Regulatable stiffness in relaxed airway smooth muscle: a target for asthma treatment?

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Raqeeb A, Jiao Y, Syyong HT, Paré PD, Seow CY. Regulatable stiffness in relaxed airway smooth muscle: a target for asthma treatment? J Appl Physiol 112: 337–346, 2012. First published October 13, 2011; doi:10.1152/japplphysiol.01036.2011.—The airway smooth muscle (ASM) layer within the airway wall modulates airway diameter and distensibility. Even in the relaxed state, the ASM layer possesses finite stiffness and limits the extent of airway distension by the radial force generated by parenchymal tethers and transmural pressure. Airway stiffness has often been attributed to passive elements, such as the extracellular matrix in the lamina reticularis, adventitia, and the smooth muscle layer that cannot be rapidly modulated by drug intervention such as ASM relaxation by β-agonists. In this study, we describe a calcium-sensitive component of ASM stiffness mediated through the Rho-kinase signaling pathway. The stiffness of ovine tracheal smooth muscle was assessed in the relaxed state under the following conditions: 1) in physiological saline solution (Krebs solution) with normal calcium concentration; 2) in calcium-free Krebs with 2 mM EGTA; 3) in Krebs with calcium entry blocker (SKF-96365); 4) in Krebs with myosin light chain kinase inhibitor (ML-7); and 5) in Krebs with Rho-kinase inhibitor (Y-27632). It was found that a substantial portion of the passive stiffness could be abolished when intracellular calcium was removed; this calcium-sensitive stiffness appeared to stem from intracellular source and was not sensitive to ML-7 inhibition of myosin light chain phosphorylation, but was sensitive to Y-27632 inhibition of Rho kinase. The results suggest that airway stiffness can be readily modulated by targeting the calcium-sensitive component of the passive stiffness within the muscle layer.

Numerous studies have demonstrated that, in healthy individuals, deep inspirations (DI) taken before experimentally induced bronchoconstriction (e.g., exposure to methacholine) protect the airways from excessive narrowing (2–4, 21). This protective effect of DI is not seen in asthmatic subjects (21). Deep inspirations before bronchoconstriction have also been shown to dilate the airways in healthy subjects (11, 19–20), but again, the beneficial effect is attenuated in asthmatic subjects (4). A recent study (16) has linked the loss of bronchodilatory effect of DI in asthma to a lack of distensibility of asthmatic airways. Understanding the mechanisms underlying airway distensibility (or stiffness) is, therefore, important in elucidating the pathophysiology of asthma.

A common approach to delineate airway distensibility is to dissect the airway stiffness into two components: active and passive. The active component is thought to associate with airway smooth muscle (ASM) activation, and it is well established that active force generation in ASM stiffens the airways (6, 18). The passive component is considered by some to associate mainly with the material properties of the nonmuscle compartments of the airway wall, such as the adventitial connective tissue and the lamina propria, and these properties can be changed through slow processes linked to chronic airway inflammation, such as airway fibrotic remodeling (or its reversal) (14). Recent studies from our laboratory have revealed a third component that contributes to airway stiffness; it originates from the airway muscle layer and yet is not related to activation of the muscle (1, 27). In those studies, we observed that, when the length of an ASM preparation was reduced to one-half from its in situ length in the relaxed state, the muscle became slack, and the passive stiffness mostly disappeared. When the muscle was stimulated periodically at the reduced length with brief (~10 s) electrical stimulations once every 5 min for ~30 min, passive stiffness returned, in many cases, to its original value (1, 27). This fast return of passive stiffness obviously is not due to airway remodeling or any fibrotic processes and represents a new component of stiffness that contributes to the overall distensibility of a relaxed airway. In the present study, the stiffness of ASM in the relaxed state is systematically examined, and the intracellular signaling pathways perturbed by pharmacological inhibitors to gain insight into the regulation of the passive stiffness.

