*Current Perspective***Current Topics in the Regulatory Mechanism Underlying the Ca^{2+} Sensitization of the Contractile Apparatus in Vascular Smooth Muscle**Katsuya Hirano^{1,*}¹*Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan*

Received February 23, 2007

Abstract. The Ca^{2+} signal is the primary determinant of the contraction of the vascular smooth muscle. However, the alteration of the Ca^{2+} sensitivity of the contractile apparatus also plays an essential role. The regulation of the myosin light chain phosphatase (MLCP) activity is considered to be the most important mechanism underlying the regulation of Ca^{2+} sensitivity. The investigations during the last 15 years have identified many proteins that participate in the regulation of the MLCP activity. Recently, the Ca^{2+} signal has also been shown to cross-talk with the mechanisms regulating the Ca^{2+} sensitivity. Consequently, Rho kinase, protein kinase C, CPI-17, and MYPT1 have all been suggested to play a physiologically important role in the regulation of the MLCP activity. We are now close to elucidating the major rules regulating the MLCP activity and the Ca^{2+} sensitivity during vascular contractions. This article will give an overview of the current understanding of the biochemical basis for the regulation of the MLCP activity, while also discussing their functional roles from a physiological point of view. I hope this article will help to develop new pharmacological strategies for the prevention and treatment of the pathological vasoconstriction often seen in vascular diseases.

Keywords: vascular smooth muscle, contraction, calcium, Ca^{2+} sensitivity, signal transduction

Introduction

The contraction of the vascular smooth muscle is primarily regulated by the intracellular Ca^{2+} signal (1). The contractile stimulations trigger an elevation of the cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) by activating either the release of Ca^{2+} from the intracellular store sites or a Ca^{2+} influx from the extracellular space (2). The elevation of $[\text{Ca}^{2+}]_i$ increases the Ca^{2+} -bound calmodulin, which then activates the myosin light chain kinase (MLCK). The phosphorylation of the 20-kDa regulatory myosin light chain (MLC20) at S19 or T18 by MLCK results in the activation of the myosin ATPase activity, thereby activating both cross-bridge cycling and the contraction (3). The termination of the contractile stimulation causes a decrease in $[\text{Ca}^{2+}]_i$, thereby

decreasing the MLCK activity. Consequently, MLC20 is dephosphorylated by the action of the MLC20 phosphatase (MLCP). The reversible phosphorylation of MLC20 thus plays an essential role in the regulation of the contraction of the vascular smooth muscle.

However, it is also well known that the relationship between the extent of the $[\text{Ca}^{2+}]_i$ elevation and the degree of the developed tension varies depending on the situation (1). In general, the receptor-mediated contractile stimulations produce a greater tension for a given elevation of $[\text{Ca}^{2+}]_i$ than the membrane depolarization. The Ca^{2+} -tension relationship also changes during the time course of the contraction; the sustained phase of the contraction is maintained by a relatively lower level of $[\text{Ca}^{2+}]_i$. When a greater contraction is produced for a given elevation of $[\text{Ca}^{2+}]_i$, this phenomenon is referred to as “ Ca^{2+} sensitization of the contractile apparatus” or “an increase in the Ca^{2+} sensitivity” (4). As a result, the contraction of the vascular smooth muscle is thus subjected to the dual regulation by the Ca^{2+} signal and the alteration of the Ca^{2+} sensitivity (Fig. 1). Precisely how the Ca^{2+} sensitivity is regulated remains elusive.

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Published online in J-STAGE: May 31, 2007

doi: 10.1254/jphs.CP0070027

Fig. 1. Dual regulation of the contraction of the vascular smooth muscle by the Ca^{2+} signal and the alteration of the Ca^{2+} sensitivity of the contractile apparatus.

