Brief Review

Vascular Smooth Muscle Contractile Elements
Cellular Regulation

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For many years the simple view was held that contractile force in smooth muscle was proportional to cytosolic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)). With the discovery that phosphorylation of myosin light chain by Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase initiated contraction, regulation of the contractile elements developed more complex properties. Molecular and biochemical investigations have identified important domains of myosin light chain kinase: light chain binding sites, catalytic core, pseudosubstrate prototope, and calmodulin-binding domain. New protein phosphatase inhibitors such as okadaic acid and calyculin A should help in the identification of the physiologically important phosphatase and potential modes of regulation. The proposal of an attached, dephosphorylated myosin cross bridge (latch bridge) that can maintain force has evoked considerable controversy about the detailed functions of the myosin phosphorylation system. The latch bridge has been defined by a model based on physiological properties but has not been identified biochemically. Thin-filament proteins have been proposed as secondary sites of regulation of contractile elements, but additional studies are needed to establish physiological roles. Changes in the Ca\(^{2+}\) sensitivity of smooth muscle contractile elements with different modes of cellular stimulation may be related to inactivation of myosin light chain kinase or activation of protein phosphatase activities. Thus, contractile elements in smooth muscle cells are not dependent solely on [Ca\(^{2+}\)]\(_i\), but use additional regulatory mechanisms. The immediate challenge is to define their relative importance and to describe molecular-biochemical properties that provide insights into proposed physiological functions. (Hypertension 1991;17:723–732)

Activation of cell surface receptors by neurotransmitters or hormones initiates a series of cellular processes leading to contraction of vascular smooth muscle. Signal transduction pathways involving regulation of Ca\(^{2+}\) influx or Ca\(^{2+}\) release from internal stores (sarcoplasmic reticulum) are involved in increasing cytoplasmic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)). In smooth muscle, like striated muscle, [Ca\(^{2+}\)]\(_i\) is a primary determinant for contraction. However, smooth muscle contractile elements have unique physiological, cellular, and biochemical properties compared with striated muscle. This Brief Review will focus on our current understanding of these properties and emphasize new areas that need additional exploration. Because the review is brief, citations are selective to emphasize various points of discussion. Many excellent research references are not listed; however, recent reviews that provide more detailed coverage of the subject are referenced.

Proteins of the Contractile Elements

Myosin

Myosin is the primary protein of smooth muscle thick filaments and is composed of two heavy chain subunits (approximately 200 kDa each) and two each of two types of light chain subunits (20 and 17 kDa, respectively). The native hexameric form of myosin is configured as an intertwined coiled-tail region embedded in thick filaments and two globular head regions that protrude from the thick filament at regular intervals to form cross bridges (Figure 1). These head regions contain distinct sites for binding to actin, ATP hydrolysis, and association of light chain subunits.

Phosphorylation of the 20-kDa regulatory light chain at serine-19 by Ca\(^{2+}\)/calmodulin–dependent myosin light chain kinase (MLCK) results in conformational changes that affect ATP hydrolysis and actin binding (Figure 1). A 100-fold increase in actin-activated MgATPase activity is due to an increase in the rate of release of the products of hydrolysis. The marked increase in MgATPase is not associated with a corresponding increase in the affinity of myosin binding to actin. Phosphorylation of the two myosin heads is random (i.e., phosphory-
Myosin phosphorylation in smooth muscle. Top: Schematic representation of the myosin molecule showing two heavy chains with respective intertwined tail regions and globular head regions. Associated with each head are one each of two types of light chains, the 20-kDa regulatory or phosphorylatable light chain (identified by "P") and the 17-kDa light chain (identified by shading). Bottom: Diagram showing pathway for initiating contraction in smooth muscle. Regulatory light chain on myosin is phosphorylated (Myosin-P) by Ca$^{2+}$/calmodulin-dependent myosin light chain kinase and dephosphorylated by a protein phosphatase. Phosphorylated myosin binds to actin, resulting in increased MgATPase activity and contraction.

