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Review Article

Regulation of force in vascular smooth muscle

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Abstract

Vascular smooth muscle contraction plays a defining role in the regulation and maintenance of blood pressure, and its deregulation is associated with many clinical syndromes including hypertension, coronary vasospasm and congestive heart failure. Over the past 20 years, there has been a growing understanding of the regulation of 20 kDa myosin light chain phosphorylation by myosin light chain kinase and myosin light chain phosphatase, the role of splice-variant isoforms of both the myosin heavy chain and the essential myosin light chain, as well as the signaling pathways involved in smooth muscle contraction under normal and pathophysiological conditions. This review will attempt to recapitulate the data in the field, primarily focusing on the contractile response of smooth muscle, and the molecular determinants responsible for the regulation of vascular tone.

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1. Introduction

Two general forms of excitation initiate contraction of smooth muscles. The initiation of contraction may occur due to innervation and consequent depolarization of the membrane's resting potential, termed electromechanical coupling, whereas activation by ligands of cell surface receptors has been termed pharmacomechanical coupling [1]. The electrical component of smooth muscle cell excitation is accounted for by action potentials, triggering the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. This rise in intracellular Ca^{2+} may be augmented by Ca^{2+}-induced Ca^{2+} release from intracellular stores (for a comprehensive review, see Bolton and coworkers [2]). On the other hand, pharmacomechanical coupling involves activation of cell surface receptors to augment the increase in Ca^{2+}, either by the release of Ca^{2+} from intracellular stores or through cell signaling-mediated mechanisms that increase the Ca^{2+} sensitivity of the contractile apparatus. For example, the Ca^{2+} response in smooth muscle cells may be modulated by α-adrenergic stimulation. Such excitation of α-adrenergic membrane receptors coupled to heterotrimeric G-proteins may activate phospholipase C (PLC) to promote hydrolysis of phosphatidylinositol bisphosphate to diacylglycerol (DAG) and inositol trisphosphate, the latter of which can serve as an agonist for inositol trisphosphate-dependent Ca^{2+} release from intracellular stores to augment intracellular Ca^{2+} concentration.

2. Myosin light chain kinase

The primary target of a rise in intracellular Ca^{2+} is believed to be calmodulin, a member of the family of EF hand Ca^{2+} -binding proteins [3]. The binding of Ca^{2+} to the four EF hands of calmodulin allows for a conformational change in Ca^{2+} calmodulin [4,5] and its subsequent interaction with myosin light chain kinase (MLCK). The COOH-terminal domain of the Ca^{2+}-calmodulin complex binds to the calmodulin-binding domain at the NH_2-terminus of MLCK, followed by the association of the NH_2-terminal domain of calmodulin with the COOH-terminus of the calmodulin-binding sequence of MLCK. The association results in a conformational change of the calmodulin-MLCK complex,

Abbreviations: cGKI, cGMP-dependent protein kinase; HSP27, Heat shock protein 27; MLC17, Acidic (MLC17b) or basic (MLC17a) 17 kDa essential myosin light chain; MLC20, Regulatory 20 kDa myosin light chain; MLCK, Myosin light chain kinase; MLCP, Myosin light chain phosphatase; MYPT1, Myosin-binding/phosphatase-targeting subunit; V_{max}, Maximum velocity of muscle shortening.

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displacing the autoinhibitory sequence of MLCK to expose the enzyme's catalytic site. This series of events leads to the activation of MLCK and a subsequent phosphorylation of Ser 19 of the 20 kDa regulatory light chain of myosin (MLC20), MLCKs only documented physiological substrate to date [6,7]. The phosphorylation of MLC20 is the primary regulatory event for the initiation of force production in smooth muscles. In vitro, myosin's sedimentation properties have been shown to change from a 10S conformation in its inactive form to a 6S conformation following phosphorylation [8]. The filamentous arrangement of myosin in the smooth muscle cell suggests that in vivo, global changes of myosin's conformation following MLC20 phosphorylation are unlikely to be as dramatic due to the steric constraints placed particularly on the myosin tail. However, with the tail domain of myosin immobilized in the thick filaments, the energy associated with the change in conformation observed in vitro might still manifest itself in vivo as a pivot along the neck region of myosin, while the tail serves as a fixed, stationary point. Additional studies have also demonstrated changes in the orientation of MLC20 as a result of phosphorylation [9], suggesting that conformational changes in myosin during smooth muscle activation merit further investigation.