The Ca^{2+} sensitivity can be regulated by either MLC20 phosphorylation-dependent or -independent mechanisms, while the phosphorylation-dependent mechanism is considered to play a major role in the regulation of Ca^{2+} sensitivity. Investigations during the last 15 years have revealed the details of the biochemical basis for the regulation of MLC20 phosphorylation (5, 6). The level of MLC20 phosphorylation is determined by the balance between the activities of the MLC20 phosphorylation and dephosphorylation. The regulation of the MLCP activity is thus considered to play a major role in the regulation of the Ca^{2+} sensitivity. The most recent investigations (7–9) have further elucidated the Ca^{2+} -dependent regulation of the MLCP activity (Fig. 1). In this review article, I would like to summarize the current understanding of the regulatory mechanisms of the MLCP activity (Fig. 2) and then discuss their physiological relevance in the regulation of Ca^{2+} sensitivity. However, the Ca^{2+} -independent mechanism of MLC20 phosphorylation (Fig. 2) will not be covered in this article. Please refer to the previous review articles for information on that topic (5, 6).

The basic properties of MLCP

MLCP is composed of a 38-kDa catalytic subunit of type 1 protein phosphatase (PP1c, δ isoform) and 110- and 20-kDa non-catalytic subunits (10, 11) (Fig. 2). The N-terminal region of the 110-kDa subunit binds to PP1c, while the C-terminal region binds to the 20-kDa subunit (11, 12). These subunit interactions help to form the heterotrimeric holoenzyme of MLCP. The 110-kDa subunit also binds to myosin, thus targeting PP1c to the myosin filaments (11). This subunit is thus referred to as the myosin phosphatase target subunit (MYPT). There are several isoforms of MYPT, and MYPT1 is the major isoform expressed in vascular smooth muscle (13). In addition to targeting MLCP to the myosin filaments,

MYPT1 is also known to increase the substrate specificity of MLCP toward myosin, and it also regulates the enzymatic activity of MLCP. MYPT1 thus plays a very important role in the regulation of the MLCP activity. The interaction between PP1c and the N-terminal region of MYPT1, which contains an ankyrin repeat domain, has been shown to increase the substrate specificity of PP1c toward myosin (10, 12). In addition, the direct interaction between MYPT1 and MLC20 or myosin also contributes to the increased specificity of PP1c toward MLC20 (10, 12). Recently, the structural basis for such a substrate specificity has been proposed by the crystallographic study (14). The observed crystal structure of the complex of PP1c and the N-terminal 299 amino acids of MYPT1 suggest that this interaction cooperatively forms a platform that favors the binding of MLC20. The platform consists of the acidic groove, the catalytic center, and the hydrophobic groove, which is believed to fit to the structure flanking the phosphorylation site of MLC20 (14). In MLC20, the basic amino acids cluster on the N-terminal side of the phosphorylation site, while the hydrophobic amino acids cluster on the C-terminal side. The basic cluster of MLC20 fits to the acidic groove of MLCP, and the hydrophobic cluster fits to the hydrophobic groove, thus positioning the phosphorylation site to the catalytic center (14). However, the crystal structure of the full-length MYPT1 still remains to be elucidated.

Inhibition of the MLCP activity by MYPT1 phosphorylation

The activity of MLCP can be regulated in three different manners (6): 1) dissociation, either gross or subtle, of the heterotrimeric structure; 2) inhibitory effect of the phosphorylation of MYPT1; and 3) inhibition by the inhibitory proteins. The heterotrimeric conformation of MLCP and the interaction between MYPT1 and myosin contribute to increasing the MLCP activity toward MLC20. Therefore, any disturbance of this structure could potentially decrease the MLCP activity. Although an example of the dissociation of MYPT1 from PP1c during the phenylephrine-induced contraction has been reported, the physiological role of the subunit dissociation in the regulation of the MLCP activity still remains to be elucidated (5, 6). On the other hand, a body of evidence has been accumulated to support the latter two mechanisms as the major mechanisms regulating the MLCP activity.

