

CALCIUM-DEPENDENT REGULATION OF RHO AND MYOSIN PHOSPHATASE IN VASCULAR SMOOTH MUSCLE

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Phosphorylation of 20 kD myosin light chain (MLC) is a critical process in eliciting smooth muscle contraction. Excitatory receptor agonists increase the extent of MLC phosphorylation by both activating myosin light chain kinase (MLCK) and inhibiting myosin phosphatase (MP). Activation of MLCK is dependent on Ca^{2+} and calmodulin, while inhibition of MP is dependent on the small guanosine triphosphatase Rho and Rho kinase. Receptor agonists were previously shown to induce Rho activation via the heterotrimeric $G_{12/13}$ protein, largely in non-muscle cells. We recently discovered the novel Ca^{2+} -dependent activation of Rho in vascular smooth muscle. This Ca^{2+} -dependent Rho activation mechanism operates upon stimulation of vascular smooth muscle by either membrane depolarization or G_q -coupled vasoconstrictor receptors. Thus, Ca^{2+} induces MLC phosphorylation through both MLCK stimulation and MP inhibition. We found that phosphoinositide 3-kinase class II α isoform (PI3K-C2 α) is involved in the Ca^{2+} -dependent Rho activation and MP inhibition. PI3K-C2 α appears to participate in regulation of vascular Rho activity and tone in vivo. These observations also indicate that PI3Ks exert isoform-specific effects on vascular tone through mechanisms involving regulation of endothelial nitric oxide production and smooth muscle MP activity.

Biomed Rev 2005; 16: 13-21.

Key words: contraction, phosphoinositide 3-kinase class II α , myosin light chain phosphorylation, Rho kinase, MYPT1

INTRODUCTION

In smooth muscle including vascular smooth muscle, binding of excitatory receptor agonists to specific cell surface receptors, most of which belong to the seven membrane-spanning type of G protein-coupled receptors, leads to activation of phospholipase C and gating of Ca^{2+} channels on the plasma membrane (1,2). The activation of phospholipase C produces the two second messengers, inositol-1,4,5-trisphosphate and 1,2-diacylglycerol. Inositol-1,4,5-trisphosphate induces Ca^{2+}

release from the intracellular Ca^{2+} pool, which, together with stimulated Ca^{2+} influx across the plasma membrane, causes an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (1-3). An increase in $[Ca^{2+}]_i$ activates the Ca^{2+} /calmodulin-dependent enzyme myosin light chain kinase (MLCK), which phosphorylates the 20 kD myosin light chain (MLC), leading to initiation of a contractile response (4). An increase in the membrane content of 1,2-diacylglycerol leads to activation of protein kinase C, which activates a contractile mechanism

Received 18 October 2005 and accepted 23 December 2005.

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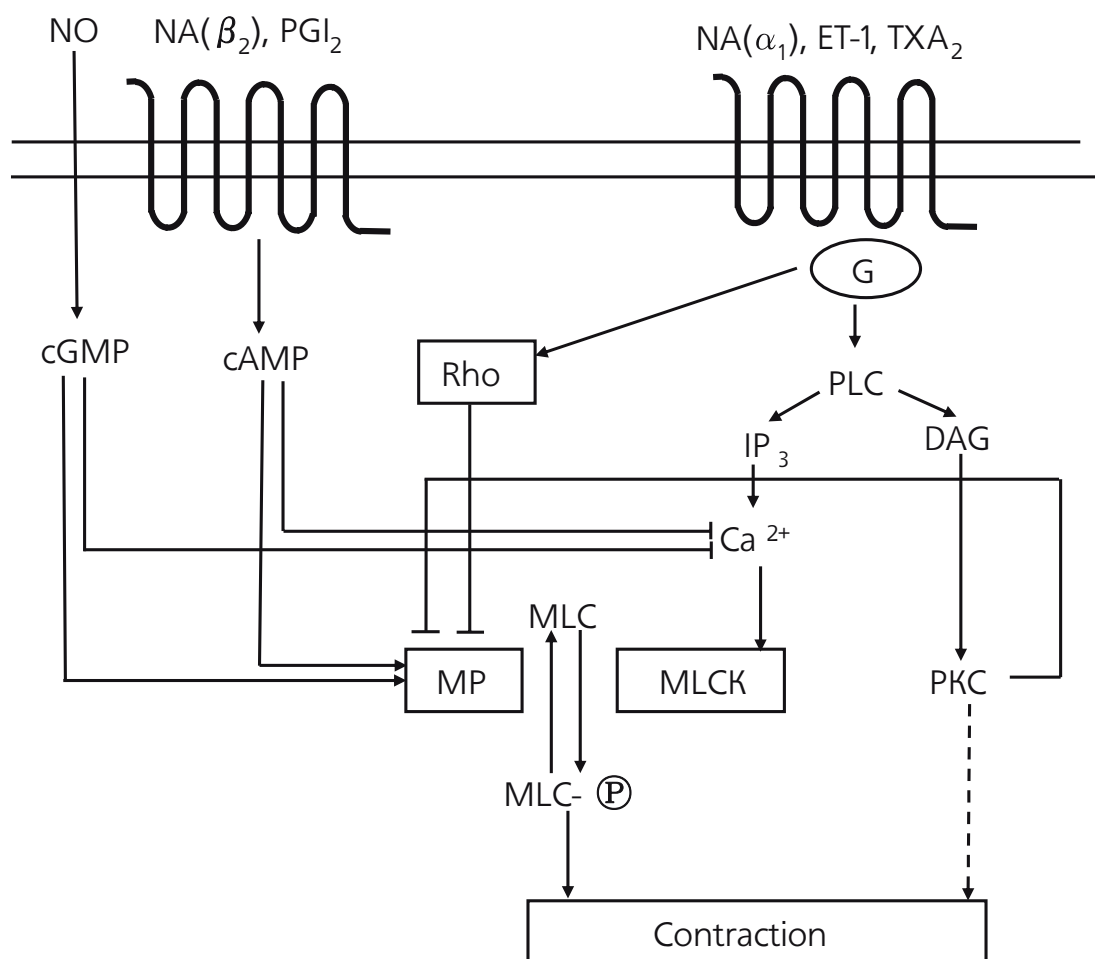


