


REVIEW

Filament evanescence of myosin II and smooth muscle function

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Smooth muscle is an integral part of hollow organs. Many of them are constantly subjected to mechanical forces that alter organ shape and modify the properties of smooth muscle. To understand the molecular mechanisms underlying smooth muscle function in its dynamic mechanical environment, a new paradigm has emerged that depicts evanescence of myosin filaments as a key mechanism for the muscle's adaptation to external forces in order to maintain optimal contractility. Unlike the bipolar myosin filaments of striated muscle, the side-polar filaments of smooth muscle appear to be less stable, capable of changing their lengths through polymerization and depolymerization (i.e., evanescence). In this review, we summarize accumulated knowledge on the structure and mechanism of filament formation of myosin II and on the influence of ionic strength, pH, ATP, myosin regulatory light chain phosphorylation, and mechanical perturbation on myosin filament stability. We discuss the scenario of intracellular pools of monomeric and filamentous myosin, length distribution of myosin filaments, and the regulatory mechanisms of filament lability in contraction and relaxation of smooth muscle. Based on recent findings, we suggest that filament evanescence is one of the fundamental mechanisms underlying smooth muscle's ability to adapt to the external environment and maintain optimal function. Finally, we briefly discuss how increased ROCK protein expression in asthma may lead to altered myosin filament stability, which may explain the lack of deep-inspiration-induced bronchodilation and bronchoprotection in asthma.

Introduction

Myosin is a superfamily of motor proteins that convert chemical energy from ATP hydrolysis to mechanical work. Cyclic interaction of myosin heads with actin filaments enables muscle contraction and cell motility. Myosin exists in all eukaryotic cells. As illustrated by an unrooted phylogenetic tree (Hodge and Cope, 2000) aided by additional information from Furusawa et al. (2000) and Salamon et al. (2003), the superfamily was once considered to contain a total of 18 classes, each assigned a Roman numeral, and >130 distinct members as a result of genetic variations. This classification was based on phylogenetic analysis of the conserved myosin motorhead (Foth et al., 2006). In more recent studies with improved sequencing analysis, including that of the less conserved tail region, it has been revealed that >70 classes exist in the myosin superfamily (Kollmar and Mühlhausen, 2017). The focus of this review is myosin II, which is found in skeletal, cardiac, and smooth muscle, as well as nonmuscle cells (Chantler et al., 2010). Smooth muscle myosin II is of particular interest in this review, but some myosin isoforms from other cell types and nonvertebrate organisms are included

in the discussion to provide the readers a more comprehensive context upon which the unique features of smooth muscle myosin II can be better appreciated.

Discovered by Kühne in 1864 (Hartman and Spudich, 2012), myosin II is the most extensively studied in the superfamily and is often referred to as the conventional myosin (Sellers, 2000). It is the only class of myosin that can form functional filaments (Chantler et al., 2010) that interact with actin to produce motility in muscle and nonmuscle cells.

Myosin thick filaments, together with actin thin filaments, form contractile units in smooth muscle. Upon stimulation, the thick and thin filaments slide relatively to each other. Within the structural confines of the contractile unit arrays, filament sliding leads to shortening of individual contractile units, which in turn causes muscle contraction. In some types of smooth muscle, myosin may primarily exist as filaments, and assembly and disassembly of myosin filaments are not required in the regulation of smooth muscle function (Horowitz et al., 1994). In other types, such as airway smooth muscle, many lines of evidence support the notion that myosin exists in monomeric and

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filamentous forms in both relaxed and activated states, and a dynamic equilibrium between the pools of monomeric and filamentous myosin can be regulated to optimize the cell function (Milton et al., 2011; Chitano et al., 2017).

Smooth muscle lines the wall of hollow organs. Some of them, such as the stomach and urinary bladder, regularly undergo large volume changes, requiring the smooth muscle cells to maintain optimal contractility over a large length range. It has been postulated that myosin filament evanescence is one of the mechanisms of this mechanical plasticity (Seow, 2005). Myosin filaments in smooth muscle can “dissolve” into monomers or oligomers and can be added into or subtracted from existing contractile unit arrays during length changes, thus allowing muscle cells to adapt to a longer or shorter length while maintaining optimal overlap of the contractile filaments within each contractile unit. In intact airway smooth muscle, ~15% of myosin is nonmuscle myosin (Halayko et al., 1996; Zhang and Gunst, 2017; Chitano et al., 2017). Nonmuscle myosin is not part of the contractile unit of smooth muscle, but its assembly from monomers into filaments appears to be necessary for the recruitment of adhesion complex to the cortical cytoskeleton of smooth muscle cells, which is required for force transmission (Zhang and Gunst, 2017). Nonmuscle myosin also exhibits signs of filament evanescence, as seen in amoeba mobility (Clarke and Spudich, 1977; Yumura and Fukui, 1985), although the regulatory mechanisms in amoeba are distinct from that of nonmuscle myosin in mammalian and vertebrate smooth muscle. This will be further discussed later.

In solution, the reversible switching between the monomeric and filamentous forms of myosin is governed by multiple factors, such as pH, ionic strength, intracellular calcium concentrations, and phosphorylation states of myosin light and heavy chains. In intact muscle, changes of cell length and other mechanical perturbations, as well as Rho-kinase (ROCK) activities within the cells, are known to play important roles in the dynamic process of filament formation and dissolution. In this review, we examine the evidence for the occurrence and regulation of myosin evanescence, as well as the relevance of this intrinsic property of smooth muscle and the potential implications of dysregulation of myosin evanescence in diseases.