MYPT1 is known to be phosphorylated at several sites (5). Among them, the phosphorylation at T696 (in human MYPT1) has been shown to be associated with the inhibition of the MLCP activity (10). Rho kinase

Fig. 2. Current overview of the mechanisms involved in the regulation of MLC20 phosphorylation. $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentrations; CPI-17, 17-kDa PKC-potiated inhibitory protein of type 1 protein phosphatase; DAG, diacylglycerol; DMPK, myotonic dystrophy protein kinase; GPCRs, G protein-coupled receptors; ILK, integrin-linked kinase; IP₃, inositol-1,4,5-trisphosphate; MLC20, 20-kDa regulatory myosin light chain; MLCK, Ca^{2+} -calmodulin-dependent myosin light chain kinase; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase target subunit 1 of MLCP; M20, 20-kDa non-catalytic subunit of MLCP; PAK, p21-activated protein kinase; PI3K-C2 α , α isoform of the class II phosphatidylinositol 3-kinase; PKC, protein kinase C; PKN, protein kinase N; PLC, phospholipase C; PP1c, a catalytic subunit of type 1 protein phosphatase; RhoGEF, GDP-GTP exchange factor of RhoA; RhoK, Rho-kinase, representing two isoforms of ROCK I and ROCK II; ZIPK, zipper-interacting protein kinase.

(RhoK) was the first kinase that was identified to catalyze the phosphorylation of this site. More kinases have subsequently been found to also phosphorylate this site (Fig. 2). RhoK can phosphorylate MYPT1 at several sites, with T696 and T853 being the two major phosphorylation sites (15). The phosphorylation of MYPT1 at T853 is thus characteristic of RhoK in comparison to the other MYPT1 kinases. In fact, RhoK shows a 3-fold preference for T853 rather than T696, while the zipper-interacting protein kinase (ZIPK)

shows a 6.7-fold preference for T696 over T853 (16). An initial study has suggested the phosphorylation at T696, but not that at T853, to be responsible for the inhibition of the MLCP activity (17). Thereafter, the phosphorylation at T853 was shown to interfere with the binding of MYPT1 to myosin (18). This effect of T853 phosphorylation could therefore decrease the phosphatase activity of MLCP toward MLC20. Recently, the phosphorylation at T853 has also been reported to contribute to the inhibition of the MLCP activity (19).

However, the reason for the apparent discrepancy between these two studies (17, 19) remains unclear. In fact, the phosphorylation sites T696 and T853 are each flanked by amino acid sequences similar to those flanking the phosphorylation site of MLC20. Namely, a basic amino acid cluster precedes the phosphorylation site, which is then followed by a hydrophobic amino acid cluster (14). This structure suggests that both regions surrounding T696 or T853 may compete with MLC20 in binding to the PP1c-MYPT1 complex, which could underlie the mechanism for the inhibition of MLCP. However, it remains unclear whether these two phosphorylation sites redundantly function as the inhibitory site or whether only one of two sites plays a physiological role. Further examination of the crystallographic structure of the complex of PP1c and the full-length MYPT1 will be necessary in order to answer these questions. Nevertheless, RhoK can induce the inhibition of the MLCP activity by the phosphorylation at either one of the two sites.

Inhibition of the MLCP activity by CPI-17

CPI-17 (17-kDa PKC-potentiated inhibitory protein of type 1 protein phosphatase) is the endogenous heat-stable inhibitory protein of the type 1 protein phosphatase, which is expressed in the smooth muscle tissues (20). CPI-17 exerts its inhibitory effect when it is phosphorylated at T38 (Fig. 2). The kinases that catalyze the phosphorylation of this inhibitory site are listed in Fig. 2. Most importantly, the kinases that phosphorylate CPI-17 largely overlap with the kinases that catalyze the inhibitory phosphorylation of MYPT1. Furthermore, some of them are known to phosphorylate MLC20 at the activation site in a Ca^{2+} -independent manner (Fig. 2).

Relative importance of MYPT1 and CPI-17 in the regulation of the MLCP activity

The expression level of MYPT1 and CPI-17 is a factor when evaluating their relative importance in the regulation of the MLCP activity. The expression level of CPI-17 is known to differ depending on the type of the smooth muscle. An extreme example is the chicken gizzard and aorta, which lacks CPI-17 (21). In these tissues, the agonist stimulations induced only a slight increase in the Ca^{2+} sensitivity. In the tissues that express CPI-17, the higher the level of the CPI-17 expression, the more PKC contributes to the Ca^{2+} sensitization. In contrast, MYPT1 is ubiquitously expressed in smooth muscle tissues.

