Invited Review

Regulation of myosin phosphorylation and myofilament Ca^{2+} sensitivity in vascular smooth muscle

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Abstract

The Ca^{2+}-dependent, reversible phosphorylation of the 20 kDa regulatory myosin light chain (MLC) plays a primary role in regulating the contraction of smooth muscle. However, it is well known that the Ca^{2+} signal is not the only factor which regulates such contraction, however, the alteration of the Ca^{2+} sensitivity in the contractile apparatus is also known to play an important role. The degree of MLC phosphorylation is determined by the balance of the activity between phosphorylation and dephosphorylation. Either the Ca^{2+}-independent activation of MLC phosphorylation or the inhibition of MLC dephosphorylation causes a greater MLC phosphorylation for a given level of Ca^{2+} signal and thereby potentiates the myofilament Ca^{2+} sensitivity. The smooth muscle myosin light chain phosphatase (MLCP) consisting of three subunits was first isolated and cloned in the early '90s. The intensive investigation thereafter has uncovered the biochemical basis for regulating the activity of MLCP. The regulation of the MLCP activity is now considered to play a critical role in regulating the myofilament Ca^{2+} sensitivity. There are three major mechanisms in the regulation of MLCP; (1) the phosphorylation of a 110 kDa regulatory subunit of MLCP (2) the conformational change of the trimeric structure, and (3) the inhibition by a smooth muscle specific inhibitor protein, CPI-17. Furthermore, some kinases have been found to phosphorylate the MLC and activate the contraction of smooth muscle in a Ca^{2+}-independent manner. Numerous protein kinases have been found to be involved in the regulation of MLC phosphorylation, and rho-kinase is one of the most frequently investigated kinases. The smooth muscle physiology is now asked to integrate the current understanding of the biochemical mechanisms and to clarify which kinases and/or proteins in the contractile apparatus play a physiological role in regulating the myofilament Ca^{2+} sensitivity and how such extracellular contractile stimulation modulates these mechanisms.

Key words: smooth muscle, calcium, contractile apparatus

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Introduction

The impaired regulation of the vascular tone plays an important role in the pathophysiology of such vascular diseases as hypertension and vasospasm. Understanding the molecular mechanism regulating the vascular tone is thus essential to establish new strategies for the prevention and treatment of vascular diseases. The contractility of vascular smooth muscle is primarily regulated by the Ca^{2+}-dependent reversible phosphorylation of the 20 kDa myosin light chain (MLC) (Hartshorne, 1987). However, it is well known that the extent of contraction obtained with a given increase in the cytosolic Ca^{2+} concentration, namely the Ca^{2+} sensitivity of the contractile apparatus, differs depending on the type of contractile stimulation (Hirano *et al.*, 1991). The change in the Ca^{2+} sensitivity of the contractile apparatus thus also plays an important role in regulating the contraction of smooth muscle (Hirano *et al.*, 1991; Somlyo and Somlyo, 1994; Ogut and Brozovich, 2003).

An intensive investigation for more than a decade has advanced our understanding of the molecular mechanism regulating the myofilament Ca^{2+} sensitivity. The regulation of the phosphorylation of the myosin light chain (MLC) for a given level of Ca^{2+} is now considered to play a primary role in regulating the Ca^{2+} sensitivity, while other mechanisms, including a latch bridge and caldesmon or calponin-mediated inhibition of actomyosin interaction, may also contribute to the regulation of Ca^{2+} sensitivity (Murphy *et al.*, 1990; Winder *et al.*, 1998; Ogut and Brozovich, 2003). The most striking achievement in the last decade is the isolation and molecular cloning of the smooth muscle myosin light chain phosphatase (MLCP) (Alessi *et al.*, 1992; Chen *et al.*, 1994; Shimizu *et al.*, 1994; Shirazi *et al.*, 1994) and the discovery of the rho-kinase-mediated inhibition of the MLCP activity (Kimura *et al.*, 1996). Since the first discovery of the phosphorylation-mediated inhibition of MLCP (Trinkle-Mulcahy *et al.*, 1995), various other kinases have been added to a list of kinases which cause an inhibition of the MLCP activity (Hirano *et al.*, 2003). The discovery of an inhibitor protein of MLCP in smooth muscle added another dimension to the regulation of the MLCP activity (Eto *et al.*, 1997; Kureishi *et al.*, 1997). MLCP has now established its central role in determining the level of MLC phosphorylation and hence the Ca^{2+} sensitivity of the contractile apparatus. Another noticeable discovery is the mechanism of the Ca^{2+}-independent phosphorylation of the MLC and the activation of myosin ATPase (Amano *et al.*, 1996; Kureishi *et al.*, 1997; Van Eyk *et al.*, 1998; Deng *et al.*, 2001; Niiro and Ikebe, 2001; Ueda *et al.*, 2002). The Ca^{2+}-dependent MLC kinase (MLCK) has long been the only kinase that phosphorylates MLC at Ser19 and Thr18 and activates the myosin ATPase activity. The discovery of the Ca^{2+}-independent MLC phosphorylation and activation of myosin allowed us to identify another mechanism regulating the Ca^{2+} sensitivity. Despite our accumulated knowledge on the biochemical basis for the regulation of MLC phosphorylation and dephosphorylation, the physiological significance of these findings and the intracellular signal transduction involved in the regulation of the Ca^{2+} sensitivity still remain to be elucidated.