MATERIALS AND METHODS

Muscle preparation and physiological saline solution. Sheep tracheas were obtained from a local abattoir. All experimental procedures were approved by the Animal Care Committee and Biosafety Committee of the University of British Columbia and conformed to the guidelines set out by the Canadian Council on Animal Care. Tracheas were removed soon after death of the sheep and put in Krebs solution (pH 7.4; 118 mM NaCl, 4 mM KCl, 1.2 mM NaH2PO4, 22.5 mM NaHCO3, 2 mM MgSO4, 2 mM MgSO4, and 2 g/l dextrose). In the laboratory, tracheas were cleaned and kept in Krebs solution at 4°C. The tracheas were good for experiments for the next 4 days, with no detectable deterioration in isometric force. Muscle strips for experiments were prepared from a tracheal ring segment of ~2 cm in length, taken from the middle portion of the trachea. Before dissection, the tracheal rings were examined under the dissecting scope for signs of muscle contracture. A wrinkled epithelial layer above the muscle layer was an indication that the ASM was in a contracted state. Only the tracheas with relaxed ASM were used for the experiments. The in situ length of the relaxed tracheal smooth muscle bundle connecting the C-shaped cartilage ring was measured. The tracheal rings were then cut open, and the adventitial connective tissue and the epithelial layer were removed from the muscle layer. Muscle strips ~5 mm long and 1.5–2 mm wide (with thickness of ~0.2–0.3 mm) were dissected, and aluminum foil clips were affixed on both ends for attachment to the force/length transducer.
Equilibration of muscle strips at reference length. The muscle strip was mounted vertically on a force-length transducer between two hooks. The bottom hook was fixed, and the upper hook was connected to the lever arm of a force-length transducer through surgical thread (size 6). The muscle strip was then immersed in a muscle bath containing Krebs solution aerated with a gas mixture of 95% O₂ and 5% CO₂ at 37°C. The 37°C bath temperature was maintained by circulating warm water through the jacket around the muscle bath. The muscle strip was stretched to its previously determined in situ length. This in situ length was used as a reference length \((L_{\text{ref}})\) for normalization of all length measurements. Muscle strips were equilibrated before starting the experiments to allow the muscle to recover from mechanical and metabolic perturbations caused by dissection, lack of perfusion, and low storage temperature. During the equilibration period, the muscle strip was activated every 5 min with a 9-s electric field stimulation (EFS; 60 Hz, 12 V, and sufficient current to produce maximal muscle response). Krebs solution in the muscle bath was replaced every 5 min with prewarmed and aerated Krebs at 37°C. Equilibration was considered complete when stimulations produced a lack of perfusion, and low storage temperature. During the equilibration period, the muscle strip was activated every 5 min with a 9-s electric field stimulation (EFS; 60 Hz, 12 V, and sufficient current to produce maximal muscle response). Krebs solution in the muscle bath was replaced every 5 min with prewarmed and aerated Krebs at 37°C. Equilibration was considered complete when stimulations produced a stable maximal isometric force \((F_{\text{max}})\) with low resting tension. The process took \(\sim 1.5 \text{ h}\).

Measurement of passive stiffness. The experiment timeline and procedure are illustrated in Fig. 1. Stiffness of the muscle was measured by applying a quick-step stretch. The speed for the stretch was \(8\% \ L_{\text{ref}}/0.01 \text{ s}\), and the stretch of \(8\% \ L_{\text{ref}}\) was maintained for 10 s before it was returned to \(L_{\text{ref}}\). The sampling rate was 1,000 Hz. The stretch amplitude and speed were chosen because the combination gave a good measurement of stiffness without affecting active force and stiffness in repeated measurements at 5-min intervals. The stretch was applied 70 s after the preceding 9-s EFS, when the isometric force had settled to the resting level. The stiffness measured was, therefore, called passive stiffness. The net force \((\text{i.e., peak force during the stretch minus the resting force})\) was divided by the step length change to obtain the passive stiffness (Fig. 1C).

Two additional parameters were measured from the force response trace during the step stretch: stress relaxation (SR) and half relaxation time \((t_{1/2})\). SR was defined as the difference between the peak force during the stretch and the plateau force at the end of the stretch. The \(t_{1/2}\) of the SR was defined as the time it took for the peak force to decline to one-half of the SR (see illustration in Fig. 1C). In materials exhibiting SR, the magnitude of the relaxation (SR) reflects the nature of interaction force among the elements within the material. In a muscle tissue, the force interaction could be in the form of ionic bonds cross-linking protein filaments or aggregates, and the rate at which the bonds are broken by shear stress is reflected in the \(t_{1/2}\).

Measurements of \(F_{\text{max}}\) and passive stiffness at different muscle lengths. The passive stiffness of muscle strips was measured at 0.5, 1.0, and 1.5 \(L_{\text{ref}}\). First, a muscle strip was equilibrated at \(L_{\text{ref}}\), and the passive stiffness was measured at this length. Then the length of the muscle was changed to either 0.5 or 1.5 \(L_{\text{ref}}\). A 25-min period was allowed for the muscle to adapt to the new length. During this time, the muscle was stimulated with 9-s EFS to obtain isometric force every 5 min; passive stiffness was measured during the relaxation phase of the 5-min interval. The muscle strip was then returned to \(L_{\text{ref}}\) and length adapted for 25 min. This procedure was repeated for the measurement at a different length (0.5 or 1.5 \(L_{\text{ref}}\)).

To measure passive stiffness under \(Ca^{2+}\)-free condition, the muscle was incubated in \(Ca^{2+}\)-free Krebs solution containing EGTA (2 mM). \(FeS\)-induced active force was abolished after 15 min in \(Ca^{2+}\)-free Krebs. Passive stiffness was measured in the same way as it was in normal Krebs.

