Viscoelastic and dynamic nonlinear properties of airway smooth muscle tissue: roles of mechanical force and the cytoskeleton

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Ito, Satoru, Arnab Majumdar, Hiroaki Kume, Kaoru Shimokata, Keiji Naruse, Kenneth R. Lutchen, Dimitrije Stamenovic, and Béla Suki. Viscoelastic and dynamic nonlinear properties of airway smooth muscle tissue: roles of mechanical force and the cytoskeleton. Am J Physiol Lung Cell Mol Physiol 290: L1227–L1237, 2006. First published January 13, 2006; doi:10.1152/ajplung.00299.2005.—The viscoelastic and dynamic nonlinear properties of guinea pig tracheal smooth muscle tissues were investigated by measuring the storage (Gʹ) and loss (Gʺ) moduli using pseudorandom small-amplitude length oscillations between 0.12 and 3.5 Hz superimposed on static strains of either 10 or 20% of initial length. The Gʹ and Gʺ spectra were interpreted using a linear viscoelastic model incorporating damping (G) and stiffness (H), respectively. Both G and H were elevated following an increase in strain from 10 to 20%. There was no change in harmonic distortion (Kd), an index of dynamic nonlinearity, between 10 and 20% strains. Application of methacholine at 10% strain significantly increased G and H while it decreased Kd. Cytochalasin D, isoproterenol, and HA-1077, a Rho-kinase inhibitor, significantly decreased both G and H but increased Kd. Following cytochalasin D, G, H, and Kd were all elevated when mean strain increased from 10 to 20%. There were no changes in hysteresivity, G/H, under any condition. We conclude that not all aspects of the viscoelastic properties of tracheal smooth muscle strips are similar to those previously observed in cultured cells. We attribute these differences to the contribution of the extracellular matrix. Additionally, using a network model, we show that the dynamic nonlinear behavior, which has not been observed in cell culture, is associated with the state of the contractile stress and may derive from active polymerization within the cytoskeleton.

Changes in the contractile ability of airway smooth muscle (ASM) play important roles in the development of bronchoconstriction and the pathophysiology of asthma. Measurements on ASM cells in culture have provided ample evidence that the viscoelastic behavior (time or frequency dependence of mechanical properties) of ASM cells is significantly altered by changes in the contractile state, mechanical forces applied to the cells, and the organization of the actin cytoskeleton (1, 12, 13, 25, 38, 46, 47). While much less is known about the detailed viscoelastic behavior of ASM cells in situ, there is evidence that the mechanics of smooth muscle tissues can be different from those of single cells (20).

When differentiated ASM cells are cultured on elastic membranes, their mechanical properties also depend on the compliance of the substratum (36). Consequently, the cells may partially lose their contractile phenotype, leading to alterations in cell physiology that may not occur in vivo and in intact ASM tissues. It has been recognized that besides the biochemical environment, the dynamic mechanical interaction of ASM cells with the extracellular matrix (ECM), mainly collagen, elastin, and proteoglycans, also has important effects on the mechanical properties of ASM cells (2, 5, 8, 19, 34). Isolated tracheal tissue strips containing ASM cells exhibit a nonlinear force-length relationship during quasistatic stretching (18, 40) as well as complicated loops during sinusoidal oscillations (15, 16, 29). However, because nonlinearities are not seen in cell culture (13), this behavior may partly be attributed to the properties of the ECM. Indeed, using computer modeling, it has recently been shown that some of the peculiar features of ASM strip mechanics can in fact be mimicked by assuming nonlinear viscoelastic properties of the ECM (5). However, little is known about how mechanical forces and changes in the contractile apparatus and cytoskeletal architecture act to influence the dynamic nonlinear behavior of ASM cells at the tissue level.

