Physical Integrity of Smooth Muscle Myosin Filaments is Enhanced by Phosphorylation of the Regulatory Myosin Light Chain

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Key Words

Myosin filament assembly • Atomic force microscopy
• Ultrasonic agitation

Abstract

Background and Aims: Smooth muscle myosin monomers self-assemble in solution to form filaments. Phosphorylation of the 20-kD regulatory myosin light chain (MLC20) enhances filament formation. It is not known whether the phosphorylated and non-phosphorylated filaments possess the same structural integrity. **Methods:** We purified myosin from bovine trachealis to form filaments, in ATP-containing zero-calcium solution during a slow dialysis that gradually reduced the ionic strength. Sufficient myosin light chain kinase and phosphatase, as well as calmodulin, were retained after the myosin purification and this enabled phosphorylation of MLC20 within 20-40s after addition of calcium to the filament suspension. The phosphorylated and non-phosphorylated filaments were then partially disassembled by ultrasonification. The extent of filament disintegration was visualized and quantified by atomic force

microscopy. **Results:** MLC20 phosphorylation reduced the diameter of the filaments and rendered the filaments more resistant to ultrasonic agitation. Electron microscopy revealed a similar reduction in filament diameter in intact smooth muscle when the cells were activated. **Conclusion:** Modification of the structural and physical properties of myosin filaments by MLC20 phosphorylation may be a key regulation step in smooth muscle where formation and dissolution of the filaments are required in the cells' adaptation to different cell length.

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Introduction

Smooth muscle myosin filaments in solution is known to be less stable than their counterparts in skeletal muscle, and it has been shown that the filaments dissolve at physiological levels of MgATP, ionic strength and pH, and that phosphorylation of the 20-kD regulatory myosin light chain (MLC20) stabilizes the filaments [1]. This may

account for the evanescence of myosin filaments *in vivo*, evidenced in the increase in filament mass upon activation of some smooth muscles [2-7]. *In vitro* studies reveal that the non-phosphorylated myosin monomers exist in a folded form, especially at low ionic strength [8-10], and phosphorylation of the light chain converts the folded myosins into extended ones, which are able to form filaments [11, 12]. *In vitro* studies also reveal that assembly of non-phosphorylated myosin monomers into filaments in solution is dependent on the monomer concentration; once a critical concentration is reached, self-assembly of filaments occurs [13]. The required critical concentration increases when ATP is present in the solution and decreases when the MLC20 is phosphorylated [10, 13]. Cross et al [14] have proposed that a tail-tip to tail-tip association of two myosin monomers is responsible for forming an anti-parallel dimer which then serves as a nucleation site for filament growth. The filaments formed in this manner may exist in two conformations, a closely packed tight conformation in equilibrium with a loosely packed conformation [14]. It is not known whether these two conformations are associated with the state of MLC20 phosphorylation.

It is increasingly recognized that myosin filament evanescence could be an important aspect of smooth muscle's adaptation to large changes in cell length [15, 16]. It is also increasingly accepted that the *in vitro* behavior of myosin filaments may have *in vivo* implications, and that MLC20 phosphorylation *in vivo* may have multiple functions including activation of actomyosin (cross-bridge) interaction [17, 18] and filament formation [19]. In this study, we examine the effects of MLC20 phosphorylation on myosin filament morphology in both intact cells and in solution, and physical integrity of the filaments in solution.

Materials and Methods

Experimental Procedures

The overall experimental procedure is briefly described in this section; detailed procedures for specific methods are described under separate subtitles. Fresh bovine tracheas were obtained from a local abattoir immediately after the animals were sacrificed. The smooth muscle tissue was carefully dissected and stored in ice-cold physiological saline solution (PSS) with the following composition (in mM): 118 NaCl, 4.5 KCl, 1.2 NaH₂PO₄, 22.5 NaHCO₃, 2 MgSO₄, 2 CaCl₂, and 2g/l dextrose. The tissue pieces were pulverized in liquid nitrogen and, after thawing, extensively washed to prepare myofibrils from which actomyosin was extracted according to the methods of Sobieszek and Bremel [20]. This actomyosin was immediately

subjected to ammonium sulphate fractionation to separate myosin and actin filament fractions as described previously by Sobieszek [21].