Myosin II structure and filament formation

As illustrated in Fig. 1, a mammalian smooth muscle myosin heavy chain (MHC) has a globular head region, a helical rod region, and a nonhelical tail region. At the base of each myosin head, there are two light chains: the essential light chain (17 kD) and the regulatory light chain (RLC; 20 kD; Rayment et al., 1993). Using enzymatic digestion with trypsin or α -chymotrypsin, fragments of myosin can be obtained as heavy meromyosin (HMM) and light meromyosin (LMM; Lowey and Holtzer, 1959). Further proteolytic digestion with papain or α -chymotrypsin cleaves HMM into S1 and S2 subfragments (Mueller and Perry, 1962). These subfragments have often been used instead of the native whole molecule in biochemical assays. The S1 fragment contains ATP-binding and actin-binding sites that are responsible for ATP hydrolysis and actin-binding that enables sliding movements between the thin and thick filaments

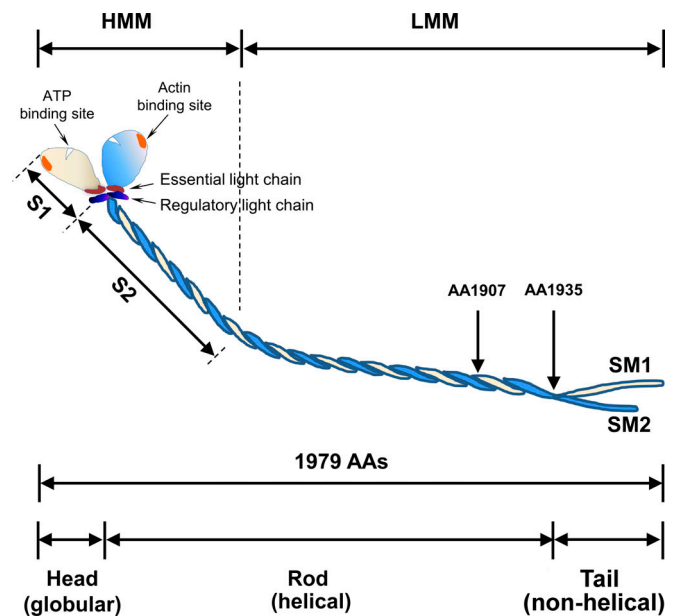


Figure 1. **Schematic diagram of a myosin II molecule.** SM1 and SM2 heterodimers are drawn for illustration purposes only. Controversy exists about whether in living cells they exist as homodimers (Kelley et al., 1992; Rovner et al., 2002) or homodimers and heterodimers by random chance (Tsao and Eddinger, 1993). S1 and S2, HMM subfragments. Adapted from Chen et al. (2018) and Kumar and Mansson (2017).

(Adelstein and Eisenberg, 1980). The genetic codes of S1 from smooth and striated muscle myosin are mostly evolutionarily conserved, with diversity in some of the amino acid (AA) sequences (Bonet et al., 1987). Studies of HMM fragments from smooth muscle have revealed important mechanisms for the phosphorylation-dependent regulation of myosin function (Seidel, 1980; Ikebe and Hartshorne, 1985b; Konishi et al., 1998; Cremo et al., 1995; Konishi et al., 2001; Ellison et al., 2003). On the other hand, the LMM fragment in both skeletal (Nyitrai et al., 1983) and smooth (Cross and Vandekerckhove, 1986) muscle has been shown to be responsible for myosin filament assembly. In fact, the important role of LMM in filament formation is seen in all types of muscle and nonmuscle myosin (Atkinson and Stewart, 1991). The AA sequence in the rod portion of the myosin molecule is highly repetitive and contains periodically distributed positively and negatively charged clusters (McLachlan and Karn, 1982) that facilitate staggered assembly of adjacent rods and give rise to the periodic spacing between the myosin heads (cross-bridges) seen on a thick filament. Four skip residuals (extra AAs) have been shown to interrupt the charge repeats on the myosin rod (Taylor et al., 2015) and change the local pitch of the coiled-coil structure and rod flexibility at that location. It is believed that the skip residuals are important in facilitating parallel and antiparallel assembly of myosin molecules into thick filaments. Also, the sequence of a 29-residue segment near the C terminus of LMM has been shown to be crucial for myosin filament formation. When this assembly competence domain is deleted from a full-length MHC (rat α -cardiac myosin), self-assembly of thick filaments is impaired (Sohn et al., 1997).

The way myosin filaments are formed could affect smooth muscle shortening, force generation (Cai et al., 1995; Sweeney, 1998), and mechanical plasticity (Seow, 2005). Regulation of filament assembly therefore has physiological consequences. The AA sequence of myosin II is of mechanistic significance in determining how the filaments are formed. As shown by cryo-electron microscopy (Scarff et al., 2020; Yang et al., 2020), each myosin molecule consists of a pair of intertwined MHC as illustrated schematically in Fig. 1. Four smooth muscle MHC isoforms have been described. In the motor domain near the ATPase site, two isoforms differ in the absence (as in tonic smooth muscle) or presence (as in phasic smooth muscle) of a 7-AA insert (Rovner et al., 1997). The two isoforms are commonly referred to as SM-A (without insert) and SM-B (with insert) and have a slight variance in AA sequence across species (Léguillette et al., 2005). The presence of the insert (SM-B) can move actin twice as fast as SM-A, as shown by in vitro motility assays (Rovner et al., 1997; Lauzon et al., 1998), but has not been reported to play a role in myosin filament assembly. At the C terminus, smooth muscle MHC contains two other distinct variants (Rovner et al., 1986; Kawamoto and Adelstein, 1987), producing isoforms of SM1 (204 kD) and SM2 (200 kD). Controversy exists over the issue of whether in living cells they exist as homodimers (Kelley et al., 1992; Rovner et al., 2002) or a random mixture of homodimers and heterodimers (Tsao and Eddinger, 1993). Mammalian smooth muscle SM1 contains a total of 1,978 AAs and is 34 AAs longer than SM2. The chicken varieties contain 1,979 AAs (Fig. 1) due to an extra residue in the tail region (Yanagisawa et al., 1987; Rovner et al., 2002). The head and rod regions consist of 1,935 AAs with the remaining in the nonhelical tail. The 1,935th AA marks the site of alternative splicing (Rovner et al., 2002) where the tail of SM1 contains 43 AAs and SM2 contains 9 AAs (Nagai et al., 1989). Using multiple monoclonal antibodies targeting different AAs in the C-terminal coiled-coil rod region of turkey gizzard smooth muscle myosin, Ikebe and colleagues (Ikebe et al., 2001) discovered that the sequence critical for the formation of filaments is within a 28-AA segment (1,907–1,935) located at the end of the LMM. Deletion of the segment increases solubility of the truncated myosin rods, similar to that observed in cardiac myosin when the assembly competent domain is deleted (Sohn et al., 1997). When this location is occupied by an antibody (mm19), myosin filament formation can be completely abolished (Ikebe et al., 2001). The immediately adjacent nonhelical tail region has also been suggested to play an important regulatory role in the filament assembly of myosin II. In vertebrate nonmuscle myosin, deletion of a 35-AA sequence in the nonhelical tail results in a 50-fold increase in the required critical concentration of rod polypeptides for filament assembly (Hodge et al., 1992). When the nonhelical tail is occupied, myosin filaments become less stable (Ford et al., 1997). In smooth muscle, Rovner and et al. (2002) have reported differential effects on myosin filament assembly by the tailpiece of SM1 and SM2 isoforms. The longer tailpiece of SM1 is associated with a greater stability in myosin filaments compared with the stability of filaments made of shorter tailpiece of SM2 (by four- to fivefold). Although the ability of SM1 and SM2 to move actin, as shown in unloaded motility assays