Myosin Light Chain Kinase

The characterization of MLCKs from a variety of tissues and animal species has led to the identification of two groups, including skeletal and smooth muscle isoforms, respectively. There are important structural and catalytic differences between these two groups. For example, smooth muscle MLCK is more substrate specific; it phosphorylates smooth muscle myosin light chain but not skeletal muscle light chain with low $K_m$ and high $V_{max}$ values. However, the striated muscle MLCK phosphorylates myosin light chain from either striated or smooth muscle tissues with similar $K_m$ and $V_{max}$ values. Synthetic peptide analogues have been used to define the substrate determinants for these two kinases.

Two groups of basic amino acid residues located amino terminal of the phosphorylatable serine in either the smooth or skeletal muscle light chain peptides are necessary for low $K_m$ values. The smooth muscle MLCK has an absolute requirement for an arginine that is three residues to the amino terminal side of the phosphorylatable serine. In skeletal muscle light chain this residue is an acidic residue (glutamic acid).

Substitution of these critical basic residues with neutral amino acids markedly increases the $K_m$ value when compared with the unsubstituted peptide. The complex nature of the substrate determinants for the smooth muscle MLCK compared with the substrate determinants of other protein kinases such as cyclic AMP (cAMP)–dependent protein kinase and protein kinase C probably accounts for the high degree of substrate specificity exhibited by this enzyme.

A comparison of the amino acid sequences of smooth and skeletal muscle MLCKs has led to the identification of several conserved regions. A central catalytic core region contains many highly conserved residues with respect to MLCKs as well as to other protein kinases (Figure 2). The percent identity of residues in the rabbit skeletal and chicken smooth muscle MLCKs to the cAMP-dependent protein kinase catalytic core is 23% and 25%, respectively. The percent identity between MLCKs from the same type of tissue (skeletal or smooth) from different species is greater than the identity between smooth versus skeletal muscle MLCKs. Recently, it has been suggested that a region immediately N-ter-
homology that has been determined from amino acid sequence of chicken smooth (CK Smooth) and rabbit skeletal (RB Skeletal) muscle myosin light chain kinases (MLCKs). Percentage comparison.

MLCKs are also an important model for Ca^2+/-calmodulin-regulated enzymes. The calmodulin binding domain is located between residues 796 and 813 of the chicken smooth muscle MLCK. Limited tryptic cleavage of smooth muscle MLCK results in successive truncations of the C-terminal with generation of a 66-kDa Ca^2+/-calmodulin-dependent MLCK, an inactive 64-kDa MLCK, and an active 61-kDa Ca^2+/-calmodulin-independent MLCK. These results indicate that MLCK contains an inhibitory region and a calmodulin-binding domain.

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MLC (1-23) S S K R A K A K T T K K R P Q

R A T S (P) N V F A

MLCK (787-804) S K D R M K K Y M A

R R K W Q K T G H A V R A

Synthetic peptides modeled after this region of the kinase were found to inhibit the kinase activity of the constitutively active 61-kDa MLCK fragment. One of these peptides (783-804) was shown to be a competitive inhibitor with respect to the light chain substrate. These results led to the hypothesis that the inhibitory region mimics the light chain substrate (i.e., is a pseudosubstrate prototpe and binds to the active site of the kinase in the absence of calmodulin) (Figure 3). Pseudosubstrates have been proposed as a mechanism of regulating the activity of other allosterically regulated enzymes. This mechanism of regulation, however, has not been directly proven for MLCK, and recent kinetic experiments indicate that a more complex model may be required.

Knowledge of the precise substrate determinants of the smooth muscle MLCK, together with the availability of complementary DNA (cDNA) clones encoding this enzyme, will provide a method for directly testing the pseudosubstrate hypothesis.

cAMP-dependent protein kinase phosphorylates MLCK purified from gizzard smooth muscle. In the absence of Ca^2+/-calmodulin, two sites (sites A and B) are phosphorylated (Figure 2) and the concentration of Ca^2+/-calmodulin required for half-maximal activation (K_{CaM}) increases 10-fold.48 In the presence of Ca^2+/-calmodulin, phosphate is incorporated into site B with no effect on MLCK activity. Similar observations have been made with MLCKs purified from a number of mammalian smooth muscles. It has been proposed that phosphorylation of site A by cAMP-dependent protein kinase could decrease the extent of MLCK activation by Ca^2+/-calmodulin with a resultant decrease in myosin light chain phosphorylation and inhibition of contraction. Thus, phosphorylation of MLCK in the regulatory site A would result in a desensitization of myosin light chain phosphorylation and thus contraction to [Ca^{2+}]. This concept will be expanded in a subsequent section.