Myosin filaments were formed from purified myosin monomers in solution during slow dialysis that gradually decreased the ionic strength of the solution. Phosphorylated and non-phosphorylated myosin filaments were grown in solutions with or without calcium. To assess filament stability, suspensions of phosphorylated and non-phosphorylated myosin filaments were subjected to brief ultrasonication and the extent of filament breakdown was visualized and quantified with atomic force microscopy (AFM). The filament length was measured using ImageJ 1.36b software (National Institutes of Health, Bethesda, USA). The time course of MLC20 phosphorylation was determined using urea/glycerol gel [22].

For visualization of myosin filaments in intact ovine trachealis using electron microscopy, smooth muscle cell bundles were dissected from the tracheas. The muscle strips were then preconditioned (equilibrated) in PSS (37°C, aerated with 95% O₂, 5% CO₂ to maintain a pH of 7.4) for one hour during which the muscles were stimulated electrically once every 5 minutes for 12 seconds (bipolar sinusoidal wave, 60 Hz, 20 V). For muscle strips fixed in the activated state, acetylcholine (10⁻⁴ M) was used for activation (after the muscles had been preconditioned) and the muscles were fixed at the plateau of contraction 2 minutes after introduction of acetylcholine into the muscle bath. The reason that ovine (instead of bovine) tissue was used for the EM examination was because the ovine tracheal smooth muscle layer possesses the ideal thickness for chemical fixation. The bovine tracheal muscle tissue was chosen for myosin purification because large amount of smooth muscle tissue could be obtained per trachea.

Purification of myosin

The purification procedure was modified from that described by Sobieszek [21]. Briefly, smooth muscle layer from bovine trachea was dissected and frozen in liquid nitrogen in small pieces. The pieces were then pulverized with mortar and pestle into a fine powder. After thawing the powder was resuspended in Bis Wash (BW) buffer (composition in mM: 40 KCl, 2 MgCl₂, 10 imidazole, 0.5 DTT, 0.5% Triton X-100, and 10 Tris base with pH adjusted to 6.6 at 4°C) using a Polytron® (a type of tissue grinder from Kinematica, Newark, USA). The tissue homogenate was spun down at 39100 x g for 30 minutes at 4°C. The resulting pellet was homogenized again in BW buffer containing 0.3% Triton and spun down as before. The resulting pellet was then homogenized in Low-ionic-strength Acto-Myosin Extraction Solution (LAMES) (composition in mM: 90 KCl, 2 EDTA, 2 EGTA, 7.5 Na₂ATP, 1 DTT, and 40 imidazole with pH adjusted to 7.2 at 4°C) and spun down. The resulting supernatant contained relatively pure actomyosin. To facilitate the separation of myosin from actin, ATP was replenished in supernatant and an excess of MgCl₂ (50 mM) was added. Actin and other contaminants were precipitated with ammonium sulphate at 40% saturation and the suspension was spun down as before. The remaining supernatant containing relatively pure myosin was precipitated at 60% ammonium sulphate saturation. The resulting pellet was

dissolved in minimal amount of BW buffer. The myosin solution was then dialyzed against 50% BW buffer overnight and then full BW buffer for an additional 3 hours. The suspension was spun down at 29325xg for 30 minutes at 4°C and the resulting pellet was re-suspended in BW buffer. In order to obtain pure myosin this process was repeated twice. Finally, the myosin pellet was frozen with liquid nitrogen and stored at -80°C until use.

Formation of myosin filaments

Frozen myosin monomers (1.3mg/ml) were dissolved in a high ionic strength solution (composition in mM: 5 EGTA, 1 Mg^{2+} , 5 MgATP, and 10 PIPES, sufficient KCl to make 500 mM ionic strength, with pH adjusted to 7.0 at 25°C). This solution was modified to create relaxing and activating solutions. In the relaxing solution, 5 mM staurosporine (Sigma-Aldrich) and 5 mM wortmannin (Sigma-Aldrich) were added to prevent MLC20 phosphorylation (relaxing solution); nothing was added to the activating solution at this point. The myosin solutions (both the relaxing and activating solutions at high ionic strength) were then dialyzed, at 4°C, against a buffer containing 2 mM $MgCl_2$ over 17 hours during which the ionic strength was decreased from 500 mM to 88 mM. Formation of myosin filaments was allowed to continue for an additional 7 hours. After dialysis, 5 mM ATP was added to the relaxing solution, whereas 100 nM microcystin (Sigma-Aldrich), 2 mM $CaCl_2$, and 5 mM ATP were added to the activating solution to induce MLC20 phosphorylation. Two minutes after introduction of calcium to the activating solution, both relaxing and activating solutions (containing myosin filaments) were divided into two portions, one was subjected to a 10-second ultrasonication (5.1 W) with Vibra-Cell™ ultrasonicator (Sonics & Materials Inc, Danbury, USA) at 4°C, the other was not sonicated (control). Aliquots of 50 μ l (out of ~1 ml) from each of the 4 solutions (relaxing, relaxing sonicated, activating, activating sonicated) were taken for AFM imaging, after dilution to the desired protein concentration.