This review article focuses on the regulation of MLC phosphorylation as a major mechanism regulating the Ca^{2+} sensitivity of the contractile apparatus. We first will update our existing knowledge of the regulatory network involved in the phosphorylation and dephosphorylation of

MLC. Next, we discuss the molecular basis for the regulation of the MLCP activity, while focusing on the phosphorylation-mediated regulation. We also discuss the upstream signal transduction that could link the contractile stimulation to the alteration of the Ca^{2+} sensitivity. Finally, we evaluate the physiological significance of our current understanding of the biochemical and molecular mechanisms regulating Ca^{2+} sensitivity, and clarify some major questions to be solved in future research.

Current Understanding of the Biochemical Basis Regarding the Regulation of Phosphorylation and Dephosphorylation of MLC

The level of MLC phosphorylation is determined by the balance between the activities of phosphorylation and dephosphorylation processes (Fig. 1). Either Ca^{2+}-independent activation of MLC phosphorylation or inhibition of MLC dephosphorylation causes an increase in the level of MLC phosphorylation for a given increase in Ca^{2+} signal, and thereby increases the myofilament Ca^{2+} sensitivity. Various mechanisms have been suggested to inhibit the MLCP activity, and they could all be classified into three mechanisms; 1) Inhibition by an alteration of the heterotrimeric structure of MLCP, 2) Inhibition by an phosphorylation of MYPT1 at a specific site, and 3) Inhibition by the inhibitor protein CPI-17 (17 kDa PKC-potentiated inhibitory protein of type 1 protein phosphatase).

Ca^{2+}-independent MLC phosphorylation and activation of myosin

The ATPase activity of smooth muscle myosin is activated upon the phosphorylation of MLC at either Ser19 or Thr18 (Hartshorne, 1987). In 1996, rho-kinase was, for the first time, found to phosphorylate MLC at Ser19 and activate myosin ATPase activity (Amano *et al.*, 1996), and later it was shown to induce smooth muscle contraction in the absence of Ca^{2+} (Kureishi *et al.*, 1997). Following this discovery, more kinases have also been found to phosphorylate MLC at the activation sites in a manner independent of the Ca^{2+} signal. Among these kinases, rho-kinase (Kureishi *et al.*, 1997), integrin-linked kinase (Deng *et al.*, 2001), p21-activated protein kinase (Van Eyk *et al.*, 1998) and zip kinase (Niiro and Ikebe, 2001) have been shown to induce smooth muscle contraction, when their catalytic domains were introduced into the permeabilized smooth muscle tissues as recombinant proteins (Fig. 1) (The contraction induced by p21-activated protein kinase was associated with an increase in the phosphorylation of caldesmon and desmin but not MLC (Van Eyk *et al.*, 1998)). The Ca^{2+}-dependent MLCK preferably phosphorylates Ser19, while integrin-linked kinase and zip kinase phosphorylates Ser19 and Thr18 with the same kinetics (Deng *et al.*, 2001; Niiro and Ikebe, 2001). Rho-kinase has been shown to phosphorylate Ser19 and Thr18 in non-muscle cells (Ueda *et al.*, 2002), while such double phosphorylation has not been reported in smooth muscle (Amano *et al.*, 1996). p21-activated protein kinase was reported to phosphorylate Ser19 but not Thr18 (Chew *et al.*, 1998; Van Eyk *et al.*, 1998).

Inhibition of the MLCP activity by altering the heterotrimeric conformaion of MLCP

MLCP is composed of three subunits: the 38 kDa catalytic subunit (PP1c), the 110 kDa non-