(Kelley et al., 1992; Rovner et al., 2002), is indistinguishable within a wide range of salt concentrations, their different abilities in maintaining myosin filament stability may play an important role in the regulation of subcellular structural malleability, a property increasingly being recognized as an intrinsic property of smooth muscle.

Chemical properties influencing myosin filament formation

The stability of myosin filaments in solution is governed by many factors, the most important of which are monomeric myosin concentration, ionic strength, pH, ATP concentration, and the state of myosin phosphorylation. The intracellular ionic strength in most organisms is ~200–300 mM (Storey, 2004). The baseline intracellular pH has been reported as 7.19 in porcine tracheal smooth muscle (Croxtton et al., 1995), 7.38 in resting human skeletal muscle (Street et al., 2001), and 7.02–7.14 in cardiac muscle from sheep, ferret, and guinea pig (Ellis and Thomas, 1976). Rovner et al. (2002) have revealed a sigmoidal relationship between percent soluble myosin and ionic strength. At pH 7.5 and in the presence of 4 mM MgCl₂, the tendency of purified unphosphorylated myosin to form filaments decreases with increasing ionic strength between 100 and 200 mM. At ionic strengths lower than 100 mM, myosin is not soluble (i.e., myosin exists exclusively as filaments). At ionic strengths higher than 200 mM, myosin mostly exists as monomers (i.e., soluble).

The presence of ATP can also influence myosin filament formation. Adding MgATP to unphosphorylated smooth muscle myosin in solution causes myosin filaments to disassemble (Onishi et al., 1978; Cross et al., 1991a, 1991b). However, lowering the pH below the physiological level can abrogate this effect of ATP. At 150 mM ionic strength and 5 mM Mg²⁺, lowering the pH from 7.0 to 6.5 or 6.2 abolishes the disassembling effect of MgATP on unphosphorylated smooth muscle myosin filaments (Kendrick-Jones et al., 1983). Finally, phosphorylation of RLC achieved by the presence of myosin light chain kinase/calmodulin-Ca²⁺ complex can provide the filaments with greater stability, such that the filaments do not disassemble even in the presence of ATP (Suzuki et al., 1978; Kendrick-Jones et al., 1983; Trybus and Lowey, 1985).

It has been suggested that myosin filament formation is a process of shifting dynamic equilibria among the folded (10S) monomers, the straight (6S) monomers, and the polymeric myosin (Fig. 2; Kendrick-Jones et al., 1987), where 10S and 6S denote the sedimentation rates in gel electrophoresis of the folded and straight monomers (10 and 6 Svedbergs, respectively). Many types of myosin, including skeletal, cardiac, and smooth muscle myosin, as well as nonmuscle myosin, can assume a folded (10S) or an extended (6S) conformation (Suzuki et al., 1978; Trybus et al., 1982; Craig et al., 1983; Smith et al., 1983; Faruqi et al., 1993; Jung et al., 2008; Yang et al., 2019; Liu et al., 2020). Smooth muscle myosin forms filaments in vitro by self-association of the rod and tail regions into flat sheets without the involvement of a core or myosin-associated proteins (Cross et al., 1991a, 1991b; Craig and Megerman, 1977). The extended 6S conformation permits the interaction between myosin rods, therefore allowing filament formation. One theory

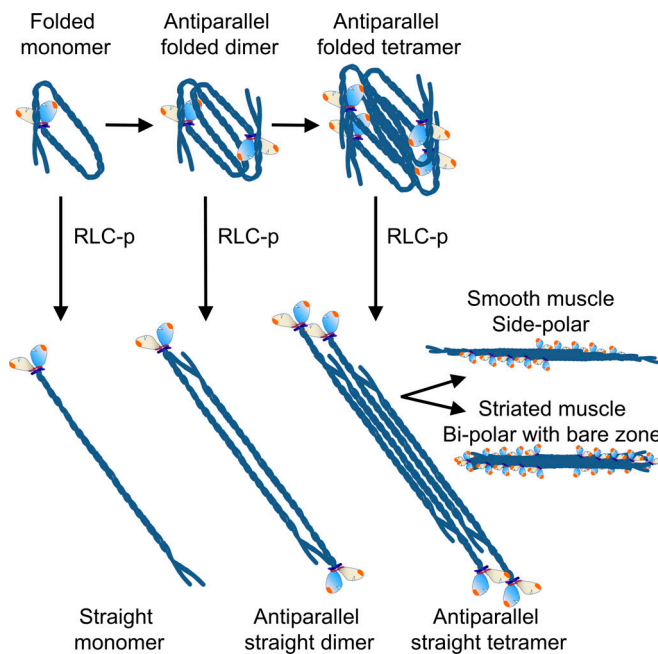


Figure 2. **Proposed model for myosin filament formation in vitro.** Unphosphorylated myosin II exists in folded monomeric form. It can assemble to form antiparallel folded dimers and then antiparallel folded tetramers. RLC phosphorylation (RLC-p) promotes unfolding. Antiparallel straight dimers and tetramers assemble to form filaments. Bipolar filaments are formed in striated muscle and side-polar filaments are formed in smooth muscle. Adapted from Dasbiswas et al. (2018), Liu et al. (2017), and Milton et al. (2011).

