CHAPTER 6
SMOOTH-MUSCLE MYOSIN II

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Abstract: The major motor protein in all hollow organs, except the heart, is smooth-muscle myosin II (SmM) and the emphasis of this chapter is on the function of SmM in differentiated smooth muscle. A sliding-filament mechanism is assumed for smooth muscle, as in striated muscle, but there are differences in smooth-muscle thick filaments with respect to assembly and stability. Various isoforms of SmM are expressed and are discussed. SmM differs from its striated muscle counterparts in the requirement for phosphorylation of the regulatory light chains to regulate motor activity. The components of myosin structure required for phosphorylation dependence are outlined. The level of SmM phosphorylation is controlled by opposing actions of the Ca2+-calmodulin-dependent myosin-light-chain kinase and myosin phosphatase. Both are subject to regulation and putative mechanisms are presented. The focus of recent research has been on regulation of myosin phosphatase and inhibition and activation are proposed. Several signaling pathways converge at the myosin phosphatase target subunit to regulate activity. Two pathways that work in opposition are inhibition via the RhoA/Rho-kinase couple and activation by cyclic nucleotides, particularly cGMP. Each is vital in smooth-muscle function and both pathways have important clinical application and are targeted by pharmacological intervention in treatment of smooth-muscle disorders.

Keywords: smooth muscle, myosin II, myosin II isoforms, myosin filaments, phosphorylation, myosin light chain kinase, myosin phosphatase, Rho-kinase, myosin phosphatase target subunit, latch

6.1. INTRODUCTION

The isolation and characterization of smooth-muscle myosin II (SmM) from many smooth muscles has been documented (for a review, see Hartshorne, 1987). The earlier studies assumed that striated-muscle myosin was the appropriate prototype (a common theme in early smooth-muscle research) and this influenced the methods used. Later, the methods were adapted to consider differences in smooth muscles (for...
example, the lower myosin content; the higher ratio of actin [and hence tropomyosin] to myosin compared to striated muscle; and a difference in solubility of skeletal and smooth muscle actomyosins) and “relatively” homogeneous preparations of SmM were obtained and characterized. Initially, it appeared that striated and SmM were indeed identical. The gross structures of each were similar; proteolytic cleavage sites also were similar and both heavy meromyosin (HMM) and subfragment1 (S1) could be generated (see Figure 6.1); and many of the enzymatic properties were similar (Hartshorne, 1987). However, a major distinction was that the activation of the Mg$^{2+}$-ATPase activity of SmM by F-actin required phosphorylation of the regulatory light chains (RLC). The discovery of this unique property originated with studies done on platelet myosin (Adelstein and Conti, 1975; Adelstein, 1978), although at this time the similarity of SmM to non-muscle (Nm) myosins was not realized. For smooth muscle it was reported independently by the laboratories of Sobieszek and colleagues (Bremel et al., 1977; Sobieszek, 1977) and by Perry and colleagues (Frearson et al., 1976). Subsequently, numerous studies have demonstrated the phosphorylation-dependence of myosins from many smooth muscles (Hartshorne, 1987). These were key discoveries and changed the concept of a major aspect of regulation for smooth muscle and subsequently Nm cells to incorporate myosin-linked regulation of contractile events via Ca$^{2+}$-dependent phosphorylation, as distinct from the actin-linked regulation in striated muscle.

6.2. SMOOTH MUSCLE MYOSIN STRUCTURE

The basic structure of SmM is similar to all myosin II isoforms and is a hexamer composed of 2 heavy chains (HC), 2 RLCs and 2 essential light chains (ELC) (Figure 6.1). The N-terminal part of each HC forms a globular S1 domain (head) containing the motor domain, each with an ATP-binding site, an actin-binding site and a regulatory domain, each with an ELC and an RLC. The rest of the HC is α-helical with the first third forming subfragment 2 (S2) and the remainder forming the coiled-coil myosin rod (aka light meromyosin, LMM) that composes the body of the thick filament. Several crystal structures for the head regions of myosin II have been solved (for a review, see Sellers, 1999). The crystal structures of vertebrate smooth-muscle (chicken) motor domain constructs with and without the ELC have been reported (Dominguez et al., 1998) and it is pointed out that the ECL plays a specific role in the mechanical properties of myosin. Details of these structures are reported elsewhere (see Chapter 2). The original report of the structure of S1 from chicken skeletal muscle remains a good model (Rayment et al., 1993a; Rayment et al., 1993b) and is represented in Figure 6.1.

6.2.1. Domain Requirements for Regulation of ATPase Activity

The actin-activated ATPase activity and motor properties of SmM and NmM are regulated by phosphorylation of Ser$^{19}$ of the RLC (Sellers, 1991; Sellers and Adelstein, 1985; Trybus and Warshaw, 1991), which is over 10 nm from the
Figure 6.1. Structure of myosin II and subfragments. Myosin II can be proteolysed to form distinct fragments. Light meromyosin (LMM) is the C-terminal two thirds of the coiled-coil tail (rod) and subfragment 2 (S2) is the remaining one-third with its N-terminus at the head-tail junction. Heavy meromyosin (HMM) contains the S2 portion of the tail plus both subfragment 1 (S1) head fragments. Each S1 heavy chain can be digested by trypsin at 2 loop regions to form fragments of 25, 50 and 23 kDa. The color for each region is matched in the atomic structure of S1, shown below. The regulatory domain contains a portion of the 23-kDa fragment of the heavy chain and the essential and regulatory light chains. The motor domain forms the remainder of the molecule (See Colour Plate 8)
catalytic site (Rayment et al., 1993b). The structural basis of this is not completely understood. Phosphorylation enhances the ATPase activity by more than a thousand fold at saturating actin concentrations (Ellison et al., 2000; Sellers, 1985). Without phosphorylation, SmM is unable to move actin filaments in an in vitro assay, whereas motion is about 0.3–1 μm/sec upon phosphorylation (Cremo et al., 1995; Sellers et al., 1985). Domain requirements for regulation have been elucidated through studies of various proteolytic and expressed subfragments of SmM and NmM. Most mutant, chimeric, and truncated constructs examined to date that have lost normal regulation have elevated ATPase activity in the unphosphorylated state and decreased activity in the phosphorylated state relative to wild type. HMM, which lacks the C-terminal two-thirds of the tail, is double-headed and regulated (Ellison et al., 2000; Ikebe and Hartshorne, 1987; Sellers, 1985), but expressed HMM constructs with truncated tails too short to form stable double-headed structures are unregulated (Matsu-ura and Ikebe, 1995; Sata et al., 1996; Trybus, 1994) as is S1 (Ikebe and Hartshorne, 1987; Konishi et al., 2001; Sellers et al., 1981) and single-headed myosin (Cremo et al., 1995; Konishi et al., 1998). Therefore, two full heads connected together with enough coiled-coil to allow for proper dimerization are required for regulation. Recent analysis of two-dimensional crystals of smooth-muscle HMM in the presence of ATP suggests a structural mechanism for phosphorylation-dependent regulation (Wendt et al., 1999; Wendt et al., 2001). Reconstructions of images to 2 nm resolution of unphosphorylated HMM showed an asymmetrical structure with an interaction between the actin-binding region of the motor domain of one head and the converter region of the motor domain of the partner head. The nucleotide state of the heads is not known. This structural motif has also been observed in tarantula thick filaments strongly suggesting that it is also present in the intact muscle (Woodhead et al., 2005). An important unanswered question is whether or not this motor–motor domain interaction is a requirement for down-regulated kinetic properties or is a consequence of some other stabilizing force. Evidence in support of the motor–motor domain interaction in down-regulation is that phosphorylation-dependent regulation is absent in a Nm HMM construct with one complete head and one head lacking the motor domain (Cremo et al., 2001). However, these data are not completely consistent with other studies of SmM constructs, which suggest that a limited level of regulation is retained in similar constructs (Konishi et al., 2001; Sweeney et al., 2000). Interaction between the motor domains was not evident in phosphorylated HMM (Wendt et al., 1999) suggesting that it is important for the down-regulation mechanism. In addition, point mutations near the regions of motor domain interactions have been shown to cause partial loss of regulation (Yamashita et al., 2000). Other interactions might also play a role. It has been proposed that full regulation of SmM requires not only interaction between the heads, but interaction of the heads with the S2 portion of the rod (Trybus et al., 1997) in the unphosphorylated-state. These data are supported by structural data, as the heads lie down on the S2 portion of the rod. Some important results remain inconsistent. A chimeric myosin with a skeletal motor (normally not regulated) and smooth regulatory/rod domains was found to be regulated (Sata et al., 1997) suggesting that the regulatory domain solely confers the phosphorylation-dependent
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regulation. This result seems inconsistent with a study of SmM showing that substitution of the actin-binding loop to the skeletal sequence can cause loss of regulation (Rovner et al., 1995). Site-specific interactions found between the two RLC in unphosphorylated HMM do not appear to be consistent with the image reconstructions (Wahlstrom et al., 2003). A conclusive picture will require further experiments.

The RLC has been the focus of mutational studies related to regulation of the ATPase (Ikebe and Morita, 1991; Ikebe et al., 1994a; Ikebe et al., 1994b; Trybus and Chatman, 1993; Trybus et al., 1994; Wu et al., 1999; Yang and Sweeney, 1995). The RLC is a calmodulin-like structure that binds largely through hydrophobic interactions with the heavy chain helix. The N-lobe of the RLC (excluding the N-terminal domain, see below) binds to a prominent bend in the heavy chain at the head–tail junction and the C-lobe binds further toward the heavy chain N-terminus (toward the motor domain). Removal of the RLC abolishes regulation (Trybus and Lowey, 1988). The hinge in the long central helix linking the two lobes of the RLC (Rayment et al., 1993b) appears to be critical (Ikebe et al., 1998). Coupling of the ATPase to motion may require flexing of this hinge region (Ikebe et al., 1998).

Studies with chimeric RLC containing either N- or C-terminal lobes from the unregulated skeletal muscle myosin implicate the C-lobe and particularly the H-helix within this lobe as critical for ability to maintain a low activity in the absence of actin (Trybus et al., 1994). The skeletal RLC N-lobe (first 6 smooth RLC residues absent) is sufficient for partial phosphorylation-induced activation if the C-lobe is intact (Trybus and Chatman, 1993). A SmM hybrid with 12 amino acids deleted (H-helix) from the RLC C-terminus had essentially the same activity regardless of actin and phosphorylation (Trybus et al., 1994; see also Ikebe et al., 1994b) as did the hybrid with 26 amino acids deleted and RLC-deficient SmM. The activities in all states were intermediate relative to native unphosphorylated- and phosphorylated-SmM. However, the motor activities were severely compromised, suggesting that the SH1 helix is in an uncoupled state (Gourinath et al., 2003). A chimeric RLC containing the C-lobe from skeletal and N-lobe from smooth sequences cannot be activated by phosphorylation (Trybus and Chatman, 1993) suggesting that important determinants for phosphate coordination reside in the RLC C-lobe. Accordingly, substitution of the smooth with the skeletal H-helix (R160 to Y amongst other substitutions) prevents activation by phosphorylation (Ikebe et al., 1994b; Trybus et al., 1994) in HMM. The extreme N-terminal region of the RLC containing the phosphorylated Ser19 is critical for regulation (Ikebe et al., 1991; Ikebe et al., 1994a). Sequential deletion of N-terminal residues 1–16 incrementally increases the unphosphorylated and decreases the phosphorylated ATPase activities. This behavior might be expected if the entire region provides stabilization energy toward the switched-off and switched-on states, respectively. Unfortunately, no electron density has been observed for the first 24 amino acids of the RLC in any of the myosin X-ray structures. These residues probably constitute an independent flexible domain (Nelson et al., 2005), and may bind to a part of the molecule that is not present in the X-ray structures (rod, other myosin head, or myosin filament binding proteins). The N-terminal domain is proximal to the
partner RLC in unphosphorylated, but not in phosphorylated HMM (Wahlstrom et al., 2003; Wu et al., 1999). It can sense the nucleotide state of the active site, but only in the context of a regulated construct, i.e., double-headed HMM, but not in the unregulated S1 construct (Mazhari et al., 2004). It transitions from a disordered to ordered structure upon phosphorylation (Nelson et al., 2005), consistent with the fact that phosphorylation protects against proteolysis of the RLC N-terminus (Jakes et al., 1976). Phosphorylation drives this domain into a more exposed and hydrophobic environment (Mazhari et al., 2004; Nelson et al., 2005). It is likely that the N-terminal domain is controlling the conformation of the head–tail junction (Ikebe and Hartshorne, 1984). The off-state is likely one in which the head–tail junction position promotes a head conformation that is downward onto the rod, such as that seen in the image reconstructions (Wendt et al., 1999, 2001) and in images obtained by atomic force microscopy (Sheng et al., 2003; Zhang et al., 1997) and by electron microscopy (Katoh et al., 1998; Konishi et al., 1998; Onishi and Wakabayashi, 1982; Onishi et al., 1983; Takahashi et al., 1999; Trybus et al., 1982). At this time further studies are needed to understand the structural mechanism for controlling the bend at the head–tail junction.

