitivity alone. When the maximum levels of myosin phosphorylation induced by carbachol at each muscle length were plotted against muscle length (Fig. 3), we found that maximum myosin phosphorylation was relatively length independent near 

\[ L_o \]

and then progressively became length dependent at muscle lengths <0.5–0.6 

\[ L_o \]. These results were consistent with the findings of Mehta et al. (16) that 

\[ \text{ACh-induced myosin phosphorylation in canine tracheal smooth muscle at 0.5, 0.7, and 1.0} \]

\[ L_o \]

could be explained by length-dependent shifts of 

\[ \text{ACh-concentration-phosphorylation relations without significant changes in the maximum level of myosin phosphorylation. These} \]

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 <0.5–0.6 

\[ L_o \]. A potential implication of these results is that muscle length may determine the number of functional muscarinic receptors on the cell membrane of airway smooth muscle cells at muscle lengths <0.5–0.6 

\[ L_o \]. The second aim of this study was to investigate the role of actin filaments in length-dependent modulation of smooth muscle activation. Several investigators hypothesized that actin filaments may serve as a mechanical transmitter of externally applied mechanical force to intracellular proteins and nuclei, thereby modulating cell function (4, 8). Cytochalasin D disrupts the supramolecular structure of actin cytoskeleton (7) and has been found to alter smooth muscle cell morphology and attenuate muscarinic receptor-mediated intracellular 

\[ \text{[Ca}^{2+}] \], myosin phosphorylation, and contractile force in airway smooth muscle at 

\[ L_2 \] (26), perhaps by constraining signal transduction proteins within microdomains (19, 22) or allosteric modulation (14, 30). If actin filaments serve as the transmitter of the mechanical signal to cellular processes in smooth muscle, then actin filament disruption by cytochalasin D should alter the length dependence of smooth muscle activation. As shown in Fig. 4A, cytochalasin D significantly attenuated carbachol-induced active force with an average of 54% loss of force, suggesting that cytochalasin D disrupted actin filaments involved in contraction. As shown in Fig. 4B, 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 similar (0.22 ± 0.08 and 0.20 ± 0.07 mol 

\[ \text{P_i} \] mol 

\[ \text{LC}^{-1} \text{L}^{-1} \]), respectively. The finding that disruption of actin filaments by cytochalasin D did not inhibit length-dependent modulation when it inhibited contraction by 54% was unexpected. These results suggest that actin filaments involved in contraction and length-dependent modulation may be differentiated by their different sensitivities to cytochalasin D. A potential implication of these results is that actin filaments bound by different actin-binding proteins (7) may be differentially involved in contraction and length-dependent modulation. The third aim of this study was to investigate the temperature sensitivity of length-dependent modulation of smooth muscle activation. Temperature is a prerequisite for myogenic response of smooth muscle (15). In this study we used temperature as a tool to compare myogenic response with length-dependent modulation. If the two phenomena are regulated by the same mechanism, then both should be highly temperature sensitive. Contrary to this prediction, lowering temperature from 37 to 23°C did not significantly change the length dependence of myosin phosphorylation (Fig. 5). Mitsui et al. (17) reported that lowering
temperature from 35 to 25°C increased steady-state myosin phosphorylation from 0.52 to 0.70 mol P/mol LC in skinned smooth muscle when [Ca\(^{2+}\)] was 10\(^{-5}\) M. However, Pawlowski and Morgan (20) did not observe any significant change in steady-state myosin phosphorylation in intact smooth muscle between 21 and 37°C. Myosin phosphorylation actually decreased significantly when temperature was lowered further to 0°C. Our results are consistent with those reported by Pawlowski and Morgan that lowering temperature did not significantly change steady-state myosin phosphorylation in intact smooth muscle. Bethel et al. (2) also found that cooling did not appear to alter the reactivity of canine tracheal smooth muscle, but statistics were not performed and myosin phosphorylation was not measured in their study. Our interpretation of these results is that skinned and intact smooth muscle preparations may be significantly different in their responses to lowering temperature. The relative temperature independence of steady-state myosin phosphorylation in intact smooth muscle suggests that myosin light chain kinase and phosphatase activities decrease proportionally in an intact smooth muscle cell when temperature is lowered from 37 to 21°C. The purpose of changing temperature in this study was to compare myogenic response and length-dependent modulation. Mechanical strain is the stimulus in myogenic contraction and the modulator in length-dependent modulation. The very different temperature sensitivities of myogenic contraction and length-dependent modulation suggest that the two apparently similar phenomena may be fundamentally different in mechanisms and/or rate-limiting steps.

In summary, results from this study have led us to conclude that 1) length-dependent modulation may be differentiated into the modulation of sensitivity and maximum response depending on the range of muscle length, 2) actin filaments involved in length-dependent modulation and contraction may be differentiated by their different sensitivities to cytochalasin D, and 3) myogenic response and length-dependent modulation may be differentiated by their different temperature sensitivities.

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REFERENCES