suggests that the intermediate 6S conformation is required for myosin filament formation and that the 10S must be converted to 6S to allow filaments to be formed (Craig et al., 1983; Kendrick-Jones et al., 1987). According to this theory, phosphorylation of the RLC converts the folded 10S into the extended 6S to favor filament formation (Craig et al., 1983). Dephosphorylation of the RLC promotes conversion of the extended 6S to the folded 10S conformation (Kendrick-Jones et al., 1987). This theory emphasizes the governing role of RLC phosphorylation in the filament formation process. An alternative theory emphasizes the roles of ionic strength and ATP concentration in determining the monomer conformation. MgATP favors the 10S conformation, while RLC phosphorylation favors the 6S conformation. However, the dependence of myosin monomer conformation on RLC phosphorylation is observed only within the range of 100–300 mM of salt concentration (Trybus and Lowey, 1984). Above 300 mM, all monomers unfold to become the extended 6S variety whether the RLC is phosphorylated or not. Below 100 mM, the monomers remain in the folded 10S conformation without reverting to the extended 6S even when RLC is phosphorylated. Indeed, it has been observed that not all the folded 10S have to be converted to the extended 6S conformation to form filaments (Trybus and Lowey, 1984) and 10S monomers can be phosphorylated and remain folded (Ikebe et al., 1983). According to this theory, the role of RLC phosphorylation is to promote myosin assembly into antiparallel dimers of 6S myosin, which become the unit structures for side-polar filaments (Craig and Megerman, 1977; Hinssen et al., 1978; Cross et al., 1991a). The

theory is consistent with recent observations of various folded conformations in nonmuscle, skeletal, cardiac, and smooth muscle myosin based on electron microscopy (Liu et al., 2017, 2018, 2020).

Fig. 2 summarizes the current understanding of the process of myosin II filament formation in vitro, according to the two theories described above. At ionic strength and pH around the physiological level, unphosphorylated myosin from skeletal, cardiac, nonmuscle, and smooth muscle forms folded monomers (with two hinge points forming a folded three-segment structure; Smith et al., 1983). Folded monomers can form antiparallel folded dimers. Antiparallel folded dimers then form antiparallel folded tetramers (see top row of Fig. 2). Because a specific region in the tail is required for filament formation and is not exposed in the folded conformation, ATP inhibits polymerization by favoring the folded 10S conformation. The presence of ATP reduces myosin polymerization in smooth muscle much more profoundly than it does in skeletal and cardiac muscle. This is one of the reasons smooth muscle myosin filaments are less stable in vivo compared with those of striated muscle. RLC phosphorylation opposes the effect of ATP and favors unfolding of any tertiary structures of monomers, dimers, or tetramers. Upon RLC phosphorylation, folded antiparallel tetramers unfold to allow more folded tetramers to bind and unfold. Multiple unfolded tetramers then entwine to form mature, longer filaments (Smith et al., 1983). Interestingly, within the first few seconds of polymerization, smooth muscle myosin can also form bipolar filaments with a central bare zone and skeletal muscle myosin can form filaments without a central bare zone (Liu et al., 2020), confirming the findings from previous studies (Koretz, 1979; Kaminer et al., 1976). A few hours into the polymerization process, skeletal muscle myosin forms the well-known bipolar filaments, while smooth muscle myosin forms side-polar thick filaments (Huxley, 1963; Craig and Megerman, 1977; Xu et al., 1996). Even though the most recent studies have revealed different intermediate structures in the process of filament formation, the opposing effects of ATP and RLC phosphorylation on filament formation remain true as described earlier. Using atomic force microscopy (Ip et al., 2007), it has been observed in solution that phosphorylated myosin filaments, purified from bovine tracheal smooth muscle, are thinner in diameter compared with unphosphorylated filaments; this perhaps reflects the transition of folded to straightened intermediate structures upon phosphorylation. They are also more resistant to physical agitation, such as that introduced by ultrasonication (Ip et al., 2007), suggesting greater structural integrity of phosphorylated myosin filaments.

Evidence for two intracellular pools of myosin

Polymerization and depolymerization of myosin in live cells can occur rapidly. Using immunoelectron microscopy, it has been observed that exposing the chemotactic stimulant cAMP to live *Dictyostelium discoideum* amoeba triggers a complete disappearance of myosin filaments within 2 min, followed by reappearance of the filaments in the next minute (Yumura and Fukui, 1985). This phenomenon of myosin evanescence can be explained by a transient cAMP-induced predominance of

phosphorylated MHC (Malchow et al., 1981). Since MHC phosphorylation prevents myosin filament formation (Kuczmarski and Spudich, 1980), onset of the chemotactic response is characterized by the disappearance of filamentous myosin. Rapid reappearance of filamentous myosin within seconds or minutes excludes the possibility of significant de novo protein synthesis and suggests that there is a reservoir of myosin monomers in the cell. The process of disappearance and reappearance of myosin filaments can be seen as evidence for the dynamic interconversion between filamentous and monomeric myosin populations.

The mechanism of myosin filamentogenesis in *D. discoideum* is different from that in vertebrate nonmuscle and smooth muscle cells. In *D. discoideum*, phosphorylation of Ser and Thr residues in the tail region of MHC destabilizes filaments, whereas dephosphorylation of these residues promotes assembly of myosin filaments (Kuczmarski and Spudich, 1980; Lück-Vielmetter et al., 1990; Egelhoff et al., 1993). Although it has been shown that RLC phosphorylation at Ser13 (Ostrow et al., 1994) regulates *D. discoideum* motor function (Griffith et al., 1987; Bosgraaf and van Haastert, 2006), a role for RLC phosphorylation in myosin II filament formation has not been described for this organism. In contrast, RLC phosphorylation has been shown to regulate filament formation in mammalian nonmuscle cells (Craig et al., 1983; Sellers and Heissler, 2019; Breckenridge et al., 2009) as well as in smooth muscle cells (Ikebe, 2008). In mammalian migratory cells, for example, a synchronized RLC phosphorylation at multiple sites controls the transient myosin filament assembly and disassembly during lamellipodia protrusion in wound healing (Aguilar-Cuenca et al., 2020). Upon growth factor stimulation, RLC at the lamellipodial edge becomes tyrosine phosphorylated at Y155. Phosphorylation at Y155 prevents the interaction between RLC and myosin hexamers, which is required for filament stabilization. As a result, myosin filaments disassemble into a reservoir of soluble, nonfilamentous myosin. As the response to growth factor proceeds, phosphorylation at Y155 plateaus while RLC phosphorylation at Ser19 becomes dominant. This drives myosin polymerization from the reservoir of soluble myosin (Aguilar-Cuenca et al., 2020).