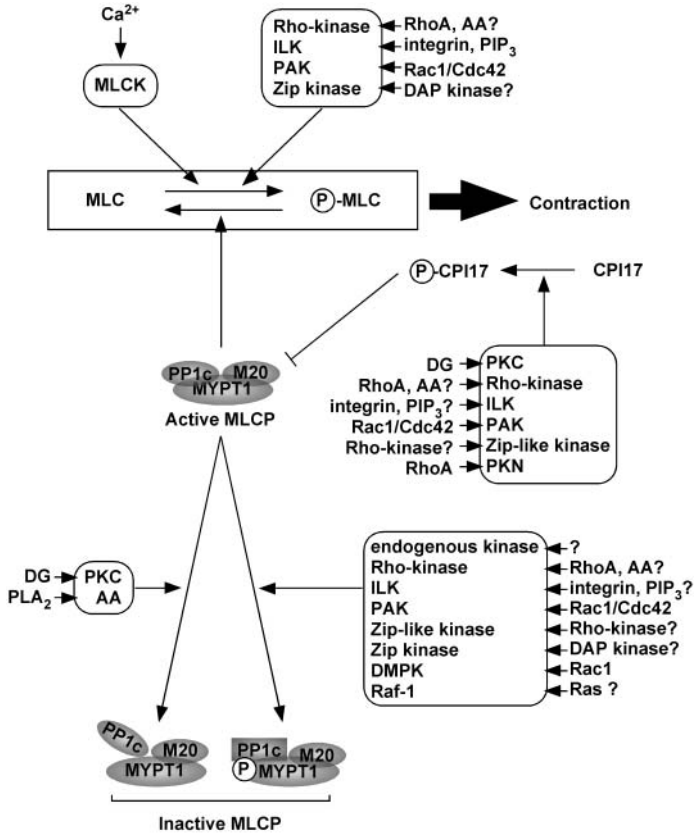

Fig. 1. Regulation of phosphorylation and the dephosphorylation of the myosin light chain in smooth muscle. AA, arachidonic acid; DAP kinase, death-associated protein kinase; DG, diacylglycerol; DMPK, myotonic dystrophy protein kinase; ILK, integrin-linked kinase; MLC, 20 kDa myosin light chain; P-MLC, phosphorylated form of MLC; MLCK, MLC kinase; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase target subunit 1 of MLCP; M20, a 20 kDa non-catalytic subunit of MLCP; PAK, p21-activated protein kinase; PKC, protein kinase C; PKN, protein kinase N; PLA_2, phospholipase A_2; PP1c, a catalytic subunit of type1 protein phosphatase; Rho-kinase, representing two isoforms; ROKα and ROKβ, or ROCKI and ROCKII; Zip-kinase; zipper-interacting protein kinase.

catalytic myosin phosphatase target subunit (MYPT1), and the 20 kDa non-catalytic subunit (M20) (Hartshorne *et al.*, 1998; Hartshorne and Hirano, 1999). MYPT1 plays an important role in targeting MLCP to myosin filaments, enhancing substrate specificity toward myosin, and regulating the enzymatic activity (Hartshorne *et al.*, 1998; Hartshorne and Hirano, 1999). The catalytic subunit PP1c is by itself capable of dephosphorylating both MLC and phosphorylase. However, its activity toward MLC increases, while its activity toward phosphorylase decreases when it complexes with MYPT1 (Alessi *et al.*, 1992; Ichikawa *et al.*, 1996a; Johnson *et al.*, 1996; Hirano *et al.*, 1997; Hartshorne *et al.*, 1998). A recent crystallographic study has revealed some structural basis for the enhancement of the catalytic activity toward MLC by MYPT1 (Terrak *et al.*, 2004). The perturbation of the quaternary structure of MLCP, either a complete dissociation

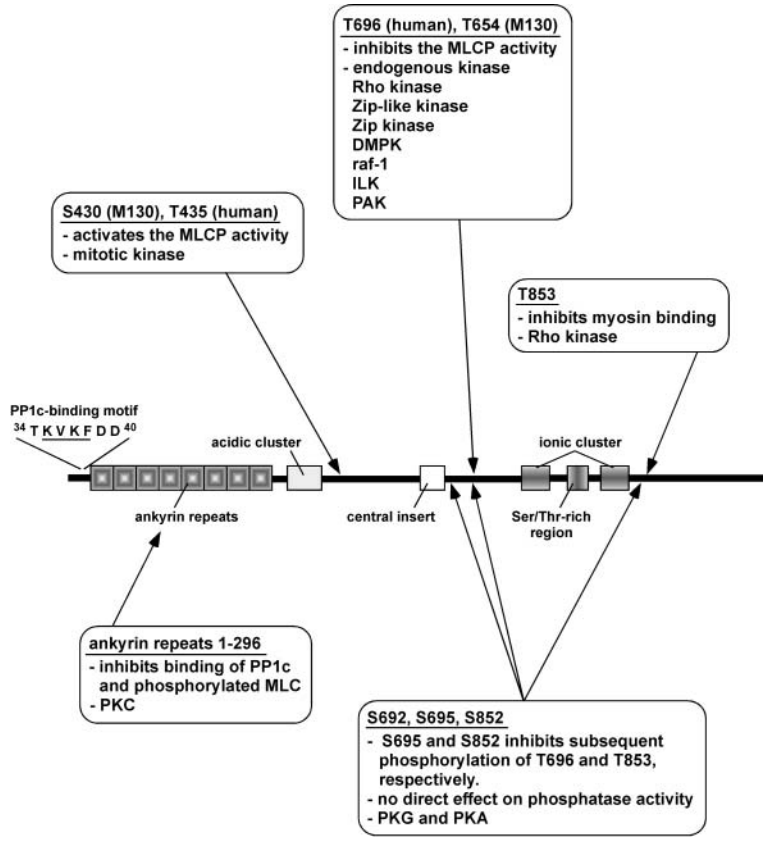

Fig. 2. Regulation of myosin phosphatase activity by phosphorylation of MYPT1. MYPT1 is schematically shown with the phosphorylation sites reported in the literature. Each phosphorylation site is annotated with its effect on the activity of myosin light chain phosphatase (MLCP) and the responsible kinases. DMPK, myotonic dystrophy protein kinase; ILK, integrin-linked kinase; MLC, 20 kDa myosin light chain; PAK, p21-activated protein kinase; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase.