Purified MLCK from smooth muscle is phosphorylated by other protein kinases as well. Protein kinase C incorporates phosphate into two sites in gizzard MLCK in the absence of Ca^2+/-calmodulin. There

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**Figure 3. Activation of myosin light chain kinase (MLCK).** Schematic illustrates two general mechanisms for activation of MLCK by Ca^{2+}/calmodulin (CaM). Pseudosubstrate mechanism (upper diagram) maintains that the active site is physically blocked by the inhibitory calmodulin binding domain. On binding of calmodulin, the active site is exposed so that light chain (LC) binds and is phosphorylated. Allosteric regulatory mechanism (lower diagram) proposes that the active site is not covered by the inhibitory calmodulin binding domain, and activation results from conformational changes in the substrate binding site that are favorable for light chain binding.
is disagreement as to whether one of these sites is identical to site A. In any case, phosphorylation by protein kinase C leads to a reduced affinity of the kinase for Ca\(^{2+}\)/calmodulin. Calmodulin-dependent protein kinase II also phosphorylates MLCK and decreases the affinity of the kinase for Ca\(^{2+}\)/calmodulin.\(^{38,39}\) Peptide mapping and sequence analysis showed that calmodulin-dependent protein kinase II phosphatase regulates regulatory site A that is also phosphorylated by cAMP-dependent protein kinase. These biochemical observations with protein kinase C and calmodulin-dependent protein kinase II are interesting in light of the previous report that contraction with carbachol or KCl was associated with an increase in \(K_{\text{Ca}}\) for MLCK in tracheal smooth muscle.\(^{40}\) Carbachol stimulation of muscarinic receptors could result in activation of both protein kinase C and calmodulin-dependent protein kinase II, whereas KCl depolarization would lead to activation of calmodulin-dependent protein kinase II. These possibilities will be discussed in a subsequent section.

The use of recombinant DNA technology is leading to a detailed molecular model of MLCKs, including properties of activation by Ca\(^{2+}\)/calmodulin and the substrate binding–catalytic mechanism. It is expected that such techniques will also be useful in analyses of secondary mechanisms of regulation involving phosphorylation of MLCK. These studies may also lead to the design of agents that can result in the specific inactivation of smooth muscle MLCK. Because of the primary role of MLCK in smooth muscle contraction, the use of such agents could provide useful pharmacological tools and potentially important therapeutic agents.

**Protein Phosphatases**

The protein phosphatase responsible for the rapid dephosphorylation of myosin light chain in vivo has not been clearly established. There are four general classes of serine/threonine protein phosphatases referred to as types 1, 2A, 2B, and 2C.\(^{41}\) Type 1 protein phosphatase binds to contractile proteins in skeletal and cardiac muscles and dephosphorylates myosin light chain in vitro.\(^{42,43}\) The catalytic subunit of type 1 phosphatase binds via an unidentified protein component referred to as the M subunit in myofibrils but binds to a different protein subunit (G subunit) in glycogen.\(^{41}\) The glycogen binding subunit undergoes multisite phosphorylation by glycogen synthase kinase 3 and cAMP-dependent protein kinase, and phosphorylation is associated with release of the catalytic subunit.\(^{44}\) Type 2A protein phosphatase represents the major soluble phosphatase activity toward myosin in cardiac muscle.\(^{45}\)

Smooth muscle contains multiple forms of protein phosphatases, which have broad overlapping substrate specificities. Bovine aortic smooth muscle myosin light chain phosphatases have been identified as soluble enzymes that have biochemical characteristics of type 2A phosphatase.\(^{41}\) A multisubunit form of the isolated catalytic subunit of aortic phosphatase 2A dephosphorylates myosin and causes relaxation of skinned fibers from porcine carotid artery and uterine smooth muscle.\(^{46}\) Gizzard smooth muscle contains four forms of myosin light chain phosphatase. Smooth muscle myosin phosphatase IV, which is relatively specific for dephosphorylation of myosin,\(^{48}\) dephosphorylates myosin light chain in skinned muscle fibers.\(^{49}\) A similar activity is present in rabbit uterine smooth muscle.\(^{50}\) This enzyme is similar to but not identical to the more general type 1 protein phosphatase.\(^{46}\) Myosin purified from gizzard smooth muscle also contains type 1-like protein phosphatase activity.\(^{51}\) The mechanism by which it binds to smooth muscle contractile elements has not been defined. Interestingly, it was recently concluded that a type 2A–like protein phosphatase bound to contractile elements in skinned smooth muscle fibers.\(^{52}\) These reports show that both types 1 and 2A protein phosphatases dephosphorylate myosin or myosin light chain in protein preparations or skinned fibers.