Determination of MLC20 phosphorylation

MLC20 phosphorylation was determined by ^{32}P incorporation from radioactive ATP (see Sobieszek et al. [23]) that was used in parallel with polyacrylamide urea-glycerol gel electrophoresis [22]. Briefly, to the myosin filament suspension, containing 0.1 mM $CaCl_2$, $\gamma^{32}P$ -ATP was added (0.5-1.0 mM) to initiate phosphorylation reaction. At the preset time points, 100 μ l of the mixed suspension was withdrawn with a positive displacement pipette and quenched in a tube containing 50 μ l of quenching solution (8.5 M urea and 40 mM of 4-(hydroxymercuril) benzoic acid) with an instant mixing on a vortex. 140 μ l of the quenched mix was spotted on 2 x 4 cm pieces of Wathman 3 MM chromatographic paper pieces that were processed for radioactivity counting as described previously [24]. After a 2-fold dilution with 8.5 M urea, the remaining 20 μ l of the mix was used for urea-glycerol PAGE analysis of the maximal phosphorylation levels of the regulatory light chain of myosin [22]. The gels were run at constant current of 10 mA for 6.5 hours using the BioRad Minigel apparatus. The bands were stained with GelCode® Blue Stain Reagent

Fig. 1. Myosin molecules from bovine tracheal smooth muscle. Purified myosin at 1 μ g/ml were diluted in relaxing solution at high ionic strength (500 mM salt) and settled onto a mica surface before they were fixed with 0.1% glutaraldehyde. The image was obtained using atomic force microscope (AFM) in tapping mode. The myosin heavy chains (grey curvy lines, ~170 nm) are seen with globular myosin heads (bright spots) attached to one end. Other spots without tails are contaminant or partially degraded proteins in the sample. Grey scale: 0-15 nm. Image size: 2 x 2 μ m.

(Pierce Biotechnology, Rockford, USA) and analyzed with an Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, USA).

Atomic force microscopy (AFM)

Myosin monomers and/or filaments were deposited on freshly cleaved mica surface for AFM imaging. Mica surface was first treated with 5 mM $MgCl_2$ for 3 minutes, and then rinsed thoroughly with distilled water, and blow dry with nitrogen gas. This gave the surface a slightly positive charge that facilitated protein adhesion onto the mica surface. Aliquots of 50 μ l of the sample solution were then pipetted onto the mica and, 5 minutes later, 10 μ l of 1% glutaraldehyde (in either relaxing or activating solution, depending on the experimental condition) were added onto the mica and let stand for additional 3 minutes. The mica was then rinsed with 90 mM ammonium acetate and then blown dry with nitrogen. Finally, the samples were placed under AFM (BioScope™, Veeco Metrology Group, Chadds Ford, USA) for imaging in either contact or tapping mode.

Electron microscopy (EM)

Tissue fixation for EM has been previously described by us [25, 26]. Briefly, after preconditioning (described above),

Fig. 2. Myosin filaments from bovine tracheal smooth muscle imaged by AFM in scanning mode. The filaments were formed in solution from purified myosin molecules in activating solution at low ionic strength (80 mM) at a concentration of 2 $\mu\text{g/ml}$. a) Height image, grey scale: 0-20 nm. b) Same image as in a, but produced from displacement signal of scanning tip of the AFM. This type of image eliminates the effect of unevenness of mica surface and produces a cleaner picture. Image size: 6.7 x 6.7 μm .