The primary purpose of this study was to characterize the viscoelastic and dynamic nonlinear properties of ASM cells at the tissue level. We hypothesized that applied mechanical forces, contractile stimulation, and changes in actin polymerization are reflected not only in the viscoelastic properties but also in the nonlinear behavior of ASM in situ. To test this hypothesis, we examined the viscoelastic and nonlinear properties of isolated tracheal smooth muscle (TSM) tissue strips in response to mechanical loading and treatments with a variety of pharmacological agents including methacholine (MCH; a contractile agonist), isoproterenol (a muscle relaxant β-agonist), cytochalasin D (an inhibitor of actin polymerization), and HA-1077 (a specific Rho-kinase inhibitor). Because, as noted above, the measured properties likely reflect the combined response of the ASM cells and ECM (5), we further hypothesized that the response of the TSM strips to certain combinations of pharmacological and mechanical stimuli will be dominated by the ASM cells. As a consequence, this may allow us to characterize the dynamic nonlinear properties of ASM cells from global measurements at the strip level. To this end, we applied pseudorandom small-amplitude length oscillations, which allowed us to simultaneously map the frequency dependence of the complex dynamic modulus (55) and extract an index of dynamic nonlinearity, called harmonic distortion, from the stress response (49, 57).

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METHODS

ASM strip preparation. Male Hartley guinea pigs (n = 12; Charles River, Boston, MA) weighing 400–450 g were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg), and tracheas were freshly isolated and placed in normal physiological solution (in mM: 3 KCl, 145 NaCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose at room temperature and pH 7.4). Tissue strips (5.0 x 0.2 x 0.8 mm) containing smooth muscle in the middle and small cartilage bands at either ends were prepared using a single-edge razor blade. The length of TSM (initial muscle length) expressed as distance between two cartilage ends was ~2.0 mm. Each cartilage end of the tissue strip was fixed by cyanoacrylate glue to small metal plates attached to straight steel wires connected to a force transducer and a lever arm. The assembly was placed in 1 ml of tissue bath horizontally. All animal procedures were approved by the Animal Care and Use Committees of Boston University.

Experimental setup. The apparatus included a servo-controlled lever arm (model 680OHP, Cambridge Tech, Watertown, MA) and a force transducer (model 403A, Aurora, ON, Canada). The lever arm was driven by a displacement signal generated by a computer. The signal was sent out from the digital/analog port of a data-acquisition board (DT2812, Data Translation, Marlborough, MA) and then smoothed with a low-pass filter (8-pole, R858L8EX, Frequency Devices, Haverhill, MA) with a cutoff frequency of 15 Hz. Both displacement and force signals were low-pass filtered at 15 Hz and sampled at 60 Hz by the computer.

The linearity and hysteresis of the measurement system itself were tested as described previously (55). The modulus of a small metal spring attached to the apparatus showed practically no frequency and amplitude dependence. Thus effects of the frequency response and nonlinearity of the apparatus itself on the viscoelastic and nonlinear properties of the TSM were negligible.

Measurement of mechanical properties. The force developed by the tissue was obtained in response to pseudorandom small-amplitude length oscillations with a peak-to-peak amplitude of 10% of the initial muscle length (55). A broadband pseudorandom displacement input signal, which allows a rapid assessment of the dynamic mechanical properties of the tissue strips was used. The displacement signal was the sum of six sinusoids (0.12, 0.29, 0.64, 1.1, 1.8, and 3.5 Hz) chosen according to the nonsum-nondifference frequency composition, such that the influence of nonlinearities on the estimated apparent complex moduli were minimized (51). The frequency composition also allowed the higher order nonlinearities (harmonic distortion and cross-talk) to be identified from frequencies that were not part of the input spectrum. The harmonic distortion index can be used to characterize dynamic tissue nonlinearities (57). The signal contained a flat power spectrum that the influence of nonlinearities on the estimated apparent complex moduli were negligible.

Network modeling. The following viscoelastic model was used to fit to the complex modulus spectra G*(ω):

\[ G^*(ω) = G'(ω) + jG''(ω) = H_{00} + j(G_{00} + R_0) \]  

where G' and G'' are the storage (elastic) and loss (frictional) moduli, respectively, \( ω \) is the circular frequency, \( j = \sqrt{-1} \) is the imaginary unit indicative of the out-of-phase behavior, and \( \omega_0 = \omega / \omega_0 \) is the normalized frequency with \( \omega \) rad/s. The parameters G and H are the tissue damping and stiffness coefficients, respectively. Note that \( \beta \) is not an independent parameter in the model as \( \beta = 2/\pi tan^{-1}(G/H) \), and it governs the frequency dependence of the real and imaginary parts of \( G^* \). A similar model was first used by Hantos et al. (21) to describe the linear viscoelasticity of parenchymal tissues of the whole lung and later modified to account for \( G^* \) of tissue strips by Yuan et al. (55) by adding a Newtonian viscous component R. The model is called “constant phase” model because usually the contribution of R is small and hence the phase angle of the modulus, tan^{-1}(G/H), is independent of frequency. The hysteresivity (17) of the tissue was defined as the ratio G/H, which is related to the relative dissipated energy in the tissue during a cycle. The model parameters were estimated by minimizing the root-mean-square error between data and model (10). We note that although the data showed signs of nonlinearity, the linear model of Eq. 1 can still be applied due to the special design of our test signal that minimizes the effects of nonlinearity of the apparent modulus as described previously (51).