The dynamic interconversion between filamentous and monomeric populations in smooth muscle cells is an important mechanism underlying smooth muscle function. If in vitro findings could be directly applied to living muscle cells, where ionic strength is ~200 mM and MgATP is present at ~5 mM, myosin molecules should primarily exist as monomers. However, filamentous myosin has been seen in smooth muscle cells in both activated and relaxed states (Somlyo et al., 1981; Qi et al., 2002). This has led to the following questions: does filamentous myosin exist in equilibrium with monomeric myosin within a smooth muscle cell, and could contractile states and other intracellular factors shift the equilibrium? Using antibodies probing for monomers (Trybus and Henry, 1989), it has been shown in intact gizzard smooth muscle that there is no difference in the amount of monomeric myosin in the relaxed or contracted state and that there is no increase in monomer concentration when the muscle is relaxed after contraction (Horowitz et al., 1994). One could conclude from these studies

that myosin exists in cells primarily in a filamentous form and the equilibrium between filamentous and monomeric myosin is static. However, other studies suggest otherwise. In anococcygeus muscle it has been found that myosin polymerization occurs during contractile activation and depolymerization occurs during relaxation (Gillis et al., 1988; Godfraind-De Becker and Gillis, 1988; Xu et al., 1997). It must be pointed out that in guinea pig taenia coli (Watanabe et al., 1993; Xu et al., 1997), no evidence of myosin filament evanescence has been found. It appears, therefore, that the extent of shift in the filament-monomer equilibrium associated with contraction and relaxation in smooth muscle is cell-type specific.

In cultured human airway smooth muscle, there is direct evidence for a significant pool of monomeric myosin in dynamic equilibrium with filamentous myosin (Milton et al., 2011). Using two antibodies, one specifically against smooth muscle monomeric myosin and the other differentially against smooth muscle filamentous myosin, as well as a peptide that prevents interaction between head and tail of folded 10S monomer, two distinct pools of myosin can be identified in cultured human airway smooth muscle cells as filamentous myosin and 10S folded monomers. By manipulating buffer composition and pH, 15–20% of total smooth muscle myosin can be converted from the monomeric pool to the filamentous pool and vice versa (Milton et al., 2011).

Distinct pools of filamentous and monomeric myosin have also been found in intact smooth muscle. In airway smooth muscle tissue preparations, we have discovered that the level of baseline RLC phosphorylation is inversely proportional to the resting muscle length (Chitano et al., 2017). Using immunostaining with mm19 antibody specific for monomeric myosin, we found significantly lower concentrations of monomers in muscle preparations fixed at longer resting lengths compared with that of muscle fixed at shorter lengths (Chitano et al., 2017). We postulate that since smooth muscle cells adapted to a longer length have more contractile units (Kuo et al., 2003), myosin from the monomeric pool could be recruited into the contractile filament lattice, where formation of filaments is favored. When smooth muscle cells are adapted to a shorter length, fewer contractile units are needed, and this could result in depolymerization of myosin filaments. Regardless of whether our interpretation is correct, the fact is, we have found a baseline concentration of myosin monomers in resting muscle, and the monomer concentration in this pool is changeable under physiological conditions (Chitano et al., 2017).

For the pool of filamentous myosin, an unanswered question is whether the myosin filaments of smooth muscle have a uniform length, like the bipolar thick filaments of striated muscle. By following thick filaments in electron micrographs of serial cross sections with thickness of 400–500 nm, Ashton et al. (1975) have found the filament length to be ~2.2 μm for rabbit vascular smooth muscle. Later studies have reported that the thick filament length measured from longitudinal sections of electron microscopy to be mostly <3 μm , but filaments as long as 8 μm have also been observed (Small, 1977); in contracted chicken gizzard cells, the filament length has been found to be ~1.6 μm in longitudinal sections (Small et al., 1990). One

problem with measuring filament length in longitudinal sections is that if the filament does not lie perfectly parallel to the surface of the section, the filament length could be underestimated. However, even with the possibility of underestimating the filament length, the finding that some filaments in longitudinal sections are longer than 2.2 μm (Small, 1977), which was considered by Ashton et al. (1975) to be a constant length for the thick filaments they measured, indicates that there is no consensus as far as myosin filament length in smooth muscle is concerned.

Using serial sections ~ 10 times thinner than those used by Ashton et al., (1975), we followed 16,587 thick filaments in ovine tracheal smooth muscle and rabbit carotid smooth muscle in both relaxed and activated states in series of consecutive electron microscopic cross sections (Liu et al., 2013). We were expecting to see a normal distribution of filament lengths in our measurements, because such a distribution has been observed in our length measurements of myosin filaments formed from purified tracheal smooth muscle myosin in solution (Ip et al., 2007). To our surprise we obtained a frequency distribution of in situ filament lengths with an exponential decay pattern; that is, the distribution skewed toward short filaments (with the majority of them < 500 nm in length), and the longer the filaments, the fewer of them were observed (Liu et al., 2013). This suggests that the pool of filamentous myosin contains thick filaments of various lengths. The finding contradicts that of Ashton et al. (1975), even though both studies used the same method of serial electron microscopy. The reason for the discrepancy is not clear. The thinner sections used in the studies of Liu et al. (2013) may provide a better resolution for length measurement and may be able to “see” a gap between two filaments aligned end to end in series, whereas with thicker serial sections the two filaments may be mistaken as one.