of the regulatory subunits from PP1c or by the subtle perturbation of the interaction among three subunits, thus can decrease the MLCP activity toward MLC. Moreover, a disturbance in such protein-protein interactions as the interactions with substrate and myosin filaments, which participated in the enhancement of the PP1c activity toward MLC, could also lead to the inhibition of MLCP. Arachidonic acid was reported to dissociate MYPT1 from PP1c, and thereby inhibit the MLCP activity and potentiate the Ca^{2+}-sensitivity of the contractile apparatus (Gong *et al.*, 1992) (Fig. 1). Protein kinase C has been shown to phosphorylate the domain of ankyrin repeats and inhibit interaction of MYPT1 with PP1c and phosphorylated MLC (Toth *et al.*, 2000) (Figs. 1 and 2). We have previously shown that the N-terminal fragment of MYPT1 served as a dominant negative mutant toward endogenous MLCP, thereby increasing MLC phosphorylation and the Ca^{2+}-sensitivity of the contractile apparatus in both intact and permeabilized smooth muscle (Zhou *et al.*, 1999; Hirano *et al.*, 2004a). Exogenously added

subunit fragments have been suggested to cause a subtle perturbation in the interaction primarily between MYPT1 and PP1c, thereby inhibiting the MLCP activity (Hirano *et al.*, 2004a).

The functional relevance of this mechanism regulating the MLCP activity has been supported by one study (Shin *et al.*, 2002). Namely, in the isolated smooth muscle cells of ferret portal vein, both MYPT1 and PP1c localized homogeneously in the cytosol under resting conditions. However, during the $PGF_{2\alpha}$-induced contraction, MYPT1 translocated to the plasma membrane within 5 min and thereafter stayed at the membrane. On the other hand, PP1c first translocated to the membrane and then subsequently returned to the cytosol. As a result, PP1c (cytosolic localization) dissociated from MYPT1 (membrane localization) during the sustained phase of the $PGF_{2\alpha}$-induced contraction. However, this phenomenon was not observed with other types of agonist stimulation (Shin *et al.*, 2002).

Regulation of the MLCP activity by phosphorylation of MYPT1

The phosphorylation of MYPT1 has been shown to be one of the major mechanisms that regulates the MLCP activity (Hartshorne *et al.*, 1998). There are several possible phosphorylation sites along the MYPT1 sequence, which cause either the activation or inhibition of MLCP (Fig. 2). The most intensively studied mechanism is the inhibition of the MLCP activity by phosphorylation at T696 (in human MYPT1). The first clue for this mechanism was obtained by a physiological study which showed the relationship between the ATPγS-induced myofilament Ca^{2+} sensitization and the thiophosphorylation of MYPT1 (Trinkle-Mulcahy *et al.*, 1995). The isolated trimeric MLCP was also found to contain some kinase activity that phosphorylates MYPT1 and inhibits the MLCP activity (Ichikawa *et al.*, 1996b). However, the molecular identification of endogenous kinase responsible for such phosphorylation remains to be clarified. Rho-kinase was the first kinase that was found to mediate the inhibition of the MLCP activity (Kimura *et al.*, 1996). Later, various other kinases were found to catalyze the phosphorylation of the inhibitory site (T696) (Fig. 2). The protein kinases that are now known to mediate the phosphorylation of the inhibitory site include endogenous kinase (Ichikawa *et al.*, 1996b), rho-kinase (Kimura *et al.*, 1996), integrin-linked kinase (Kiss *et al.*, 2002; Muranyi *et al.*, 2002), p21-activated protein kinase (Takizawa *et al.*, 2002), zip-like kinase (MacDonald *et al.*, 2001a), zip kinase (Niiro and Ikebe, 2001), myotonic dystrophy protein kinase (Muranyi *et al.*, 2001) and raf-1 (Broustas *et al.*, 2002).

The phosphorylation of MYPT1 at other sites has been shown to alter the protein-protein interaction. One such phosphorylation site is T853. Rho-kinase has been shown to phosphorylate several sites, while T696 and T853 are the two major phosphorylation sites (Feng *et al.*, 1999b; Kawano *et al.*, 1999; Velasco *et al.*, 2002). The phosphorylation of T853 by rho-kinase has been shown to dissociate MYPT1 and myosin (Velasco *et al.*, 2002), while it has no direct inhibitory effect on the MLCP activity (Feng *et al.*, 1999b). However, the interaction between MYPT1 and myosin may contribute to the enhancement of the MLCP activity toward myosin. Therefore, the phosphorylation of T853 by rho-kinase may decrease the MLCP activity toward myosin. Protein kinase C has been shown to phosphorylate T34 adjacent to the PP1c-binding motif (Toth *et al.*, 2000; Cohen, 2002). However, this phosphorylation was shown to have no effect on the MLCP activity. Protein kinase C also phosphorylates the ankyrin repeats

domain (Toth *et al.*, 2000). This domain mediates the interactions of MYPT1 with PP1c and phosphorylated MLC, thereby contributing to the enhancement of the MLCP activity toward MLC (Hartshorne *et al.*, 1998; Hartshorne and Hirano, 1999). The phosphorylation of the domain of ankyrin repeats by protein kinase C was shown to inhibit these interactions, thereby reducing the MLCP activity toward myosin.