Characterizing dynamic nonlinearity. When applying a single sinusoidal input signal, the degree of system nonlinearity can be characterized by the amount of output energy appearing at frequencies other than that of the input. More generally, when the input is broadband containing several sine waves, the nonlinearity can be quantified by the harmonic distortion index \( K_d \), which quantifies the amount of both harmonic distortion and cross-talk in the output signal resulting from system nonlinearities (49). For a broadband input, the \( K_d \) is defined as:

\[ K_d = \sqrt{P_{TOT}/P_{OUT}} \]  

where \( P_{TOT} \) is the total output power and \( P_{IN} \) is the power at noninput frequencies, i.e., the output power due to system nonlinearities only. The values of \( K_d \) were also corrected for nonzero energy at noninput frequencies due to noise (57). The advantage of using \( K_d \) is that it can be calculated from a single spectrum, whereas the traditional method of characterizing nonlinearity requires the measurement of the moduli at several distinct amplitudes. In a linear system, \( K_d \) is zero.

Network modeling. Because the actin cytoskeletal network is the major determinant of the mechanical properties of living cells (13, 26, 47), we developed a simple nonlinear elastic network model of the cytoskeleton within a single smooth muscle cell. The line elements were nonlinear springs arranged in a hexagonal lattice for convenience. Two opposite sides, the top and bottom rows, of the lattice were fixed, while the lateral and the internal nodes were free to move. The node coordinates were first randomized, the initial length of each spring was then set to the length of the spring in this randomized configuration, which defined the stress-free configuration of the network. Next, the network was stretched uniaxially by 20% strain in the

random length oscillations on a static strain of 10%. Following four recordings of control measurements at 10% static strain, the static strain was increased to 20% and the measurements were repeated in the same muscle strip. Next, one of the following pharmacological agents, MCh (Sigma), isoproterenol (Sigma), HA-1077 (Tocris, Ellisville, MO), or cytochalasin D (Tocris) was added to the tissue bath and kept there for 15–30 min while the tissue was held at 10% strain and at room temperature. Following equilibration, force-length oscillatory data were collected both at 10 and 20% static strains. To obtain time-matched control measurements, the tissue strips were kept in normal solution for 20 min, and the measurements were performed at 10% static strain.

Viscoelastic modeling. The following viscoelastic model was used to fit to the complex modulus spectra G*(ω):
vertical direction. Because the mechanical properties of tracheal strips were measured such that the length oscillations were superimposed on a static initial strain, during simulations the mean length of the network model was kept constant. The network was then further stretched by superimposing on the static initial stretch of 20% a single cycle of sinusoidal oscillations of 5% in strain amplitude. At five points along the sine wave, the total potential energy of the network was minimized using simulated annealing (9). The total force developed by the network was obtained at each strain as the sum of the forces exerted by the springs at the top boundary in the direction of the strain. The stress was simply calculated as the total force divided by the width of the network (thickness was taken to be unity), and the full stress-strain cycle was reconstructed. Using Fourier analysis, the stiffness and the harmonic distortion were calculated as from the experimental data.

First, the stiffness and the harmonic distortion were calculated for the control condition. Next, the response of ASM cells to contractile agonists such as MCh was mimicked by increasing the number of parallel network elements compared with baseline, which is equivalent to actin polymerization as reported previously (1, 22). To mimic the effects of cytochalasin D, the number of parallel network elements was reduced, which is equivalent to actin depolymerization.