muscle preparations were fixed with the primary fixing solution (see below for details) for 15min while they were still attached to the experimental apparatus that monitored isometric force. Care was taken not to physically perturb the tissue during the initial fixation. The tissue was then removed from the apparatus and cut into small cubes and immersed in the primary fixing solution for an additional 2hrs at 4°C. The primary fixing solution contained 2% glutaraldehyde, 2% paraformaldehyde, and 2% tannic acid in 0.1 M sodium cacodylate buffer. In the process of secondary fixation, the small tissue cubes were put in 1% OsO_4 in 0.1 M sodium cacodylate buffer for 2hrs. The tissue was then stained with 1% uranyl acetate, dehydrated with increasing concentrations of ethanol and embedded in resin (TAAB 812 mix). The resin blocks were sectioned with a diamond knife to obtain sections of ~ 90 nm of thickness. The sections (on copper grids) were further stained with 1% uranyl acetate and Reynolds lead citrate. Images of thin sections of smooth muscle cells were obtained using a FEI Technai 12 Twin transmission electron microscope.

Diameters of the myosin filaments were measured in micrographs of cell cross-sections with the help of the image analysis program Image Pro-Plus 3.0.

Statistical Analysis

For analysis of distributions of filament lengths, Kruskal-Wallis test (JMPIN; SAS Institute Inc., Cary, USA) was used to evaluate the significance of differences among the distributions. Comparison of means was done by ANOVA. Values reported in this study were means $\pm$ standard errors of the means.

Results

Purification of myosin and filament assembly

As described in Methods, a purified native-like myosin preparation was obtained from bovine tracheal smooth muscle. To check the quality of the myosin molecules after the purification process, the molecules were imaged under atomic force microscope (AFM). Fig. 1 shows individual non-phosphorylated myosin molecules in solution of high ionic strength (500 mM salt). The head and tail regions of the molecules can be easily identified, suggesting that the molecules remained intact in terms of their secondary and tertiary structures.

The purified myosin molecules were able to form filaments when the ionic strength of the solution was lowered, especially when the MLC20 was phosphorylated, as shown in Fig. 2. The purified protein also contained calmodulin, myosin light chain kinase and phosphatase, and the myosin filaments could be phosphorylated by addition of calcium, and dephosphorylation occurred when MgATP became depleted over time due to its hydrolysis by myosin. The time course of phosphorylation is shown in Fig. 3, which indicates that in our preparation, MLC20 phosphorylation reaches a maximal plateau between 0.5-10min. The experiments described immediately below were performed within this time period.

Fig. 3. Time course of myosin phosphorylation and dephosphorylation determined by urea-glycerol PAGE (A) and $\gamma^{32}\text{P}$ incorporation in radioactive counts per thousand per minute (cpm $^{-3}$) (B). MLC20a and MLC20b are two forms of non-phosphorylated MLC20. P-MLC20a and P-MLC20b are two forms of phosphorylated MLC20. MLC17a and MLC17b are two forms of essential light chain of the myosin.

Testing the physical integrity of myosin filaments

Filaments with phosphorylated and non-phosphorylated MLC20 were subjected to a brief period (10s) of ultrasonication, and the extent of filament disassembly was examined under AFM. Greater extent of filament breakdown in the non-phosphorylated group compared with the phosphorylated group is shown in Fig. 4. The breakdown was quantified by measuring the distribution of filament length before and after ultrasonication in both groups, and the results are summarized in Fig. 5. The total number of filaments measured for each group was 6269 (non-phosphorylated), 10105 (non-phosphorylated and sonicated), 4555 (phosphorylated), and 7532 (phosphorylated and sonicated). Because the experiments were repeated 3 times using tracheal smooth muscle from 3 animals, the measurements from each animal were first averaged before the means ($n=3$) were averaged.

Measurement of the number and diameter of phosphorylated and non-phosphorylated filaments

As shown in Fig. 6a and 6b, myosin filaments formed in the relaxing solution appear to have larger diameters than those formed in the activating solution. Also, the number of filaments in the activating solution appears to be greater compared with that in the relaxing solution. Counting the number of filaments in $10 \times 10 \mu$ areas (images) randomly sampled by AFM, we found a significant increase in filament number in the activating solution (169 ± 4) compared with that in the relaxing solution (50 ± 2). (20 images were used for each group that included data from 2 separate experiments using different animals, $p < 0.05$).