Measurement of redevelopment of active force and passive stiffness after strain-induced softening at \(L_{\text{ref}}\). Length oscillation was applied to the muscle preparation in the relaxed state at \(L_{\text{ref}}\) to induce softening of the tissue so that the redevelopment of passive stiffness could be examined. The strain-induced softening (or strain softening) protocol consisted of sinusoidal length stretches of 40% \(L_{\text{ref}}\) (peak-to-peak amplitude) at a frequency of 0.25 Hz for 1 min. The strain compliance of the lever system accounted for \(~35\%\) of the length change; the actual strain applied to the muscle was, therefore, \(~35\%\).

The oscillation protocol was not designed to mimic the respiration pattern, but rather to induce measurable strain softening without

![Fig. 1. Schematic illustration of the protocols used for measuring passive stiffness of airway smooth muscle (ASM) under different conditions. A: time line of experiments. After the muscle had been equilibrated and adapted at reference length \((L_{\text{ref}})\), the reference electric field stimulation (EFS)-induced force and passive stiffness were measured (at the time labeled “Reference”) and used later for data normalization. For control observations, length oscillation was applied to soften the muscle; the force and passive stiffness were measured in each of the six 5-min cycles (30-min period). For the treatment group, \(Ca^{2+}\)-free Krebs or blocker/inhibitor were used to perturb the signaling pathways in muscle activation; the break in the time line indicates a variable time period allowed for the intervention to take effect. The dots along the time line indicate EFS. B: events within the 5-min intervals. EFS was applied to the muscle at the beginning of the interval to obtain the active force (top trace), and the passive stiffness was measured with a step stretch (bottom trace) after the force had returned to baseline. C: details of the force response (top trace) to a length step (bottom trace). \(\Delta L\), amplitude of length step; \(\Delta F\), peak response of force; SR, stress relaxation; \(t_{1/2}\), half relaxation time. The passive stiffness is defined as \(\Delta F/\Delta L\).](http://jap.physiology.org/doi/10.1152/japplphysiol.01036.2011)
causing permanent damage to the muscle. Immediately after the length oscillation, passive stiffness was measured (first stiffness measurement after strain softening). The muscle was then stimulated (9-s EFS) to obtain active force (first force measurement after strain softening). The second stiffness measurement was done 1 min after the first EFS when the muscle was completely relaxed. This was then followed by a period of repeated EFS at 5-min intervals for 30 min, during which both active force and passive stiffness were measured every 5 min.

Reference values were first established for active force and passive stiffness. These were obtained immediately after the muscle had been equilibrated and length-adapted at $L_{\text{ref}}$. The muscle was then subject to the 1-min strain-softening protocol, followed by 30 min of recovery. After recovery, for one set of experiments, the muscle was again strain softened and allowed to recover. The purpose of this experiment was to test the repeatability of the measurements. The reference values were used later for normalization of measurements of force and stiffness done with the same muscle under different test conditions. The test conditions included incubation of muscle in the following: i) Ca$^{2+}$-free Krebs; ii) 50 μM SKF-96365, a nonspecific Ca$^{2+}$ entry blocker; iii) 50 μM ML-7, a myosin light chain kinase (MLCK) inhibitor; and iv) 10 μM Y-27632, a Rho-kinase inhibitor. The test condition is indicated in Fig. 1A under the “Treatment” period. The incubation time varied for each condition and was indicated in the RESULTS section. All reference values were obtained before the muscle was subject to any intervention that includes length oscillation, exposure to Ca$^{2+}$-free Krebs, or incubation with blockers/inhibitors.

Statistical analysis. Data with error bars represent means ± SE. Two-way ANOVA was used for most of the statistical analyses. Some comparisons were done by paired T-tests. All statistical analyses were performed using Prism 4 (GraphPad Software, San Diego CA), and a $P < 0.05$ was considered to be sufficient to reject the null hypothesis.

RESULTS

Calcium-sensitive passive stiffness at different muscle lengths. Figure 2 shows the EFS-induced force (A) and passive stiffness (B) as functions of muscle length in the presence and absence of calcium. As expected, incubation of the muscle in Ca$^{2+}$-free Krebs solution abolished the EFS-induced force. In the absence of calcium, substantial decrease in passive stiffness was observed at all lengths (Fig. 2B). Note that the decrease in stiffness was not due to strain softening in this experiment: it occurred on its own after replacement of normal Krebs with Ca$^{2+}$-free Krebs (Fig. 2C). The averaged values from the measurements at $L_{\text{ref}}$ were used as the reference values to which other measurements at different lengths were normalized. Data plotted in Fig. 2 came from two groups of measurements. One started at $L_{\text{ref}}$, changed to 0.5 $L_{\text{ref}}$, and back to $L_{\text{ref}}$; $n = 7$ for this group. The reference force and stiffness for this group were 182.6 ± 11.8 kPa and 68.9 ± 7.0 kPa/mm, respectively. The other started at $L_{\text{ref}}$, changed to 1.5 $L_{\text{ref}}$, and back to $L_{\text{ref}}$; $n = 5$ for this group. The reference force and stiffness for this group were 209.2 ± 18.0 kPa and 54.8 ± 2.4 kPa/mm, respectively.