Next, we examined the possible contribution of the ECM to the mechanics of the TSM strip by using a multiscale network model containing elements with mechanical properties that were similar to either muscle cells (muscle-like springs) or connective tissue fibers (connective tissue-like springs). To do this, the actin network in each muscle cell was represented by single spring with second-order polynomial nonlinearities in its force-length relationship. The polynomial coefficients were chosen so that the muscle-like springs had a decreasing $K_d$-H relationship similar to that in Fig. 6A. The tissue-like spring had a force-length relationship with the polynomial coefficients adjusted so that these springs displayed a $K_d$-H relationship in which $K_d$ increased with increasing H as observed in the lung parenchyma (56). This network was then cyclically stretched, and its $K_d$-H relationship was determined numerically. Finally, to mimic the effects of pharmacological agents on the muscle cells, the linear spring constant of the muscle-like springs was varied.

Statistical analysis: The number (n) of strips tested varied under different conditions. All data were expressed as means ± SD. Either t-test or ANOVA followed by post hoc analysis using Dunnett test was used to evaluate the significance of differences between means, with $P < 0.05$ as the level of significance using SigmaStat (SPSS). Statistical analysis was performed according to guidelines for reporting statistics in journals published by the American Physiological Society (11).

RESULTS

Effects of static stretch. Representative traces of the displacement and force signals during pseudorandom length oscillations around 10% static strain in normal solution are shown in Fig. 1. Examples of the fits of the constant phase model (Eq. 1) to the G′ and G″ spectra as a function of frequency are shown in Fig. 2A, corresponding to the two different static strain levels (10 and 20%). Both G′ and G″ depended on frequency and increased in magnitude with increasing static strain. The viscoelastic model of Eq. 1 fits the data well at both static strains. The population averages of the various parameters extracted from the data are shown in Fig. 2B (n = 10). Increasing static strain from 10 to 20% significantly elevated the coefficients of both tissue damping G ($P = 0.002$) and stiffness H ($P = 0.002$). While hysteresivity was slightly reduced by increasing the static strain, the decrease in hysteresivity did not reach statistical significance. The Newtonian viscous parameter R also increased significantly ($P = 0.002$). Furthermore, the relative changes in R closely followed the pattern of G under all conditions tested with a regression $G = -0.13 \pm 7.43^*r (r = 0.89, P < 0.001)$. Hence, the parameter R is not described separately from G and its interpretation is retained in DISCUSSION. The harmonic distortion $K_d$ did not change between 10 and 20% strain levels.

Contractile stimulation with MCh. The G′ and G″ spectra were fit well by the model at 10% strain both in the control condition and after the treatment with 10 μM MCh (Fig. 3A). Both G′ and G″ were frequency dependent and highly elevated by MCh compared with the control. Comparisons of the viscoelastic properties (G, H, and hysteresivity) and nonlinearity ($K_d$) at 10% static strain in the presence (n = 6) or absence (n = 6) of MCh are shown in Fig. 3B. Treatment with MCh significantly increased G ($P = 0.01$) and H ($P = 0.01$), but it significantly decreased $K_d$ ($P = 0.01$). In contrast to cell culture studies where hysteresivity was sensitive to stimuli (13), the values of hysteresivity here were not significantly affected by MCh. In the presence of MCh at 20% strain level, the samples developed very high forces. Due to a limitation of the force transducer, these data could not be obtained in this study.

Effects of relaxing agents and inhibition of actin polymerization. Representative cases of the data and model fits of G′ and G″ as a function of frequency at 10% static strain in the control condition and after the treatment with isoproterenol or cytochalasin D are shown in Fig. 4A. Compared with control, both G′ and G″ decreased in the presence of isoproterenol or cytochalasin D treatment in agreement with data obtained in cell cultures (14, 47). Comparisons between the average values...
of G, H, hysteresivity, and \( K_d \) at 10% strain before and after treatment with 10 \( \mu \text{M} \) cytochalasin D \((n = 5)\), 10 \( \mu \text{M} \) isoproterenol \((n = 5)\), or 100 \( \mu \text{M} \) HA-1077 \((n = 5)\) are shown in Fig. 4B. When the TSM tissue strips were kept in normal solution at 10% strain for 20 min, the values of mechanical properties (control values) were not affected \((n = 5)\). The values of G and H were significantly decreased by cytochalasin D, isoproterenol, and HA-1077 compared with the time-matched control values \((P < 0.05)\). The \( K_d \) values were significantly increased by isoproterenol, cytochalasin D, and HA-1077 \((P < 0.05)\). Again, contrary to cell culture studies \((14, 32)\), hysteresivity was not significantly altered by these agents.