Measurements of the width of the filaments confirmed that the difference was statistically significant ($p < 0.05$). The results are summarized in Fig. 7a. We have noticed in our electron micrographs of cross-sections of

Fig. 4. Effect of brief ultrasonification on myosin filament stability. a) Filaments formed in relaxing solution with a protein concentration of 2 $\mu\text{g/ml}$. b) Same filaments as in a, after 10s of ultrasonification. c) Filaments formed in activating solution with a protein concentration of 0.5 $\mu\text{g/ml}$. d) Same filaments as in c, after 10s of ultrasonification. Image size: 10 x 10 μm . Grey scale: 0-25 nm.

Fig. 5. Distribution of filament length before and after ultrasonification in terms of fractions of the total number of myosin filaments measured versus the filament length under the 4 conditions described in Fig. 4.

airway smooth muscle that the diameters of myosin filaments in cells fixed in the relaxed state appear larger compared with those fixed in the activated state [27]; an example is shown in Fig. 6c, and 6d. We however have never quantified this difference. In this study we measured the diameter of 120 myosin filaments in EM cross-sections

in 3 cells (40 per cell) from tracheal smooth muscle fixed in the relaxed state and equal number of filaments in cells fixed in the activated state, and showed that the difference was statistically significant ($p < 0.05$). The results are summarized in Fig. 7b. The absolute diameters of the two groups are different. Over estimation of diameter

Fig. 6. Myosin filaments imaged by AFM and electron microscopy (EM) showing the difference in filament diameter in the relaxed and activated state. a) Filaments formed in relaxing solution at low ionic strength (88 mM salt, 0.5 $\mu\text{g}/\text{ml}$). Image size: 5 x 5 μm . b) Filaments formed in activating solution under the same conditions. Image size: 5 x 5 μm . c) Electron micrograph showing a cross-section of a ovine tracheal smooth muscle cell fixed in the *relaxed* state. Image size: 0.5 x 0.5 μm . d) Electron micrograph showing a cross-section of a ovine tracheal smooth muscle cell fixed in the *activated* state. Arrows indicate some selected myosin filaments. Image size: 0.5 x 0.5 μm .

for filaments formed in solution by AFM is an artifact due to the relatively large scanning tip of the AFM compared with the real filament diameter. The diameter ratios (activated/relaxed) are 0.66 and 0.63 for the filaments in solution and in intact cells, respectively. The height measurements by AFM indicated that the average diameter of the filaments laying flat on mica was slight less than 20 nm.

Discussion

The role of phosphorylation of MLC20 in activating the actomyosin interaction in smooth muscle is well known. For myosin in solution, its phosphorylation promotes formation of myosin filaments [10, 13]. The *in vivo* significance of this *in vitro* effect of MLC20 phosphorylation has not been fully explored. The

Fig. 7. Measurement of myosin filament diameter in the four groups described in Fig. 6. Note that the diameter is reduced in the activated state in both measurements using EM and AFM. See text for more details. “*” denotes statistically significant difference ($p < 0.05$) from relative controls (relaxed filaments).

demonstration that the number of myosin filaments increases during contractile activation in some smooth muscle cells [2-7] suggests that MLC20 phosphorylation may have a second important *in vivo* function, and that is, to facilitate formation of myosin filaments. The observation in intact airway smooth muscle that inhibition of MLC20 phosphorylation abolishes myosin filament reassembly [19] also supports the notion that the *in vitro* dependence of filament formation on MLC20 phosphorylation [10, 13] is likely taking place *in vivo*.

Structural changes and structural integrity of the phosphorylated versus non-phosphorylated myosin filaments are little known. Results from the present study demonstrated that these filaments are different, in both morphology and physical properties.

Purified myosin and filament formation

Myosin molecules from tracheal smooth muscle (Fig. 1) are morphologically similar to those from turkey gizzard [28], except, under our experiment condition, the two globular heads (S1) of the molecule were closely apposed (Fig. 1). The difference could be because of the high ionic strength of our solution; it also could be due to the intrinsic difference in the two isoforms. The close apposition of myosin heads is thought to indicate the conformation of myosin in the inactivated state [28]. We did not see the folded tails of non-phosphorylated myosin monomers [8-10], instead, we observed extended tails of the molecules (Fig. 1), as did Sheng et al [28]. This could be due to the high ionic strength solution we used, although there may be other reasons.

