Mechanical strain increases velocity and extent of shortening in cultured airway smooth muscle cells

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ABNORMAL MECHANICAL STRESS is associated with increased mass and contractility of smooth muscle in airway, vascular, and visceral tissues. One of several mechanisms proposed to explain smooth muscle hyperresponsiveness in these disease states is that there is an increase in the contractility of individual cells. Because mechanical strain may alter multiple cell types and extracellular factors, in vivo tissue preparations are less than ideal when trying to determine the specific effects of strain on smooth muscle. Therefore, we have developed an in vitro system subjecting cultured canine airway smooth muscle (ASM) cells to mechanical strain in the form of cyclic deformational strain (passive stretch; see Refs. 22–24). In this system, strain induced increases in cell content and activity of contractile proteins (23, 24) and also increased organization of cytoskeletal elements in a manner dependent on tyrosine kinase activation (22), suggesting that mechanical stress may contribute to smooth muscle dysfunction dependent of other extracellular factors. This system eliminates many confounding variables found in vivo and so is well suited for studies of individual components of the contractile apparatus in hyperresponsive smooth muscle independent of changes in the extracellular matrix and humoral factors.

Smooth muscle contraction is initiated when the 20-kDa regulatory light chain of myosin (LC20) is phosphorylated by myosin light chain kinase (MLCK). This activates actomyosin ATPase and cross-bridge cycling, resulting in force production and cell shortening. Increases in cell content of MLCK have been predicted to increase the rate and extent of LC20 phosphorylation and thus lead to increases in the velocity of shortening of activated muscle cells (3, 6). Conversely, an increase in the content and organization of cytoskeletal elements has been predicted to act as an internal impedance to shortening, the passive elastic component (PEC; see Refs. 10 and 20). Because our previous studies have shown strain-induced increases in MLCK content, activity, and stress filament production (22–24), we compared the rate and extent of unloaded shortening of individual cells subjected to mechanical strain and otherwise identical cells grown under physically static conditions. This system measures contractility of individual cells while eliminating influence from the extracellular matrix and thus may indicate potential contributions of airway pathology specific to the muscle.

METHODS

Cell culture. Trachealis muscle was harvested from adult mongrel dogs killed after cardiac electrophysiological studies as previously described (22–24). The trachealis muscle was minced and digested in collagenase, elastase, and soybean trypsin inhibitor, filtered, and washed in Hanks' buffered saline. Cells were seeded into flasks at a density of 5 x 10⁴ cells/cm² in a 1:1 mixture of DMEM-Ham's F-12 and 10% FBS (GIBCO). Cells were passaged between days 5 and 8 when 80–90% confluent. Cells were characterized as >95% smooth muscle cells by indirect immunostaining for smooth muscle actin (Sigma, St. Louis, MO), and absence of epithelial cell contamination was confirmed by absence of staining for keratin AE1/AE11 (Boehringer Mannheim, Indianapolis, IN). Primary, first- and second-passage cells were used for the studies.

Strain protocol. To subject cells to mechanical strain, they were passaged onto flexible Silastic membranes coated with collagen type I in six-well plates (Flexcell, McKeesport, PA). The plates were then placed on a vacuum manifold triggered to cycle periodically by computer software (Flexcell). The vacuum was set to cause 10% deformation of the membranes for 2 s followed by 2 s of relaxation. This regimen was maintained continuously for 12 days. Confluent growth of the monolayer was usually noted by days 8–9, and serum-free medium was used for 48 h before contractility studies.

Single-cell contractility. All cell contractility studies were done at room temperature. To determine the rate of unloaded...
cell shortening, membranes were cut from the wells, and segments of the membrane were transferred to the moveable stage of a Nikon inverted microscope. Cells were bathed in physiological saline (in mM: 118 NaCl, 4.6 KCl, 1.2 K2HPO4, 1.2 MgSO4, 2.5 CaCl2, 22.5 NaHCO3, and 10 HEPES, pH 7.4) using a continuous flow-through system. Individual cells were identified and gently lifted off the membrane with a micromanipulator, leaving one end of the cell attached to prevent it from floating away during solution changes. To minimize movement artifact and variations in contraction due to direction of fluid movement against the cells, cells oriented parallel to the direction of the flow of the solution were selected, and the end of the cell downstream from the flow of solution was lifted from the membrane. To minimize variations in cell phenotype that might affect contractility, in both strain and control populations, cells with similar morphology (i.e., spindle shaped) and lengths (125 and 175 µm) were selected. Cells were stimulated to contract by changing the solution from physiological saline to 90 mM KCl (in mM: 30 NaCl, 90 KCl, 1.2 MgCl2, 2.5 CaCl2, 25 NaHCO3, and 1.15 dextrose, pH 7.4). The cell contraction was video recorded for later analysis. Only cells with readily visible landmarks that were not obscured by movement or folding were used for analysis, as described by Dougherty and Driska (7). Because some cells were noted to contract simply with manipulation, probably as a result of damaging the cell membrane, only cells in which shortening coincided with the addition of KCl were analyzed. Between 10 and 15 cells were recorded per experiment. In each experiment, cells from identical sources and passage not exposed to strain were used as controls.