3. Myosin light chain phosphatase

As a means to balance MLCK activity, smooth muscle myosin's actin-activated Mg^{2+}-ATPase is attenuated by dephosphorylation of MLC20 by myosin light chain phosphatase (MLCP), a type 1 protein phosphatase [10–12]. The regulation of smooth muscle myosin activity through balanced MLC20 phosphorylation and dephosphorylation distinguishes this class of myosin II from striated muscle myosin II isoforms, which are not dependent on MLC20 phosphorylation for activation. In fact, since actomyosin activation in smooth muscles is dependent on downstream phosphorylation events pursuant to the intracellular influx of Ca^{2+} or agonist stimulation, its slower kinetics of contraction are easily understood in contrast to striated muscles, wherein Ca^{2+} acts directly at the level of the actomyosin crossbridges.

Protein purification and cDNA cloning techniques demonstrated the MLCP holoenzyme to be a heterotrimer, consisting of a ~110 kDa myosin-binding subunit (MYPT1), a 37-kDa catalytic subunit as well as a 20-kDa subunit of unknown function to date [10–12]. The selectivity of the phosphatase for phosphorylated myosin is conferred by the myosin-binding subunit, likely potentiating the dephosphorylation of myosin by the catalytic subunit as determined by competition assays [12]. Interestingly, the myosin-binding subunit shows isoform diversity, with two alternatively spliced exons—one central and another near the 3' end of the transcript, responsible for the expression of up to four unique isoforms. With characterization of the heterotrimeric enzyme, the smooth muscle myosin phosphatase was subsequently recognized to be a G-protein-regulated enzyme, confirming earlier expectations [13,14]. As it stands, the contribution of the myosin-binding subunit isoforms to MLCP activity and smooth muscle contractility is newly emerging.

3.1. Ca^{2+} sensitization

One of the driving observations in characterizing MLCP activity was the demonstration that for a given intracellular Ca^{2+}concentration, the force generated was variable depending on the method of muscle activation. In fact, it was observed that agonist stimulation yielded higher force for a given Ca^{2+} concentration, when compared to depolarization [15–17], and the increased interest in MLCP regulation has stemmed from its role in this observed Ca^{2+} sensitization. Experiments have demonstrated that activated G-proteins are involved in the signaling pathway for Ca^{2+} sensitization, since GTPγS, a non-hydrolyzable GTP analog, increases MLC20 phosphorylation and potentiates force. GTPγS was also shown to decrease the rate of MLC20 dephosphorylation, consistent with increased muscle tone and predictive of an overall decrease in MLCP activity [14]. Independent observations demonstrated that incubation of rabbit portal vein with ATPγS in a low Ca^{2+} buffer resulted in an increase of Ca^{2+} sensitivity. In the same study, ATPγS was demonstrated to lead to thiophosphorylation of the myosin-binding subunit of MLCP [18]. This presumably decreased the holoenzyme's activity, resulting in greater MLC20 phosphorylation for a given Ca^{2+} concentration, rationalizing the ensuing Ca^{2+} sensitization. This further suggested that phosphorylation is the driving reversible event for MLCP's regulation. Subsequent experiments suggested that G-protein-mediated Ca^{2+} sensitization, and the consequent decrease of MLCP activity were a result of the RhoA-GTP-activated Rho-kinase pathway leading to the phosphorylation of the myosin-binding subunit of MLCP (Fig. 1). RhoA is a monomeric G-protein that is active in its GTP-bound form, a transition that is facilitated by Rho-guanine nucleotide exchange factors that support the exchange of nucleotide to activate RhoA-GDP to RhoA-GTP [19]. RhoA's candidacy as a primary regulator involved in Ca^{2+} sensitization was in agreement with the ability of GTPγS to Ca^{2+} sensitize smooth muscle preparations. Experiments delineating the pathway involved demonstrated that activated RhoA-GTP might, in turn, bind to and activate Rho-kinase, a serine/threonine kinase [20]. Phosphorylation of the myosin-binding subunit of MLCP by Rho-kinase has been shown to inhibit MLCP activity [21]. This results in a decrease in overall phosphatase activity, allowing the MLCK:MLCP balance to favor increased MLC20 phosphorylation, the net result being an increase in force for a given submaximal intracellular Ca^{2+} concentration. To date, this remains the best-characterized pathway for agonist-induced Ca^{2+} sensitization. The decrease in MLCP activity due to Rho-kinase activation has been attributed to phosphorylation of two residues on the COOH-terminal half of the myosin-binding subunit, Thr695 and 850 [22]. However, recent experiments have suggested that phosphorylation of