Effects of static stretch on relaxed tissue. An increase in static strain from 10 to 20% resulted in statistically significant increases, respectively, in G, H, and \( K_d \) in the presence of 10 \( \mu \text{M} \) cytochalasin D \((P = 0.003, P = 0.003, P = 0.004\), respectively\), 10 \( \mu \text{M} \) isoproterenol \((P = 0.02, P = 0.003, P = 0.002\), respectively\), and 100 \( \mu \text{M} \) HA-1077 \((P = 0.03, P = 0.03, P = 0.003\), respectively\; Fig. 5). There were no significant changes in hysteresivity following an increase in static strain in any experimental condition.

Relationship between stiffness and dynamic nonlinearity. To investigate the relationship among mechanical force, ASM contractility, and dynamic nonlinearity, the relative changes in the parameters H or G and \( K_d \) were plotted against each other corresponding to all experimental conditions. The changes in H and \( K_d \) were expressed relative to their values in normal solution at 10% strain. Contractile stimulation by MCh increased H with a reduction in \( K_d \) (Fig. 6A). While each of the muscle relaxing agents (isoproterenol, HA-1077, and cytochalasin D) decreased H, interestingly, they increased \( K_d \) (Fig. 6A). Loading the muscle strip by increasing the static strain from 10 to 20% increased H with no significant change in \( K_d \) in the control condition (Fig. 6B). However, after treatment with isoproterenol, cytochalasin D, or HA-1077, the values of \( K_d \) also increased with static stretch (Fig. 6B). These data suggest that at a given level of static strain, application of various pharmacological agents leads to an inverse relationship between H and \( K_d \). The relationship between the relative changes in G and \( K_d \) was similar to the H–\( K_d \) relationship as shown Fig. 6, C and D. Moreover, due to the tight correlation between G and R, the...
G-$K_d$ relationship and the R-$K_d$ relationship were similar (data not shown).

**Network simulations.** The network configurations corresponding to a single muscle cell in the control, the contracted, and the relaxed states are shown in Fig. 7A. When the response to contractile agonists such as MCh was simulated, the network had more parallel actin fibers that mimicked polymerization as reported previously (1, 22) and hence the total load was distributed among many parallel fibers. When the response to cytochalasin D was simulated, the total load was carried only by a few parallel fibers as a result of depolymerization. Stiffness is proportional to the number of parallel fibers. Thus the stiffness of the network increased when parallel fibers were added and decreased when parallel fibers were removed. As shown in Fig. 7B, the calculated normalized harmonic distortion was consistent with a hyperbolic function of the normalized stiffness similar to the experimental data obtained in ASM strips (Fig. 6A).

To examine the effects of the presence of connective tissue in the TSM strip, the network of muscle-like and connective tissue-like elements was cyclically stretched while the ratio of muscle-like to tissue-like elements was varied. The $K_d$-$H$ relationship of the network of muscle and tissue elements shows a complex behavior due to the coupling of disparate nonlinearities (Fig. 8). When all elements in the network were muscle-like (0% tissue), we obtained a $K_d$-$H$ relationship (solid line, Fig. 8) that was similar to the behavior of a single cell as in Fig. 7B. The small difference in behavior between the single cell and the network of cells is due to reorientation of the elements in the network during stretching. To mimic the contribution of the ECM, first 25% of the muscle-like elements were randomly replaced by connective tissue-like elements. The resulting network showed a decreasing $K_d$-$H$ relationship for small values of $H$ and an increasing $K_d$-$H$ relationship for large values of $H$ (dashed line, Fig. 8). This behavior is even more pronounced when the fraction of connective tissue-like elements in the network was increased to 75% (dotted line, Fig. 8).

**DISCUSSION**

In the present study, the dynamic mechanical properties of TSM tissues isolated from guinea pigs were characterized by applying pseudorandom small-amplitude length oscillations...
over a range of physiological frequencies. The main findings are that 1) both the storage and loss moduli, \( G' \) and \( G'' \), respectively, displayed a characteristic frequency dependence conforming to a viscoelastic model description similar to that found for cells in culture and the lung parenchyma; 2) dynamic tissue stiffness increased with muscle loading (static stretch) and contractile stimulation; 3) stiffness was reduced by muscle relaxants or by an inhibitor of actin polymerization; and 4) in contrast to less contractile tissues such as the lung parenchyma, the dynamic nonlinear behavior was inversely related to dynamic stiffness.