To determine the time course of cell shortening, video recordings were played back through a computer capable of image capture (capture rate = 30 frames/s) and analysis. The computer cursor was used to draw a line through the long axis of the cell, and software computed the length of this line (Fig. 1). Cell lengths were measured from time 0 (= just before shortening) at regular intervals over 4 min. Irregular length-time curves were taken as an indication of movement artifact and discarded (7). Cell lengths are expressed as a fraction of the original length so that at any time (x), length = Lx/L0 where L0 was the original length. Because cell shortening profiles (length-time data) appeared biphasic, data were analyzed by computer software by least-squares method for best fit to a double-exponential equation (SigmaPlot; Jandel, San Rafael, CA).

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f(x) = ae^{-bx} + ce^{-dx}
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where f(x) is the relative cell length, x is in seconds, b and d are rate constants for the fast and slow components, respectively, of length change (in s\(^{-1}\)), and a and c are constants relative to the magnitude of the first and second components, respectively. The maximal velocity of shortening (\(V_{\text{max}} = 1/b\)) as well as the rate of the second, slower phase of shortening (1/d) and the length at transition between \(V_{\text{max}}\) and the second phase of shortening (1 - c) were thus calculated. The minimal length of the cell (L_{min}) was taken as the length at 4 min after response to KCl. Data from individual cells were discarded if the R value calculated for best fit by least squares to the above equation was < 0.9.

![Fig. 1. Example of cell manipulation and length measurement. Cells lying parallel to the direction of fluid flow were identified, and one end was left attached to the membrane while the remainder of the cell (filled arrow) was lifted free using a micromanipulator (open arrow). Cell images were captured and timed, and the length of a line drawn on the cell was measured by computer software.](image)

![Fig. 2. Mean length-time relationship of a population of second-passage cells subjected to 10 days of strain (n = 13) compared with identical cells not subjected to strain as control (Cont; n = 15). After being lifted from the cell membrane, cells were stimulated to contract with 90 mM KCl. Length at regular time points (L_x) is expressed as a fraction of the resting cell length (L_0); means ± SE are shown.](image)
Western blot analysis of MLCK. To determine if strain increased cell content of MLCK, strain cells were compared with control cells by SDS-PAGE and Western blotting. Monolayers were washed three times in PBS and disrupted by scraping in lysis buffer (1 mM EDTA, 3 mM Na3PO4, 20 mM MOPS, 1% Triton X-100, 1 mM MgCl2, 0.25 mM phenylmethylsulfonfyl fluoride, and 10 µg/ml leupeptin; pH 6.3). Lysates were boiled in sample buffer (50 mM Tris base, pH 6.3, 33 mM SDS, 10% glycerol, and 0.01% bromphenol blue) for 3 min and centrifuged, and the supernatant was collected. Samples were normalized for total protein and loaded onto 7.5 × 10-cm 10% polyacrylamide gels. After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore). Mouse anti-smooth muscle MLCK IgG (Sigma) was applied followed by horseradish peroxidase-conjugated goat anti-mouse IgG. The blots were developed by enhanced chemiluminescence (ECL; SuperSignal; Pierce, Rockford, IL), and proteins were detected on radiographic paper. Protein amount per lane was compared by densitometry of the radiographic images (Scion 5000; USB, Cleveland, OH).