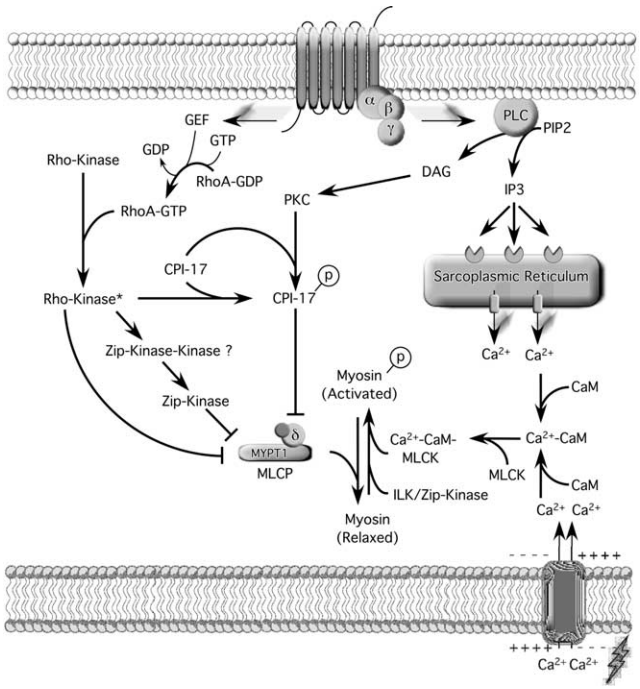

Fig. 1. Schematic overview of voltage-dependent and agonist-dependent regulation of smooth muscle contraction. Electromechanical innervation through voltage-dependent Ca^{2+}channels (lower membrane) is shown to increase intracellular Ca^{2+} to activate MLCK to increase myosin phosphorylation and initiate contraction. Pharmacomechanical coupling through membrane receptors (upper membrane) activates G-protein-dependent pathways to modulate the activity of MLCP through several possible pathways controlled by Rho-kinase, Zip-kinase and ILK. Conversely, activation of PLC additionally activates inositol trisphosphate (IP3)-dependent Ca^{2+} release from the sarcoplasmic reticulum and PKC through DAG to modulate MLCP activity.

the myosin-binding subunit of MLCP is not necessary to observe GTPγS-dependent force enhancement [23]. Furthermore, it remains unclear whether Rho-kinase treats all myosin-binding subunit isoforms as equivalent substrates; diversity in its catalytic activity against MYPT1 isoforms may provide another point for modulation of contractility.