Figure 1. The signaling mechanisms for smooth muscle contraction. Vasoconstrictors such as noradrenaline (NA) acting on α_1 receptors, endothelin-1 (ET-1), and thromboxane A₂ (TXA₂) act on G protein-coupled receptors to stimulate phospholipase C (PLC) and Rho. PLC stimulation leads to Ca^{2+} -mobilization and accumulation of membrane 1,2-diacylglycerol (DAG), resulting in the activation of the calmodulin-dependent enzyme myosin light chain kinase (MLCK) and protein kinase C (PKC). MLCK phosphorylates MLC and initiates contraction. Rho activation leads to myosin phosphatase (MP) inhibition, potentiating Ca^{2+} -dependent MLC phosphorylation. PKC contributes to contraction through inhibition of MP and/or independently of MP. Vasodilators such as nitric oxide (NO), cyclic nucleotides cAMP and cGMP, NA (acting on β_2 receptors), and prostacyclin (PGI₂) inhibit MLC phosphorylation and contraction through decreasing the intracellular Ca^{2+} concentration and stimulation of MP. The mechanism of PKC-induced MLC phosphorylation-independent contraction remains unknown (dotted line).

independently of or synergistically with an increase in the $[\text{Ca}^{2+}]_i$; (Fig. 1) (1,3).

Phosphorylation state of MLC is determined by the activities of the de-phosphorylating enzyme myosin phosphatase (MP) as well as by the phosphorylating enzyme MLCK (5,6). The smooth muscle-specific MP consists of three subunits: 38 kD catalytic subunit (PP1 δ), 110 kD myosin targeting regulatory subunit (MYPT1/MBS) and 20 kD regulatory subunit (M20) (Fig. 2) (7). Recent evidence indicates that the activity of MP

is regulated by both constrictors and relaxants; constrictors inhibit MP activity whereas relaxants stimulate it (3,5,6). Thus, excitatory receptor agonists such as noradrenaline and endothelin not only activate MLCK, but also inhibit MP, resulting in efficient phosphorylation of MLC. Conversely, relaxants such as the cyclic GMP-elevating natriuretic peptides inhibit MLCK and stimulate MP, resulting in an effective reduction of MLC phosphorylation (Fig. 1). Accumulated data showed importance of the small G protein Rho as a switching

molecule for regulating MP activity (3,8). In fact, excitatory receptor agonists induce activation of Rho in smooth muscle. In non-muscle cells, Rho regulates actin cytoskeletal assembly, controlling cell shape changes, migration and adhesion (9). In many cell types, a particular family of the heterotrimeric G proteins named G_{12/13} plays an important role in the regulation of Rho activity (10). Unexpectedly, we recently discovered that membrane depolarization induced Rho activation in a Ca²⁺-dependent manner, as potently as excitatory receptor agonists (11). This discovery provided considerable insight into understanding the regulatory mechanisms of MP and Ca²⁺-dependent contraction in smooth muscle. In this review, we will focus on the mechanisms of Rho regulation and Rho-dependent MP inhibition in vascular smooth muscle, with particular emphasis on the novel Ca²⁺-dependent Rho and MP regulation mechanism.