The controversy over the issue of whether smooth muscle myosin filaments have a constant length or not (Somlyo, 2015; Seow, 2015) has deeper implications. For bipolar filaments such as those seen in striated muscle, a uniform length for the thick filaments is a must to ensure a uniform dimension for the contractile units. For side-polar filaments, the requirement for a uniform length is not needed. In a contractile unit where a thick filament is sandwiched by thin filaments, the thick filament can be as long as the contractile unit, or it can be replaced by several shorter filaments lying in series end to end within the contractile unit (Liu et al., 2013; Lan et al., 2015). Myosin filament structures (bipolar or side polar) therefore determine the configuration of contractile units. Also, short filaments like myosin dimers and tetramers (each acts as a ratcheting motor) may be redistributed more easily within the cytoskeleton when muscle cells alter their shape in adaptation to external forces.

Myosin filament evanescence in smooth muscle at rest and during activation

As discussed in previous sections, the intracellular chemical environment of smooth muscle does not favor myosin filament formation (Rovner et al., 2002), especially in the relaxed state (Suzuki et al., 1978; Craig et al., 1983; Kendrick-Jones et al., 1987; Trybus et al., 1982; Trybus and Lowey, 1984). And yet myosin

filaments are present in smooth muscle cells whether or not the RLC is phosphorylated (Somlyo et al., 1981; Qi et al., 2002). One explanation of the puzzle is that the physical environment in smooth muscle cells facilitates myosin polymerization. It is known that myosin filament formation within actin filament lattice is helped by the close contact of myosin with actin (Mahajan et al., 1989; Applegate and Pardee, 1992). The actin-filament-binding protein caldesmon is able to cross-link actin and myosin in the relaxed state (Katayama et al., 1995; Wang, 2001) and stabilize myosin filaments. Telokin and 38k protein may also regulate myosin filament formation in relaxed smooth muscle. Telokin has been suggested to stabilize dephosphorylated myosin (Shirinsky et al., 1993; Kudryashov et al., 2002), while 38k protein aids the assembly of dephosphorylated myosin into filaments (Okagaki et al., 2000). Formation of myosin filament is also driven by the concentration of myosin monomers. If the concentration of monomeric myosin is sufficiently high, polymerization of myosin will occur even without RLC phosphorylation (Kendrick-Jones et al., 1987). A local high concentration of myosin may be found in the actin filament lattice of smooth muscle.

While the presence of myosin filaments in relaxed smooth muscle is not a controversy, and, as discussed in the previous section, there is strong evidence supporting the presence of two pools of myosin, monomeric and filamentous, a valid question is whether the mass of filamentous myosin could be augmented at the expense of the mass of monomeric myosin and vice versa. As discussed in the previous section, dynamic equilibrium appears to exist in some smooth muscle cell types and not in others. The following discussion will focus mainly on airway smooth muscle, a cell type that exhibits high degrees of myosin evanescence.

Although myosin filaments are present in relaxed smooth muscle cells, they appear to be unstable. By subjecting relaxed airway smooth muscle to repeated stretch and release (within the length range experienced by the muscle in the lung during a deep inspiration [DI]), a substantial decrease in the myosin filament mass can be induced (Kuo et al., 2001). The decrease in filament mass is associated with a proportional decrease in the ability of the muscle to generate force, suggesting that mechanical perturbation is able to cause depolymerization of myosin into monomers. Furthermore, if the muscle is allowed to recover under static conditions, myosin filament mass recovers, along with the muscle's ability to generate force (Kuo et al., 2001). Activation of airway smooth muscle can also shift the equilibrium between the monomeric and filamentous myosin pools in favor of filament formation (Herrera et al., 2002). Using birefringence as an index of filament mass and with more refined equipment and improved time resolution, the time course of change in myosin filament density has been delineated in porcine trachealis (Smolensky et al., 2005). The time course of increase in myosin filament density follows closely the time course of rise of isometric force (induced by 0.1 mM acetylcholine); during relaxation, the birefringence signal decreases but lags behind the decrease in isometric force. This suggests that the rate of decrease in force after stimulation is governed by the state of actomyosin interaction and also the rate of myosin depolymerization. In intact airway smooth muscle cells, it has

been shown that during electrical field stimulation, inhibition of RLC phosphorylation without affecting Ca^{+2} transient by wortmannin abolishes the increase in birefringence, suggesting that RLC phosphorylation is the dominating mechanism for promoting myosin filament formation (Smolensky et al., 2007). In addition, diphosphorylation at both Ser19 and Thr18 (Ikebe and Hartshorne, 1985a) promotes both actin-activated Mg^{2+} -ATPase enzyme activity and stability of myosin filaments significantly more than monophosphorylation at Ser19 (Ikebe et al., 1988a).

Length adaptation in smooth muscle

Unlike striated muscle, smooth muscle is able to function over a very large length range. For example, the cell length of rabbit bladder smooth muscle in a full bladder is more than seven times its own length when the bladder is empty (Uvelius, 1976). The ability of smooth muscle to generate force over such a large length range is believed to stem from a mechanism called length adaptation (Bai et al., 2004). Even in airway smooth muscle, which is not known to require a large functional length range, the length adaptive behavior is preserved (Pratusevich et al., 1995; Gunst et al., 1995).

Length adaptation is a process in which smooth muscle regains its contractility after a large change in muscle length or other forms of mechanical perturbation such as length or force oscillation (Bai et al., 2004). Although the underlying mechanism is not entirely clear, length adaptation is known to produce a shift in the muscle's length-force relationship (Herrera et al., 2005; Wu et al., 2008). In striated muscle, the length-force relationship is determined by the sarcomeric structure (Gordon et al., 1966). The striated muscle length-force relationship is a static one, reflecting the static structure of its contractile apparatus under normal conditions. The observed shifts in the length-force relationship in smooth muscle during length adaptation suggests that the structure of the contractile apparatus underlying the length-force relationship can be readily altered. Myosin evanescence is likely an important part of the structural malleability of the contractile apparatus of smooth muscle.