As activated Rho-kinase is predominantly associated with the cell membrane, its participation in the mechanism for phosphorylation of a myofibril-associated phosphatase is unclear. Morgan's group [24] has demonstrated that prostaglandin F2α stimulation of freshly dispersed single smooth muscle cells results in a Rho-kinase-dependent phosphorylation and translocation of MLCP from the cytosol to the cell membrane. Once translocated, phosphorylated MYPT1 and the catalytic subunit dissociate, decreasing MLC phosphatase activity. The data are consistent with reports of a spatial activation and distribution of MLC20 phosphorylation during agonist activation [25]. These investigators demonstrated that agonist stimulation leads to a RhoA-dependent temporal and spatial difference in MLC20 phosphorylation. Subsequent to a global rise in phosphorylated MLC20 levels with stimulation, the cortical region of the cell retained MLC20 phosphorylation levels, whereas MLC20 phosphorylation levels fell in the central region of the cell.

Others have demonstrated the presence of a unique kinase, termed Zip-like kinase (also referred to as MYPT1 kinase), which co-localizes with MLCP and may, therefore, participate in Ca^{2+} sensitization. Zip-like kinase is phosphorylated following activation of a Rho-kinase-dependent pathway during carbachol stimulation of rabbit bladder, and the phosphorylated Zip-like kinase can, in turn, phosphorylate the myosin-binding subunit at Thr-695, considerably faster than Rho-kinase [26]. The addition of activated MYPT1 kinase to β-escin skinned smooth muscle results in a Ca^{2+}-independent contraction [27]. This direct effect of activated Zip-like kinase in the absence of RhoA stimulation suggests that it is indeed downstream of Rho-kinase in the pathway for Ca^{2+} sensitization. However, Niiro and Ikebe [28] have demonstrated that MYPT1 is a poor substrate for Zip-like kinase, and that Zip-like kinase directly phosphorylates MLC20. A similar mechanism leading to direct MLC20 phosphorylation for Ca^{2+}-independent contractions has independently been suggested for integrin-linked kinase (ILK) [29]. Further experiments are clearly required to consolidate the roles of the newly identified kinases within the signaling pathways controlling MLC20 phosphorylation (see Fig. 1). Other potential mechanisms have also been proposed for Ca^{2+} sensitization. CPI-17 is a small protein that serves as a substrate for both Rho-kinase and protein kinase C (PKC) [30]. Phosphorylated CPI-17 binds to the catalytic subunit of MLCP to inhibit the enzyme's activity [31]. In addition, the magnitude of Ca^{2+} sensitization has been correlated with CPI-17 expression [32]. Thus, Ca^{2+} sensitization may involve Rho-kinase- or PKC-mediated phosphorylation of CPI-17 to increase MLC20 phosphorylation and force. Although CPI-17 is abundantly expressed in mammalian smooth muscle, the protein has not been found to be expressed in avian smooth muscle (unpublished observations), suggesting that CPI-17 may participate in a unique aspect of the mechanism for force maintenance only in mammalian smooth muscle.

Another potential pathway for Ca^{2+}sensitization involves arachidonic acid. Activation of heterotrimeric G-proteins have been shown to lead to the production of arachidonic acid [33], which interacts with MYPT1 to dissociate the MLCP holoenzyme, and consequently, inhibit phosphatase activity. Alternatively, arachidonic acid has been proposed to activate a kinase that may phosphorylate MYPT1 to inhibit phosphatase activity [34]. Thus, agonist stimulation via the production of arachidonic acid could lead to an inhibition of MLC phosphatase activity and consequent Ca^{2+} sensitization.