There have been some recent attempts to identify the type of protein phosphatase that dephosphorylates myosin light chain in smooth muscle cells. Calyculin A is more effective than okadaic acid in eliciting a contraction in smooth muscle fibers.\(^{51}\) Calyculin A inhibits type 1 protein phosphatase more effectively than type 2, suggesting that type 1 is the dominant protein phosphatase that dephosphorylates myosin in smooth muscle. Although it seems likely that at least one of the protein phosphatases (type 1 or 2A) dephosphorylates myosin in smooth muscle, the possibility cannot be ignored that both enzymes are involved. Additional investigations may also reveal interesting regulatory mechanisms.

**Thin Filament Proteins**

Although there is general agreement that myosin light chain phosphorylation plays a major role in regulating smooth muscle contraction, there has been considerable effort to investigate other potential regulatory mechanisms, the existence of which are suggested by results obtained from physiological and pharmacological experiments with intact tissues (see below). Such observations have stimulated interest in identifying proteins in actin thin filaments that could alter interactions between myosin cross bridges and actin.

Caldesmon is a long, flexible 93-kDa protein with both ends associated with the shaft of the thin filament.\(^{53}\) It inhibits the actin-activated MgATPase activity of phosphorylated myosin, and its inhibitory activity is reversed by Ca\(^{2+}\)/calmodulin.\(^{54,55}\) From these types of studies a mechanism for controlling actin and myosin interactions was proposed: caldesmon binds to actin in the absence of Ca\(^{2+}\) and to calmodulin in the presence of Ca\(^{2+}\) (i.e., Ca\(^{2+}\)/calmodulin reverses the inhibition). Subsequent biochemical experiments have shown that caldesmon contains binding sites to myosin as well as to actin.
tropomyosin, and Ca\textsuperscript{2+}/calmodulin.\textsuperscript{56–58} Furthermore, phosphorylation of caldesmon reverses its inhibitory activity on actomyosin MgATPase activity.\textsuperscript{59} Although caldesmon is phosphorylated in intact smooth muscle, phosphopeptide maps showed that the phosphorylation sites were distinct from those obtained with purified caldesmon phosphorylated by protein kinase C or the multifunctional Ca\textsuperscript{2+}/calmodulin–dependent protein kinase II.\textsuperscript{60} The functional properties of caldesmon phosphorylated in intact smooth muscle were not identified.

Caldesmon, a recently discovered 34-kDa actin-binding protein in smooth muscle, has also been proposed as a candidate for thin filament regulation of actin–myosin interactions.\textsuperscript{61} It binds to tropomyosin and actin and inhibits actin-activated MgATPase activity of phosphorylated smooth muscle myosin. This protein also binds to Ca\textsuperscript{2+}/calmodulin. Phosphorylation of purified calponin by protein kinase C or Ca\textsuperscript{2+}/calmodulin–dependent protein kinase II reverses the inhibition of MgATPase activity of myosin.\textsuperscript{62} These biochemical properties are similar to those described for caldesmon.

There are two primary problems in understanding the physiological importance of caldesmon and calponin. First, the affinities of caldesmon\textsuperscript{63} and calponin (M. Walsh, personal communication, September 1990) for Ca\textsuperscript{2+}/calmodulin appear to be two or three orders of magnitude weaker than the affinity of Ca\textsuperscript{2+}/calmodulin for MLCK. Thus, it seems unlikely that calmodulin will bind to these proteins at low cytosolic Ca\textsuperscript{2+} concentrations that are sufficient for MLCK activation. Second, addition of a partially proteolyzed calmodulin-independent MLCK to skinned fibers in the absence of Ca\textsuperscript{2+} results in contraction.\textsuperscript{64} Similar results have been obtained with microinjection of calmodulin-independent MLCK into single smooth muscle cells.\textsuperscript{65} Under these conditions, caldesmon or calponin would be expected to inhibit contraction induced by phosphorylated myosin. Additional physiological investigations are needed to establish a role for the thin-filament proteins in regulating actin–myosin interactions in smooth muscle.