Redevelopment of active force and passive stiffness following strain softening at $L_{\text{ref}}$. In this group of experiments, strain softening was used to transiently reduce muscle force and stiffness, and $L_{\text{ref}}$ was the only length at which changes in force and stiffness were examined in this group. The passive stiffness measured at the end of the protocol (30 min) was designated as the redeveloped stiffness, to differentiate from the stiffness maintained by ASM not subject to strain softening. Strain softening caused both the EFS-induced force and passive stiffness to decrease substantially. To assess whether the stiffness decrease was reproducible, we conducted the following control experiment. We applied the strain-softening protocol to the same muscle preparation twice during a 60-min period. Active force and passive stiffness of the muscle recovered completely in 30 min after the first length oscillation (solid symbols in Fig. 3). The muscle was then subject to another length oscillation that reduced the force and stiffness to about the same extent as the first oscillation; the force and stiffness both recovered completely again in 30 min (open symbols in Fig. 3). The reference force and stiffness values for this group of experiments were 153.7 ± 9.3 kPa and 87.4 ± 6.3 kPa/mm, respectively ($n = 7$). The results indicated that the stiffness decrease was completely reversible.
Ca\textsuperscript{2+} and force oscillation). The dotted lines indicate the force and stiffness reference values measured before the muscle was strain-softened for the first time. Values are means ± SE.

The data points shown in Fig. 4 at time 0 represent the first measurements immediately after length oscillation under different conditions. For Fig. 4, A and B, replacement of normal Krebs solution with Ca\textsuperscript{2+}-free Krebs took place 15 min before length oscillation was applied to the muscle (see MATERIALS AND METHODS for the protocol). After 10 min in Ca\textsuperscript{2+}-free Krebs, the active force was completely abolished (data not shown), and there was no redevelopment of active force post oscillation (Fig. 4A); the redeveloped stiffness (at the end of the 30-min recovery period) was reduced by ~34% compared with the control obtained at the same time point (Fig. 4B). This stiffness is not different from the stiffness maintained under the same (Ca\textsuperscript{2+}-free) condition shown in Fig. 2C after 10 min (P > 0.05), indicating that, with or without strain-induced softening, at equilibrium, the passive stiffness under Ca\textsuperscript{2+}-free condition is the same. The reference force and stiffness values for this group of experiments were 173.2 ± 12.3 kPa and 60.2 ± 3.5 kPa/mm, respectively (n = 7).

For Fig. 4, C and D, 50 µM SKF-96365 was added to the normal Krebs (to block Ca\textsuperscript{2+} entry into the muscle cells) 30–60 min (the time it took to abolish the active force) before the length oscillation; there was no recovery of force after oscillation (Fig. 4C). The redeveloped stiffness was reduced in the presence of SKF (Fig. 4D) to the same extent as that in the Ca\textsuperscript{2+}-free Krebs (Fig. 4B) (P = 0.9925, two-way ANOVA). The reference force and stiffness values for this group of experiments were 180.3 ± 11.1 kPa and 80.5 ± 8.5 kPa/mm, respectively (n = 6).

Inhibition of MLCK by ML-7 (50 µM, 90-min incubation) completely eliminated active force (Fig. 5A), but the redevelopment of passive stiffness was unaffected (Fig. 5B) (P = 0.8061, two-way ANOVA). Note that these experiments were done with normal Krebs. Even though the active force was abolished, we continued to stimulate the muscle with EFS at 5-min intervals to make sure that the signaling pathways were activated during the recovery period. The reference force and stiffness values for this group of experiments were 179.9 ± 12.4 kPa and 92.4 ± 8.5 kPa/mm, respectively (n = 4).

Inhibition of Rho-kinase by Y-27632 (10 µM, 25 min incubation) reduced the redeveloped active force by ~48%, and the redeveloped passive stiffness by ~18% (Fig. 6). Compared with the results obtained under Ca\textsuperscript{2+}-free condition (Fig. 4), where there was a 100% reduction in active force and ~34% reduction in passive stiffness, inhibition of Rho kinase with 10 µM Y-27632 reduced both the active force and the calcium-sensitive portion of the passive stiffness by approximately one-half. The reference force and stiffness values for this group of experiments were 233.3 ± 15.7 kPa and 78.9 ± 9.4 kPa/mm, respectively (n = 4).