3.2. Ca^{2+} desensitization

In addition to its role in Ca^{2+} sensitization, MLCP has also been demonstrated to be a target for nitric oxide (NO)-mediated relaxation of vascular smooth muscle. NO acts on the intracellular, soluble guanylate cyclase to increase the protein's activity and raise intracellular cyclic GMP concentration [35]. Cyclic GMP has a well-characterized intracellular target in cGMP-dependent protein kinase (cGKI), and has

been shown to interact with the COOH-terminal region of MYPT1 through its NH_2-terminal domain that contains a leucine zipper [36]. Interestingly, MYPT1 has also been shown to express isoforms containing a leucine zipper at the COOH-terminus of the protein, a consequence of exclusion of a 31 nucleotide exon in the transcript of MYPT1 [34,37]. This leucine zipper motif has been shown to allow specific interaction with the amino-terminus leucine zipper of cGMP-dependent protein kinase Iα (cGKIα). The association was found to lead to an activation of MLC phosphatase activity by cGKIα, and potentiated MLCP's ability to dephosphorylate MLC20 [36]. This pathway was validated further by in vivo experiments demonstrating that only smooth muscles expressing myosin-binding subunit isoforms containing the leucine zipper motif were sensitive to relaxation by 8-bromo-cGMP, a membrane permeable cGMP analog [37]. The expression of leucine zipper positive isoforms of MYPT1 has been demonstrated to be tissue specific and developmentally regulated [37], which could suggest that the diversity in the sensitivity to NO-mediated vasodilatation is determined in part by the relative expression of the leucine zipper-positive isoforms of MYPT1.

4. Fast and slow contractile properties

The contractile properties of smooth muscle can be broadly classified as phasic (or fast) and tonic (or slow) [1]. For phasic smooth muscle, it has been well documented that the rates of force activation and relaxation are relatively rapid and the maximum velocity of muscle shortening (V_{max}) is relatively fast. Tonic smooth muscle maintains a resting tone or force has slower rates of force activation and relaxation, as well as a slower V_{max}. Although the focus of intense research, the molecular mechanism(s) responsible for the diverse contractile characteristics in smooth muscles are just slowly being elucidated. It is very likely that the contractile properties observed are due to differences in both messenger signaling and actomyosin crossbridge kinetics. However, the fast and slow contractile properties of smooth muscles remain after activation of skinned, thiophosphorylated smooth muscle tissue strips with photolytic release of caged MgATP [38]. Such an observation suggests that the differences in the contractile properties are predominantly due to the kinetics of the actomyosin crossbridge cycle.

4.1. Myosin heavy chain

Smooth muscle myosin is a type II myosin, a hexamer composed of two intertwined heavy chains, two 20 kDa regulatory light chains and two essential 17 kDa light chains (MLC17). High-resolution structure determination has shown that the structure of myosin II isoforms from smooth and striated muscles are very similar [39,40], in agreement with the general similarity in their heavy chain and light chain compositions. The smooth muscle myosin heavy chain gene encodes a protein with an elongated, coiled-coil rod at the COOH-terminus that extends into a neck region connecting the myosin tail to the NH_2-terminal globular motor domain that houses the actin binding and catalytic sites of the enzyme. The two sets of light chains associate non-covalently with the neck region of the heavy chains, with the essential light chains being more proximal to the globular motor domain. A single gene encodes the smooth muscle myosin heavy chain with two pairs of alternatively spliced exons near the 5' and 3' ends of the gene. Two isoforms, named SM-1 and SM-2, differ by expression of two unique alternatively spliced exons encoding either a 43 (SM-1) or 9 (SM-2) amino acids sequence at the COOH-terminal tail [41,42]. Their contribution to contractility remains under investigation using both in vitro and cellular systems [43,44]. In the motility assay, SM-1 and SM-2 myosins do not demonstrate significant differences in the speed with which they propel actin filaments. Recent evidence suggests that the two isoforms may have unique contributions to thick filament assembly by favoring homodimer vs. heterodimer formation [45]. Therefore, the SM-1 and SM-2 isoforms of the myosin heavy chain may have more pertinent contributions to the organization of thick filaments in the cell, rather than the kinetics of smooth muscle contractility. This finding may be an initial clue to interpreting the correlation between SM-1/SM-2 expression level and final cell length following contraction of single smooth muscle cells [44].