**Cellular Mechanisms Regulating Physiological Responses**

**Myosin Cross Bridges and Contraction**

A simplified scheme for Ca\textsuperscript{2+} activation of contractile elements may be proposed from biochemical investigations (Figure 1). Ca\textsuperscript{2+} binds to calmodulin and this complex then binds to and activates MLCK. The activated Ca\textsuperscript{2+}/calmodulin/MLCK phosphorylates the regulatory light chain subunit of myosin, which leads to an increase in actin-activated myosin MgATPase activity. Physiologically, myosin light chain phosphorylation would be associated with attachment of cross bridges to actin and the subsequent development of force and increase in maximal shortening velocity. A decrease in Ca\textsuperscript{2+} inactivation of MLCK and dephosphorylation of myosin light chain by a protein phosphatase would lead to relaxation. Physiological investigations have shown that the general scheme described above does not adequately describe cellular processes involved in smooth muscle contraction.\textsuperscript{1,4,3,6} Stimulation of many smooth muscles with various agonists elicits an increase in [Ca\textsuperscript{2+}], and myosin light chain phosphorylation that subsequently declines to low levels while developed force is maintained. The decline in myosin light chain phosphorylation has also been correlated with a decrease in maximal velocity of shortening, an estimate of cross-bridge cycling rates. Force maintenance with reduced myosin light chain phosphorylation and maximal shortening velocity has been referred to as a latch state by Murphy and his colleagues.\textsuperscript{67} These observations have been extended with the development of a quantitative model whereby a phosphorylated myosin cross bridge may become dephosphorylated while attached to actin. It is proposed that the dephosphorylated cross bridge (latch bridge) detaches much more slowly than the phosphorylated cross bridge and thus may be responsible for force maintenance at low levels of myosin light chain phosphorylation.\textsuperscript{14} Central to this model is the assumption that free and attached cross bridges are substrates for MLCK and myosin light chain phosphatase and that the turnover of phosphate in myosin light chain occurs at a high rate. Estimated maximal rates of phosphorylation (1 sec\textsuperscript{−1}) and dephosphorylation (0.25 sec\textsuperscript{−1}) are consistent with the model.\textsuperscript{68} The latch bridge model is proposed from physiological experiments and to date there have been no biochemical studies that directly demonstrate this unique form of the myosin cross bridge. Many reports on the correlations between [Ca\textsuperscript{2+}], myosin light chain phosphorylation, maximal shortening velocity, and force in the hog carotid artery are consistent with the latch bridge model as the primary cellular mechanism regulating contractile properties.\textsuperscript{14} However, it should be pointed out that the latch bridge hypothesis does not necessarily exclude secondary mechanisms of regulation.

An alternative explanation for the latch state is that there is a single population of force-bearing myosin cross bridges rather than two populations. This model requires additional Ca\textsuperscript{2+}-dependent mechanisms to regulate cross-bridge turnover rates and maintenance of force. A number of observations in other smooth muscles, including various types of vascular tissues, indicate that there may be other important regulatory processes in addition to myosin light chain phosphorylation. Although phosphorylation of myosin heavy or light chains by protein kinases (such as protein kinase C) was proposed to represent a secondary mechanism of regulation, physiological evidence has been to the contrary.\textsuperscript{69–72} In ferret aortic smooth muscle tissues, 12-deoxyphorbol, 13-isobutyrate 20-acetate, an activator of protein kinase C, induced a slow contraction of similar magnitude to that induced by K\textsuperscript{+} without significantly...
increasing light chain phosphorylation. In canine tracheal smooth muscle, myosin light chain phosphorylation was completely dissociated from force development when muscles were stimulated with carbachol in Ca²⁺-free physiological salt solution and contracted by readmission of CaCl₂. The addition of okadaic acid, a protein phosphatase inhibitor isolated from the marine sponge Halichondria, to bovine tracheal smooth muscle contracted with carbachol resulted in a decrease in [Ca²⁺], and relaxation in spite of maintenance of myosin light chain phosphorylation at high levels. Phosphopeptide mapping slowed phosphorus-32 incorporation only in serine-19, the site normally phosphorylated by MLCK. This observation was important since phosphorylation of myosin light chain at distinct sites by protein kinase C does not activate myosin MgATPase activity. A dissociation between the extent of myosin light chain phosphorylation and maximal shortening velocity has also been observed.