Several lines of evidence support the physiological importance of CPI-17 in the regulation of the Ca^{2+}

sensitivity during the contraction. First, the agonist stimulations induce only a marginal increase in the Ca^{2+} sensitivity in a chicken artery that lacks CPI-17 (21). Second, the phosphorylation level of CPI-17 was increased by the contractile stimulations. In contrast, the phosphorylation of MYPT1 at T696 remained unchanged (22, 23). However, the phosphorylation of MYPT1 at T853 was also increased (22, 23). Under resting conditions, CPI-17 stayed mostly unphosphorylated, while MYPT1 was significantly phosphorylated at both T696 and T853. The degree of the CPI-17 phosphorylation was thus closely correlated with that of the MLC20 phosphorylation. These observations therefore suggested that the phosphorylation of CPI-17 plays a major role in the Ca^{2+} sensitization under physiological situations. The phosphorylation of MYPT1 at T696 may not play a regulatory role, while the phosphorylation at T853 may play a role in the inhibition of the MLCP activity during the contraction.

The relative importance of MYPT1 and CPI-17 may also differ with physiological or pathophysiological situations. The MLC20 phosphorylation and the contraction of the cultured smooth muscle cells depend on the Rho-RhoK pathway to a greater extent than that seen in the freshly isolated arterial strips (24, 25). The contractile stimulation increased the level of phosphorylation of both MYPT1 at T696 and CPI-17 at T38 in cultured smooth muscle (25). The physiological relevance of these observations in the cultured smooth muscle cells remains to be validated. However, the smooth muscle cells in the culture may represent the phenotype of the smooth muscle seen in vascular regions. Furthermore, the RhoK inhibitor significantly inhibited the contractile response and decreased the blood pressure in the hypertensive model (25, 26). The Rho-RhoK-MYPT1 pathway may thus play a more important role in pathophysiological situations than in physiological situations.

Ca^{2+} -dependent Ca^{2+} sensitization: cross-talk between the Ca^{2+} signal and the mechanisms regulating the Ca^{2+} sensitivity

The Ca^{2+} -dependent activation of RhoA has been demonstrated in the rabbit aorta (7). Both high K^{+} -depolarization and noradrenaline increased the GTP-bound form of RhoA in a manner sensitive to either the removal of the extracellular Ca^{2+} , the treatment with Ca^{2+} channel blockers, or the depletion of the Ca^{2+} store (7). However, the mechanism underlying the Ca^{2+} -dependent activation of RhoA remains unclear. Recently, the α isoform of the class II phosphatidylinositol 3-kinase (PI3K-C2 α) has been proposed to be involved in the Ca^{2+} -dependent RhoA activation (8) (Fig. 2). However,

how PI3K-C2 α activates RhoA; whether or not any GDT-GTP exchange factor (GEF) is involved; and which GEF is involved, if any, remain unknown. Recently, the phosphorylation of CPI-17 has also been shown to take place in a Ca²⁺-dependent manner (9). CPI-17 was rapidly phosphorylated in the early phase of the phenylephrine-induced contraction in the rabbit femoral artery, with a similar time course of the [Ca²⁺]_i elevation and MLC20 phosphorylation. The Ca²⁺-dependent protein kinase C (PKC) was suggested to mediate this rapid phosphorylation of CPI-17 (Fig. 2). In the later phase of the contraction, CPI-17 was shown to be phosphorylated by RhoK and PKC in a Ca²⁺-independent manner. Consequently, the mechanisms regulating the MLCP activity and the Ca²⁺ sensitivity may differ with the phase of the contraction.