Slow dialysis to lower gradually the ionic strength produced long filaments with tapered ends (Fig. 2). Note that short filaments (~0.5-0.6 μm) could be formed if the ionic strength of the solution was lowered rapidly (results not shown). The short filaments are likely not the same as the longer filaments found in intact cells [29].

Structural integrity and morphology of myosin filaments

Ultrasonication was used to agitate the solution containing myosin filaments. The level of energy for breaking up the filaments was chosen so that some degree of filament disassembly could be observed in both the phosphorylated and the non-phosphorylated groups. The different extents of filament dissolution were quantified by measuring the lengths of the filaments under four conditions (see Fig. 4). We used the fractional length distributions (normalized to the total number of filaments measured) to gauge the extent of filament disassembly,

as shown in Fig. 5. The distribution shifts are all statistically significant ($p < 0.05$) except for the distributions of relaxed versus activated group ($p = 0.13$). The different distributions of the two sonicated groups demonstrated that phosphorylated myosin filaments are more resistant to ultrasonic agitation; we interpret this as due to a tighter packing of myosin molecules in the filament structure that also made the filaments more stable.

The notion of thick filaments in tight and flared (loosely packed) conformations originates from Cross et al [14]. In their observation, two myosin molecules form a dimer (tail-to-tail), and in the tight conformation, these dimers with straight tails are packed into a sheet, whereas in the flared conformation, the curvy tails result in a loosely packed filament with an increased filament diameter. Our hypothesis that phosphorylated myosin filaments are formed with more tightly packed myosin molecules is supported by the measurement of the diameters of myosin filaments in the relaxed and activated states in both synthetic filaments in solution and native filaments in intact muscle cells (Fig. 7). However, it is not clear whether the relaxed and activated filaments from our experiments are the same as the tightly and loosely packed filaments described by Cross et al [14], because the experimental conditions used in the two studies are different, and the status of MLC20 phosphorylation was not determined in the studies by Cross et al [14]. A recent study [30] provided another explanation for the difference in diameter of myosin filaments. It was suggested that the apparent smaller diameter of the filaments in the activated muscle is due to crossbridges moving away from the myosin filament backbone and attaching to the actin filaments. The resolution of our filament image was not high enough to resolve crossbridges on the filaments; our results therefore cannot offer support to their explanation. We favor the explanation that the increased filament integrity after MLC20 phosphorylation is due to a change in the mechanical properties of the filament backbone, and is related to the observed change in the filament diameter that reflects a more tightly packed filament structure.

We also suggest that the tight and loose conformations of the filaments associated respectively with phosphorylated and non-phosphorylated MLC20 occur both in solution and in intact cells. Our previous studies [25] showed that large amplitude length oscillation applied to relaxed smooth muscle strips could induce partial dissolution of myosin filaments in intact cells, implying that the filaments *in vivo* are unstable. Therefore the lability of non-phosphorylated myosin filaments may serve an important physiological role, as discussed below.

Myosin filament lability and its physiological significance:

The large volume change of hollow organs such as urinary bladder and stomach suggests that the smooth muscle cells lining the organ wall are able to function over a large length range. By fixing the rabbit urinary bladders at different volumes, Uvelius [31] has demonstrated a remarkable length range of smooth muscle cells associated with the different volumes. Many studies using different types of smooth muscle have also shown that the muscle is able to generate maximal or near maximal force over a large length range [32-35]. It is believed that structural malleability of the contractile and cytoskeletal filaments in smooth muscle is what enables the muscle to adapt and function at the extremes of cell lengths [7, 26, 34, 36-39]. A key component of the cell malleability may be related to the ability of the myosin

filaments to dissolve and reform at different intracellular locations as the cell changes its length [15], in order to maintain optimal overlap between the myosin and actin filaments and thus optimal function of the cell. If evanescence of myosin filaments is part of the mechanism of cell adaptation, then it is important that the stability of the filaments can be regulated. Here we propose that MLC20 phosphorylation is a key step in that regulation.

Acknowledgements

Special thanks to Pitt Meadows Meats Limited (Pitt Meadows, BC) for the supply of fresh bovine and ovine tracheas in kind support for this research project. This study was supported by operating grants from Canadian Institutes of Health Research (MOP-13271, MOP-4725).