LC20 phosphorylation. In separate experiments, MLCK activity was estimated by measuring the degree of LC20 phosphorylation after exposure of cell monolayers to contraction buffer (90 mM KCl) by the method of Hathaway and Haeberle (11) with modification. Before treatment with KCl, medium was removed, and cells were washed in buffered saline. Contraction buffer was instilled into the wells, and the reaction was stopped by aspirating the contraction buffer and quickly coating the cells with 10% TCA in acetone and 10 mM dithiothreitol (DTT). Cells were then kept at −80°C for 1 h. Protein precipitates were scraped from the wells and washed three times with water. The protein was resuspended in 8.5 M urea, 20 mM Tris, 23 mM glycine, 10 mM DTT, and 250 mM sucrose on a shaking vortexer for 1 h. Thick minigels (1.5 mm) containing 10% polyacrylamide, 5% bis-acrylamide, and 40% glycerol were prephoresed at 300 V for 1 h with 2 mM thioglycolic acid added to the reservoir buffer. Protein samples were then loaded onto the minigels with fresh reservoir buffer, DTT, and thioglycolic acid and were electrophoresed at 450 V for 2 h. Proteins were transferred to Immobilon-P paper (250 mA, 1 h) and labeled with anti-LC20 antibodies (Sigma) and peroxidase-conjugated goat anti-mouse IgG antibodies (Jackson Immunoresearch Laborato-

Fig. 3. Mean values of parameters derived by fitting the length-time relationships of contracting cells to a double-exponential equation. A: maximal velocity of shortening (Vmax); B: maximal extent of shortening (Lmin); C: rate of late velocity of shortening; D: cell length at which transition from Vmax occurred (Transition). Lx/Lo, length at time x divided by optimal length. Strain cells from primary (P), first (P1), and second (P2) passages all demonstrated a greater Vmax. There was a tendency toward greater extent of shortening in strain cells, although this reached statistical significance only in second-passage cells (P2). Transitional lengths and the delayed phase of shortening were not different between strain and control cells. *P < 0.05, t-test; 3 populations per passage, minimum 10 cells per experiment.
Although Ld cells (Fig. 3) shortening were not different between strain and control cultured ASM cells without evidence for an increase in MLCK were accompanied by increased extent of LC20 phosphorylation consistent with our earlier findings, these differences in strain cells when determined by Western blotting and ECL detection (Fig. 4; see Refs. 23 and 24). Also strain cells when determined by Western blotting and ECL detection (Fig. 4; see Refs. 23 and 24). Also that MLCK quantity was significantly greater in the strain compared with 0.007 cell length/s^2 for control; Fig. 3A). Although L_{min} tended to be less for the strain cells than for the control cells, this reached statistical significance (P < 0.05) only for second-passage cells (Fig. 3B). The rate of the second phase of shortening (Fig. 3C) and the length at transition from V_{max} to the second phase of shortening were not different between strain and control cells (Fig. 3D).

MLCK determination and LC20 phosphorylation. Findings in this study confirmed our earlier reports that MLCK quantity was significantly greater in the strain cells when determined by Western blotting and ECL detection (Fig. 4; see Refs. 23 and 24). Also consistent with our earlier findings, these differences in MLCK were accompanied by increased extent of LC20 phosphorylation in the strain cells when they were exposed to KCl (Fig. 5, A and B). The extent of LC20 phosphorylation was also greater in the strain cells at baseline before addition of KCl.

**DISCUSSION**

The major finding of this study is that mechanical strain increased the V_{max} of unloaded shortening of cultured ASM cells without evidence for an increase in the internal impedance to cell shortening. This correlated with increased cellular content of MLCK, which in turn accounted for increased phosphorylation rates of the regulatory light chain of myosin (LC20). Strain-induced increases in MLCK content and corresponding increases in the extent of phosphorylation of LC20 are consistent with our previous studies showing increased MLCK content and LC20 phosphorylation and adds to these findings by correlating them with increased shortening velocities (23, 24). In these previous studies, the increase in LC20 phosphorylation was in proportion to increases in MLCK content so that specific activity did not appear to account for increased kinase activity. These data are in agreement with studies correlating increased shortening velocities to increased MLCK content in whole tissue preparations (8, 13), skinned smooth muscle fibers (3), and single ASM cells (17). The use of a cultured cell system in these studies eliminates the possibilities of alterations in load from the extracellular matrix or influences from other cell types from consideration for changes in cell contractility. These mechanical parameters were consistent through two culture passages, demonstrating retention of the con-
tractile phenotype through multiple doublings of the parent cell population.