Signal transduction pathways involved in the regulation of the MLCP activity and the Ca²⁺ sensitivity in vascular tissues

The findings of recent investigations have helped to advance our understanding of the biochemical basis for the regulation of the MLCP activity and MLC20 phosphorylation. However, how these mechanisms are regulated by the contractile stimuli and which components play a physiologically relevant role in the vascular tissues remains to be elucidated. For example, the upstream regulators for the integrin-linked kinase, p21-activated protein kinase, protein kinase N, myotonic dystrophy protein kinase, and Raf-1, which were reported to phosphorylate MYPT1 or CPI-17 (Fig. 2), have not yet been identified in the context of the smooth muscle contraction. Their physiological relevance in the regulation of the Ca²⁺ sensitivity thus still remains speculative. Few studies have addressed the question of which G proteins are involved in Ca²⁺ sensitivity. One study of the cultured smooth muscles cells demonstrated that the agonist stimulation engaged G $\alpha_{12/13}$, thereby inducing MLC20 phosphorylation in a manner dependent on the Rho-RhoK pathway (27). This observation is consistent with the role of G $\alpha_{12/13}$ in the activation of RhoGEF (Fig. 2). However, the signal transduction pathway that plays a dominant role in the MLC20 phosphorylation may differ between in vivo conditions and in culture (24). Furthermore, other G proteins, such as G $\alpha_{q/11}$, can activate RhoGEF (Fig. 2). Therefore, which G protein plays a physiological role in terms of the Ca²⁺ sensitization during the contraction of the vascular tissues still remains to be elucidated.

Several factors should be taken into account when considering the physiological relevance of each component involved in the regulation of MLC20 phosphoryla-

tion (Fig. 2). These include the level of expression, subcellular localization, and the enzymatic properties, such as the K_m and k_{cat} values. CPI-17 is a typical example of the issue of tissue-specific expression, as discussed above. MYPT1 has been found to interact with the GTP form of RhoA, while MYPT1 binds to the myosin filaments (6, 28). Therefore, RhoK could be activated in close proximity to its substrates, MYPT1 and MLC20. However, this claim may be challenged by another notion that the activation of RhoA requires lipid modification, and therefore membrane localization is considered to be a prerequisite for RhoA activation. In this case, RhoK would also be activated at the plasma membrane. Consequently, this situation makes it difficult for RhoK to phosphorylate MYPT1 and MLC20, which are localized on the contractile apparatus. Recently, ZIPK has been suggested to fill this gap (Fig. 2). RhoK has a 1.7-fold preference for ZIPK over MYPT1, and it phosphorylates and thereby activates ZIPK (16). ZIPK is thus considered to function as an effector molecule of RhoK, and it directly executes the phosphorylation of MYPT1. However, how ZIPK shuttles between RhoK at the plasma membrane and MYPT1 on the contractile apparatus still remains unknown. An increase in the phosphorylation of MYPT1 during the contraction was preferably observed on T853 rather than T696 (7, 8, 22, 29). This fact suggests that RhoK, but not ZIPK, may therefore be a kinase mediating MYPT1 phosphorylation during the contraction. Furthermore, ZIPK seems to prefer MLC20 over MYPT1 as a substrate, thus suggesting that ZIPK plays a physiological role as a Ca²⁺-independent MLC20 kinase (Fig. 2) rather than a MYPT1 kinase (23).

CPI-17 can be phosphorylated and activated by several kinases (Fig. 2). The agonist-induced phosphorylation of CPI-17 has been shown to be inhibited by either RhoK inhibitors or PKC inhibitors, depending on the type of agonist and the vascular tissues (22, 29, 30). Furthermore, the contribution of RhoK and PKC to the CPI-17 phosphorylation differed with the phase of contraction, i.e., early vs late phase (9). As a result, both PKC and RhoK could be a physiologically relevant kinase that phosphorylates CPI-17, while their relative contribution varies depending on the type of agonist and the type of vascular tissue.

Concluding remarks

Our understanding of the biochemical basis for the regulation of the MLCP activity and MLC20 phosphorylation has been advanced to a great extent during the last 15 years (Fig. 2). Recent studies of muscle physiology have revealed that RhoK, PKC, CPI-17, and

Fig. 3. The major pathways mediating the inhibition of the MLCP activity and the Ca²⁺ sensitization during contractile stimulations. Abbreviations are identical to those in Fig. 2.