References

- 1 Suzuki H, Onishi H, Takahashi K, Watanabe S: Structure and function of chicken gizzard myosin. (1978) *J Biochem (Tokyo)* 1978;84:1529-1542.
- 2 Gillis JM, Cao ML, Godfraind-De Becker A: Density of myosin filaments in the rat anococcygeus muscle, at rest and in contraction. II. *J Muscle Res Cell Motil* 1988;9:18-29.
- 3 Godfraind-De Becker A, Gillis JM: Analysis of the birefringence of the smooth muscle anococcygeus of the rat, at rest and in contraction. I. *J Muscle Res Cell Motil* 1988;9:9-17.
- 4 Watanabe M, Takemori S, Yagi N: X-ray diffraction study on mammalian visceral smooth muscles in resting and activated states. *J Muscle Res Cell Motil* 1993;14:469-475.
- 5 Xu JQ, Gillis JM, Craig R: Polymerization of myosin on activation of rat anococcygeus smooth muscle. *J Muscle Res Cell Motil* 1997;18:381-393.
- 6 Herrera AM, Kuo KH, Seow CY: Influence of calcium on myosin thick filament formation in intact airway smooth muscle. *Am J Physiol Cell Physiol* 2002;282:C310-C316.
- 7 Kuo KH, Herrera AM, Wang L, Pare PD, Ford LE, Stephens NL, Seow CY: Structure-function correlation in airway smooth muscle adapted to different lengths. *Am J Physiol Cell Physiol* 2003;285:C384-C390.
- 8 Onishi H, Wakabayashi T: Electron microscopic studies of myosin molecules from chicken gizzard muscle I: the formation of the intramolecular loop in the myosin tail. *J Biochem (Tokyo)* 1982;92:871-879.
- 9 Trybus KM, Lowey S: Conformational states of smooth muscle myosin. Effects of light chain phosphorylation and ionic strength. *J Biol Chem* 1984;259:8564-8571.
- 10 Trybus KM, Lowey SJ: Assembly of smooth muscle myosin minifilaments: effects of phosphorylation and nucleotide binding. *Cell Biol* 1987;105:3007-3019.
- 11 Craig R, Smith R, Kendrick-Jones J: Light-chain phosphorylation controls the conformation of vertebrate non-muscle and smooth muscle myosin molecules. *Nature* 1983;302:436-439.
- 12 Smith RC, Cande WZ, Craig R, Tooth PJ, Scholey JM, Kendrick-Jones J: Regulation of myosin filament assembly by light-chain phosphorylation. *Philos Trans R Soc Lond B Biol Sci* 1983;302:73-82.
- 13 Kendrick-Jones J, Smith RC, Craig R, Citi S: Polymerization of vertebrate non-muscle and smooth muscle myosins. *J Mol Biol* 1987;198:241-252.
- 14 Cross RA, Geeves MA, Kendrick-Jones J: A nucleation—elongation mechanism for the self-assembly of side polar sheets of smooth muscle myosin. *EMBO J* 1991;10:747-756.
- 15 Seow CY: Myosin filament assembly in an ever-changing myofilament lattice of smooth muscle. *Am J Physiol Cell Physiol* 2005;289:C1363-C1368.
- 16 Seow CY: Biophysics: Fashionable cells. *Nature* 2005;435:1172-1173.
- 17 Sobieszek A: Ca-linked phosphorylation of a light chain of vertebrate smooth-muscle myosin. *Eur J Biochem* 1977;73:477-483.