**SR and t\textsubscript{1/2} after a quick step increase in length.** Figure 7 shows SR and the t\textsubscript{1/2} (after the quick step stretch) measured at the end of recovery (30 min) after strain softening for all control and test conditions shown in Figs. 4–6. The respective force and stiffness values associated with the measurements (of SR and t\textsubscript{1/2}) are shown as data pairs at the 30-min time point in Figs. 4–6. For quantification of the magnitudes of SR (SR was defined in Fig. 1 as the peak force obtained during stretch minus the horizontal baseline force at the end of the stretch, between 9–10 s of the step stretch), values obtained under test conditions were normalized by the corresponding values obtained under control conditions and were expressed as fractions of the control values to reduce interpreparation difference, because the test and control observations were always made in the same muscle preparation. An example is illustrated in Fig. 7A. The solid and shaded traces were obtained by trace averaging (to reduce noise in individual traces) from six traces recorded under each condition (i.e., normal and Ca\textsuperscript{2+}-free Krebs). The raw data (the trace records) came from stiffness measurements made after recovery from strain softening under normal and Ca\textsuperscript{2+}-free conditions. (The stiffness values are plotted in Fig. 4B at the 30-min time point for both conditions). The t\textsubscript{1/2} was measured as illustrated in Fig. 1. To visualize difference in t\textsubscript{1/2}, the solid and shaded traces were first normalized to their own maximal SR and then superimposed at both the beginning and end of the trace records (Fig. 7B). Note that, in Fig. 7, A and B, only 0.3 s out of 10 s of the records are shown; also note that there is little difference in force at 0.3 and 10 s after the stretch, i.e., the force declined to a semiplateau soon after the stretch. Figure 7C summarizes the results obtained under all test conditions. Note that each test condition is matched with its own control condition, because different muscle preparations were used for each paired measurements. One obvious observation is that the magnitude of SR is correlated to the t\textsubscript{1/2} of relaxation, and they, in turn, are
correlated to the calcium-sensitive portion of the passive stiffness (Fig. 8).

The time course of SR can be described by a power-law function. As shown in Fig. 9, the reference SR shown in Fig. 7A (solid trace) could be fit with a power-law function of the form:

\[ S(t) = S_0 - \alpha t^\beta, \]

where \( S(t) \) is the SR as a function of time, \( S_0 \) is the peak stress response at time 0, and \( \alpha \) and \( \beta \) are constants. Although the goodness of fit (\( r^2 = 0.9341 \)) may be acceptable statistically, the best fit is still not accurate enough to be used to calculate mathematically the rate of stress decline over time. We, therefore, decided not to apply the power-law analysis to the SR and instead focused on direct measurement of the amplitude of SR and the \( t_{1/2} \) from the force records, as described above for Fig. 7, B and C.

**DISCUSSION**

In this study, we have identified a component of ASM stiffness that is independent of active actomyosin cross-bridge interaction, and that its origin appears to be intracellular. This stiffness component is not sensitive to ML-7 inhibition of MLC phosphorylation, but is subject to regulation by the signaling pathways involved in normal muscle activation.

The role of airway stiffness in lung function. Airways are embedded in, and mechanically tethered to, lung parenchyma, and, as the lung volume fluctuates due to tidal breathing and occasional DI, airway diameter varies accordingly. It has been known for a long time that the airways of asthmatic subjects are less distensible than normal (10, 26), and it has been shown recently that the lack of airway distensibility may underlie the lack of DI-induced bronchodilation seen in severe asthmatic subjects (16). The degree of airway distension is determined by tethering forces, transmural pressure, and airway stiffness. Conventionally, airway stiffness is dissected into two components: one stems from the active actomyosin cross-bridge interaction that can be inhibited by inhibiting the contractile force of ASM (e.g., by \( \beta_2 \)-agonist); the other is considered passive and is thought to originate mainly from extracellular elements of the airway wall. It is thought that this component of passive stiffness cannot be readily modulated without altering the airway wall structure, for example, through airway remodeling or its reversal.
The present study identified a new component of airway stiffness that is not associated with cross-bridge activation (i.e., it can be decoupled from active force) and yet can be readily altered by perturbing an intracellular signaling pathway. As shown in Fig. 2B, this regulatable stiffness exists in ASM over a large length range (see the difference between the solid and open symbols, Fig. 2B): at in situ length \((L_{ref})\), it accounts for over 25% of the total “passive” stiffness, and at 0.5 \(L_{ref}\), it accounts for \(\sim 40\%\) of the stiffness. The potential to modulate the regulatable stiffness, therefore, has significant implications with respect to airway response to oscillatory strains and to lung function.