MYPT1 are the most physiologically important molecules participating in the regulation of MLCP activity (Fig. 3). RhoK phosphorylates CPI-17 (T38) and MYPT1 (T853>T696), thereby inhibiting the MLCP activity. RhoK may not directly phosphorylate MYPT1, but indirectly does by phosphorylating and thereby activating ZIPK. ZIPK then directly mediates the phosphorylation of MYPT1 (T696). CPI-17 can be phosphorylated not only by PKC but also RhoK. The relative importance of PKC and RhoK may differ depending on the type of contractile stimulation and vascular tissues. Moreover, the Ca²⁺ signal cross-signals to the mechanism of the Ca²⁺ sensitivity, by activating either the RhoA-RhoK pathway or the PKC pathway (Fig. 3). We are now close to elucidating the major rules regulating the MLCP activity and the Ca²⁺ sensitivity under physiological situations. However, the mechanisms regulating the Ca²⁺ sensitivity under the pathophysiological situations may differ from those involved under the physiological conditions. Elucidating the mechanisms and the signaling molecules underlying the Ca²⁺ sensitization during the pathological vasoconstriction, such as vasospasms and the increased vascular tone, would be the next step in the field of vascular medicine. This will help us to not only understand the vascular pathophysiology but also develop new pharmacological strategies for the prevention and treatment of pathological vasoconstriction.

Acknowledgments

We thank Mr. Brian Quinn for linguistic comments and help with the manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research (No.17590744) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a grant from the Yokoyama Rinsho Yakuri Foundation.

References

- Hirano K, Hirano M, Abe S, Kanaide H. In: Sperelakis N, Kuriyama H, editors. Ion channels of vascular smooth muscle cells and endothelial cells. New York: Elsevier; 1991. p. 93–105.
- Karaki H, Ozaki H, Hori M, Mitsui-Saito M, Amano K, Harada K, et al. Calcium movements, distribution, and functions in smooth muscle. *Pharmacol Rev.* 1997;49:157–230.
- Hartshorne DJ. In: Johnson LR, editor. Physiology of the gastrointestinal tract. 2nd ed. New York: Raven Press; 1987. p. 423–482.
- Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature.* 1994;372:231–236.
- Hirano K, Hirano M, Kanaide H. Regulation of myosin phosphorylation and myofilament Ca²⁺ sensitivity in vascular smooth muscle. *J Smooth Muscle Res.* 2004;40:219–236.
- Hirano K, Dmitry DN, Hirano M, Nishimura J, Kanaide H. Protein kinase network in the regulation of phosphorylation and dephosphorylation of smooth muscle myosin light chain. *Mol Cell Biochem.* 2003;248:105–114.
- Sakurada S, Takawa N, Sugimoto N, Wang Y, Seto M, Sasaki Y, et al. Ca²⁺-dependent activation of Rho and Rho kinase in membrane depolarization-induced and receptor stimulation-induced vascular smooth muscle contraction. *Circ Res.* 2003;93:548–556.
- Wang Y, Yoshioka K, Azam MA, Takawa N, Sakurada S, Kayaba Y, et al. Class II phosphoinositide 3-kinase α -isoform regulates Rho, myosin phosphatase and contraction in vascular smooth muscle. *Biochem J.* 2006;394:581–592.
- Dimopoulos GJ, Semba S, Kitazawa K, Eto M, Kitazawa T. Ca²⁺-dependent rapid Ca²⁺ sensitization of contraction in arterial smooth muscle. *Circ Res.* 2007;100:121–129.
- Hartshorne DJ, Ito M, Erdodi F. Myosin light chain phosphatase: subunit composition, interactions and regulation. *J Muscle Res Cell Motil.* 1998;19:325–341.
- Hartshorne DJ, Hirano K. Interactions of protein phosphatase type 1, with a focus on myosin phosphatase. *Mol Cell Biochem.* 1999;190:79–84.
- Hirano K, Phan BC, Hartshorne DJ. Interactions of the subunits of smooth muscle myosin phosphatase. *J Biol Chem.* 1997; 272:3683–3688.
- Hartshorne DJ, Ito M, Erdodi F. Role of protein phosphatase type 1 in contractile functions: myosin phosphatase. *J Biol Chem.* 2004;279:37211–37214.
- Terrak M, Kerff F, Langsetmo K, Tao T, Dominguez R. Structural basis of protein phosphatase 1 regulation. *Nature.* 2004;429:780–784.
- Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T, Ito M, et al. Phosphorylation of myosin-binding subunit (MBS) of