Viscoelastic properties. Several previous works have focused on cultured ASM cells because of their usefulness and availability for molecular techniques (25, 35). Using the magnetic twisting cytometry technique and atomic force microscopy, the viscoelastic mechanical behavior of cultured ASM cells has been characterized in detail (1, 13, 14, 25, 32, 38, 42). These studies have confirmed that the contractile response observed at the tissue level is characteristic of many of the fundamental mechanical features of ASM cells in culture (15, 16).

To function as a cohesive tissue, smooth muscle cells need to maintain a complex interaction with the ECM via the integrins and cell-cell interactions at contact sites through tight junctions or gap junctions (6, 26). Thus the biochemical and mechanical interactions between neighboring cells and the ECM are likely important factors altering the shortening and force developing characteristics of ASM that play crucial roles in airway hyperreactivity of asthmatic individuals (7, 8, 34). Using canine TSM tissues, Fredberg et al. (15, 16) measured the mechanical properties including active force, stiffness, and hysteresivity during contraction by applying sinusoidal length oscillations at a frequency of 1 Hz. To our knowledge, this is the first study to investigate the viscoelastic properties characterized by \( G' \) and \( G'' \) at the level of intact ASM tissue strips using pseudorandom length oscillations, a technique that had been applied to parenchymal tissue strips (55, 56). We found that the \( G' \) and \( G'' \) spectra increased with frequency that was consistent with the constant phase model originally developed for parenchymal tissues based on whole lung measurement (21). This description of the ASM strip was accurate under all conditions.

Fig. 4. A: representative examples of \( G' \) and \( G'' \) as a function of frequency and model fits (solid, dashed, and dotted lines) in control and in the presence of 10 \( \mu M \) isoproterenol (ISO) or 10 \( \mu M \) cytochalasin D (CytD). B: population means \pm SD of \( G, H, hysteresivity, R, \) and \( K_d \) in control and in the presence of 10 \( \mu M \) CytD, 10 \( \mu M \) ISO, or 100 \( \mu M \) HA-1077 corresponding to 10% static strain. *\( P < 0.05 \) compared with the values of control.
pharmacological perturbations and at both static strain levels applied in the present study (Figs. 2–5). It is also noteworthy that the mathematical form of this model is equivalent to that used by Fabry et al. (13) to account for the viscoelastic properties of cultured ASM cells.

Both tissue stiffness (H) and damping (G) increased during contractile stimulation with MCh challenge and decreased during muscle relaxant isoproterenol, consistent with the previous studies in TSM tissues (15) and in cultured ASM cells (14, 25). Moreover, cytochalasin D and Rho-kinase inhibition with HA-1077, both of which inhibit contractility of ASM cells (27, 33, 53), mimicked the effects of isoproterenol on tissue mechanics as reported in cultured ASM cells (1). Thus our results indicate that the viscoelastic properties, specifically the dynamic stiffness, are associated with contractile stress at the tissue level similar to previous findings using cultured cells (38, 47).

Fig. 5. Effects of static stretch on the mechanical properties in the presence of relaxing agents. Means ± SD of G, H, hysteresivity, R, and Kd corresponding to 10 and 20% static strain levels after treatment with 10 μM CytD, 10 μM ISO, or 100 μM HA-1077. *P < 0.05 compared with the values corresponding to 10% static strain.
ization and myosin phosphorylation contribute to the viscoelastic properties of ASM tissues via alterations in the contractile prestress. However, the roles of other cytoskeletal proteins such as microtubules and intermediate filaments, which also contribute to the mechanical behavior of ASM cells (46, 52), have not been investigated in the present study.

The parameter R represents a Newtonian viscous term in the model (Eq. 1). Interestingly, the value of R showed a significant correlation with G under all conditions. Fabry et al. (13) reported that in cell culture, \( \frac{G}{R} \) approached a slope of 1 on a log-log graph at higher frequencies, which is consistent with the R term in Eq. 1. While numerical values were not reported, we estimated from their data the crossover frequency at which the R term started to dominate (50–100 Hz) and calculated that the ratio G/R was between 2 and 5. The fact that our G/R ratio was 7.43 suggests that the ECM may contribute to this ratio. Indeed, based on the data of Yuan et al. (56) on parenchymal tissue strip, which is dominated by the properties of the connective tissue, the G/R was between 40 and 150. The strong relationship between G and R therefore implies that the physical mechanisms responsible for G also influence R and that this relationship is different in ASM strip than in cell culture possibly due to the contribution of ECM.