In addition to the negative regulation of MLCP as discussed above, the phosphorylation of MYPT1 was also associated with positive regulation (Fig. 2). MYPT1 was found to undergo mitosis-specific phosphorylation (Totsukawa *et al.*, 1999). MYPT1 phosphorylated in a mitosis-specific manner showed a higher binding activity toward myosin, and it was also associated with an increase in the MLCP activity. Recently, cyclic nucleotide-dependent protein kinases, protein kinase G and protein kinase A have been reported to phosphorylate MYPT1 at three sites; S692, S695 and S852 (Wooldridge *et al.*, 2004) (Fig. 2). S695 and S852 are adjacent to the phosphorylation sites of rho-kinase; S696 and S853, respectively. The phosphorylation of MYPT1 by protein kinase G has been shown to have no direct effect on the MLCP activity (Nakamura *et al.*, 1999). However, the phosphorylation of MYPT1 by protein kinase G or protein kinase A inhibited the subsequent phosphorylation by rho-kinase, and vice versa (Wooldridge *et al.*, 2004). Therefore, the phosphorylation by protein kinase G and protein kinase A antagonizes the rho-kinase-induced inhibition of the MLCP activity.

Inhibition of MLCP by CPI-17

No inhibitor protein of PP1c such as inhibitor-1 and DARPP-32 (48) had been identified in smooth muscle, until CPI-17 was isolated (Eto *et al.*, 1995). CPI-17 is expressed in both vascular and visceral smooth muscle tissues, and it exerts its inhibitory activity toward PP1c upon the phosphorylation at T38 (Eto *et al.*, 1995; Eto *et al.*, 1997). It was originally identified as a substrate for protein kinase C, however, more kinases were also found to phosphorylate CPI-17 at T38 (Fig. 1). It is now conceivable that the activity of CPI-17 as a PP1c inhibitor is also regulated by multiple kinases. These kinases include rho-kinase (Koyama *et al.*, 2000), integrin-linked kinase (Ti Deng *et al.*, 2002), p21-activated protein kinase (Takizawa *et al.*, 2002), protein kinase N (Hamaguchi *et al.*, 2000), and zip-like kinase (MacDonald *et al.*, 2001b).

Signal Transduction Regulating Ca^{2+} Sensitivity in Vascular Smooth Muscle

As discussed above, a recent investigation has helped to clarify the biochemical basis for the regulation of MLC phosphorylation and dephosphorylation. The involvement of a complex network of protein kinases and protein-protein interactions in the regulation of the Ca^{2+} sensitivity is now evident. However, the intracellular signal transduction that connects the contractile (or relaxant) stimulations to such molecular networks regulating the Ca^{2+} sensitivity has yet to be elucidated. For example, although a number of reports have suggested the involvement of a rhoA-rho-kinase pathway in the regulation of the vascular tone and myofilament Ca^{2+} sensitivity, it remains to be elucidated as to how this pathway is activated by the contractile stimulations that cause Ca^{2+} sensitization. Recently, the α subunits of heterotrimeric G protein G_{12} and G_{13} were shown to activate a family of unique rho guanine

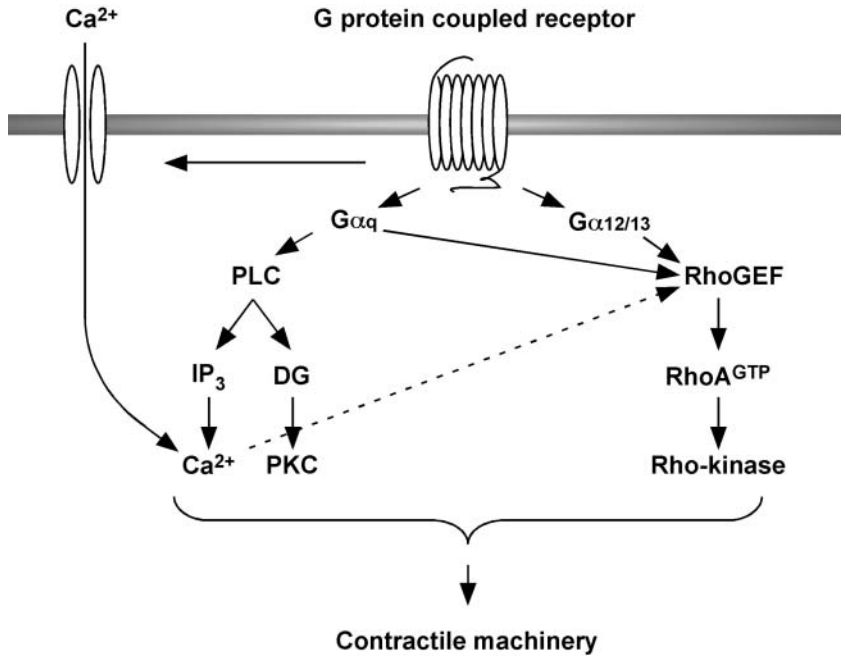

Fig. 3. Signal transduction pathway leading to activation of a rhoA-rho-kinase pathway and an increase in the Ca^{2+} signal. DG, diacylglycerol; GEF, guanine nucleotide exchange factor; IP_3, inositol 3,4,5-trisphosphate; PLC, phospholipase C; PKC, protein kinase C.