The system employed in these studies to subject cultured cells to mechanical strain has been previously described and was chosen to approximate the amount of mechanical stress imposed on ASM during normal respiratory motion of the airways (22–24). This system is limited in that the strain is imposed only on the resting surface rather than on the three dimensions encountered by cells embedded in extracellular matrix and so may underestimate the total forces on individual cells. Although the use of cultured cells eliminates many in vivo factors that influence smooth muscle function, the range of phenotype of individual cells makes it important to select cells of uniform length and morphology and to test a sufficient number of cells to ensure adequate sampling. Contractility of cultured cells has been reported in other studies (10, 16). One other study utilizing cultured ASM cells was able to correlate changes in specific contractile proteins induced by prolonged serum-free conditions with relevant mechanical parameters (17).

The increased $V_{\text{max}}$ found in the strain cells correlated with increased cell content of MLCK and LC$_{20}$ phosphorylation. Because the activity of MLCK determines the rate and extent of LC$_{20}$ phosphorylation, which in turn is necessary for cross-bridge cycling, the velocity of shortening is believed to reflect the extent of MLCK activity (3, 14).

The relationship of the extent of LC$_{20}$ phosphorylation with changes in $V_{\text{max}}$ has also been reported in isolated chicken gizzard smooth muscle cells, with a maximal effect when the extent of LC$_{20}$ phosphorylation reached 65%, comparable to our findings (3). Increased ASM cell content of MLCK may have clinical relevance to disease states as evidenced by animal studies showing similar increases in ASM function and contractile enzymes induced by immunosensitization, which accompany increases in $V_{\text{max}}$ (2, 8, 13), and from limited clinical observations (1, 5). Although there are insufficient data to confirm changes in MLCK levels as causative in all ASM disease, more rapid contraction of the muscle (increased $V_{\text{max}}$) as a result of increased MLCK has been suggested as an explanation for clinically evident impairment of normal stretch-induced relaxation in asthmatics (21).

Mechanisms other than increases in MLCK content that may contribute to increased $V_{\text{max}}$ and LC$_{20}$ phosphorylation include decreased myosin phosphatase activity. We have reported such decreases in myosin phosphatase activity in ASM cells exposed to strain (24). There is evidence for strain-induced Rho activation (25), which may decrease myosin phosphatase activity through Rho kinase (15). Because total phosphorylation of LC$_{20}$ would be a sum of MLCK and myosin light chain phosphatase activities within the cell, decreases in phosphatase activity may contribute to more rapid shortening. Mechanical strain also increases the content of smooth muscle-specific isoforms of myosin heavy chain (19, 24). However, velocity of shortening appears to be independent of the amount or the isoforms of smooth muscle myosin heavy chain expressed (14, 18). Increased myosin content per cell would instead be predicted to increase the magnitude of force rather than the velocity of shortening.

The hypothesis that strain-induced organization of cytoskeletal elements might decrease the extent of cell shortening by increasing an internal impedance to shortening from the PEC (10, 20) was not supported in this study. Recently, studies have shown that cell stiffness determined by impedance to twisting of magnetic microspheres adhered to cell membrane increases with organization of stress filaments by pharmacologically induced cell contraction (12). An increase in impedance from the PEC of the strain cells in the present study may have been obscured because of the inertial effects of increased LC$_{20}$ activation and increased shortening velocity. Activation of LC$_{20}$ might then need to be normalized before increased impedance to shortening could be assessed. It is also conceivable that parallel orientation of stress fibers along the long axis of the cell rather than random array seen in nonstressed ASM cells allows more efficient contraction rather than impeding shortening. The methods used in this study to test the contribution of stress filaments to an internal load of contraction also have limitations. When cells are lifted from the substratum, focal adhesions must be broken and may cause stress fibers to disassemble. Finally, the internal load of smooth muscle cells is thought to be a function mainly of intermediate filaments (4, 20). Although we and others have reported increased organization of stress fibers, we were unable to find increased numbers of intermediate filaments on transmission micrographs of strain ASM cells and only small differences in the components that compose intermediate filaments (desmin and vimentin), so there was no evidence that the filaments proposed to compose the PEC are increased by strain (22).

Both clinical observations and laboratory studies suggest that, in certain diseases, abnormal mechanical stress increases both mass and contractility of smooth muscle. Although increased mass of smooth muscle is seen in these diseases, there is debate as to the cellular mechanisms for increased smooth muscle contractility. Alterations in the tissue load against which the ASM contracts have also been proposed as a mechanism for hyperresponsive airways (5). Use of cultured cells demonstrates that increases in ASM contractility can occur independently of extracellular matrix components and are attributable to intrinsic changes of the cells. The system used in these studies offers the advantages of studying these questions without confounding variables found with either animal models or tissue strips.

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REFERENCES