- myosin phosphatase by Rho-kinase in vivo. *J Cell Biol.* 1999;147:1023–1037.
- 16 Hagerty L, Weitzel DH, Chambers J, Fortner CN, Brush MH, Loiselle D, et al. ROCK1 phosphorylates and activates zipper-interacting protein kinase. *J Biol Chem.* 2007;282:4884–4893.
 - 17 Feng J, Ito M, Ichikawa K, Isaka N, Nishikawa M, Hartshorne DJ, et al. Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J Biol Chem.* 1999;274:37385–37390.
 - 18 Velasco G, Armstrong C, Morrice N, Frame S, Cohen P. Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin. *FEBS Lett.* 2002;527:101–104.
 - 19 Muranyi A, Derkach D, Erdodi F, Kiss A, Ito M, Hartshorne DJ. Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: inhibitory effects and occurrence in A7r5 cells. *FEBS Lett.* 2005;579:6611–6615.
 - 20 Eto M, Leach C, Tountas NA, Brautigan DL. Phosphoprotein inhibitors of protein phosphatase-1. *Methods Enzymol.* 2003;366:243–260.
 - 21 Kitazawa T, Polzin AN, Eto M. CPI-17-deficient smooth muscle of chicken. *J Physiol.* 2004;557:515–528.
 - 22 Kitazawa T, Eto M, Woodsome TP, Khalequzzaman M. Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca²⁺ sensitization in rabbit smooth muscle. *J Physiol.* 2003;546:879–889.
 - 23 Niino N, Ikebe M. Zipper-interacting protein kinase induces Ca²⁺-free smooth muscle contraction via myosin light chain phosphorylation. *J Biol Chem.* 2001;276:29567–29574.
 - 24 Bi D, Nishimura J, Niino N, Hirano K, Kanaide H. Contractile properties of the cultured vascular smooth muscle cells: the crucial role played by RhoA in the regulation of contractility. *Circ Res.* 2005;96:890–897.
 - 25 Seko T, Ito M, Kureishi Y, Okamoto R, Moriki N, Onishi K, et al. Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ Res.* 2003;92:411–418.
 - 26 Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature.* 1997;389:990–994.
 - 27 Gohla A, Schultz G, Offermanns S. Role for G₁₂/G₁₃ in agonist-induced vascular smooth muscle cell contraction. *Circ Res.* 2000;87:221–227.
 - 28 Hirano M, Niino N, Hirano K, Nishimura J, Hartshorne DJ, Kanaide H. Expression, subcellular localization, and cloning of the 130-kDa regulatory subunit of myosin phosphatase in porcine aortic endothelial cells. *Biochem Biophys Res Commun.* 1999;254:490–496.
 - 29 Niino N, Koga Y, Ikebe M. Agonist-induced changes in the phosphorylation of the myosin-binding subunit of myosin light chain phosphatase and CPI17, two regulatory factors of myosin light chain phosphatase, in smooth muscle. *Biochem J.* 2003;369:117–128.
 - 30 Azam MA, Yoshioka K, Ohkura S, Takuwa N, Sugimoto N, Sato K, et al. Ca²⁺-Independent, inhibitory effects of cyclic adenosine 5'-monophosphate on Ca²⁺ regulation of phosphoinositide 3-kinase C2α, Rho, and myosin phosphatase in vascular smooth muscle. *J Pharmacol Exp Ther.* 2007;320:907–916.