nucleotide exchange factors (GEFs), such as p115rhoGEF, PDZrhoGEF and LARG (leukemia-associated rhoGEF), which contains a regulator of G protein signaling (RGS) domain (Hart *et al.*, 1998; Kozasa *et al.*, 1998; Wang *et al.*, 2004) (Fig. 3). Later, $G_{\alpha q}/G_{\alpha 11}$ was also reported to activate rhoGEFs (Chikumi *et al.*, 2002; Vogt *et al.*, 2003). Furthermore, the increase in cytosolic Ca^{2+} concentrations induced by either membrane depolarization or receptor stimulation has been shown to activate the rhoA-rho-kinase pathway and contribute to the regulation of the Ca^{2+} sensitivity (Sakurada *et al.*, 2003). However, the mechanism of Ca^{2+}-mediated activation of rhoA and the identification of the GEF involved, if any, remains to be elucidated. As a result, regarding the rhoA-rho-kinase pathway, the possible existence of signaling pathways that connect the contractile stimulation to the Ca^{2+} sensitivity has become experimentally evident (Fig. 3). However, regarding the other signaling molecules that were summarized to participate in the regulation of the MLC phosphorylation (Fig. 1), the upstream signaling pathways still remain largely unknown.

Unsolved Questions Regarding the Regulation of Myofilament Ca^{2+} Sensitivity

Q1. Which kinase plays a physiological role in regulating the phosphorylation level of MLC?

As shown in Fig. 1, many kinases have been reported to induce the phosphorylation of MYPT1 and CPI-17 at the inhibitory site, thereby contributing to the inhibition of the MLCP

activity. Some kinases, such as rho-kinase, integrin-linked kinase and p21-activated protein kinase, have been shown to phosphorylate both MYPT1 and CPI-17. Furthermore, they have also been shown to directly phosphorylate MLC in a Ca^{2+}-independent manner. However, it remains to be clarified as to which kinase(s) plays a physiological role in the regulation of myofilament Ca^{2+} sensitivity. It is also possible that all kinases listed in Fig. 1 may play a functional role but under different physiological conditions or pathological conditions.

There could be several possible approaches to answer these questions. One approach is to develop specific kinase inhibitors. However, there are limitations to the currently available pharmacological inhibitors. Many kinase inhibitors antagonize ATP binding to the catalytic domain of the kinases (Davis *et al.*, 2000; Bain *et al.*, 2003). The structure of such a domain is relatively similar to each other. Previous reports recommended the use of two structurally unrelated inhibitors of the protein kinase (Davis *et al.*, 2000; Bain *et al.*, 2003). However, this is not applicable to all kinases, and a perfect specificity could not be expected for most such inhibitors. It has recently been reported that the peptide inhibitor can be designed for any kinase based on the sequence of kinase itself (Niv *et al.*, 2004). Such inhibitors are expected to possess a high specificity toward the target kinase. However, the impermeability of the peptide through the plasma membrane is one major obstacle to overcome when applying this type of inhibitor to the vascular physiology. The lipid modification of the peptide, such as an attachment of the palmitate lipid, has been used to introduce the membrane impermeable peptides (Covic *et al.*, 2001). The *Clostridium botulinum* exoenzyme C3 has been introduced into intact vascular tissue as a fusion protein with the B fragment of diphtheria toxin, which thus allows the introduction of C3 into intact cells which contain diphtheria toxin receptors (Fujihara *et al.*, 1997). Recently, a group of peptides, termed cell-penetrating peptides, has been successfully used to introduce cell-impermeable molecules such as peptide and nucleotide into the intact cells (Lindgren *et al.*, 2000; Schwarze and Dowdy, 2000). These peptides include those seen in human immunodeficiency viral transcription factor Tat protein, drosophila transcription factor anntenapedia and herpes simplex virus-1 DNA binding protein VP22 (Lindgren *et al.*, 2000; Schwarze and Dowdy, 2000). The cell-penetrating peptide-mediated protein transduction has also been suggested to be independent of both cell surface receptor and ATP (Lindgren *et al.*, 2000; Schwarze and Dowdy, 2000). This protein transduction technique is thus anticipated to be applicable to various types of cells. We and others have successfully applied this protein transduction technique to the vascular biology (Sauzeau *et al.*, 2001; Hirano *et al.*, 2004a; Hirano *et al.*, 2004b; Koga *et al.*, 2004). The specific peptide inhibitor can be introduced using cell-penetrating peptides as previously proposed (Niv *et al.*, 2004). Other approach to investigate the physiological role of kinases in the regulation of myofilament Ca^{2+} sensitivity is to analyze the vascular tissues that are specifically deficient in a certain kinase due to the gene knockout or gene silencing by RNA interference. RNA interference may be difficult to simply apply to the vascular tissue. Gene knockout animals may give a useful and definite answer to the role of the target kinase. However, they may be too cumbersome to conduct an evaluation of all candidate kinases.