The ELC is not required to maintain the off-state, whereas it is important in maintaining a fully on state with respect to both ATPase and motility (Katoh and Morita, 1996; Trybus, 1994). It is interesting to compare the Ca$^{2+}$-regulated scallop myosin (Szent-Gyorgyi et al., 1996; Szent-Gyorgyi et al., 1999) to phosphorylation-regulated myosins. Ca$^{2+}$ turns on scallop myosin and binds to the ELC, which mediates interactions of the ELC with the RLC (Houdusse and Cohen, 1996; Xie et al., 1994). If Ca$^{2+}$ and phosphorylation-dependent regulatory mechanisms are parallel, then one might expect that an interaction between the SmM ELC and RLC would be critical for activation by phosphorylation. Because the ELC is not required to maintain the off-state, it might be that this state does not require such interactions between the ELC and RLC. These questions remain open.

### 6.2.2. Smooth-muscle Myosin Filaments

SmM, like other regulated myosins, can adopt many different conformations. The conformation in the cell from which force is generated is a filamentous array of hundreds of myosin molecules interacting between the rod or tail domains. SmM tends to form side-polar filaments (Craig and Megerman, 1977; Trybus, 1991; Xu et al., 1996), which means that the heads emerge from the right side of the filament with one sense and the left side of the filament with the opposite sense (Figure 6.2A). The rods on one side are arranged in parallel (facing the same way) and they are staggered by 14.3 nm. The other side of the filament is built in the same manner. The two sides interact, with the rods in an antiparallel interaction with the C-termini abutted. A myosin from the left side of the sheet interacts with the myosin above it from the right side with a 14.3 nm overlap. Filaments are close packed flat sheets (Figure 6.2B). These sheets interact by their bare faces into stacks of two to three. This structural arrangement allows actin interaction on both sides
Figure 6.2. Side polar filament structure. A. A single smooth-muscle myosin II side polar filament, showing the 14.3 nm overlap of the ends of the myosin heavy chain tails. B. General 3D arrangement of stacked multiple smooth-muscle myosin sheets. Figure not to scale

of the sheet and may partially explain the remarkable ability of smooth muscles to lengthen, without the actin-myosin interaction going beyond overlap (nonfunctional state). Also, this arrangement allows the ends of the filaments to serve as sites from which monomers can attach. Skeletal muscle filaments (bipolar) do not have these attributes. Therefore, these features of smooth-muscle myosin filament structure appear to be adapted well for reversible polymerization.

Earlier it was hypothesized (Needham, 1964) that myosin filaments in smooth muscle completely dissolve in relaxed muscle and then polymerize on muscle activation. This hypothesis has been tested thoroughly and has now been discarded. Thick filaments are present in relaxed smooth muscles in which the SmM RLC is predominately unphosphorylated (Cooke and Fay, 1972; Garamvolgyi et al., 1971, 1973; Gillis et al., 1988; Godfraind-De Becker and Gillis, 1988; Hodgkinson et al., 1995; Somlyo et al., 1973; Somlyo et al., 1981; Watanabe et al., 1993; Xu et al., 1997). However, in some smooth-muscle types, there is strong evidence that filaments can undergo a reversible process of polymerization and depolymerization, also termed evanescence (Seow, 2005).

Labile SmM filaments have been detected using many different techniques in many muscle types. Significant increases in myosin filament density upon smooth-muscle activation have been observed in rat anococcygeus (Gillis et al., 1988; Godfraind-De Becker and Gillis, 1988; Watanabe et al., 1993; Xu et al., 1997) and porcine trachea (Herrera et al., 2002), whereas no changes were observed in guinea pig taenia coli (Tsukita et al., 1982; Watanabe et al., 1993; Xu et al., 1997), rabbit portal vein (Somlyo et al., 1981) and chicken gizzard (Horowitz et al., 1994). It appears that the extent to which myosin can dynamically cycle between filaments and some other form not detected as filaments depends upon the muscle type. In the airway, there is a large increase in myosin filament density (within seconds) when the muscle is activated (Herrera et al., 2002). This suggests that there is a
non-filamentous pool of myosin in relaxed muscle that is primed for assembly into filaments in response to activation. The nature of this non-filamentous pool and how it is regulated at the molecular level in not known, but important clues come from in vitro studies (see below). SmM filament stability may be due not only to differences in the SM1 and SM2 myosin isoforms (see isoforms section), but also to differences in the expression of myosin filament stabilizing proteins. The formation of thick filaments at the appropriate place and time may be stabilized by the thin filament protein, caldesmon, that is known to bind to both actin and myosin (Wang, 2001). In addition, the level of telokin may be important (see below). Telokin was suggested to stabilize filaments of dephosphorylated myosin against dissociation by ATP (Shirinsky et al., 1993) via binding of its acidic C-terminus to the S1–S2 region of myosin (Silver et al., 1997). One effect of the interaction was to suppress formation of the 10S state (see below) favoring filament formation by 6S myosin (Masato et al., 1997). However, the level of telokin in tonic muscle is low, compared to phasic muscle (Gallagher and Herring, 1991). The 38k protein (Okagaki et al., 2000) may also contribute to filament stability. Airway SmM filaments can be depolymerized in the resting state (Herrera et al., 2002; Kuo et al., 2003; Kuo et al., 2001; Qi et al., 2002) and this may reflect a very low resting activity and low resting RLC phosphorylation levels. Anococcygeus muscle, primarily phasic but with a small component of tonic activity, has a long low resting activity between contractions, and it shows evidence of labile filaments (see above). In contrast, taenia coli has the ability to spontaneously contract and therefore is probably never completely relaxed. Force and thick-filament density both fall by about 20–30% after repeated passive ± 30% length changes (oscillation) to relaxed porcine trachea smooth muscle. This amplitude of stretch is similar to that experienced by airways during a deep inspiration (Fredberg et al., 1999). Exposure to subsequent tetani increased the force and filament density to initial values. This suggests that the mechanism for force reduction after passive length changes may be due to myosin thick filament depolymerization. The magnitude of force reduction (prior to adaptation) is proportional to the amplitude and duration of the length oscillation (Wang et al., 2000). Thus, the labile nature of SmM filaments is likely to be partially responsible for the unique mechanical properties of smooth muscles, with the extent of filament stability tuned for the specific function of each muscle type.

6.2.3. Other Conformations of Smooth Muscle

Myosin – 10S and 6S States

In vitro studies strongly support the idea that SmM filaments may be labile in muscle. Under physiological conditions in the presence of ATP, SmM is in a dynamic equilibrium between filaments and an intramolecularly hairpin-folded or bent monomeric conformation called 10S (S for Svedberg; earlier literature for 6S/10S conformers given in Hartshorne, 1987). RLC phosphorylation not only activates the SmM ATPase activity, but it strongly promotes filament assembly in vitro. Phosphorylated SmM is stabilized in filaments, whereas unphosphorylated
SmM is stabilized in the folded 10S conformation. The relative amount of each conformation is defined by the critical monomer concentration (Ankrett et al., 1991), the myosin concentration at which filaments form. Many isoforms of myosin II can adopt the 10S conformation, including those from vertebrate smooth muscle (Hartshorne, 1987; Onishi and Wakabayashi, 1982; Onishi et al., 1983; Suzuki et al., 1985; Trybus et al., 1982; Trybus and Lowey, 1984), molluscan striated (Ankrett et al., 1991; Takahashi et al., 1989), vertebrate striated (Katoh et al., 1998), vertebrate cardiac (Takahashi et al., 1999) and vertebrate cytoplasmic sources (Barylko et al., 1986; Citi and Kendrick-Jones, 1986; 1987; Citi et al., 1989; Craig et al., 1983; Helbing et al., 1993; Higashihara et al., 1989; Korn and Hammer, 1988; Scholey et al., 1980; Takeuchi and Ishimura, 1985). The 10S conformations of the regulated myosins (smooth, nonmuscle and molluscan) are much more stable and therefore are more likely to be physiologically relevant. It is possible that the extent of filament assembly in various muscle types is correlated to the stability of the 10S conformation in situ.

Other motors such as myosin I (Stoffler and Bahler, 1998), myosin V (Liu et al., 2006), and kinesin (Hackney et al., 1992) have been shown to form self-inhibited folded structures similar to 10S myosin II. Therefore, the ability to adopt such folded structures may be an evolutionarily conserved property of motor proteins. Since 10S is a fully assembled form of myosin that can be rapidly assembled into filaments, by unfolding transiently to the 6S extended monomer (Cross et al., 1991; Rosenfeld et al., 1994), it may be a storage or trafficked form of myosin ready for participation in the force generating process (Cross, 1988; Trybus, 1991). ATP would not be wasted because 10S “traps” ADP-Pi at the active site (Cross et al., 1986). This means that the off-rate for Pi is extremely slow and the molecule is functional, but catalytically inactive. The ATP turnover rate of the 10S conformer is \( \sim 0.0002 \text{sec}^{-1} \). 10S binds extremely weakly to actin (Olney et al., 1996), so it would not compete with filamentous myosin for actin-binding sites in the cell. There has been only one study attempting to detect 10S in smooth muscle (Horowitz et al., 1994). 10S-specific monoclonal antibodies detected a small amount of 10S in adult gizzard, but the amount was unchanged upon contraction, and did not change from relaxed to contracted conditions in the permeabilized muscle. Interestingly, the amount of 10S increased substantially when the permeabilized embryonic gizzard tissue was exposed to “10S-promoting” buffer conditions. This finding supports the idea that tissues undergoing remodeling may have a greater capacity for reversible polymerization than fully differentiated tissues. It would be productive to extend these studies to a tonic muscle (rather than phasic) in which reversible myosin polymerization has been verified by other methods (see above).

Since 10S myosin is trapped in the ADP-Pi state, it is of interest as a structural analog of the pre-power stroke state. Earlier studies with sedimentation velocity indicated that 10S myosin was a compact form compared to the extended 6S state (stable in vitro only at non-physiological high ionic strength). Electron micrographs of shadowed and negatively-stained single molecules (Katoh et al., 1998; Konishi et al., 1998; Onishi and Wakabayashi, 1982; Onishi et al., 1983; Takahashi
et al., 1999; Trybus et al., 1982) show that the heads are bent down towards the tail and that the tail is bent at least twice into three regions of approximately equal length. Cryo-EM data show that dephosphorylated smooth HMM (Wendt et al., 1999; Wendt et al., 2001) and 10S (Liu et al., 2003) both have the same motor–motor interacting motif described above, although this technique did not give certain information about the location of the tail. From conventional EM and from atomic force microscopy (Sheng et al., 2003; Zhang et al., 1997), the first bend in the tail or rod is at the S2-LMM junction and the second bend appears to interact with the regulatory domains. The location of the second bending region coincides with two closely spaced potential bends characterized by their low propensity for coiled-coil structure (Olney et al., 1996). These two closely spaced bends interact with the RLC at the N- and C-lobes (Olney et al., 1996; Salzameda et al., 2006) and potentially other sites. The tail-regulatory domain interaction is consistent with the fact that both the ELC and the RLC, and specifically the RLC N-terminus, are required for 10S stability (Ikebe et al., 1994a; Trybus and Lowey, 1988). Single-headed SmM can fold, but only at unusually low ionic strength (Olney et al., 1996). Therefore, the presence of both heads appears to strongly stabilize the interaction of the tail. Since both motor domains are required to adopt the off state (Cremo et al., 2001), it appears that the tail binds to the heads in the off-state or the ADP-Pi trapped state (Olney et al., 1996). This is consistent with mutagenesis data showing that myosin hybrids that cannot adopt the off-state are less stable in the 10S conformation (Ikebe et al., 1994a). It will be of interest to compare and contrast the structural mechanisms for stabilizing the off-state for the various myosin isoforms and for other motors that can form self-inhibited states.