18	Small JV, Sobieszek A: Ca-regulation of mammalian smooth muscle actomyosin via a kinase-phosphatase-dependent phosphorylation and dephosphorylation of the 20 000-Mr light chain of myosin. <i>Eur J Biochem</i> 1977;76:521-530.	24	Sobieszek A, Babiychuk EB, Ortner B, Borkowski J: Purification and characterization of a kinase-associated, myofibrillar smooth muscle myosin light chain phosphatase possessing a calmodulin-targeting subunit. <i>J Biol Chem</i> 1997;272:7027-7033.	31	Uvelius B: Isometric and isotonic length-tension relations and variations in cell length in longitudinal smooth muscle from rabbit urinary bladder. <i>Acta Physiol Scand</i> 1976;97:1-12.
19	Qi D, Mitchell RW, Burdyla T, Ford LE, Kuo KH, Seow CY: Myosin light chain phosphorylation facilitates in vivo myosin filament reassembly after mechanical perturbation. <i>Am J Physiol Cell Physiol</i> 2002;282:C1298-C1305.	25	Kuo KH, Wang L, Pare PD, Ford LE, Seow CY: Myosin thick filament lability induced by mechanical strain in airway smooth muscle. <i>J Appl Physiol</i> 2001;90:1811-1816.	32	Harris DE, Warshaw DM: Length vs. active force relationship in single isolated smooth muscle cells. <i>Am J Physiol</i> 1991;260:C1104-C1112.
20	Sobieszek A, Bremel RD: Preparation and properties of vertebrate smooth-muscle myofibrils and actomyosin. <i>Eur J Biochem</i> 1975;55:49-60.	26	Herrera AM, McParland BE, Bienkowska A, Tait R, Pare PD, Seow CY: 'Sarcomeres' of smooth muscle: functional characteristics and ultrastructural evidence. <i>J Cell Sci</i> 2005;118:2381-2392.	33	Pratusevich VR, Seow CY, Ford LE: Plasticity in canine airway smooth muscle. <i>J Gen Physiol</i> 1995;105:73-94.
21	Sobieszek A: Smooth muscle myosin: Molecule conformation, filament assembly and associated regulatory enzymes. In: <i>Airways Smooth Muscle: Biochemical Control of Contraction and Relaxation</i> , edited by D. Raeburn and M. A. Gienbycz, Basel:Birkhauser Verlag, 1994;p. 1-29	27	Kuo KH, Herrera AM, Seow CY: Ultrastructure of airway smooth muscle. <i>Respir Physiol Neurobiol</i> 2003;137:197-208.	34	Wang L, Pare PD, Seow CY: Selected contribution: effect of chronic passive length change on airway smooth muscle length-tension relationship. <i>J Appl Physiol</i> 2001;90:734-740.
22	Sobieszek A, Jertschin P: Urea-glycerol-acrylamide gel electrophoresis of acidic low molecular weight muscle proteins: Rapid determination of myosin light chain phosphorylation in myosin, actomyosin and whole muscle samples. <i>Electrophoresis</i> 1986;7:417-425.	28	Sheng S, Gao Y, Khromov AS, Somlyo AV, Somlyo AP, Shao Z: Cryo-atomic force microscopy of unphosphorylated and thiophosphorylated single smooth muscle myosin molecules. <i>J Biol Chem</i> 2003;278:39892-39896.	35	McParland BE, Tait RR, Pare PD, Seow CY: The role of airway smooth muscle during an attack of asthma simulated in vitro. <i>Am J Respir Cell Mol Biol</i> 2005;33:500-504.
23	Sobieszek A, Andrucho OY, Grabarek Z, Kulikova N, Liebetrau C, Matusovsky OS: Modulation of myosin filament activation by telokin in smooth muscle liberation of myosin kinase and phosphatase from supramolecular complexes. <i>Biophys Chem</i> 2005;113:25-40.	29	Small V: Studies on isolated smooth muscle cells: the contractile apparatus. <i>J Cell Sci</i> 1977;24:327-349.	36	Gunst SJ, Meiss RA, Wu MF, Rowe M: Mechanisms for the mechanical plasticity of tracheal smooth muscle. <i>Am J Physiol</i> 1995;268:C1267-C1276.
		30	Smolensky AV, Gilbert SH, Harger-Allen M, Ford LE: Inhibition of myosin light-chain phosphorylation inverts the birefringence response of porcine airway smooth muscle. <i>J Physiol</i> 2007;578:563-568.	37	Fabry B, Maksym GN, Butler JP, Glogauer M, Navajas D, Fredberg JJ: Scaling the microrheology of living cells. <i>Phys Rev Lett</i> 2001;87:148102.
				38	Bursac P, Lenormand G, Fabry B, Oliver M, Weitz DA, Viasnoff V, Butler JP, and Fredberg JJ: Cytoskeletal remodelling and slow dynamics in the living cell. <i>Nat Mater</i> 2005;4:557-561.
				39	Deng L, Trepat X, Butler JP, Millet E, Morgan KG, Weitz DA, Fredberg JJ: Fast and slow dynamics of the cytoskeleton. <i>Nat Mater</i> 2006;5:636-640.