The subcellular localization of the kinases could be another factor to consider when evaluating their physiological role. The localization in the myofilaments may be a prerequisite

to play a physiological role in the regulation of myofilament Ca^{2+} sensitivity. But it is not necessarily a constant, fixed or exclusive localization. Even a transient localization of a subpopulation of kinase may be sufficient to play a regulatory role. Zip-like kinase has been isolated from the myofilaments (MacDonald *et al.*, 2001a). The endogenous kinase (Fig. 1) was discovered in the preparation of MLCP isolated from the myofilaments (34b), and it could thus become localized in the myofilaments. RhoA has been found to directly interact with MYPT1 (Kimura *et al.*, 1996). Therefore, rho-kinase could be activated at the proximity of MLCP. On the other hand, since rhoA localizes in the plasma membrane when it is activated (Takai *et al.*, 2001), rho-kinase also localizes in the plasma membrane (MacDonald *et al.*, 2001a). The subcellular localization of rho-kinase during the contraction thus remains to be elucidated. Integrin-linked kinase was originally discovered as an enzyme that binds to the cytoplasmic domain of β-integrins (Hannigan *et al.*, 1996). However, a subcellular fractionation approach identified two populations of integrin-linked kinase; one associated with integrin and the other retained in myofilaments (Deng *et al.*, 2001). The latter subpopulation could play a physiological role in the regulation of the myofilament Ca^{2+} sensitivity. Zip kinase contains a couple of nuclear localization signals, but the majority of this kinase was found to be localized in the cytoplasm in non-muscle cells (Shani *et al.*, 2004).

Q2. Relative contribution of MYPT1 and CPI-17 to the regulation of Ca^{2+} sensitivity

Several reports have suggested that the relative importance of MYPT1 and CPI-17 in inhibiting MLCP could differ depending on the type of tissue (Woodsome *et al.*, 2001; Sakurada *et al.*, 2003; Kitazawa *et al.*, 2003; Niiro *et al.*, 2003; Kitazawa *et al.*, 2004). First, the expression level of CPI-17 has been shown to vary depending on the species and the type of smooth muscle (Woodsome *et al.*, 2001; Kitazawa *et al.*, 2003; Kitazawa *et al.*, 2004). The smooth muscle tissue of the chicken including the aorta and gizzard are extreme examples that do not express CPI-17 at a level detectable by immunoblot or polymerase chain reaction analyses (Kitazawa *et al.*, 2004). The Ca^{2+} sensitization induced by agonist stimulation in the chicken tissues was smaller than that seen in such CPI-17-rich tissues as the rabbit arteries (Kitazawa *et al.*, 2004). In rabbit tissue, the level of CPI-17 varies depending on the type of tissue, and the expression ratio of CPI-17 to MLCP was found to correlate with the Ca^{2+} sensitization mediated by a PKC activator (Woodsome *et al.*, 2001). An higher the expression of CPI-1 expression, more PKC contributed to the Ca^{2+} sensitization.

The phosphorylation of MYPT1 at T696 has been shown to increase in response to $PGF_{2\alpha}$ in a manner dependent on the presence of rho-kinase in the rabbit aorta (Ito *et al.*, 2003). On the other hand, the relative contribution of MYPT1 and CPI-17 to the regulation of the MLCP activity has been studied in the tissues expressing both MYPT1 and CPI-17 (Kitazawa *et al.*, 2003; Niiro *et al.*, 2003). In the rabbit femoral artery (Niiro *et al.*, 2003), rabbit portal vein (Kitazawa *et al.*, 2003) and rabbit vas deference (Kitazawa *et al.*, 2003), the agonist stimulations or GTPγS have been shown to increase the level of phosphorylation of CPI-17 at T38. However, the level of phosphorylation of MYPT1 at T696 remaines unchanged, while the phosphorylation of MYPT1 at T853 increased. Under resting conditions, CPI-17 was scarcely phosphorylated, while MPYT1 was significantly phosphorylated both at T696 and T853. Accordingly, the

phosphorylation of CPI-17 at T38 was suggested to play a major role in the Ca^{2+}-sensitization during agonist-induced contractions (Kitazawa *et al.*, 2003; Niiro *et al.*, 2003). The phosphorylation of MYPT1 at T853 has been shown to have no direct effect on the MLCP activity (Feng *et al.*, 1999b). However, it has been shown to interfere with myosin binding (Velasco *et al.*, 2002). The increased phosphorylation at this site may also contribute to some degree to the Ca^{2+} sensitization during the agonist-induced contraction (Kitazawa *et al.*, 2003). On the other hand, the phosphorylation of MYPT1 at T696 seen under resting conditions may play a fundamental role in determining the basal level of the MLCP activity, and hence the basal vascular tone. This phosphorylation has been shown to be resistant to the inhibitors of rho-kinase (Kitazawa *et al.*, 2003; Niiro *et al.*, 2003). Kinases other than rho-kinase, such as those shown in Figs. 1 and 2, may thus play a functional role in phosphorylating MYPT1 at an inhibitory site under resting conditions, thus helping to determine the basal activity of MLCP.

Q3. Physiological significance of Ca^{2+}-independent phosphorylation of MLC and activation of myosin