**Calcium and length dependence of muscle stiffness at resting state.** As suggested by the present results, the resting stiffness of ASM bundle can be divided into two components: regulatable and true passive components. Here we define “passive” as being not regulatable by calcium signaling pathways that lead to muscle contraction. Because we did not measure intracellular calcium directly, we assume that abolishment of active force (by removing extracellular calcium or blocking calcium entry into the muscle cell) signals complete removal of intracellular calcium or reduction of the internal store of calcium to such an extent that the calcium-related signaling pathways cannot be activated. The extracellular matrix of the muscle bundle, which is composed mainly of collagen and elastin fibers (8, 12), likely contributes to at least some of the true (nonregulatable) passive stiffness seen in this study. Evidence supporting this claim is that this component of the passive stiffness is insensitive to calcium, be it intracellular or extracellular calcium (Fig. 4). This passive stiffness, however, is length dependent (Fig. 2B), it increased significantly (one-way ANOVA, \(P < 0.01\)) over the length range from 0.5 to 1.5 \(L_{ref}\) after length adaptation.

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**Fig. 6.** Redevelopment of active force and passive stiffness after inhibition of Rho kinase by Y-27632 (10 \(\mu\)M). The solid circles (●) represent control observations without Rho-kinase inhibition. The open circles (○) are data obtained with Y-27632. A: the redeveloped active force was reduced by 48% at the end of the 30-min recovery period in the presence of Y-27632. ***\(P < 0.001\) (two-way ANOVA). B: the redeveloped passive stiffness at the end of the recovery period was reduced by 18% in the presence of Y-27632. **\(P < 0.01\) (two-way ANOVA). Values are means ± SE.

**Fig. 7.** SR after a quick (8% \(L_{ref}\) in 0.01 s) step increase in length. A: time course of SR in normal Krebs (solid trace) and Ca\(^{2+}\)-free Krebs (shaded trace), normalized to the maximal SR \((S_{max})\) obtained under the control condition (i.e., normal Krebs). Only 0.3 s of the 10-s recording is shown, because the rest of the traces were virtually flat. The peak force (first plotted data point) for each trace corresponds to the data point obtained at the end of the stretch, i.e., 10 ms after the initiation of the length step. B: same traces as in A, but normalized to maximal SR of each trace. This was done to compare the rate of relaxation \((t_{1/2})\) under each condition. C: summary of the results of SR and \(t_{1/2}\) under each of the test conditions compared with the reference condition (normal Krebs). Except for the ML-7 values, all values of SR and \(t_{1/2}\) are significantly different from their respective reference values. **\(P < 0.01\) and ***\(P < 0.001\) (paired T-test). Values are means ± SE.
The difference between passive stiffness obtained in the presence and absence of calcium (Fig. 2B, solid and open circles) is relatively constant. If we assume that the components responsible for the regulatable (calcium-sensitive) and nonregulatable (calcium-insensitive) passive stiffness act in parallel, then we can say that the regulatable passive stiffness is relatively independent of muscle length, especially at lengths less than \(L_{\text{ref}}\) (Fig. 2B). This suggests that the regulatable stiffness does not likely stem from static structures, such as the collagen-elastin extracellular matrix, but instead originates from dynamic interactions among intracellular elements (such as cross-linking) that can be broken and reestablished after a length change. There is evidence that passive tension in detrusor smooth muscle can be maintained by actomyosin interaction in the resting state, but it is likely that a large part of the regulatable stiffness could stem from multiple (cross-bridge and noncross-bridge) origins, and, therefore, it could be regulated through multiple branches of the signaling network. It appears that the pathway involving Rho-kinase activation is important in the generation of the passive stiffness. Figure 6 shows that, when active force was reduced by about one-half by the Rho-kinase inhibitor Y-27632 (10 \(\mu\)M), the calcium-sensitive passive stiffness was also reduced by about one-half.

**Could the calcium-sensitive passive stiffness reside in the extracellular matrix?** The calcium-sensitive passive stiffness revealed in Fig. 2 was demonstrated by removing both intracellular and extracellular calcium. This raises a question of whether the extracellular stiffness is calcium sensitive. When calcium is present in the Krebs solution, the muscle can be strain-softened by length oscillations. Both the active force and passive stiffness recovered fully after 30 min, during which the muscle was repeatedly (every 5 min) and briefly (9 s) stimulated by EFS to produce \(F_{\text{max}}\) (at \(L_{\text{ref}}\)) (Fig. 4, A and B, solid circles). When calcium is removed from Krebs, active force was abolished, but the passive stiffness recovered partially (Fig. 4, A and B, open circles). The difference between the recovered passive stiffness, with and without calcium, represents the calcium-sensitive component of the passive stiffness, and this component is quantitatively the same as that shown in Fig. 2B at \(L_{\text{ref}}\).

By blocking calcium entry into the smooth muscle cells using SKF, while leaving the extracellular calcium unchanged, identical results were obtained (compare Fig. 4, A and B with C and D). This suggests that the regulatable stiffness is independent of extracellular calcium, and that this stiffness originates from intracellular sources. However, due to the nonspecificity of SKF action, contribution to the regulatable stiffness from extracellular source cannot be definitively excluded. Direct measurement of intracellular calcium or the use of single-cell preparation is needed to address this issue.