6.2.4. Isoforms of Smooth Muscle Myosin

Isoforms of both the HCs and LCs have been described (for reviews, see Somlyo, 1993; Eddinger and Meer, 2007). SmM HC is expressed by a single gene located on human chromosome 16 (16q12.1; Matsuoka et al., 1993). Various isoforms of the HC are generated as a result of alternative splicing of the message at the 3’ and 5’ termini. The former generates 2 HCs differing at their C-termini. These were detected in Murphy’s laboratory (Rovner et al., 1986) by SDS-electrophoresis and termed SM1 (∼205 kDa) and SM2 (∼200 kDa). The difference was shown to reside in the LMM fragment (Eddinger and Murphy, 1988). Cloning and sequence analysis (Nagai et al., 1989; Babij and Periasamy, 1989) showed that the C-termini were distinct. Unique sequences of 43 residues and 9 residues were determined for SM1 and SM2, respectively. In addition, differences in splicing at the 5’ end were found (Hamada et al., 1990) and later shown to reflect either the presence or absence of a 7-residue insert in the myosin head close to the ATP-binding site at the 25/50 kDa junction (Kelley et al., 1993; White et al., 1993). The insert is located 28 residues to the C-terminal side of the GXXGXG motif in the loop 1 region (see Figure 6.1). Its presence correlated with a higher velocity of movement of actin filaments and increased Mg^{2+}-ATPase activity (Kelley et al., 1993).
Two ELC isoforms were detected by electrophoresis (Cavaille et al., 1986; Helper et al., 1988; Hasegawa et al., 1988) and later sequenced from cDNA libraries of smooth muscle and Nm sources (Nabeshima et al., 1987; Lenz et al., 1989) and by protein sequencing (Hasegawa and Morita, 1992). The 2 isoforms are products of a single gene generated by alternative splicing and utilization of different exons (exon 6 in smooth muscle and exon 7 for NmM; Lenz et al., 1989). The product is 151 residues that differ in the C-terminal 9 residues that contains 5 differences in mammalian ELC and 4 differences for avian ELC (Nabeshima et al., 1987). (Sequences in this region may vary slightly in different reports.) The more acidic muscle ELC ( termed LC 17a or LC17gi [for gizzard] and Nm ELC (LC 17b) are both present in smooth-muscle preparations (Cavaille et al., 1986; Hasegawa et al., 1988; Helper et al., 1988). In general, myosin of tonic smooth muscles (e.g., those of major arteries) contain both ELCs, but myosin of phasic smooth muscles (e.g., stomach, jejunum and gizzard) contain predominantly the muscle-specific ELC (Helper et al., 1988) and it was proposed that the ratio of ELC isoforms influenced ATPase activity (Hasegawa and Morita, 1992). Isoforms of the RLC (LC20) have been observed. In addition to changes in pi caused by phosphorylation (at Ser19, Ser19 and Thr18, and at the PKC sites, see below) other variants were found in smooth muscle of arteries (Mougios and Barany, 1986), uterus (Barany et al., 1987) and carotid (Gaylinn et al., 1989). These represent the muscle and Nm isoforms, but unlike ELC are the products of 2 genes (Taubman et al., 1987; Kumar et al., 1989). It is tempting to correlate expression of Nm RLC with that of the Nm HC, as suggested by Gaylinn et al. (1989). This relationship may not apply in Nm cells and it was shown that in platelets both muscle and Nm RLCs are present, but only Nm HCs. It is known that NmM is present in differentiated smooth muscle (Gaylinn et al., 1989) and is higher in proliferating and developing smooth-muscle cells (Eddinger and Murphy, 1991; Monical et al., 1993; Frid et al., 1993). The muscle/non-muscle phenotype may also vary during physiological and pathological vascular remodeling (Sartore et al., 1994). A new isoform of RLC was found in embryonic chicken gizzard that differed in 10 residues from the adult form (Inoue et al., 1989).

For the head insert (the 5’ insert; also termed SMA minus insert, or SMB plus insert) its presence increased actin-activated ATPase activity and motility (Kelley et al., 1993; Rovner et al., 1997) and increased ATPase and maximum velocity of shortening (DiSanto et al., 1997). In general, these changes were independent of the ELC content. Eddinger and Meer (2001) noted, however, that although the presence of the insert increased shortening velocity other mechanisms were involved. On the other hand, it was suggested that the 5’ insert had no effect on contractile properties of smooth muscle (Haase and Morano, 1996). At the molecular level, it was proposed that the presence of the insert increased the ADP off rate and reduced the affinity of the myosin head for ADP (Rovner et al., 1997). This idea is consistent with the suggested role of ADP in tonic muscle and with the latch state. Other proposals pertinent to the latch state, were: that the higher content of Nm ELC in tonic muscle increased affinity for ADP and thus contributed to the
latch state (Fuglsang et al., 1993); and that in the dephosphorylated state where the ELC acts as a repressor the ELC isoforms modulate ADP affinity and thereby the rate of cross-bridge cooperative cycling (Matthew et al., 1998). The case for a lack of function for ELC isoforms include those references quoted above for ATPase activity etc., and the finding that in single smooth-muscle cells the ratio of ELC isoforms was not a determinant of shortening velocity (Eddinger et al., 2000).

There is still speculation on the role of the C-terminal HC isoforms (3’ C-terminal addition). Although the 2 isoforms, SM1 and SM2, are expressed at different levels in various smooth muscles there is no consensus (see discussion in Eddinger and Meer, 2007) that the different SM1/SM2 ratios alter functional properties of the smooth muscle (e.g., for pregnant and non-pregnant uteri; Sparrow et al., 1988) or actomyosin ATPase activity and movement of actin filaments (Samuel et al., 1992; Kelley and Adelstein, 1994). SM1 and SM2 may be restricted to homodimers (Kelley et al., 1992; Kelley and Adelstein, 1994) or, found as both homo-and heterodimers (Tsao and Eddinger, 1993). Both the SM1 and SM2 isoforms can also contain the head insert (Kelley and Adelstein, 1994). The 43-residue extension of the HC at the C-terminus (found in SM1) is non-helical and was thought to modify filament formation. However, there is some controversy concerning which part of the C-terminal HC is critical in filament formation. Removal of approximately 4 kDa from the C-terminus of SM1 by proteolysis enhanced filament aggregation of the digested myosin and the stabilized filament formation modified enzymatic activity (Ikebe et al., 1991). Thus, the suggestion that the C-terminal coiled-coil region was required, rather than the non-helical extension (Ikebe et al., 2001). However, in native myosin the longer tail piece found in SM1 confers greater filament stability (Rovner et al., 2002). In addition, LMM derived from SM1 and SM2 showed differences in solubility and paracrystal formation (Quevillon-Cheruel et al., 1999). Thus, the alternative view is that the non-helical portion influences differences between SM1 and SM2 in regard to filament formation. Possibly the 2 isoforms influence plasticity and assembly and disassembly of thick filaments (Seow, 2005), but further work is necessary to establish this. In addition, if the SM1/SM2 isoforms are important for in vivo stability of filaments the formation of homodimers and/or heterodimers is an important consideration. The C-terminus of Acanthamoeba myosin II is critical for filament formation, although here the proteolytic removal of the C-terminal fragment, containing 3 phosphorylation sites, abolishes filament formation (Kuznicki et al., 1985). In smooth-muscle cells from bovine aorta it was found that the HCs were phosphorylated (Kawamoto and Adelstein, 1988) and this was traced to an acidic region of the C-terminal extension containing a casein kinase II site (Kelley and Adelstein, 1990; Fukui and Morita, 1996; see Figure 6.3). A second, but less effective HC kinase, possibly PKC, also was detected. The casein kinase II site is conserved in SM1 and non-muscle myosins and argues in favor of a myosin-linked physiological role for casein kinase II in cell function, possibly in filament assembly in smooth-muscle cell proliferation (Kelley and Adelstein, 1990).
A. HC Head Insert

Subfragment 1:

![Diagram of smooth-muscle myosin II isoforms]

B. HC Tail Insert (rat)

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM1</td>
<td>KSKLR—RGNEASFVPSSRAAGRRVIENTDG(S)EEEM</td>
</tr>
<tr>
<td></td>
<td>DARDSDFNGTAKE — 1979</td>
</tr>
<tr>
<td>SM2</td>
<td>KSKLR—GPPQETSQ — 1945</td>
</tr>
</tbody>
</table>

C. ELC

<table>
<thead>
<tr>
<th>Type</th>
<th>Aorta</th>
<th>Gizzard</th>
</tr>
</thead>
<tbody>
<tr>
<td>17a --Muscle</td>
<td>ELVRMVLMG</td>
<td></td>
</tr>
<tr>
<td>Muscle (gizzard)</td>
<td>ELVRMVLNG</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type</th>
<th>Aorta</th>
<th>Fibroblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>17b --Non-muscle</td>
<td>AFVRMILSG</td>
<td></td>
</tr>
<tr>
<td>Non-muscle (fibroblast)</td>
<td>AFVRHILSF</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.3. Summary of inserts for smooth-muscle myosin II isoforms. A. The 7-residue insert located in the S1 heavy chain region at loop 1. B. The C-terminal sequences for the SM1 and SM2 isoforms. The Ser in parenthesis in the SM1 sequence indicates the casein kinase II phosphorylation site. The number of total heavy-chain residues assumes the presence of the 7-residue head insert. C. Inserts over a 9-residue sequence for the muscle and non-muscle forms of the essential light chain (ELC). For references, see text.

Thus, although several isoforms of SmM can be assembled (for summary, see Figure 6.3), there is controversy concerning the physiological roles for these isoforms. An attractive scenario is that the content of the 7-residue head insert and the proportion of muscle to Nm ELC may underlie the different physiological properties of phasic and tonic muscles. (Phasic muscles, including bladder, portal vein, longitudinal layer of ileum and gizzard, generate action potentials and respond with a rapid transient contraction. Tonic muscles, including most major arteries and trachealis, respond to many agonists [pharmacomechanical coupling, Somlyo and Somlyo, 1968; 1994] with slow and sustained contractions.) A higher content of the head insert and the muscle ELC would be expected in the phasic muscle myosin (Somlyo and Somlyo, 1994). In this context consider also that the levels of MLCK
and MP are higher in phasic muscle (Gong et al., 1992). The presence/absence of the head insert appears to modify the active site of SmM and there is a body of evidence to support the claim that its presence increases ATPase activity in vitro and velocity of shortening in muscle fibers. However, the observed changes do not fully rationalize the variations in shortening velocity found in different smooth muscles. As pointed out by Murphy et al. (1997) much of the observed diversity in power output should be attributed to the regulatory mechanisms modulating cross-bridge cycling rates rather than to variations in isoform expression.

6.3. PHOSPHORYLATION OF SMOOTH-MUSCLE MYOSIN

6.3.1. Basic Mechanism

It is generally accepted that phosphorylation of the RLC (2 mol P/mol myosin) initiates contraction in smooth muscle and motile events or structural changes in Nm (for reviews, see Hartshorne, 1987; Somlyo and Somlyo, 1994). The fundamental mechanism of contraction in smooth and striated muscle is similar and involves cyclic cross-bridge interactions with F-actin in the thin filaments and the transition between weakly-bound (myosin plus the ATP hydrolysis products, Pi and ADP) and strongly-bound (following the release of Pi) states. It was shown several years ago that phosphorylation or thiophosphorylation of RLCs in smooth-muscle fibers induced contraction even in the absence of Ca\(^{2+}\) (Walsh et al., 1982) and that RLC phosphorylation was closely correlated to shortening velocity (Dillon et al., 1981). The kinetic step regulated by phosphorylation followed ATP hydrolysis and was suggested to be either Pi release or a preceding step (Sellars et al., 1982; Somlyo and Somlyo, 1994). An additional connection being that the rate of force development in permeabilized smooth-muscle fibers reflected the rate of RLC phosphorylation (Somlyo et al., 1988).