The phosphatase inhibitors such as okadaic acid and microcystine have been shown to induce contraction in the absence of Ca^{2+} in the permeabilized smooth muscle tissues (Ozaki *et al.*, 1987; Kureishi *et al.*, 1999; Niiro and Ikebe, 2001). This Ca^{2+}-independent contraction was also shown to be resistant to such MLCK inhibitors as wortmannin, ML-9 and HA1077 (Kureishi *et al.*, 1999; Niiro and Ikebe, 2001). It is thus highly possible that the Ca^{2+}-independent phosphorylation of MLC and activation of myosin play a functional role in such Ca^{2+}-independent contraction. Furthermore, the Ca^{2+}-independent contraction induced by the phosphatase inhibitors has been shown to be resistant to the inhibitors of rho-kinase (Y27632 and HA-1077) and protein kinase C (H-7, calphostin C, chelerythrine) (Kureishi *et al.*, 1999; Niiro and Ikebe, 2001). Rho-kinase may thus not play a physiological role in either the Ca^{2+}-independent phosphorylation of MLC or activation of contraction. This finding is consistent with the fact that rho-kinase has a higher specificity toward MYPT1 than MLC (the Km value for MYPT1 and MLC is 0.1–0.2 μM and 2.5–5 μM, respectively) (Feng *et al.*, 1999a). As a result, integrin-linked kinase, p21-activated protein kinase and zip kinase remain candidate kinases (Fig. 1). Nevertheless, exactly which kinase plays a physiological role in the Ca^{2+}-independent phosphorylation of MLC and contraction remains to be identified. On the other hand, smooth muscle contraction is usually associated with an elevation of the cytosolic Ca^{2+} level under physiological situations. It thus remains to be determined whether Ca^{2+}-independent MLC phosphorylation contributes to the usual contraction associated with an elevation of the Ca^{2+} signal. The development of specific inhibitors of the candidate kinases, and the establishment of the smooth muscle tissue which is specifically deficient in a certain kinase may therefore be required before these questions can be answered.

Q4. Upstream signal transduction regulating Ca^{2+} sensitivity

The intensive investigations have clarified a number of molecules that are involved in the regulation of MLC phosphorylation and dephosphorylation as listed in Fig. 1. However, the intracellular signal transduction linking the contractile stimulation to such regulatory molecules remains largely unknown. This remains the most important question to be clarified in the

smooth muscle physiology and pathophysiology.

As discussed above, a recent study has clarified a $G_{12/13}$ or G_q-rhoGEF pathway that connect the G protein coupled receptor stimulation to the rhoA-rho-kinase system in non-muscle cells (Hart *et al.*, 1998; Kozasa *et al.*, 1998; Chikumi *et al.*, 2002; Vogt *et al.*, 2003). Whether such a pathway plays a functional role in the contraction of smooth muscle and the Ca^{2+} sensitization remains to be solved. The rho-kinase purified from smooth muscle tissues has also been shown to be activated by arachidonic acid (Feng *et al.*, 1999a). However, the physiological significance of arachidonic acid-induced activation of rho-kinase remains to be determined.

The activity of p21-activated protein kinase and myotonic dystrophy protein kinase is dependent on rac1 and cdc42. The p21-activated protein kinases have been shown to phosphorylate not only MLC (Chew *et al.*, 1998; Van Eyk *et al.*, 1998), MYPT1 (Takizawa *et al.*, 2002) and CPI-17 (Takizawa *et al.*, 2002) (Fig. 1), but also MLCK (Sanders *et al.*, 1999; Goeckeler *et al.*, 2000; Wirth *et al.*, 2003) and caldesmon (Van Eyk *et al.*, 1998). The muscarinic acetylcholine receptor m2 has been shown to activate p21-activated protein kinase 1 via G_{i3}, rac1/cdc42, and thereby inhibit the MLCK activity in intestinal smooth muscle (Murthy *et al.*, 2003).

Integrin-linked kinase is known to be activated by phosphatidylinositol 3,4,5-trisphosphate (Delcommenne *et al.*, 1998). However, phosphatidylinositol 3,4,5-trisphosphate has been shown to have no effect on the phosphorylation of myosin by smooth muscle integrin-linked kinase (Deng *et al.*, 2001).

Death-associated protein kinase, DAP kinase, has recently been shown to phosphorylate zip kinase and regulate its subcellular localization and oligomerization status (Shani *et al.*, 2004). Zip kinase is localized both in the nucleus and cytoplasm under resting conditions with a majority in the cytoplasm, while it predominantly localizes in the cytoplasm upon phosphorylation by DAP kinase. DAP kinase has been shown to be activated by various signals inducing cell death such as interferon-γ and transforming growth factor-β (Cohen *et al.*, 1997; Inbal *et al.*, 1997; Jang *et al.*, 2002; Shani *et al.*, 2004). The role of DAP kinase in the contraction of smooth muscle thus remains to be determined.

Concluding Remarks

The intensive investigations in the last decade have advanced our understanding of the protein networks regulating the phosphorylation and dephosphorylation of MLC, especially the molecular mechanism regulating the MLCP activity. They have also provided us with numerous proteins that may play a physiological role in the regulation of myofilament Ca^{2+} sensitivity. However, it remains largely unknown regarding which molecule plays a functionally significant role. First, some of the identified molecules may not play a physiological role in the regulation of smooth muscle contraction. The phosphorylation-dependent activation of myosin is an essential step to regulate not only the contraction of smooth muscle but also cell motility, cytoskeletal organization and secretion in non-muscle cells. Therefore, some of the biochemical mechanisms discussed so far may play a physiological role in such cell functions other than the contraction of smooth muscle. We should first identify which molecules are involved in the

regulation of the myofilament Ca^{2+} sensitivity and the contraction of smooth muscle. The next question is to elucidate the intracellular signals regulating the contraction of smooth muscle. These signals may differ depending on such factors as; vascular vs. visceral smooth muscle, physiological contraction vs. pathological contraction, phasic muscle vs. tonic muscle etc.

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