**Regulatable passive stiffness and the myosin light chain phosphorylation.** Figure 5 shows that, when MLCK was inhibited by ML-7, active force was abolished (Fig. 5A), suggesting that phosphorylation of the regulatory myosin light chain was prevented during stimulation by EFS. However, passive stiffness was unaffected (Fig. 5B). This indicates that active force and passive stiffness can be separated, and that active cycling of the cross bridges (or active force generation) is not required for the recovery of regulatable passive stiffness. If actomyosin interaction constitutes part of the regulatable passive stiffness, then it follows that this interaction can occur without myosin light chain phosphorylation.

**What regulates the regulatable passive stiffness?** The regulatable passive stiffness could stem from multiple (cross-bridge and noncross-bridge) origins, and, therefore, it could be regulated through multiple branches of the signaling network. It appears that the pathway involving Rho-kinase activation is important in the generation of the passive stiffness. Figure 6 shows that, when active force was reduced by about one-half by the Rho-kinase inhibitor Y-27632 (10 \(\mu\)M), the calcium-sensitive passive stiffness was also reduced by about one-half.
The calculation of the decrease in passive stiffness was based on the fact that calcium removal resulted in \( \sim 34\% \) decrease in the stiffness (Fig. 4), and Y-27632 resulted in \( \sim 18\% \) decrease in the stiffness (Fig. 6). We did not increase the concentration of Y-27632 beyond 10 \( \mu \)M because of the concern about nonspecific effect of the inhibitor at high concentrations. We did not measure Rho-kinase activity directly in this study and, therefore, do not know how much of the kinase activity was inhibited with the concentration of Y-27632 used. However, the results suggest that Rho-kinase activation is crucial for the generation of the regulatable passive stiffness, and, taken together with the results from the ML-7 experiments (Fig. 5), they indicate that the inhibition of myosin light chain phosphatase (MLCP) by Rho-kinase activation is not likely the path that leads to generation of the regulatable passive stiffness, because the stiffness is independent of the degree of the light chain phosphorylation. This implies that other pathways downstream from the Rho kinase are responsible for the generation of the regulatable passive stiffness.

Amplitude and rate of SR. It has been reported that, in relaxed ASM strips, SR after a sudden change in length can be described by a power-law process (9). The reference SR (solid trace, Fig. 7A) could be fit with a power-law function, as shown in Fig. 9. It should be pointed out that the time course of SR does not necessarily possess a horizontal asymptote, and that, in fitting the data, the power-law curve was allowed to go below the baseline of force “plateau”. Although the goodness of fit \( r^2 = 0.9341 \) may be acceptable statistically, the best fit is still not good enough to differentiate small variations in \( t_{1/2} \) (Fig. 7B). Our analysis of SR was, therefore, concentrated on the parameters that we could quantify more accurately from direct measurements of force records, that is, the amplitude and half-time of the SR (Fig. 7C).

As shown in Fig. 8, changes in the calcium-sensitive passive stiffness are highly correlated with the changes in the amplitude and \( t_{1/2} \). Although the power-law description was considered not accurate for distinguishing the small difference in \( t_{1/2} \), it nevertheless can account for the bulk of the SR (Fig. 9). This suggests that smooth muscle tissue behaves like a non-Newtonian soft material (7), and that creep or SR in the tissue involves disruption and reorganization of intracellular elements bound together by weakly interacting forces. If we assume that these weak forces represent ionic bonds cross-linking subcellular elements, then the rate of SR can be interpreted as the rate at which the bonds are broken. The good correlation shown in Fig. 8 suggests that the reduced calcium-sensitive passive stiffness due to Rho-kinase inhibition, for example, may be due to a reduction in the number of the cross-linking ionic bonds that, in turn, results in the reduced peak stress response during the step stretch. Another interpretation is that, under the test conditions that reduced the passive stiffness, the strength of the ionic interaction may be weakened so that, during a step stretch, the peak stress response is reduced (as reflected in the reduced stiffness and amplitude of SR), and the rate of bond breaking increased (as reflected in the reduced \( t_{1/2} \)).

Relations to earlier studies. It has been shown that the resistance to stretch (or stiffness) in resting smooth muscle is calcium dependent, and that the source of this stiffness could be the actomyosin cross bridges that remain attached in the relaxed state (22). The present study confirmed the existence of the calcium-sensitive passive stiffness in ASM; however, as to the source of the stiffness, the present results suggest that intracellular elements other than the cross bridges may contribute to the bulk of the stiffness.

It has been shown in detrusor smooth muscle that the generation of passive stiffness is dependent on Rho-kinase activation (23), similar to what we found in ASM (Fig. 6). However, the Rho-kinase-dependent passive stiffness in the detrusor smooth muscle, once developed, can be maintained even in the absence of calcium (23). In ASM, this stiffness can be abolished by simply incubating the muscle in calcium-free Krebs (Fig. 2C). What underlies this difference is not clear; it could be due to species difference or different smooth muscle types.