The major site of phosphorylation on RLC is Ser\(^{19}\). The sequence around Ser\(^{19}\) is important for recognition and specificity of MLCK. The Arg residue at the P-3 position is critical and is thought to interact with acidic residues in the catalytic core of MLCK. Hydrophobic residues to the C-terminal side of Ser\(^{19}\) (P+1, P+2 and P+3) also are important. Other residues in the N-terminal half of RLC may be involved in substrate recognition (for a review, see Stull et al., 1998). Ser\(^{19}\) is the preferred site of phosphorylation, but at high concentrations of MLCK under in vitro conditions Thr\(^{18}\) also is phosphorylated. Dual phosphorylation increased actin-activated ATPase activity (Ikebe and Hartshorne, 1987; Tanaka et al., 1985; Ikebe et al., 1986) and doubled Vmax with little effect on actin affinity (Ikebe et al., 1988). However, whether diphosphorylation of RLC has an important physiological role is still in question. The level of diphosphorylation observed on stimulation of smooth-muscle fibers is usually low (e.g., Miller-Hance et al., 1988; Colburn et al., 1988; Harada et al., 1995) and in addition, double phosphorylation had no effect on velocities of SmM or actin movement in motility assays (Umemoto et al., 1989; Bresnick et al., 1995). Diphosphorylation of RLC is frequently observed in Nm cells and several kinases other than
MLCK readily phosphorylate Thr\textsuperscript{18} (see below). For example, phosphorylation of NmM by Rho-kinase (ROK) usually results in double phosphorylation. Again, its purpose is not established, but since double phosphorylation of SmM increases its tendency to aggregate (Ikebe et al., 1988), it might be speculated that in Nm cells its role is to increase attachment or localization of myosin to cytoskeletal structures.

The level of RLC phosphorylation is controlled by opposing activities of myosin-light-chain kinase (MLCK) and myosin-light-chain phosphatase, or myosin phosphatase (MP). MLCK is specific for Ser\textsuperscript{19} and to a lesser extent Thr\textsuperscript{18} of the RLCs (thus a “dedicated” kinase) and its activity is regulated primarily by binding and activation of the Ca\textsuperscript{2+}–calmodulin (Ca\textsuperscript{2+}-CaM) complex (see below). MP is not Ca\textsuperscript{2+}-dependent, but can be regulated and both inhibition and activation of activity have been claimed (see below). Clearly, if the [Ca\textsuperscript{2+}] is held constant and either the MLCK or MP activities are altered, the level of RLC phosphorylation will change, resulting in a shift of the so-termed “Ca\textsuperscript{2+} sensitivity” (see below).

6.3.2. Myosin Light Chain Kinase

In smooth muscle the MLCK gene expresses 3 transcripts due to alternate promoters. These are the short and long forms of MLCK and the C-terminus of MLCK expressed as an independent protein, telokin. The mass for the short MLCK varies between 101 kDa and 129 kDa, although higher values are reported from SDS electrophoresis. The long MLCK contains an additional N-terminal extension and is not usually found in differentiated (contractile phenotype) smooth muscle. It is also referred to as embryonic, non-muscle, endothelial or 210-kDa MLCK. The domain structure of short MLCK was evident from the original report by Guerriero et al. (1986) and an unusual feature of the smooth-muscle and Nm forms of MLCK was later found to include the motifs, designated I and II, found in twitchin, an unc-22 (for uncoordinated) gene product (Benian et al., 1989) and titin (Labeit et al., 1990). Short MLCK was found to have 3 type II repeats identified as IgG folds (Ig domains) and 1 type-I repeat, a fibronectin (FN) module. One of the Ig folds is located in telokin and from its crystal structure was shown to be a β-barrel structure similar to the CH\textsubscript{2} domain of the immunoglobulin fragment, Fc (Holden et al., 1992). Other domains, or regions, include the kinase domain and to its C-terminal side an autoinhibitory, or pseudosubstrate (PS) sequence that overlaps with the CaM-binding site.

An early observation was that MLCK bound to F-actin (Dabrowska et al., 1982) and an obvious question was which part of the MLCK molecule was responsible for this interaction? An actin-binding peptide was isolated and corresponded to the N-terminal sequence 1–114 of the short form (Kanoh et al., 1993). Subsequently, a unique structural motif was identified that was found to be necessary for high affinity binding to F-actin. In short MLCK, 3 of these units were found at residues 2–7, 30–35 and 58–63 and were termed DFRXXL motifs (Smith et al., 1999). Skeletal muscle MLCK does not bind actin with high affinity and does not contain
these motifs. In another report, the actin-binding site was suggested to be residues 2–42 and this peptide would contain the first 2 DFRXXL motifs (Ye et al., 1997). An additional 2 DFRXXL motifs (and 6 Ig repeats) were found in long MLCK and it was suggested that these motifs conferred to the long form an increased affinity of actin binding (Smith et al., 2002). Each DFRXXL motif binds to one actin monomer, thus the short form binds to 3 actin monomers and the long form to 5. A reconstruction of the interaction of MLCK with F-actin indicated the binding of MLCK would not interfere with other key actin-binding proteins, e.g., myosin, tropomyosin, caldesmon and calponin (Hatch et al., 2001). There is still an unresolved issue that binding of MLCK to native filaments might be strengthened by interaction with an additional thin filament component (Lin et al., 1999).

If MLCK remains attached to the thin filament during the contractile cycle of smooth muscle, how might this affect myosin phosphorylation? A rough estimate of [MLCK] in gizzard (a phasic muscle) is 3 μM (Dabrowska et al., 1982), but would be lower in tonic muscles (Gong et al., 1992). Concentrations of actin and myosin heads (i.e., RLC) are about 0.8 mM and 80–100 μM, respectively. This translates to stoichiometries of about 1:270 for MLCK: actin and 1: 27–30 for MLCK: RLC. (The latter level is relatively high and under optimum conditions determined in vitro [excess Ca\(^{2+}\)-CaM, unlimited access, etc.] would rapidly phosphorylate all myosin.) Even allowing for preferential localization of MLCK to the contractile domain in the smooth-muscle cell (as opposed to structural domains; Small et al., 1986), the binding of MLCK to actin would limit distribution of MLCK to a few molecules of MLCK/thin filament. Assuming a thin filament of 1 μm (thin filaments in smooth muscle may be longer, but are difficult to measure; Somlyo, 1980) there would be about 2 molecules of MLCK/thin filament. Is the MLCK randomly distributed, but fixed, along the thin filament or can it move, is there a specific location, e.g., at either end and is another component(s) involved in binding or localization? These intriguing questions remain open. If there is a fixed location for MLCK, this would logically restrict access to the myosin heads (RLCs) and may limit phosphorylation levels and it might also be expected that increased RLC phosphorylation requires relative sliding of the thin and thick filaments. The restricted access of thin-filament bound MLCK is compensated for, to some extent, by the larger number of thin filaments surrounding the thick filament in smooth muscle (12–16:1; Somlyo, 1980) compared to skeletal muscle (6:1). One observation that suggests that all of the myosin heads are accessible to MLCK is that thiophosphorylation levels (using ATP\(\gamma\)S) of RLC (the thiophosphate group is resistant to phosphatase activity) often are considerably higher than phosphorylation levels (using ATP) and can approach 100% (Kenney et al., 1990).

If MLCK is attached to the thin filament via the 3 DFRXXL motifs (for short MLCK) can the kinase domain reach the Ser\(^{19}\) phosphorylation site on the RLC of myosin on the thick filament? To the C-terminal side of the actin-binding motifs are 2 Ig domains and 1 FN module. These would extend about 130 Å (Lin et al., 1999) and to this should be added the distance to the cleft between the 2 lobes in the catalytic core. If the surface-to-surface distance between the thin and thick filament...
is about 150 Å (Somlyo, 1980), then the extension of MLCK away from the thin filament seems adequate to reach the phosphorylation sites (Stull et al., 1998).

On the C-terminal side of the 2 lobes comprising the catalytic/kinase domain is the regulatory domain. This consists of a sequence resembling the RLC phosphorylation site, i.e., around Ser19, with an emphasis on basic residues that overlap with the CaM-binding site. The simplest interpretation is that in the absence of CaM, the PS sequence binds to the catalytic domain and inhibits activity (i.e., the PS mechanism found in many kinases; Pearson et al., 1988). When CaM binds to MLCK this interaction is displaced and the kinase becomes active. Many techniques have been applied to define the PS and CaM-binding sequences. From sequential proteolysis (Ikebe et al., 1987) it was determined that both the PS and CaM-binding site were contained in residues 787–815 (numbering for chicken gizzard MLCK; Pearson et al., 1988). Several synthetic peptides incorporating all or parts of this sequence were found to be effective inhibitors of MLCK and the sequence 783 to 807 is a potent inhibitor of MLCK in the nM range (Foster et al., 1990). These inhibitors are extremely useful experimentally and can be adapted for cell delivery (Wu et al., 2003). Several other studies have attempted to define the PS sequence more precisely. Ito et al. (1991) suggested that the sequence Tyr794 to Trp800 was critical for inhibition; however, the essential elements for inhibition are not unequivocally established. From X-ray and neutron scattering studies it was shown that the inhibitory (PS) sequence interacts with the larger C-terminal lobe of the catalytic core and on binding Ca2+-CaM there is a conformational collapse where the PS moves away from the catalytic core (Krueger et al., 1997). The location of the CaM-binding site may be predicted based on known sequences from several CaM-binding sites in which the site is flanked by 2 hydrophobic residues separated by approximately a 12-residue amphipathic helix (Ikura et al., 1992). This would place the CaM-binding site of gizzard MLCK between Trp800 and Leu813 and therefore overlapping with the PS sequence. The binding of Ca2+-CaM involves interaction of the hydrophobic patches between the pairs of EF hands (exposed in the presence of Ca2+) with the flanking hydrophobic residues on the CaM-binding site and the collapse of the central helix (the flexible tether) of CaM to allow the 2 lobes to come together (Persechini and Kretsinger, 1988).

6.3.2.1. Phosphorylation of myosin light chain kinase

The dominant mechanism for regulation of MLCK is via Ca2+-CaM, but if other mechanisms could modulate MLCK activity at fixed [Ca2+], this also would alter Ca2+-sensitivity. Any change may result from modification of either MLCK or CaM.

Initially it was found that the cAMP-dependent protein kinase (PKA) phosphorylated MLCK at 2 sites in the absence of CaM and 1 site in its presence (Adelstein et al., 1978; Conti and Adelstein, 1981) and that phosphorylation decreased MLCK activity by reducing KCaM, i.e., higher concentrations of CaM were required to activate MLCK. The 2 sites were Ser815 and Ser828 (Lukas et al., 1986; Payne et al., 1986; Pearson et al., 1988). The site responsible for influencing KCaM,
Ser\textsuperscript{815}, site A, is located at the C-terminal edge of the CaM-binding site. Two other kinases phosphorylate site A with similar in-vitro effects on K\textsubscript{CaM}, namely Ca\textsuperscript{2+}/CaM-dependent protein kinase II (CaM K II; Hashimoto and Soderling, 1990; Ikebe and Reardon, 1990; Tansey et al., 1992) and protein kinase C (PKC; Ikebe et al., 1987; Nishikawa et al., 1985). Following a report that p21-activated kinase (PAK) inhibited MLCK in Nm cells (Sanders et al., 1999), it was found that addition of PAK1 to smooth-muscle fibers attenuated contraction by phosphorylating and inhibiting MLCK (Wirth et al., 2003). The extent to which PAK is involved in normal smooth-muscle function is not known. Other kinases modifying MLCK activity, include: PAK2 in endothelial cells (Goeckeler et al., 2000); a mitogen-activated kinase and cyclin-dependent kinase-1 (Morrison et al., 1996); and activation via the extracellular signal-regulated kinase (ERK; Nguyen et al., 1999). It was also claimed that MLCK can autophosphorylate (Tokui et al., 1995).

Most of the interest with respect to physiological relevance to differentiated smooth muscle focused on the potential influence of phosphorylation of site A. The candidates are PKA, PKC and CaM K II. The initial concern with a theory that modified K\textsubscript{CaM} was that since CaM is such an abundant protein (often 0.1% of total cellular protein) the cytoplasmic levels could be in the order of 10–20 \(\mu\text{M}\) if all of the CaM is accessible/free. The K\textsubscript{CaM} for various CaM-binding sites, including MLCK is in the nM range (Ikura et al., 1992) and a shift in K\textsubscript{CaM} of 10–20 (as suggested by in vitro studies) would not be recognized and the MLCK would still be saturated by CaM. Obviously, the level of Ca\textsuperscript{2+}-CaM depends on the [Ca\textsuperscript{2+}], but this would be 10\textsuperscript{−7}–10\textsuperscript{−6} M. However, it is suggested that the free [CaM] is considerably less than the total level and most of it is sequestered by various CaM-binding proteins (Luby-Phelps et al., 1995; Persechini and Cronk, 1999). It was proposed that in Nm cells the free [CaM] was only about 1/1000 of the total [CaM] and this would bring the free level down to less than 50 nM (Romoser et al., 1997). Thus, the K\textsubscript{CaM} shift mechanism may be feasible. For PKA the situation is controversial. It was found that \(\beta\)-adrenergic relaxation did not involve an increase in K\textsubscript{CaM}, although at high concentrations of isoproterenol, K\textsubscript{CaM} did increase slightly (for a review, see Stull et al., 1991). A regulatory role for PKC by phosphorylating MLCK, also is unlikely. Addition of the \(\varepsilon\)-isoenzyme of PKC causes a Ca\textsuperscript{2+}-independent contraction in vascular smooth muscle and this is not consistent with inhibition of MLCK as a sole mechanism (Horowitz et al., 1996). There is evidence in favor of CaMK II influencing Ca\textsuperscript{2+}-sensitivity via MLCK phosphorylation (Tansey et al., 1992), but the Ca\textsuperscript{2+} requirements for CaM K II are higher than MLCK (Tansey et al., 1994) and it not certain whether this would constitute a normal regulatory function, or a negative feed-back mechanism for higher [Ca\textsuperscript{2+}].