It is generally agreed that myosin light chain phosphorylation plays an important role in initiating smooth muscle contraction. However, there is not general agreement on the cellular mechanisms involved in force maintenance with low levels of myosin light chain phosphorylation and maximal shortening velocity. Based on published reports from many laboratories, it appears that the latch bridge hypothesis does not uniquely describe all mechanisms regulating smooth muscle contractile elements and that other Ca²⁺-dependent or perhaps Ca²⁺-independent processes act in concert with the myosin light chain phosphorylation system (Figure 4).

**Ca²⁺-Dependent Myosin Phosphorylation**

The content of MLCK and calmodulin in smooth muscle cells is important in relation to Ca²⁺ regulation of myosin light chain phosphorylation. The total cellular content of calmodulin and MLCK is about 40 and 4 µM, respectively. Because these values are 10,000- and 1,000-fold greater than the affinity of Ca²⁺/calmodulin for MLCK, low [Ca²⁺] levels are sufficient for kinase activation. In resting smooth muscle cells [Ca²⁺], is generally about 140 nM. An increase to only 250–300 nM results in half-maximal light chain phosphorylation in agonist-stimulated smooth muscle, indicating a highly sensitive Ca²⁺-dependent process. Thus, [Ca²⁺], is a primary determinant for myosin light chain phosphorylation.

The relation between [Ca²⁺], and force in smooth muscle, however, is not simple. Although smooth muscle is actively developing force, it is expected that the kinetic properties of changes in [Ca²⁺], myosin light chain phosphorylation/dephosphorylation and force development will result in varying relations. However, even under steady-state conditions, unique relations have been described that are dependent on the type of stimulus. For example, during force maintenance Morgan and Morgan in 1984 found that phenylephrine produced a larger increase in force relative to [Ca²⁺], compared with the responses obtained with K⁺ depolarization. Thus, the contractile elements appeared to be more sensitive to the effects of [Ca²⁺], with agonist stimulation. From a number of other studies, it is apparent that the sensitivity of the contractile response (force) to [Ca²⁺], is not invariant.

Rembold and Murphy in 1988 examined the cellular basis for the apparent change in Ca²⁺ sensitivity. They found that there was a single relation between steady-state stresses and the extent of myosin light chain phosphorylation on stimulation with agonists and K⁺. However, agonists resulted in a greater extent of myosin light chain phosphorylation at a given [Ca²⁺], value compared with the responses obtained with K⁺ (i.e., there was an increase in the Ca²⁺ sensitivity of myosin light chain phosphorylation with agonists).

Differences in the Ca²⁺-dependence of myosin light chain phosphorylation in smooth muscle implies that the ratio of MLCK to phosphatase activity is not constant. At a specific [Ca²⁺], value, an increase in the extent of myosin light chain phosphorylation could result from an increase in MLCK activity, a decrease in phosphatase activity toward myosin light chain, or both (Figure 5). Opposite changes in enzymatic activities would occur if there were a decrease in myosin light chain phosphorylation at a specific [Ca²⁺]. Thus, the relation between cytosolic [Ca²⁺] and the extent of myosin light chain phosphorylation may be variable due to secondary mechanisms of regulation of kinase and phosphatase activities.
As mentioned before, MLCK is activated by calmodulin in a Ca\textsuperscript{2+}-dependent manner. There are no biochemical or physiological reports suggesting that the kinase activity may be increased due to increasing the affinity of MLCK for Ca\textsuperscript{2+}/calmodulin. However, as discussed above, MLCK can be phosphorylated at site A, and this phosphorylation decreases the affinity of the enzyme for Ca\textsuperscript{2+}/calmodulin. The predicted physiological consequence of this phosphorylation reaction is to shift the Ca\textsuperscript{2+}-myosin light chain phosphorylation relation to the right, resulting in desensitization of the phosphorylation reaction to Ca\textsuperscript{2+} (Figure 5).