Additional myosin heavy chain isoform diversity is generated by alternative splicing of a 21 nucleotide exon nearer to the 5' end of the transcript. The unique seven amino acids encoded by this exon lie within the surface loop spanning the ATP-binding pocket of the myosin head [42,46]. Myosin heavy chain isoforms with inclusion of this seven amino-acid stretch are termed SM-B isoforms and are predominantly expressed in visceral smooth muscles in contrast to their relatively low levels in vascular smooth muscles. In keeping with this observation, myosin heavy chain isoform diversity at the NH_2-terminus has been hypothesized as a determinant of the tonic and phasic contractile properties of smooth muscle [46–48]. Studies using the laser trap to characterize the SM-A and SM-B isoforms have shown the SM-A isoforms to demonstrate a longer duty cycle, a reflection of the time myosin remains attached to actin during the power-stroke [47]. This would reasonably imply an increase in shortening speed for smooth muscles primarily expressing SM-B isoforms of the smooth muscle myosin heavy chain due to their reduced time spent attached to actin during the actomyosin crossbridge cycle. In fact, this hypothesis has been strengthened by comparisons of shortening velocities from phasic and tonic smooth muscles [49], from isolated single smooth muscle cells with differing SM-A/SM-B expression levels [50] and an SM-B knockout mouse model [51].

It is certainly of interest to characterize the catalytic activity of the various myosin isoforms, and more importantly, their contribution to the contractility of intact smooth muscle tissues. However, the isoform heterogeneity observed in

smooth muscles [48] complicates comparisons of contractility between muscles expressing different myosin isoforms. Quite often, such heterogeneity excludes unequivocal assessment of the contribution of specific myosin isoforms to contractility. This limitation has been circumvented by the use of transgenic mouse lines, notably knockout mice deficient in smooth muscle myosin heavy chain expression [52] or specific targeting of exons to knockout SM-B myosin heavy chain isoform expression in favor of SM-A [51]. Surprisingly, muscle strips from embryos of smooth muscle myosin heavy chain knockout mice demonstrated the ability to contract in response to depolarization. However, the initial, transient phasic component of force generation during contraction was lost, in favor of slower, but sustained force activation. The researchers suggested that although smooth muscle myosin isoforms are required to initiate the rapid force development at activation, non-muscle myosin was sufficient to sustain smooth muscle contractions. However, the knockout offspring died soon after birth, demonstrating that smooth muscle myosin heavy chain function and expression is developmentally regulated and become dominant after birth. Deletion of the exon encoding the seven amino-acid insert near the ATP-binding pocket of the myosin head resulted in smooth muscles impaired in force production and rate of tension regeneration, suggesting that the SM-B isoforms of the smooth muscle myosin heavy chain are important in determining faster kinetics of force production.

4.2. The essential myosin light chain

Additionally, isoform diversity of MLC17 associated with the neck region of the myosin heavy chain increases the heterogeneity of myosin isoforms. Two splice variants of MLC17 have been found to be associated with smooth muscle myosin, and they differ by inclusion (acidic isoform or MLC17b) or exclusion (basic isoform or MLC17a) of a 39 bp exon [53]. In general, vascular smooth muscles with tonic contractile properties display a higher percentage of acidic MLC17b expression vs. basic MLC17a [54,55]. Studies have shown a correlation between increased acidic MLC17b expression and reduced actin-activated Mg^{2+}-ATPase activity of myosin [56] as well as decreased V_{max} in tissue strips [54]. This has been independently demonstrated following chemical exchange of MLC17 isoforms in permeabilized smooth muscles, wherein reconstitution with basic MLC17a increased both the velocity of shortening and the rate of force activation [57]. At the level of the single smooth muscle cell, recent data have shown both agreement and disagreement with results obtained from smooth muscle tissue strips. Eddinger and coworkers [58] found no relationship between MLC17 isoform expression levels and the velocity of shortening. On the other hand, the influence of MLC17a and MLC17b in modulating the rate of force development was confirmed at the level of intact single smooth muscle cells through overexpression of the endogenous or exogenous MLC17 isoform in cultured aorta or gizzard smooth muscle cells [59]. It was shown therein and elsewhere [60] that phasic and tonic force activation profiles of smooth muscle cells rely heavily on the expression level of MLC17a (phasic) or MLC17b (tonic). This finding is supported by experiments demonstrating a change from fast to slow force activation kinetics for cultured gizzard smooth muscle cells following an endothelin-1-induced increase in acidic MLC17b isoform expression [55]. Proposals in explaining the function of MLC17a in modulating the rate of force development have hypothesized that MLC17 isoforms may differentially affect the stiffness of the myosin lever arm, accounting for the changes in force development kinetics. Additional experiments will be required to expound on this hypothesis.