In striated muscle, changes in the sarcomeric structure, such as the overlap between the thick and thin filaments, offer important clues for explaining the muscle's mechanical function, such as the ability to generate force (Gordon et al., 1966). A fruitful approach in striated muscle research has been to first delineate the change in its sarcomeric structure so that the corresponding functional change can be understood as the mechanical manifestation of the structural change. Because of its "smoothness," smooth muscle reveals almost no sign of change in its intracellular structure that could be used to explain the functional change. A reversed approach is therefore often adopted in smooth muscle research; that is, to predict changes in structure from available functional data.

Functional changes in smooth muscle associated with length adaptation, besides the shift in length-force relationship mentioned above (Herrera et al., 2005), are the linear increase in absolute shortening velocity and power output with adapted muscle length, while isometric force remains the same before and after (but not during) length adaptation (Pratusevich et al., 1995). Note that in relative terms (i.e., after normalization of

velocity by muscle length), the relative velocity and power output would be the same before and after length adaptation. In addition, length adaptation does not alter the shape of the muscle's force-velocity relationship, suggesting that the dynamics of actomyosin interaction in the so called cross-bridge cycle has not been affected by the process of length adaptation (Seow, 2013). Based on the observed changes in function of smooth muscle before and after length adaptation, the simplest model that could explain the structural changes associated with length adaptation is that, while the basic structure of individual contractile units remains the same, the number of these units connected in series in an array that spans the cell length can be altered. That is, in a muscle adapted to a longer length, more contractile units are added to the array; and in a muscle adapted to a shorter length, some contractile units are deleted from the array. By doing so, optimal overlap between the myosin and actin filaments within the contractile units can be maintained. This is schematically illustrated in Fig. 3. Although this model explains the changes in mechanical properties of smooth muscle measured at different adapted lengths, the mechanism underlying the proposed change in the structure of the contractile apparatus is still largely unknown.

The observation that length oscillation led to a decrease in myosin filament mass (Kuo et al., 2001) suggests that mechanical strain could be the first signal that triggers dissolution of myosin filaments, the initial step of length adaptation. It is not clear whether filament depolymerization is a direct consequence of shear strain or indirectly through a cascade of signals initiated by the strain, such as that mediated by integrins and cAMP (Alenghat et al., 2009).

Unlike in striated muscle, individual contractile units in smooth muscle cannot be identified in longitudinal sections from electron microscopy (Kuo and Seow, 2004). The adaptation model (Fig. 3) therefore cannot be verified by counting the number of contractile units in series in muscle cells adapted to different lengths. However, in electron micrographs of cell cross sections, myosin filament density (defined by the number of myosin filaments per cell cross-sectional area excluding the area occupied by organelles) can be accurately quantified in smooth muscle. Because both the filament number and length contribute to the filament density (i.e., the probability of myosin filaments in a cell intersected by a random cross section is proportional to the number and average length of the filaments), the cross-sectional density of myosin filaments is a measure of myosin filament mass. Because smooth muscle cell volume is conserved when the cell is stretched to different lengths (Kuo et al., 2003), doubling the cell length would reduce the cell cross-sectional area to half. If we assume that myosin filament mass is constant at different cell lengths and the filaments are evenly distributed within the cell, then the filament density at any cell cross section in cells set at different lengths would be the same. In the study by Kuo et al. (2003), the filament densities measured at different cell lengths are not the same. In fact, adapting the muscle to twice its original length leads to a ~67% increase in the filament density. The same amount of increase in absolute shortening velocity and power output is also observed in the same muscle undergone the same length adaptation.

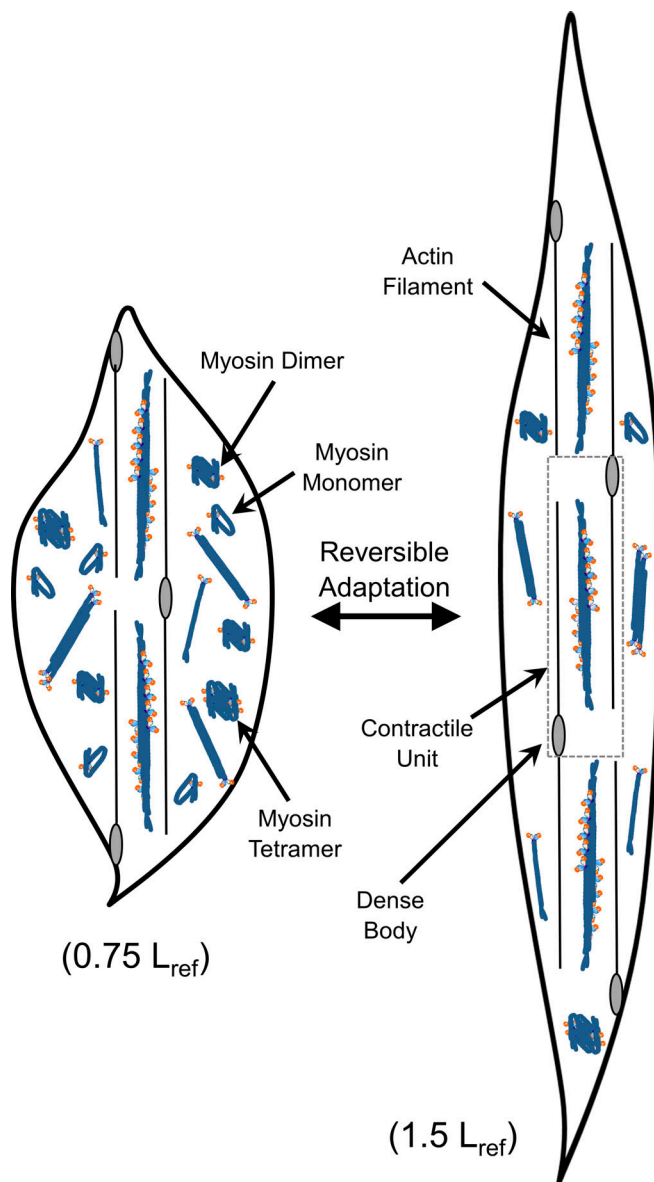


Figure 3. **Proposed model for myosin filament evanescence during reversible length adaptation process.** For illustration purposes, there are two and three contractile units in series in the muscle cell adapted to 0.75 and 1.5 reference length (L_{ref}), respectively (see text for description of the model of a contractile unit). Smooth muscle adapted to a shorter length ($0.75 L_{ref}$) contains higher concentrations of monomers, dimers, and tetramers in both folded and straightened conformations outside of the contractile units compared with the one adapted to a longer length ($1.5 L_{ref}$), which contains more filamentous myosin. Nonmuscle myosin intermediates are not shown, as they do not participate in the adaptation process. Dotted lines highlight a contractile unit. Adapted from Chitano et al. (2017) and Kuo et al. (2003).