Fredberg et al. (16) provided experimental evidence that hysteresivity of ASM is directly associated with shortening velocity, which reflects the cross-bridge cycling rate during contraction and hence the metabolic state of the cells. More recent interpretation of the hysteresivity is based on cell culture studies and involves the hypothesis that the cytoskeletal network behaves as a soft glassy material which provides a link between stiffness and hysteresivity (16). In the present study, we did not find any statistically significant change in hysteresivity due to pharmacological agents or mechanical stretching. This is in sharp contrast with the data obtained in living cultured ASM cells that showed that hysteresivity significantly decreased with increasing contractility (38, 47). To address this discrepancy, we note that each dynamic measurement was assessed after the baseline isometric force was stabilized. Hence, we did not record the transient changes observed by Fredberg et al. (15, 16).

Effects of static stretch. Mechanical forces generated by stretch play an important role in regulating the structure, function, and metabolism of the respiratory system (28, 37). The parenchymal strip preparation represents a model with large ECM contributions to mechanical behavior (50, 55, 56). Recently, Salerno et al. (39) examined the effects of different oscillatory strain amplitudes on mechanical properties in constricted and nonconstricted parenchymal strips and clearly demonstrated that the ECM is the primary contributor to alteration by strain amplitudes. On the other hand, the major contributors to mechanical behavior of ASM cells are contractile and cytoskeletal proteins within the cells (1, 12, 13, 47). Because the TSM is composed of both active ASM cells and ECM, components of the ECM are expected to contribute to
the mechanical behavior in response to mechanical stretch in TSM tissues (5). Previous results on the static stress-strain relationship have revealed that static stiffness increased with loading of TSM tissues (18, 40). Recently, Rosenblatt et al. (38) developed a stretchable cell culture substrate device and demonstrated that an increase in cell distension caused an increase in dynamic stiffness in human cultured ASM cells. One explanation for the relationship between stiffness and stretching of cells in culture is that the cytoskeletal distending stress is a key determinant of the viscoelastic properties of the cells. In the present study, we observed that the TSM tissues became stiffer after muscle loading (static stretching) without noticeable change in hysteresivity. Although we applied pseudorandom length oscillations only for 30 s after the static stretch was increased, we cannot rule out the effects of cytoskeletal remodeling, which occurs after a long period of oscillatory muscle loading (12, 43, 44). Such polymerization would increase hysteresivity of the cells (14). However, static stretch is known to slightly decrease the hysteresivity of the connective tissue elements likely due to the contribution of collagen (56). These two competing mechanisms within the tissue strip may have been the reason that we observed no change in hysteresivity. Another possibility is that the various cells in the TSM had responded slightly differently to stretch and the pharmacological stimulations and that this heterogeneity of response resulted in little change at the level of the strip.

**Dynamic nonlinearity.** The rheological properties of living tissues exhibit two distinct types of nonlinearities: nonlinear elastic properties characterized by the quasistatic stress-strain curve and dynamic nonlinearities characterized by the dependence of the dynamic moduli on strain amplitude (31, 41, 55). The static stress-strain behavior of TSM tissues also exhibits nonlinearities (18, 40). However, nonlinearities are not found in cell cultures (13). While the reason for this discrepancy is unclear, possible explanations include cell-cell interactions, cell-ECM interactions, cell orientation (random in cell culture vs. nearly axial in TSM), or differences in the geometry of cell deformation (local in cell culture vs. global in TSM). In the present study, we assessed the dynamic nonlinear behavior of ASM tissues by measuring the harmonic distortion index $K_d$ of the stress in response to length oscillations. When the input strain is sinusoidal, $K_d$ characterizes the extent to which the stress-strain loop is "banana shaped" or, in other words, deviates from a perfect ellipse as expected for the ideal sinusoid (49). Our pseudorandom length perturbation signal contains six sine waves whose frequencies are selected to ensure smooth $G'$ and $G''$ spectra at the input frequencies (51), whereas $K_d$ is calculated from the energy at the intermediate frequencies (57) which allows the calculation of the $G'$ and $G''$ spectra as well as $K_d$ from a single measurement.