Recent studies from our laboratory have shown that the passive stiffness of ASM can be abolished by a sudden 50% reduction of muscle length in the resting state (1). A sudden large change in length, therefore, has a similar effect to strain-induced softening by length oscillation, which was used in this study. It was observed that passive stiffness recovered partially to a plateau level \( \sim 1 \) h after the muscle length was halved (1); this recovered passive stiffness could be abolished by removal of calcium, but not by inhibition of Rho-kinase by Y-27632 (3 \( \mu \)M) (1). This may appear to be contrary to our present finding regarding the effect of Y-27632 (Fig. 6), but the difference actually reflects a very interesting property of the calcium-sensitive passive stiffness. That is, once the passive stiffness is redeveloped, its maintenance depends on the presence of calcium, but is insensitive to Rho-kinase inhibition. When Rho kinase is inhibited before the redevelopment of stiffness, the redevelopment is inhibited. Therefore, a logical conclusion is that the redevelopment and maintenance of the calcium-sensitive passive stiffness are governed by different mechanisms.

It has been shown that, like other soft materials, smooth muscle cells can be softened by shear stress (24) in a solidlike to a fluidlike transition. This phenomenon can be explained by weak interactions among the stress-bearing elements within the cells, such as proteins or protein aggregates that form a network of force transmission, which can be disrupted by external agitation, such as shearing (24). In cultured human bladder smooth muscle cells, a transient stretch has been shown to lead to rapid decrease in cell stiffness and depolymerization of actin filaments, followed by a slower recovery of the stiffness and actin repolymerization (5). This suggests that cell stiffness could reside in the cytoskeletal actin filament network that consists of actin filaments and actin-binding proteins, such as \( \alpha \)-actinin and filamin. Surprisingly, in that study (5), the redevelopment of cell stiffness was not sensitive to a variety of inhibitors of enzymes involved in the cell signaling, including Rho-kinase inhibitor Y-27632. The reason for the discrepancy with our finding (Fig. 6) is not clear, but it could be due to differences in muscle preparation (cultured cells vs. muscle bundle) and muscle type. It could also be due to differences in the methods for estimating the stiffness; the oscillation method used by Chen et al. (5) separates the elastic and viscous properties, whereas in our measurements these properties were mixed together.

Inhibition of Rho-kinase by Y-27632 can lead to significant disruption of the signaling pathways in smooth muscle cells. Rho kinase has direct and indirect inhibitory effects on the MLCP and also on enzymes that regulate actin polymerization.
and filament stabilization (15). The effect of Y-27632 that we have observed in the present study is not likely related to the MLCP pathway, because we only examined the passive response of the muscle, in the absence of any active force generation. The effects that we have observed are likely related to the perturbation of the signaling pathways that regulate the cytoskeletal organization. We speculate that the cross-linking of cytoskeletal filaments (that alters the stiffness of the filament network) may be one of the targets of Rho kinase.

A recent study from our laboratory (27) has shown that dense bodies in ASM form cablelike structures that run parallel with the contractile filaments and the axis of force transmission, and that these “cables” are able to bear resting tension. An interesting property of these cablelike structures is that they are able to adjust their length according to the muscle cell length during length adaptation. This ability may underlie the recovery of passive cell stiffness lost transiently after a large step decrease (50%) in cell length (27). The dense body cables, therefore, can be considered as another possible source for the regulatable passive stiffness described in the present study.

Significance to airway physiology and asthma. A major difference between asthmatic subjects and nonasthmatic subjects is in their response to DIs. Specifically, nonasthmatic subjects benefit from DIs in terms of attenuation of subsequent bronchoconstriction and reversal of established bronchoconstriction; the benefit, on the other hand, is largely diminished or totally lost in asthmatic subjects (2–4, 21). A difference in airway distensibility likely contributes to the difference seen in asthmatic subjects and nonasthmatic subjects in their response to DIs (16). Reduction of ASM tone will decrease airway stiffness (6, 18). However, even without tone, the smooth muscle layer still contributes to airway distensibility. In ASM strips adapted to short lengths, upon restretching the muscle back to its in situ length, the passive tension often exceeds that of active tension (13, 25). Airway distensibility, therefore, can be largely determined by the passive tension of the smooth muscle layer, especially if the airway is chronically narrowed and the smooth muscle adapted to an abnormally short length. Acute reversal of passive stiffness associated with the smooth muscle layer, however, has never been considered a strategy for treatment of asthma, because of the conventional belief that the stiffness is associated with the relatively permanent structures within the airway wall. Our present finding shows that a substantial component of the passive stiffness that resides within the smooth muscle cells is calcium sensitive and is regulated by signaling pathways associated with cell activation. This paves the ground for future search of drug targets that could lead to an acute decrease in airway stiffness and restoration of the bronchoprotective and bronchodilatory effects of DI in asthmatic subjects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