6.3.2.2. Phosphorylation of Calmodulin

The other possibility for altered function with the MLCK scheme is by phosphorylation of CaM. This has been recognized for many years and both Ser/Thr and Tyr phosphorylation occur (for a review, see Benaim and Villalobo, 2002). The Ser/Thr kinases are casein kinase II (CKII) and MLCK (an exception to the dedicated role
of MLCK). The latter phosphorylates CaM in the presence of a polybasic peptide at predominantly Thr\textsuperscript{29}, but the phospho-CaM was indistinguishable from control CaM in activating MLCK (Davis et al., 1996). On the other hand, phosphorylation by CKII (Nakajo et al., 1988) does decrease binding to CaM targets, including MLCK (Sacks et al., 1992; Leclerc et al., 1999). Major sites of phosphorylation are Thr\textsuperscript{79}, Ser\textsuperscript{81} and Ser\textsuperscript{101} (Benaim and Villalobo, 2002). It is difficult, however, to incorporate activation of CKII (usually by polybasic compounds) during the normal contractile cycle of differentiated smooth muscle.

Several Tyr kinases have been reported to phosphorylate CaM (Benaim and Villalobo, 2002). These include receptor-linked Tyr kinases (InsR and EGFR) and non-receptor kinases (members of the Src family). The effect of Tyr phosphorylation was not studied with MLCK, but it was reported that phosphorylation of Tyr\textsuperscript{99} by InsR reduced its ability to activate phosphodiesterase (Williams et al., 1994). An unusual finding was phosphorylation of CaM by InsR also required a polybasic compound (Sacks and McDonald, 1989).

At the moment there is no evidence to implicate CaM phosphorylation in the signaling cascades of differentiated smooth muscle. Even the intriguing possibility that Src might be involved is speculative. Phosphorylation of CaM by InsR may be involved in nuclear function (Sacks and McDonald, 1989) and as such may be important in proliferating smooth-muscle cells.

6.3.3. Other Kinases that Phosphorylate Smooth-muscle Myosin

There are many kinases that phosphorylate smooth-muscle and Nm myosin II. Table 6.1 is not comprehensive, but gives an idea of the range of kinases and by implication various signaling pathways that could lead to myosin phosphorylation. In differentiated smooth muscle only a few of these kinases should be considered. Obviously, MLCK is central, but in addition, there are interesting scenarios involving ROK, ZIPK and ILK. Although there are several reports that PKC can induce a Ca\textsuperscript{2+}-free, slow contraction, the underlying mechanism(s) is not clear. It is unlikely that phosphorylation of myosin is involved (Walker et al., 1998) as the PKC sites on the RLC do not increase ATPase activity, but reduce subsequent phosphorylation by MLCK (Nishikawa et al., 1984; Ikebe et al., 1987) and thus should favor relaxation. One suggestion is that PKC induces contraction by phosphorylation of calponin or caldesmon (Allen and Walsh, 1994).

Evidence that other kinases may phosphorylate myosin under certain conditions is provided by the MLCK knock-out (KO) mice. Blood vessels from -/- MLCK KO mice from embryonic to term, permeabilized with α-toxin, developed force in response to increasing [Ca\textsuperscript{2+}], relaxed normally and showed Ca\textsuperscript{2+}-sensitization in response to GTP\textgamma{S}. Stimulation of MLCK -/- cells by lysophosphatidic acid induced RLC phosphorylation. Thus, it was argued that the MLCK -/- smooth muscles utilize and possibly upregulate an unknown kinase. From the fiber data this appeared to be Ca\textsuperscript{2+}-dependent, but it could also be regulated by an upstream Ca\textsuperscript{2+}-dependent step (Somlyo
Table 6.1. Kinases that phosphorylate the RLC of Myosin II

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Site of RLC phosphorylated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin Light Chain Kinase (MLCK)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt; &gt; Thr&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Ikebe et al., 1988</td>
</tr>
<tr>
<td>Protein Kinase C (PKC)</td>
<td>Ser&lt;sup&gt;1&lt;/sup&gt;, Ser&lt;sup&gt;2&lt;/sup&gt;, Thr&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Ikebe et al., 1987</td>
</tr>
<tr>
<td>CaM-dependent Kinase I and II (CaM II) (CaM I)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;</td>
<td>Edelman et al., 1990</td>
</tr>
<tr>
<td>Rho-associated kinase (ROK; Rho-Kinase)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;, Thr&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Amano et al., 1996</td>
</tr>
<tr>
<td>Leucine Zipper Interacting Kinase (ZIPK)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;, Thr&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Murata-Hori et al., 1999</td>
</tr>
<tr>
<td>Integrin-linked Kinase (ILK)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;, Thr&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Wilson et al., 2005</td>
</tr>
<tr>
<td>Death-Associated Kinase (DAP-kinase)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;, Thr&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Bialik et al., 2004</td>
</tr>
<tr>
<td>p21-activated Kinase (PAK)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt; (in vitro)</td>
<td>Van Eyk et al., 1998</td>
</tr>
<tr>
<td>Cyclin-p34 cdc2 (Cdc2 Kinase)</td>
<td>Ser&lt;sup&gt;1&lt;/sup&gt;, Ser&lt;sup&gt;2&lt;/sup&gt;, Thr&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Satterwhite et al., 1992</td>
</tr>
<tr>
<td>Citron Kinase</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;, Thr&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Yamashiro et al., 2003</td>
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<tr>
<td>P90 Ribosomal S6 Kinase-2 (RSK-2)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;</td>
<td>Suizu et al., 2000</td>
</tr>
<tr>
<td>Aurora/Ipl1p-related kinase (AIM-1)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;</td>
<td>Murata-Hori et al., 2000</td>
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<tr>
<td>Mitogen-activated protein kinase-2 (MAPKAP; Kinase2)</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt;</td>
<td>Komatsu and Hosoya, 1996</td>
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<tr>
<td>Casein kinase II</td>
<td>Thr&lt;sup&gt;134&lt;/sup&gt; (LC)</td>
<td>Tashiro et al., 1984</td>
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<tr>
<td>Phosphorylase kinase</td>
<td>Ser&lt;sup&gt;19&lt;/sup&gt; (LC)</td>
<td>Singh et al., 1983</td>
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<tr>
<td>Twitchin</td>
<td>Thr&lt;sup&gt;15&lt;/sup&gt; (analogous to Ser&lt;sup&gt;19&lt;/sup&gt;) (LC)</td>
<td>Heierhorst et al., 1995</td>
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</table>

<sup>1</sup>Most of the kinases listed above phosphorylate intact myosin, but use of isolated light chain is designated LC.

et al., 2004). The occurrence and utilization of this unidentified myosin kinase is not known, but these data clearly indicate that myosin kinase(s) other than MLCK exist.

6.3.3.1. Rho-associated kinase

There are 2 isoforms of Rho-kinase (ROK), α and β (termed ROKα = ROCK II and ROKβ = ROCK I = p160ROCK). The major form in gizzard smooth muscle is the α isoform (Feng et al., 199a) and both isoforms are found in airway smooth muscle (Yoshii et al., 1999). The kinase is ~160 kDa and consists of an N-terminal kinase domain, a central coiled-coil region that includes the RhoA-binding site and a C-terminal pleckstrin homology domain with an insertion of a cysteine-rich zinc finger (for a review, see Zhao and Manser, 2005). ROK is a member of the AGC family that includes PKB/Akt and PKC. Activation of ROK is via binding of GTP.RhoA and displacement of the PS/autoinhibitory segment. The inactive
GDP-RhoA is held in the resting state as a complex with the guanine-nucleotide-dissociation inhibitor (GDI) and following stimulation is released by the activated guanine-nucleotide-exchange factor (GEF) as GTP. RhoA (for a review, see Somlyo and Somlyo, 2000). Many agonists, via receptors linked to Go12, Go13 and Go4, can trigger activation of GEF(s) and thus activation of ROK (for a review, see Somlyo and Somlyo, 2003).

Under in-vitro conditions ROK directly phosphorylates SmM and increases actin-activated ATPase activity (Amano et al., 1996). Also, introduction of a constitutively active fragment of ROK to permeabilized smooth-muscle fibers induced contraction (Kureishi et al., 1997). However under physiological conditions the direct phosphorylation of SmM by ROK is unlikely to contribute to contraction. A more compelling role for direct phosphorylation of myosin II is found in Nm cells where it is involved in cytoskeletal organization. In addition to myosin II there are many more targets for ROK, including: LIM kinase, adducin, the ezrin-radixin-moesin family, vimentin, etc. and these are important for various aspects of cytoskeletal structure and cell attachments (Riento and Ridley, 2003).

Given the above view, why is ROK important for smooth-muscle function? This reflects the phosphorylation by ROK of other targets, namely, the myosin-phosphatase-target subunit (MYPT1), a phosphorylation-dependent inhibitory protein of MP (CPI-17) and ZIPK.

6.3.3.2. Leucine zipper-interacting protein kinase

Early studies on MP found that the holoenzyme isolated from gizzard contained an endogenous kinase that phosphorylated MYPT1 and inhibited phosphatase activity (Ichikawa et al., 1996). This was identified as ZIPK (also termed MYPT1-kinase, ZIP-like kinase and DAPK3; MacDonald et al., 2001a), a member of the DAPK/DRAK family. The human enzyme has 454 amino acids, ∼52.5 kDa, an N-terminal kinase domain, a helical (?) C-terminal domain and a putative leucine zipper motif (for a review, see Haystead, 2005). The form isolated from bladder (∼32 kDa; MacDonald et al., 2001a) was thought to be a proteolytic fragment of the full length kinase as only the 52.5 construct (message) was found in vascular smooth muscle (Endo et al., 2004).

ZIPK has several potentially important targets. It phosphorylates several sites on MYPT1, including an inhibitory site (Thr695; Borman et al., 2002); it phosphorylates and increases inhibitory potency of CPI-17 (MacDonald et al., 2001b); and as noted above, directly phosphorylates RLC on Ser19 and Thr18. The addition of a recombinant ZIPK to permeabilized smooth-muscle fibers induced a Ca2+-independent contraction (Niirro and Ikebe, 2001) that was suggested to reflect direct phosphorylation of myosin. In addition, it was hypothesized that ZIPK may phosphorylate RLC in detrusor smooth muscle at longer muscle lengths (Ratz and Miner, 2003). Thus, ZIPK could influence contractile behavior of smooth muscle in essentially 2 ways: by direct phosphorylation of myosin; and by inhibition of MP by phosphorylation of MYPT1 and/or CPI-17. The original idea was that the role of ZIPK in cell function was as a regulator of apoptosis, but more diverse functions are now
accepted (for a review, see Scheidtmann, 2007). The kinase is normally localized in the nucleus, but on complex formation with Par-4 (prostate apoptosis response-4), the kinase moves out of the nucleus and colocalizes with the actin cytoskeleton. It is thought that the subcellular localization of ZIPK and other family members might be a regulatory mechanism to induce or prevent apoptosis (for a review, see Kogel et al., 2001). However, would this method of regulation apply to smooth muscle? A recent finding suggests that its regulation is linked to the RhoA signaling pathway in that ROK phosphorylates and activates ZIPK (Hagerty et al., 2007). One of the problems inherent to the RhoA signaling pathway is that activation of RhoA is thought to promote translocation and attachment via its isoprenyl moieties to the cell membrane and thus ROK also would be membrane linked. The question then becomes, how does the activated ROK have access to all of its targets? Many are also membrane-bound, but probably not myosin, MYPT1 or CPI-17. The presence of a shuttle kinase, i.e., ZIPK, could solve this problem. In addition, it was suggested that ZIPK activity may be modulated by the Ca\(^{2+}\)-CaM-dependent death-associated protein kinase (DAP kinase; Shani et al., 2004) and by autophosphorylation (Graves et al., 2005).