Using $K_d$, Yuan et al. (55) examined the effects of MCh on the dynamic nonlinear behavior of parenchymal tissue strips isolated from guinea pigs. They found that both $H$ and $K_d$ increased after MCh challenge or following passive stretching. The mechanical properties of the parenchyma are primarily determined by the ECM consisting of the collagen-elastin fiber network, and it is the collagen that is mostly responsible for the nonlinear behavior (50). The nonlinearity is influenced by the internal prestress on the ECM fibers that can be modulated by the tone or contraction of interstitial cells. On the other hand, the nonlinear behavior of TSM strips is likely influenced both by the ECM and the cells. Because the density of contractile cells in the TSM strip is significantly larger than in the parenchyma, one would expect that the dynamic nonlinear behavior of TSM strips is dominated by the properties of ASM cells. However, in the relaxed condition where the ASM cells are inactivated, the contribution of the ECM to the nonlinearity may be important. Moreover, disruption of the actin network in TSM caused ~30% reduction in $H$ (Fig. 4B), whereas in cell...
culture, the disruption of actin network reduces the stiffness by nearly an order of magnitude (13). Thus the contribution of the ECM to the observed nonlinearity perhaps depends on the contractile state of the muscle cells.

The possible contribution of the ECM to the mechanics of the TSM strip was investigated by using a multiscale network model which has different amounts of tissue-like spring mimicking the properties of the ECM (Fig. 8). When 25 or 75% of the muscle-like elements were randomly replaced by connective tissue-like elements, the resulting network showed first a decreasing $K_d$-$H$ relationship for small values of $H$, and an increasing $K_d$-$H$ relationship for large values of $H$ (dashed and dotted lines, Fig. 8). This suggests that the mechanical and nonlinear properties of such a network are dominated by the behavior of the muscle cells at low activation. However, once the muscle cells stiffen beyond a critical threshold, the connective tissue fibers become increasingly more stretched and their nonlinearities begin to dominate the response of the network. Estimates of the ECM content of ASM strips were reported to be between 30 and 60% (34). Therefore, one would expect a behavior somewhere in between 25 and 75% connective tissue-like spring cases. However, the measured $K_d$-$H$ relationship in TSM strips does not show an increasing $K_d$ with increasing $H$ (Fig. 6A). Thus, based on these simulation results, we speculate that the dynamic nonlinear behavior of TSM strips mostly reflects the mechanical force distribution in the cytoskeletal actin network rather than the contribution of the ECM.

In light of the above analysis, we may now interpret the results in Fig. 6 in terms of our simple network model of the actin cytoskeleton in the ASM cells (Fig. 7A). The primary assumption was that MCh induced polymerization, whereas the relaxing agents induced depolymerization of the actin network as reported previously (1, 22–24). As can be seen from the colors representing the magnitude of force in Fig. 7A, when the response to MCh was simulated, the total load was distributed among many parallel fibers. Alternatively, when the response to cytochalasin D was simulated, the total load was carried only by a few parallel fibers. Because stiffness is proportional to the number of parallel fibers, during polymerization the stiffness goes up. However, the force per fiber is reduced compared with control and the relaxed tissue. On the other hand, in the relaxed state, stiffness goes down because of the fewer number of parallel fibers, whereas force per fiber goes up. Because a higher average force on the fibers induces stronger nonlinearity, the $K_d$ decreases during contraction and increases during relaxation. We note that this dynamic nonlinear behavior is distinct from the quasistatic stiffening behavior of biological polymer networks (48). We conclude that the dynamic nonlinear behavior of TSM strips mostly reflects the average mechanical force distribution in the cytoskeletal actin network, which can be assessed using the harmonic distortion index at the level of the tissue strip.

In summary, we characterized the viscoelastic and the dynamic nonlinear properties of TSM tissues isolated from guinea pigs. We found that the basic viscoelastic properties are similar to those seen in cell culture. However, we identified important differences including the hysteretic properties and how they change in response to mechanical force or pharmacological agents. Additionally, the dynamic nonlinear behavior that has not been observed in cultured cells is likely associated with the state of the contractile prestress and may derive from active polymerization within the actin cytoskeleton. The nature of this dynamic nonlinear behavior of the ASM tissues may play a role in airway narrowing in asthma.

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