The adaptation model (Fig. 3) predicts that the myosin filament mass should have a linear relationship with muscle length, just like the linear relationships absolute shortening velocity and power output have with the muscle length. This assumes that if more contractile units are needed in muscles adapted to a longer length (and vice versa for adapting to a shorter length), then more myosin filaments need to be synthesized, presumably from the monomeric myosin pool (Fig. 3). In the studies by Kuo et al.

(2003), the relationships among myosin filament mass, shortening velocity, power output, and muscle length have been delineated and shown to fit exactly the model prediction. The rate of ATP hydrolysis has also been shown to have the same linear relationship with muscle length, which is an additional piece of evidence supporting the notion that there are more contractile units (and hence more myosin filaments) in muscles adapted to longer lengths that results in more energy consumption (Kuo et al., 2003). Later studies have provided direct evidence that the monomeric myosin concentration decreases linearly with adapted muscle length (Chitano et al., 2017). The increased myosin polymerization seen at longer muscle lengths is therefore linked to a decrease in the monomeric myosin pool. Although it is not clear how myosin monomers are recruited to actin filament lattices to form filaments and vice versa, it is reasonable to assume that myosin evanescence plays a key role in this process of length adaptation. Specifically, through depolymerization, myosin monomers and oligomers may be more easily redistributed to different intracellular locations to form filaments.

Relevance to asthma

From the discussion in the previous sections, we learned that if a sufficiently large stretch is applied to airway smooth muscle, it will cause a transient depolymerization of myosin filaments and temporarily reduce the ability of the muscle to generate force (Wang et al., 2000; Kuo et al., 2001). It appears that this unique feature of myosin filament lability in airway smooth muscle may at least in part be responsible for the phenomena of DI-induced bronchodilation and bronchoprotection. In bronchochallenged healthy subjects, it is known that DI leads to bronchodilation (Skloot et al., 1995; Scichilone et al., 2001). It is also known that DIs taken immediately before bronchochallenge can lead to a reduction in the severity of subsequently induced bronchoconstriction; this effect is known as bronchoprotection (Malmberg et al., 1993; Scichilone et al., 2001; Skloot and Toghias, 2003). Interestingly, one of the hallmark features of asthma is the failure of DI to protect against bronchoconstriction in asthmatics (Fish et al., 1977; Hida et al., 1984; Brown et al., 2003; Skloot and Toghias, 2003). Measurement of mechanical properties of tracheal smooth muscle tissue from asthmatics has revealed a different response. The reduction in force generation by DI-mimicking oscillations is less in airway smooth muscle from asthmatic patients than that from nonasthmatic controls (Chin et al., 2012). This observation is consistent with the clinical observation of absent bronchoprotection in asthmatics.

Examination of factors influencing myosin filament evanescence in live smooth muscle cells reveals that ROCK regulates myosin filament stability. ROCK is known to promote RLC phosphorylation by its inhibitory effect on myosin light chain phosphatase. As mentioned above, RLC phosphorylated myosin filaments are more stable than unphosphorylated filaments such that they do not readily disintegrate in response to physical agitation (Ip et al., 2007). Diphosphorylation of RLC at both Ser19 and Thr18 sites further enhances myosin filament stability (Ikebe et al., 1988a, 1988b). Inhibition of ROCK activity in force-matched intact airway smooth muscle preparations result in a

dramatic reduction in myosin thick filament density (Lan et al., 2015). This reduction in filament density could be due to some filaments being completely dissolved into monomers and some filaments being only reduced to shorter fragments such that they are not detectable under an electron microscope while maintaining their force-generating ability (Lan et al., 2015). These observations have led to a postulation that ROCK is involved in myosin filament formation and regulation of filament length. Fragmented myosin filaments due to ROCK inhibition could have functional consequences on smooth muscle response to mechanical oscillations, such as those imposed by a DI. In the same study, Lan et al. (2015) show a greater loss in force due to mechanical oscillation in ROCK-inhibited muscle. The authors propose that fragmented myosin filaments could be lost from the actin filament lattice (more easily than long filaments) during large-amplitude DI-mimicking oscillations, leading to loss of contractility.

It has been reported recently that the protein expression of ROCK (both ROCK1 and ROCK2 isoforms) is up-regulated in airway smooth muscle and pulmonary blood vessels of asthmatics (Wang et al., 2020a). The protein p116^{Rip}, which inhibits the RhoA-ROCK pathway, was also found to be reduced in airway smooth muscle of asthmatics (Komatsu et al., 2020). Both discoveries support a new phenotype of asthma in which ROCK signaling is augmented. Besides many other implications on airway smooth muscle contractility and stiffness (Wang et al., 2020b), this new phenotype could serve as one of the underlying mechanisms for increased myosin filament stability and failure of protection against bronchoconstriction by DI in asthmatics.

Conclusions

Lability of filamentous myosin II in smooth muscle is crucial for proper function of the muscle. Filament lability is determined by intracellular chemical environment, contractile stimulation, and the associated signaling pathways regulating RLC phosphorylation, as well as the strains associated with changes in cell dimension. Besides its role in regulating contractility, myosin filament lability is also indispensable in the process of length adaptation in smooth muscle. The labile nature of smooth muscle myosin filament underlies the phenomenon of myosin evanescence and contributes to cellular malleability of smooth muscle. This malleability is required for normal function of the muscle but may be altered in a diseased state. Increased ROCK expression in asthma may increase myosin filament stability and thereby underlie the failure of DI-induced bronchodilation and bronchoprotection in asthma.

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