5. Force maintenance

It has been documented that vascular smooth muscles have the ability to maintain force for extended periods of time with a low rate of energy utilization. The initial observations correlating force production with MLC20 phosphorylation were confounded by experiments demonstrating a maintenance of tone in the face of falling or low MLC20 phosphorylation levels and decreasing actin-activated myosin Mg^{2+}-ATPase activity [17,61–63]. One early hypothesis suggested that force was maintained through dephosphorylated myosins attached to actin in a force-generating state [64]. Such an observation rationally suggests that crossbridges in vascular smooth muscle may segregate into two large populations—one population of rapidly cycling crossbridges responsible for the initial force activation and the other set of slowly cycling crossbridges playing a predominant role in force maintenance. Interestingly, the sustained phase of smooth muscle contraction can be inhibited with Y-27632, the Rho-kinase inhibitor [65]. It opens the possibility that the slowly cycling crossbridges working during agonist-induced force maintenance may be additionally regulated by Rho-kinase. Experiments with the smooth muscle myosin heavy chain knockout mice have led credence to the idea that during force maintenance, there are two crossbridge populations—one rapidly cycling and the other cycling slowly. Bladder tissue from the knockout animal lacks the rapid, phasic phase of force development, but contracts slowly to a steady-state level with force maintenance [52]. Since the force maintenance phase of smooth muscle contraction is conserved in the knockout mice, this suggests that non-muscle myosin, possibly regulated by the Rho-kinase pathway, is a major factor in the mechanism for force maintenance and may represent the slowly cycling crossbridge population. Nonetheless, these finding were in embryonic bladder tissue, as the homozygous offspring of these mice died shortly after birth. This not only cements smooth muscle myosin II's importance in muscle contraction during early development and adulthood, but also raises the possibility that this model may not be entirely reflective of force maintenance in the adult.

Another mechanism of force maintenance has been suggested from comparisons of the contractile properties of

tonic and phasic smooth muscles. The rate of ADP release from smooth muscle myosin is dependent on MLC20 phosphorylation [66], and phosphorylated crossbridges in tonic smooth muscles may exhibit stronger ADP binding compared to phasic smooth muscles, rationalizing a longer duty cycle and facilitating force maintenance [49]. This would be in agreement with independent research suggesting that dephosphorylated but ADP-bound crossbridges play a large role in force maintenance during low MLC20 phosphorylation levels [67]. Thus, force maintenance may also be facilitated by a slowing in the rate of ADP dissociation from smooth muscle myosin, and a resulting increase in the number of crossbridges populating the strong binding, force producing, ADP-bound state of actomyosin.