Based on biochemical data, phosphorylation of MLCK by cAMP-dependent protein kinase was proposed to lead to smooth muscle relaxation. It was subsequently shown that a 17-fold stimulation of cAMP formation with forskolin resulted in MLCK phosphorylation in phosphorus-32–labeled tracheal smooth muscle.\textsuperscript{84} The sites of phosphorylation were not identified. However, treatment of tracheal smooth muscles with isoproterenol (a \(\beta\)-agonist that increases cAMP levels) at a concentration sufficient for relaxation had no effect on the Ca\textsuperscript{2+}/calmodulin activation properties of MLCK.\textsuperscript{40} At a high concentration (5 \(\mu\)M) of isoproterenol, however, \(K_{\text{CAM}}\) increased less than twofold. Interestingly, \(K_{\text{CAM}}\) increased more with carbachol or KCl treatments, both of which resulted in contraction.\textsuperscript{40} Although sites of phosphorylation in MLCK were not identified, the conclusion was reached that \(\beta\)-adrenergic relaxation does not require an increase in \(K_{\text{CAM}}\) for MLCK.

Recent investigations have provided new insights into the cellular properties of MLCK phosphorylation.\textsuperscript{85} Under control conditions, MLCK contains about 0.9 mol \(^{32}\)P/mol kinase. Treatment of tracheal tissues with carbachol, KCl, isoproterenol, or phorbol 12,13-dibutyrate increased the extent of kinase phosphorylation. Six primary phosphopeptides (A–F) of MLCK, identified by two-dimensional phosphopeptide mapping, contained different amounts of \(^{32}\)P depending on experimental conditions. Site A was phosphorylated to an appreciable extent (up to 0.8 mol \(^{32}\)P incorporated/mol of site A) only with carbachol or KCl, agents that increase \([\text{Ca}^{2+}]_i\), and result in contraction of smooth muscle. These results show that cAMP-dependent protein kinase and protein kinase C do not affect smooth muscle contractility by phosphorylating site A in MLCK.

Under steady-state conditions of contraction, site A is phosphorylated to a greater extent with \(K^+\) depolarization than with agonist stimulation, and the extent of site A phosphorylation correlated to increases in the concentration of Ca\textsuperscript{2+}/calmodulin required for activation (\(K_{\text{CAM}}\)).\textsuperscript{88} Although these recent observations are consistent with the notion that the differences in the Ca\textsuperscript{2+} dependencies of myosin light chain phosphorylation between \(K^+\) and agonists are related to MLCK phosphorylation, additional experiments are needed to demonstrate the change in Ca\textsuperscript{2+} sensitivity within smooth muscle cells. It is also crucial that the kinase that phosphorylates MLCK be identified so that cellular and biochemical mechanisms of regulation may be understood.

Another potential mechanism for changing the Ca\textsuperscript{2+} sensitivity of myosin light chain phosphorylation involves regulation of protein phosphatase activity. As discussed in the previous section, evidence has been presented that protein phosphatase types 1 and 2A dephosphorylate smooth muscle myosin.\textsuperscript{51,52} The activities of these protein phosphatases are regulated in other tissues by inhibitory proteins and phosphorylation of inhibitory proteins and regulatory subunits.\textsuperscript{41}

A guanine nucleotide–binding protein (G protein) may also be involved in regulation of protein phosphatase activity toward smooth muscle myosin. GTP γS increases the sensitivity of the contractile response to activation by Ca\textsuperscript{2+} in permeabilized smooth muscle.\textsuperscript{87–89} Recent experiments have shown that the increase in tension appears to be mediated by inhibition of protein phosphatase activity toward myosin light chain.\textsuperscript{90}

The potential regulation of protein phosphatase activity toward myosin light chain broadens the scope of mechanisms for affecting smooth muscle contractility. This new area of investigation should provide important information since myosin is most likely dephosphorylated by protein phosphatases that have broad substrate specificities. Thus, results from these studies could provide insights into regulation of dephosphorylation of many proteins, including proteins present in tissues other than smooth muscle. Furthermore, the identification of which protein phosphatase dephosphorylates smooth muscle myosin in vivo and the establishment of modes of regul-
lation may reveal potential approaches for the development of therapeutic agents.

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