6.3.3.3. Integrin-linked kinase

Integrin-linked kinase was identified using the 2-hybrid screen and \(\beta 1\) integrin as bait. It has 4 ankyrin repeats towards the N-terminus, followed by a pleckstrin homology motif and a C-terminal domain containing an unusual catalytic domain and sites for interaction with several other proteins, including integrin. It is located at focal adhesions and fibrillar adhesions in complex with other proteins involved in connection to the actin cytoskeleton and these interactions are consistent with its role in cell attachment and spreading. In addition, it is also involved in cell signaling and via phosphorylation inhibits glycogen-synthase kinase 3 and activates PKB/Akt (for a review, see Wu and Dedhar, 2001).

Slow contraction of permeabilized smooth-muscle fibers has been observed at low [Ca\(^{2+}\)]. This contraction is accompanied by RLC phosphorylation at Ser\(^{19}\) and Thr\(^{18}\) and does not involve MLCK (Weber et al., 1999). One candidate for the pertinent kinase is ZIPK (see above), but also it was claimed that ILK was involved (Deng et al., 2001; Wilson et al., 2005). Based on the association of ILK with integrin it would be expected that ILK is restricted to the membrane fraction, but Deng et al. (2001) found an additional sink associated with the “myofibrillar” fraction of gizzard. ILK could also influence contractile behavior of smooth muscle via inhibition of MP activity and its ability to phosphorylate MYPT1 (Muranyi et al., 2002) and /or CPI-17 (Deng et al., 2002).

6.4. MYOSIN PHOSPHATASE

The discovery and charactization of myosin phosphatase (MP; also termed smooth-muscle-protein phosphatase-1, SMPP-1) is reviewed elsewhere (Hartshorne et al., 2004; Somlyo and Somlyo, 2003).
Initially, it was thought that MP was unregulated and therefore, based on the Ca\textsuperscript{2+}-dependence of MLCK that a fixed relationship between myosin phosphorylation, [Ca\textsuperscript{2+}] and force should exist. This was not realized. Part of the dilemma was solved when it was discovered that MP could be regulated and that this could shift the [Ca\textsuperscript{2+}]/RLC phosphorylation relationship. Both inhibition and activation of MP have been proposed (Hartshorne et al., 2004; Somlyo and Somlyo, 2003). At constant suboptimal [Ca\textsuperscript{2+}] and assumed constant MLCK activity, inhibition of MP would increase RLC phosphorylation, i.e., Ca\textsuperscript{2+}-sensitization, and activation of MP would decrease RLC phosphorylation, i.e., Ca\textsuperscript{2+}-desensitization. This forms an important consideration in smooth-muscle function and many contractile agonists induce Ca\textsuperscript{2+}-sensitization, while cyclic nucleotides in general (cAMP and cGMP) cause Ca\textsuperscript{2+}-desensitization. A challenge for future research is to understand the molecular basis for these effects and at the moment only an incomplete picture is available.

6.4.1. Structure of Myosin Phosphatase

In the original characterization of MP it was described as a heterotrimer (1:1:1) composed of a catalytic type 1 phosphatase subunit, the δ isoform, ~38 kDa (PP1cδ); a regulatory subunit, myosin phosphatase target subunit (MYPT1, also called myosin-binding subunit, [MBS]), ~110 kDa; and a small subunit, ~20 kDa (M20). The latter has no clear role in MP function and there is no compelling evidence to consider M20 as an integral component of MP, but for historical reasons it is included as a subunit. Its presence with the isolated MP may reflect binding to MYPT1 (see below). MP, in contrast to MLCK, has several substrates in addition to RLC and many of these also are substrates for ROK, including: the ERM family (Kawano et al., 1999); adducin (Kimura et al., 1998); Tau and MAP2 (Amano et al., 2003); elongation factor-1α (Izawa et al., 2000); the tumor suppressor protein, merlin (Jin et al., 2006); and histone deacetylase 7 (Parra et al., 2007) (see also the proteins that bind to MYPT1). Clearly, MP is involved in a wide range of cell functions, in addition to dephosphorylation of P-myosin and a more general title would be more appropriate, e.g., multi-purpose myosin phosphatase (MPMP)!

An outstanding problem with MP is that its cell location in differentiated smooth muscle is not established and as discussed below it may have varied locations. It is assumed to bind to myosin, i.e., thick filaments, but there is no direct evidence for this.

6.4.1.1. Myosin phosphatase target subunit

This subunit is critical for regulation of PP1 activity and also for localization to various targets. The targeted disruption of the MYPT1 gene is embryonic lethal (Okamoto et al., 2005). Various MYPT1s have been cloned and sequenced and differ only in regions of alternative splicing of the single gene product. In general, the molecule is hydrophilic with no extensive hydrophobic patches. The N-terminal end of the molecule is characterized by 8 ankyrin (AN) repeats, residues 39–296. Each
repeat is \(\sim 33\) residues and shows a conserved structure forming a \(\beta\)-hairpin-helix-loop-helix (\(\beta_2\alpha_2\)) unit (Sedgewick and Smerdon, 1999). This is designed for interaction with target proteins of which several are known (Bennett and Baines, 2001). Thus, a general role for this section of the molecule is to act as an interactive platform.

At the N-terminal edge of the AN repeats is the PP1c-binding motif, residues 35–38, found in most PP1c–binding proteins and often referred to as the RVXF motif. A consensus sequence is \((R/K)X_1(V/I)X_2(F/W)\), where large hydrophobes are excluded at the \(X_1\) and \(X_2\) regions (for a review, see Cohen, 2002). Other features of the MYPT1 molecule include: an acidic patch, residues 326–372; 2 ionic regions in the area 710–850; a Ser/Thr rich region, residues 773–795 (human isoform numbering); several nuclear localization signals in both the N- and C-terminal regions (Wu et al., 2005); and a C-terminal coiled-coil region distinct from the leucine zipper (LZ) sequences found in some isoforms. In addition, there are several phosphorylation sites, notably the inhibitory sites, Thr\(_{696}\) and Thr\(_{853}\), which are phosphorylated by several kinases including ROK (see below).

The MYPT1 gene (on human chromosome 12q15–q21.2) appears to be a housekeeping gene, as MYPT1 is found in many tissues but at higher concentrations in smooth muscles (in portal vein [MYPT1] is \(\sim 1.2 \mu\)M). Isoforms are generated by cassette-type alternative splicing of the single gene pre-mRNA and result in the presence or absence of a central insert and the C-terminal LZ. The isoforms originally described from chicken gizzard, the M130 and M133 isoforms (Shimizu et al., 1994) differ by a 123-nucleotide insert arising from a single exon (Dirksen et al., 2000). In rat the situation is more complex (Dirksen et al., 2000) and several central isoforms are generated by differing expression of 2 exons (13 and 14). The variable central insert region contains a putative cdc2 site (in exon 13, Ser\(_{601}\)), but other potential phosphorylation sites are not present. In addition, isoforms are generated by alternative splicing of exon 23. Skipping of this exon codes for the LZ+ MYPT1 and inclusion of the exon introduces a premature stop codon and generates the LZ-MYPT1 (Khatri et al., 2001). These isoforms may be developmentally modulated (Khatri et al., 2001; Payne et al., 2006) and their expression in smooth-muscle disorders may be altered (e.g., portal hypertension; Payne et al., 2004; or vasoconstriction during heart failure, Karim et al., 2004). Many studies focused on the presence/absence of the LZ sequences since these have been implicated in the response to cGMP (see below).

A family of proteins with some similarity to MYPT1 (presence of AN repeats and the RVXF motif) have been described. MYPT2 is a distinct gene product (human chromosome 1q32) and is found predominantly in striated muscle and brain. Two isoforms are known (MYPT2A and B). It differs mainly in the N-terminal sequence, but overall its properties are similar to MYPT1 (Hartshorne et al., 2004) and it is assumed to carry out a similar function in striated muscle to that of MYPT1 in smooth muscle. Other family members include: MBS 85; MYPT3; and TIMAP (Hartshorne et al., 2004). However, the function(s) of these PP1c-binding proteins are not established.
An important feature of MYPT1 is that it provides a platform for many interactions and these underlie the variety of functions in which MYPT1 is involved. A central interaction is with PP1cδ and binding to MYPT1 increases phosphatase activity with myosin II as substrate and inhibits activity with phosphorylase a. Several regions of the molecule are involved in a hierarchy of interactions. An anchor point is binding to the RVXF motif and studies using surface plasmon resonance suggest additional interactions with residues 1–22, the AN repeats and residues 304–511 (Tóth et al., 2000). An important contribution to understanding the PP1c-MYPT1 interaction was the resolution of the crystal structure for a complex of MYPT1_{1–299} and PP1cδ (Terrak et al., 2004). The pivotal interaction of the RVXF motif with a hydrophobic pocket on PP1c was documented and several other interactions indicated. The N-terminal sequence, residues 1–34, forms an arm that wraps around PP1c to reach the base of the Y-shaped groove of PP1c (in which the catalytic site is located) and may interact with or modulate reaction with the substrate. Interactions with AN repeats 1, 5, 6 and 7 also are indicated, but these are suggested to play only a limited role in binding affinity and may modulate the catalytic activity of PP1c and increase substrate specificity. It is also suggested that the similarity of sequence around the 2 inhibitory sites on MYPT1 and around Ser^{19} of the RLC might favor interaction of the inhibitory regions with the catalytic site of PP1c (i.e., a PS/autoinhibitory mechanism). In summary, these results indicate that the multiple interactions of the MYPT1-PP1c complex extend the catalytic cleft and increase specificity for the substrate, phosphorylated myosin (P-myosin).

A second interaction that obviously is central to the role of MP is binding of MYPT1 to P-myosin, but this remains a controversial point. One view is that P-myosin or P-RLC binds to the N-terminal AN repeats, possibly repeats 6–8 (Hirano et al., 1997) and that in the presence of ATP binding to the dephosphorylated substrate does not occur. Clearly, interaction of PP1c and P-RLC is essential. The other view is that myosin binds to the C-terminal region (excluding the 72 C-terminal residues; Johnson et al., 1997) and is reduced on phosphorylation of Thr^{853} (Velasco et al., 2002). Simultaneous binding of P-myosin to both the N- and C-terminal regions could occur if the myosin head binds to the N-terminus and the myosin rod to the C-terminus. Other interactions with the AN repeats of MYPT1 include: adducin (α, β and γ); Tau and MAP2.

A surprising feature of MYPT1 is that the C-terminal half also is involved in multiple interactions. With respect to smooth-muscle function a few of these should be noted (Hartshorne et al., 2004). GTP-RhoA, but not GDP-RhoA, binds close to the C-terminus and thus may represent an alternative docking site in addition to the plasmalemma for RhoA and ROK. Acidic phospholipids target the C-terminal one-third of the chicken M133 isoform and inhibits MP activity. This may facilitate membrane localization of MYPT1. The M20 subunit binds to the C-terminal segment, residues 934–1006 of human MYPT1, probably via coiled-coil interactions (Langsetmo et al., 2001), but does not involve the LZ motifs. Several interactions involve the C-terminal LZ repeats and include: interaction of the LZ repeats of cGMP-dependent protein kinase (PKG) with the LZ motifs of MYPT1;
binding of PDZ2 domains of interleukin 16 precursor proteins to the C-terminal 30 residues of MYPT1; and, interaction of a coiled–coil domain in myosin phosphatase RhoA-interacting protein (M-RIP; found in vascular smooth muscle) with the LZ motifs in MYPT1 (Surks et al., 2003). Subsequently, it was shown that M-RIP was a RhoA-GAP and that it activated MP activity via binding to myosin and MYPT1 (Koga and Ikebe, 2005). Obviously, many of the proteins that bind MYPT1 are substrates for MP (see below). The many interactions outlined above indicate that MYPT1 could be involved in several cellular functions and may be found in different cellular locations. With the differentiated smooth-muscle phenotype the range of functions may be more restricted, but in proliferating smooth muscle and Nm cells a greater repertoire might be expected.