6. The thin filament and the cytoskeleton

The roles of the thin filament [68] and the cytoskeleton [69] in smooth muscle contraction have been recently reviewed; therefore, we will only present a brief overview in this section. Despite numerous studies, the role of the thin filament for force regulation in smooth muscle remains controversial [70–72]. Both caldesmon [73,74] and calponin [75,76] have been suggested to modulate smooth muscle activation. Caldesmon is able to bind actin and inhibit actomyosin ATPase activity [77,78]. These observations have been extended to show that caldesmon is capable of regulating force in smooth muscle [73,79]. Caldesmon's inhibitory effect on the actomyosin ATPase activity can be reversed by phosphorylation [80,81], providing an important means for regulation of its inhibitory activity. Also relevant to its regulation, caldesmon has been shown to be phosphorylated by both PKC [82] and MAP kinase [83,84], although differences in the magnitude of caldesmon phosphorylation have been reported. Adam et al. [85] were only able to demonstrate a modest increase in caldesmon phosphorylation, whereas Morgan's group [86] has demonstrated a time-dependent, 3-5-fold increase in caldesmon phosphorylation following α-agonist activation of ferret aorta. Although independent studies have confirmed the phosphorylation of caldesmon during agonist stimulation of both tracheal [87] and pulmonary arterial smooth muscle [88], the event was unrelated to force production [88]. Additional experiments have demonstrated that differences in caldesmon content among tissues affect force at submaximal levels of Ca^{2+} activation [74]. Thus, further experiments are required to define the role of caldesmon in smooth muscle force regulation.

Calponin is also a thin filament-associated protein that may influence smooth muscle contraction. Two isoforms, h1- and h2-calponin, have been found to be expressed in smooth muscles. H1-calponin has been suggested to influence smooth muscle V_{max} [72,89], but others have not found a correlation of V_{max} and h1-calponin expression [90]. Similarly, h1-calponin has been reported to both decrease [91] or not affect maximal force [72,89]. These conclusions are additionally confounded by the changes in thin filament protein content observed in the h1-calponin knockout mice [72], leaving discrepancies in the observed function of h1-calponin that still require reconciliation. Recent experiments have suggested that h2-calponin may play a more prominent role in the regulation of the actin cytoskeleton [92,93], suggesting that it does not have a direct contribution to the actomyosin crossbridge cycle during cell motility.

6.1. Cytoskeleton

There is also evidence that a secondary system outside of the thin and thick filament domains may play a role in force maintenance. Agonist-induced phosphorylation of the membrane-associated dense plaque proteins, paxillin and talin, have been shown to correlate with force production [94]. Further, paxillin antisense has been demonstrated to neither affect the expression of other cytoskeletal proteins nor MLC20 phosphorylation, but result in a reversible decrease in both paxillin expression and force in response to both KCl and acetylcholine stimulation [95]. These authors suggest that attachment of actin filaments to membrane-associated dense plaques is regulated during smooth muscle contraction by the PKC-mediated phosphorylation of membrane-associated dense plaque proteins. Therefore, force maintenance would have a large component that would require reorganization of actin filaments to support load.

Others have demonstrated that both extracellular signal-regulated kinase (ERK) MAP kinase and p38 MAP kinase are activated during agonist stimulation of smooth muscle [87]. The activation of ERK MAP kinase leads to downstream phosphorylation of caldesmon, while p38 MAP kinase does not phosphorylate caldesmon, but rather increases phosphorylation and activation of heat shock protein 27 (HSP27) via a MAPKAP kinase 2 pathway [88]. In agreement with these findings, inhibition of p38 MAP kinase resulted in a reduction in HSP27 phosphorylation and consequent decreases in both the rate of increase and maximal force. Interestingly, HSP27 is known to regulate actin polymerization (for review, see [69]), once again suggesting that remodeling of the actin cytoskeleton may participate in the mechanism for force maintenance during smooth muscle contraction.

Therefore, the contribution of thin filament-associated proteins and the cytoskeleton in the regulation and maintenance smooth muscle contraction will require further clarification. However, with emerging biophysical, molecular and protein chemistry techniques, the progress in elucidating the mechanisms responsible for smooth muscle contraction will inevitably be swift.

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