6.4.1.2. Small non-catalytic subunit

The M20 subunit was cloned from chicken gizzard and 2 splicing variants were found of 161 (M18) and 186 (M21) residues. Only the latter contained the C-terminal LZ motifs. Expression of M18/M21 is tissue specific and M18 and M21 are the major isoforms of gizzard and mammalian tissues, respectively (Mabuchi et al., 1999). An interesting suggestion was that since the C-terminal 120 residues of M21 are similar to the C-terminus of MYPT2 (91% identity, 100% similarity) M21 might be generated from the MYPT2 gene (Moorhead et al., 1998). This was confirmed for 2 heart-specific isoforms (hsM21A and B) and reflects transcription of the 2 isoforms from the MYPT2 gene via a heart–specific promoter in intron 13 (Arimura et al., 2001). This situation is similar to the generation of telokin from the smooth-muscle MLCK gene. The role(s) of M20 is not clear. It does not affect phosphatase activity of MYPT1 plus PP1c. Suggested roles are: modification of Ca$^{2+}$-sensitivity in renal artery and cardiac myocytes; binding to a myosin dimer; and regulating microtubule dynamics (Hartshorne et al., 2004).

6.4.1.3. Regulation of myosin phosphatase: inhibition

Initially, it was found that stimulation with several agonists caused inhibition of MP and that in many instances this was linked to RhoA and thus ROK (review; Somlyo and Somlyo, 2003). The link to MYPT1 originated with the finding that in permeabilized portal vein incubation with ATPγS induced Ca$^{2+}$-sensitization and concomitant thiophosphorylation of MYPT1 (Trinkle-Mulcahy et al., 1995). Subsequently, it was shown that phosphorylation of MYPT1 by a co-purifying (endogenous) kinase inhibited the gizzard MP holoenzyme (Ichikawa et al., 1996). As discussed above the endogenous kinase was identified as ZIPK. It was found also that ROK could phosphorylate MYPT1 and inhibit MP activity (Amano et al., 1996). Initially, the inhibitory phosphorylation site was restricted to Thr$^{696}$ (Feng et al., 1999b), but it was subsequently found that phosphorylation at Thr$^{853}$ also was inhibitory (Murányi et al., 2005). Activity of MP with other substrates also is inhibited by phosphorylation of MYPT1 (Feng et al., 1999b). These sites are phosphorylated by several other kinases, e.g., ILK (possibly only Thr$^{696}$; Muranyi et al., 2002), myotonic dystrophy protein kinase (Murányi et al., 2001), myotonic
dystrophy kinase-related Cdc42 binding kinase (Tan et al., 2001), PAK (only at Thr<sup>696</sup>; Takizawa et al., 2002a) and Raf-1 (Broustas et al., 2002). Thus, there is the possibility that several signaling pathways could converge at MYPT1 and inhibit MP activity. The possible connection between ZIPK and ROK has been mentioned above. Whether several signaling mechanisms can regulate MP activity is an active area for future research, but there is considerable evidence to support a major role for ROK in smooth-muscle function (for a review, see Somlyo and Somlyo, 2003). An important tool for many of these studies is the availability of reasonably selective ROK and protein kinase C-related protein kinase (PRK family) inhibitors (see Davies et al., 2000; Riento and Ridley, 2003) including Y-27632 (at higher concentrations will also inhibit PKCα and PKN; Uehata et al., 1997) and HA-1077, or analogs (Asano et al., 1989).

The important role of RhoA/ROK in smooth-muscle function and the availability of inhibitors for ROK led to the recognition of altered RhoA signaling in several smooth-muscle disorders. These include: hypertension, vasospasm, asthma, preterm labor, and erectile dysfunction (for a review, see Somlyo and Somlyo, 2003). Two new aminofurazan-based inhibitors of ROK have been developed that are effective in the nM range and reduce blood pressure in DOCA-salt-induced hypertensive rats (Doe et al., 2007).

Other signaling pathways may lead to activation of ROK and bypass RhoA. Arachidonic acid induced Ca<sup>2+</sup>-sensitization of permeabilized smooth-muscle fibers (Fu et al., 1998) and it was proposed that the lipid dissociated the MP holoenzyme and thus reduced MP activity (Gong et al., 1992). An alternative explanation is that arachidonic acid directly activated ROK independent of RhoA and phosphorylated MYPT1 (Araki et al., 2001). Only the native ROK was activated by arachidonic acid and not an N-terminal constitutively-active fragment, suggesting that activation was due to removal of the autoinhibitory (C-terminal) region of ROK. The possibility that arachidonic acid can act as a second messenger in smooth-muscle function increases the range of agonists to include those linked to phospholipase A<sub>2</sub>. The Ca<sup>2+</sup>-independent phospholipase A<sub>2</sub> is thought to be important in this regard (Guo et al., 2003). Sphingosylphosphorylcholine also was proposed to directly activate ROK and be involved in Ca<sup>2+</sup>-sensitization of cerebral artery (Shirao et al., 2002) and may provide a link to effects of cholesterol on Ca<sup>2+</sup>-sensitization of vascular smooth muscle (Morikage et al., 2006).

The molecular basis for inhibition with phosphorylated MYPT1 is not known. The simplest idea is that the phosphorylation sites act as competitors for the phosphorylated light chain, a pseudosubstrate mechanism. The similarity around the 2 phosphorylation sites on MYPT1 and the RLC Ser<sup>19</sup> site has been pointed out. While this cannot be eliminated there are several problems. Fragments of MYPT1 phosphorylated by ROK inhibit PP1c (Muranyi et al., 2002), but are not effective inhibitors (compared to peptides from the PS region of MLCK). PP1c and the phosphorylation sites are at different ends of the molecule and would necessitate either bending or formation of a head-to-tail dimer or oligomer. The full-length recombinant MYPT1 was shown to form a dimer, probably via interaction of the
coiled-coil regions in the C-terminus and thus would be a head-to-head dimer (Langsetmo et al., 2001). Other mechanisms are possible, such as binding of the phosphorylation sites to regions modifying the catalytic site (see above discussion on crystal structure), or phosphorylation-dependent dissociation of PP1c and MYPT1, or dissociation of substrate from MYPT1 following phosphorylation.

Several sites for ROK on MYPT1 were identified, but the major sites are Thr\textsuperscript{696} and Thr\textsuperscript{853}. Whether one site or the other is preferred is not clear. Several reports are consistent with a putative regulatory role for Thr\textsuperscript{696}, but Thr\textsuperscript{853} also has been claimed to be an important site and is suggested to be a useful indicator of ROK activity in vivo (see Murányi et al., 2005). As discussed above, several kinases can phosphorylate both sites and thus several signaling pathways can be involved. A distinction between the 2 sites may offer a mechanism for integration of different pathways converging at MYPT1. So far there is no mechanism that would provide a molecular rationale for discrimination between the 2 sites.

An emerging theme is that the many functions of MYPT1 may be associated with its translocation to various cell locations. It is known that MYPT1 binds to several proteins and these could provide a scaffolding function. It is not known whether there are specific scaffolding proteins for MYPT1, for example equivalent to the AKAPS involved in PKA functions. The translocation of MYPT1 was shown originally in isolated portal vein cells. It was found that after stimulation by prostaglandin F\textsubscript{2\alpha}, the MYPT1-PP1c complex was translocated to the membrane and this was followed by dissociation of PP1c to cause a reduced level of phosphatase activity for P-myosin. The RLC phosphorylation profile showed sustained levels of phosphorylation consistent with the lower MP activity. It was also suggested that phosphorylation of Thr\textsuperscript{696} by ROK initiated the translocation. In contrast, another agonist, phenylephrine, did not induce translocation and this was reflected by a more transient profile of RLC phosphorylation (Shin et al., 2002). More recently it was found that in colonic smooth-muscle cells activation of both RhoA and PKC pathways by acetylcholine resulted in phosphorylation of MYPT1 at Thr\textsuperscript{696}, inhibition of MP activity and translocation of MYPT1 to the particulate fraction where it was associated with HSP27 (Patil and Bitar, 2006). In Nm cells in the presence of okadaic acid translocation of MYPT1 depended on the site of phosphorylation. Phosphorylation at Thr\textsuperscript{696} induced a membrane location and phosphorylation at Thr\textsuperscript{853}-induced translocation to the nucleus (Lontay et al., 2005). One point (of many) to resolve is whether the various isoforms of MYPT1 show different cell locations.

An inhibitory protein of 17–20 kDa was isolated by Eto et al. (1995) from porcine aorta and termed “PKC-potentiated inhibitory protein phosphatase-1 inhibitory protein” (CPI-17; Senba et al., 1999). It is located in several smooth muscles, in brain (Eto et al., 1997) and in platelets (Watanabe et al., 2001). The sequences of mammalian CPI-17 are well conserved, especially in the N-terminal half, residues 1–67 encoded by exon 1 (Yamawaki et al., 2001). A splicing variant, lacking 27 residues (68–94) encoded by exon 2 is also expressed in human aorta (Yamawaki et al., 2001). In contrast to other PP1 inhibitors, e.g., inhibitor 1, CPI-17 can inhibit
the MP holoenzyme, although it is slightly more sensitive to the isolated PP1 catalytic subunit (Erdodi et al., 2003). Phosphorylation of Thr\textsuperscript{38} enhances inhibitory potency of CPI-17 (about a thousand fold; Eto et al., 1997) and a minimum inhibitory domain was proposed for residues 35–120 and is necessary for recognition by MP (Hayashi et al., 2001). The specificity of CPI-17 for MP is thought to reside in its interaction with the PP1c catalytic site modified by MYPT1 (Ohki et al., 2001). Type 2A, 2B and 2C phosphatases dephosphorylated phospho-CPI-17, but not type 1, thus MP would not reverse inhibition by CPI-17 (Takizawa et al., 2002b). Other phosphorylation sites on CPI-17 have been identified (MacDonald et al., 2001b; Dubois et al., 2003).

Initially, it was reported that PKC phosphorylated CPI-17 and could therefore be responsible for the PKC-induced Ca\textsuperscript{2+}-sensitization of tonic smooth muscle (Woodsome et al., 2001). It was shown in femoral artery that CPI-17 was phosphorylated in response to several stimulants, including histamine and GTP\textgamma{}S, but inhibitors of both ROK and PKC reduced contraction and phosphorylation of CPI-17 (Kitazawa et al., 2000) and thus suggested participation of at least 2 signaling pathways. The importance of PKC-induced Ca\textsuperscript{2+}-sensitization is discussed elsewhere (Somlyo and Somlyo, 2003), but it is clear that several pathways can target CPI-17 to modify MP activity. This idea is supported by the findings that several kinases can phosphorylate Thr\textsuperscript{38} of CPI-17 to increase inhibitory potency, including: ROK (Koyama et al., 2000); PKN (Hamaguchi et al., 2000); ZIPK (MacDonald et al., 2001); PAK (Takizawa et al., 2002a); and ILK (Deng et al., 2002). These kinases are in general expected to promote a contractile response and thus inhibition of MP activity is consistent with suggested roles. (It should also be pointed out that several of these kinases also phosphorylate MYPT1 at inhibitory sites.) It is surprising that CPI-17 also is phosphorylated by PKA and PKG and increases inhibition (Erdodi et al., 2003). The effect of cyclic nucleotides, cAMP and cGMP, in smooth muscle is to promote relaxation and thus the role(s) of PKA/PKG with CPI-17 is not clear. In fiber studies it was found that PKG activation led to CPI-17 dephosphorylation (Bonnevier and Arner, 2004), although prolonged exposure to nitroprusside resulted in phosphorylation of CPI-17 (Etter et al., 2001).

The interaction or integration of the 2 modes of inhibition involving phosphorylation of either CPI-17 or MYPT1 is not fully resolved. One suggestion is that there is a temporal distinction. On stimulation of femoral artery with phenylephrine there was a rapid Ca\textsuperscript{2+} and PKC–dependent phosphorylation of CPI-17 followed by a slower phosphorylation of MYPT1 (Dimopoulos et al., 2007). CPI-17 is found in higher concentrations in tonic muscle than in phasic muscle and the opposite is true for MYPT1. Approximate concentrations of CPI-17 in femoral artery (tonic), portal vein and vas deferens (both phasic) were 6.7, 4.4, and 0.8 μM, respectively (Woodsome et al., 2001). The relative importance of the mechanisms would therefore depend on the tissue or cell examined. Other phosphorylation-dependent inhibitors have been identified (phosphatase holoenzyme inhibitor-1 [PHI-1; Eto et al., 1999]; PKC-dependent protein phosphatase 1 inhibitor [KEPI;
Liu et al., 2002]; and gut and brain phosphatase inhibitor [GBPI; Liu et al., 2004]) and these may have functions analogous to CPI-17. For example, in chicken smooth muscle CPI-17 is absent, but PHI-1 may act as a substitute (El-Touhky et al., 2005).

6.4.1.4. Regulation of myosin phosphatase: activation

The molecular basis for activation of MP in smooth-muscle cells is not established. Part of the problem is that it is not clear whether there is net activation (above a control level) or just a decrease in the level of inhibition. Examples where net activation is demonstrated are limited and include activation of the MYPT1-myosin-PP1c complex by M-RIP (Koga and Ikebe, 2005) and activation of MP via phosphorylation of MYPT1 in the region of Ser$^{435}$ by a mitosis-specific kinase, probably cdc2 (Totsukawa et al., 1999). The latter is suggested to reflect an increased affinity of MYPT1 for myosin II.

Much of the interest in activation of MP stems from earlier observations that cGMP increased MP activity in permeabilized smooth-muscle fibers (Wu et al., 1996; Lee et al., 1997). This is of considerable clinical interest in that it involves the NO/cGMP/PKG pathway that is important in smooth-muscle relaxation. In general, the effects of cyclic nucleotides oppose those of the RhoA/ROK pathway. The effects of cGMP in smooth muscle are complex and involve reduction of [Ca$^{2+}$], by activating Ca$^{2+}$-dependent K$^+$ channels, inhibiting activation of PLC, increasing Ca$^{2+}$ uptake into the SR and inhibiting Ca$^{2+}$ release from the SR by IP$_3$ (Lincoln et al., 2001). However, the effect on [Ca$^{2+}$] would be neutralized in the permeabilized fibers and thus another mechanism must be operative. This was suggested to be activation of MP. Direct phosphorylation of MYPT1 by PKG (Nakamura et al., 1999) or PKA (Muranyi et al., 2002) does not activate MP activity and thus other components are involved. An interesting development was the discovery that PKG binds to MYPT1 via LZ motifs on each (Surks et al., 1999; Surks et al., 2003) and it was found that only those smooth muscles expressing the LZ+ MYPT1 isoform were sensitive to relaxation by cGMP. It was shown that in chicken the LZ+ form was expressed in tonic muscle and the LZ- in phasic muscle (Khatri et al., 2001). In a model of portal hypertension it was suggested that the more phasic muscles reverted to a tonic phenotype with a corresponding reduction in [MYPT1] and a switch from LZ- to LZ+ (Payne et al., 2004). The MYPT1 profile also changed in small resistance arteries subject to altered blood flow (Zhang and Fisher, 2007). Initially, the change to both high and low flow in a rat model reduced [MYPT1] and caused a switch to the LZ+ isoform. At longer times the high flow state restored [MYPT1] and reverted to the LZ-isoform. In contrast, in the low flow state there was an increase in the LZ+ isoform and an enhanced sensitivity to NO donors. (Other workers using the same model showed that a change to both high or low blood flow caused extensive changes in gene expression [Wesselman et al., 2004] notably in myosin, RhoA and ROK. Whether any perturbation of the status quo, e.g., stretch, osmotic stress, hypoxia, induce changes in the expressed gene profile remains to be determined.) Thus, there are several examples
in which sensitivity to NO/cGMP is linked to expression of LZ+ MYPT1 and a reasonable assumption (as claimed by Surks et al., 1999) is that this reflects binding of PKG to MYPT1 via LZ motifs. However, although the requirement for the LZ motif was acknowledged it was suggested (Huang et al., 2004) that the actual interaction of PKG and MYPT1 did not involve the LZ motifs on MYPT1, but possibly the coiled-coil regions (that are present in all MYPT1 isoforms). The mechanism that links PKG and activation of MP activity to the LZ motifs, therefore, remains an enigma. It is possible that other proteins are involved but such are not identified.

One suggestion that does not involve LZ motifs is that phosphorylation of MYPT1 at the PKA/PKG sites, Ser$^{695}$ and Ser$^{852}$, blocks subsequent phosphorylation at the inhibitory sites (Wooldridge et al., 2004). This mechanism would prevent inhibition, rather than effecting a net activation. A similar effect would be elicited by inactivation of RhoA or pathways upstream of RhoA (assuming that ROK plays a pivotal role). Several reports support this idea. Both PKA (Dong et al., 1998; Goeckeler and Wysolmerski, 2005) and PKG (Sauzeau et al., 2000; Sawada et al., 2001) phosphorylate RhoA at Ser$^{188}$ and block activation. The block was suggested to be via enhanced interaction with the Rho-GDI (Lang et al., 1996) rather than a direct effect on RhoA, or GAP activity or geranylgeranyl transferase activity (Ellerbroek et al., 2003). A decrease in RhoA activation may also reflect a decrease in upstream signaling by phosphorylation with PKA and inhibition of the Go$_{13}$ receptor (Manganello et al., 2003). However, it is accepted that many agonists and hence many receptors, including Go$_q$, lead to activation of RhoA (Somlyo and Somlyo, 2003). The net result of a decrease in GTP.RhoA would be lower ROK activity and reduced inhibition of MP. Other signaling mechanisms, e.g., ILK should not be affected unless there is cross talk between the pathways. Other suggested targets for PKG include the calponin homology-associated smooth-muscle protein (Borman et al., 2004) and heat-shock protein 20 (Rembold et al., 2000).

Telokin is an independently expressed protein derived from the MLCK gene and contains the C-terminal domain of MLCK (Ito et al., 1989; Gallagher and Herring, 1991). Its expression is specific to smooth muscles (Herring and Smith, 1996; 1997) and it is higher in phasic than tonic muscle. It is phosphorylated by PKA and/or PKG and was proposed to be involved in cyclic nucleotide-dependent Ca$^{2+}$-desensitization (Wu et al., 1998). It causes a dose-dependent relaxation and a reduction in RLC phosphorylation in smooth-muscle fibers and this effect is enhanced by phosphorylation at Ser$^{13}$ (Walker et al., 2001). How telokin induces relaxation, presumably by activation of MP, is not known, but part of the mechanism could be a cGMP-induced translocation following phosphorylation of telokin at Ser$^{13}$ and/or Ser$^{19}$ to smooth-muscle cell membranes (Komatsu et al., 2002). Several other kinases can phosphorylate telokin (Krymsky et al., 2001). Telokin may also influence MLCK and it was proposed to modulate oligomerization of MLCK (Nieznanski and Sobieszek, 1997) and influence activity of MLCK (Silver et al., 1997).
6.5. LATCH MECHANISM

While the biochemical properties of SmM and the regulatory components fix a framework on which contractile behavior is based, it is difficult to fully account for the observed physiological data in either phasic or tonic muscle and additional factors have been implicated. These include the thin-filament proteins, caldesmon (Wang, 2001) and calponin (Allen and Walsh, 1994), structural considerations and the probability that several of the signaling molecules are targeted to specific areas or structures in the cell. One physiological observation that is struggling for a biochemical identity is latch.

In skeletal muscle the number of cycling cross bridges (CB) is determined by Ca\(^2+\) binding to troponin C allowing attachment of the myosin heads to actin. In smooth muscle the situation is more complex and changes in shortening velocities under various conditions led to the idea that not only the number of attached CBs could be regulated, but also CB cycling rates. Early studies carried out in Murphy’s laboratory with carotid artery (a tonic muscle) showed that shortening velocity depended on RLC phosphorylation and also that following dephosphorylation at lower [Ca\(^{2+}\)], CBs remained attached and maintained tension (Dillon et al., 1981). These slowly or non-cycling CBs characterize the latch state. These latch bridges do not redevelop tension when released from their original length. A similar state of tension maintenance and low energy cost, termed catch, has been known for many years in molluscan smooth muscle. A model proposed to account for latch suggested 2 types of CB attachments: a cycling phosphorylated CB; and a non-cycling dephosphorylated attached CB. The only Ca\(^2+\)-dependent regulation is phosphorylation of RLCs by MLCK. Both types of CBs are subject to dephosphorylation by MP and the latch bridge is formed by dephosphorylation of an attached CB. These maintain tension and have low-energy cost. The detachment of the latch bridge is the rate-limiting step (Hai and Murphy, 1988). More recently, this model has been refined to include regulation of MP activity and cooperativity in attachment between phosphorylated and dephosphorylated CBs (Murphy and Rembold, 2005).

An important question is why a dephosphorylated myosin head would cycle more slowly, and thus apply force to the actin filament more economically, than a phosphorylated, active myosin head. A unique property of SmM is its high affinity for ADP for the acto-myosin complex (Cremo and Geeves, 1998). If strain could delay the dissociation of ADP preferentially from unphosphorylated cross-bridges, they would remain attached and generate tension without low ATP consumption. A candidate for the strain-dependence of ADP release and thus a molecular basis for the latch state was seen in a conformational change in smooth myosin S1 (a single myosin head) bound to actin. Cryo-electron microscopy (Whittaker et al., 1995) and electron paramagnetic resonance (EPR) of S1-decorated gizzard muscle (Gollub et al., 1996) revealed an axial rotation of the regulatory domain toward the "barbed" end of the actin filament upon MgADP release. This rotation was in the direction expected for the power stroke and was not seen with skeletal S1 (Gollub et al., 1996). EPR experiments in gizzard muscle monitoring the rotation of the RLC also showed
that the regulatory domain rotated upon MgADP release (Gollub et al., 1999). The data suggest that the affinity for ADP of phosphorylated heads is weaker than unphosphorylated heads in the muscle. Thus, MgADP has more power to keep the unphosphorylated heads in the pre-rotation position and would have to wait for that rotation, opposed by strain, to release the MgADP to complete a cycle. Strain on myosin heads and MgADP binding may serve to delay detachment from actin, reducing energy consumption and prolonging occupancy of a post-power stroke, force-generating state of the myosin head, thus enhancing the economy of tension maintenance.

6.6. SUMMARY AND PERSPECTIVES

Since smooth-muscle myosin II was recognized as distinct from striated muscle myosins there has been remarkable progress toward establishing its properties and role in smooth-muscle function. Each discovery opened new areas for research and these have impacted a broad range of related disciplines. For each of the topics discussed above this is apparent, but might be best illustrated by the phosphorylation mechanism. Identification of regulation of smooth-muscle myosin by phosphorylation was a key factor for understanding smooth-muscle function and also had a strong influence on understanding non-muscle cell function. Extensive research focused on the regulatory mechanisms involved in myosin phosphorylation. Initial efforts were to characterize MLCK and via its calmodulin dependence to determine its role in the contraction-relaxation profiles for smooth muscle. As discussed above part of this was successful, but the variation in cross-bridge cycling rate occurring in latch needs additional data on the kinetic steps involved. The characterization of MLCK was accompanied by the realization that phosphorylation-dependent activation of myosin II and the role of MLCK was widespread and could be applied to many cell types. In general, MLCK plays a dominant role in smooth-muscle function, but in non-muscle cells other kinases may be recruited for myosin phosphorylation, notably ROK.

The other half of the regulatory couple, myosin phosphatase, showed a similar, albeit more recent progression. The smooth-muscle enzyme was characterized first and this was later adapted to non-muscle function. Important points being that the major phosphatase-targeting subunit and the catalytic PP1 subunit were present in most eukaryotic cells and therefore would constitute a similar mechanism in muscle and non-muscle cells. The later discovery that MP could be regulated was a major event and narrowed the gap between biochemical expectations and physiological behavior. The proposal that MP could be inhibited or activated channeled much of the research into investigations on these regulatory mechanisms. Several signaling pathways have been implicated, but two major features stand out and these are: that for inhibition of MP the RhoA/ROK couple plays a dominant role; and for activation of MP the NO/cGMP/PKG pathway is crucial. Each has generated considerable clinical interest and for the former many clinical tests or treatments
involve use of ROK inhibitors in smooth-muscle disorders (e.g., hypertension and asthma). For the latter, phosphodiesterase inhibitors are used to maintain cGMP levels (as in treatment for erectile dysfunction). In addition, the possibility of raising or maintaining cGMP levels would facilitate relaxation of vascular muscle. Despite the wealth of data accumulated, there are many areas that are not yet understood. With the interplay of several signaling pathways centered on MP regulation it is not obvious which mechanisms operate under a given set of conditions. Are some pathways redundant or is each associated with a specific role? At the molecule level, several aspects of the regulatory mechanisms remain to be resolved.

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