MOLECULAR MECHANISMS OF RHO-MEDIATED CALCIUM SENSITIZATION

The development of membrane-permeabilized smooth muscle preparation contributed to our understanding of contractile mechanisms. In smooth muscle permeabilized with staphylococcal α -toxin and β -escin, excitatory receptor agonists including phenylephrine and thromboxane A₂ potentiated contraction at a constant [Ca²⁺]_i, thus sensitizing contraction to Ca²⁺ (12,13). This potentiation of contraction was accompanied by an increase in the extent of MLC phosphorylation. The potentiating actions of receptor agonists required GTP, and were mimicked and inhibited by the non-hydrolyzable guanine nucleotides, GTP γ S and GDP β S, suggesting the involvement of a G protein in the potentiation of MLC phosphorylation and contraction. The first evidence for the role of Rho in smooth muscle contraction was provided by the observation that GTP γ S-induced enhancement of Ca²⁺-induced contraction in permeabilized vascular smooth muscle was abolished by Staphylococcal exoenzyme epidermal cell differentiation inhibitor (EDIN) and Clostridium botulinum exotoxin C3 (14), both of which specifically ADP-ribosylate and inactivate Rho. However, it was unknown how Rho was involved in smooth muscle contraction. We demonstrated for the first time that pretreatment of permeabilized vascular smooth muscle cells with C3 completely inhibited GTP γ S enhancement of Ca²⁺-induced MLC phosphorylation (8), indicating that Rho was involved in Ca²⁺-induced MLC phosphorylation. Our observation implied the two possibilities, i.e. Rho might somehow potentiate MLCK-catalyzed phosphorylation of MLC and/or Rho might inhibit MP-catalyzed de-phosphorylation of MLC. We pursued both

possibilities and discovered that Rho mediated inhibition of MP but not potentiation of MLCK activity, thus resulting in potentiation of MLC phosphorylation (8,15) (Fig. 1).

Active Rho directly binds to and regulates activities of several target proteins, which mediate many of Rho actions. These include the serine/threonine kinase Rho kinase/ROCK/ROK, protein kinase N (PKN) and Citron kinase, and the non-kinase proteins mDia, Rhotekin and RhoGDI (9,16). Kimura *et al* (17) demonstrated that Rho kinase, but not PKN, phosphorylated the 110 kD MYPT1 subunit of purified myosin phosphatase holoenzyme *in vitro*. Importantly, Rho kinase-catalyzed phosphorylation of MYPT1 decreased phosphatase activity *in vitro*. Feng *et al* (18) identified Thr⁶⁹⁵ of MYPT1 (numbering of chicken M133 isoform) as an inhibitory phosphorylation site. Rho kinase also phosphorylates MYPT1 at Thr⁸⁵⁰ phosphorylation, which is shown to inhibit the binding of MP to myosin (19). Recent investigations (20,21) demonstrated that receptor agonists increased the extent of MYPT1 phosphorylation more definitely at Thr⁸⁵⁰ rather than at Thr⁶⁹⁵. We showed that the two structurally different Rho kinase inhibitors Y27632 and HA1077 abolished GTP γ S-induced potentiation of Ca²⁺-induced MLC phosphorylation in permeabilized smooth muscle cells, but not MLC phosphorylation induced by Ca²⁺ alone (15). Moreover, we found that HA1077 abolished GTP γ S-induced inhibition of MP activity by the immunoprecipitation-phosphatase assay *in vivo*. The prevention of GTP γ S-induced MP suppression by the Rho kinase inhibitor was accompanied by inhibition of GTP γ S-induced MYPT1 phosphorylation although the phosphorylation site of MYPT1 was not determined (15). Recent investigations (20,21) demonstrated that the Rho kinase inhibitors suppressed MYPT1 phosphorylation at Thr⁸⁵⁰ *in vivo*. Altogether, these observations indicate that Rho kinase mediates Rho-dependent MP inhibition through mechanisms involving MYPT1 phosphorylation in smooth muscle (Fig. 2).

The inhibitory phosphorylation site of MYPT1 (Thr⁶⁹⁵), was also shown to be phosphorylated *in vitro* by other kinases including ZIP/MYPT1-kinase (22) and integrin-linked kinase (ILK) (23). However, it is not known whether these kinases are involved in regulation of smooth muscle myosin phosphatase activity *in vivo*. It is either unknown whether Rho is somehow linked to regulation of activities of these kinases.

Protein kinase C-potentiated phosphatase inhibitor of 17 kD (CPI-17) is an endogenous smooth muscle-specific inhibitor protein for myosin phosphatase (24). In particular, CPI-17 is abundantly expressed in vascular smooth muscle. The phosphatase-inhibitory activity of CPI-17 is very low in its non-

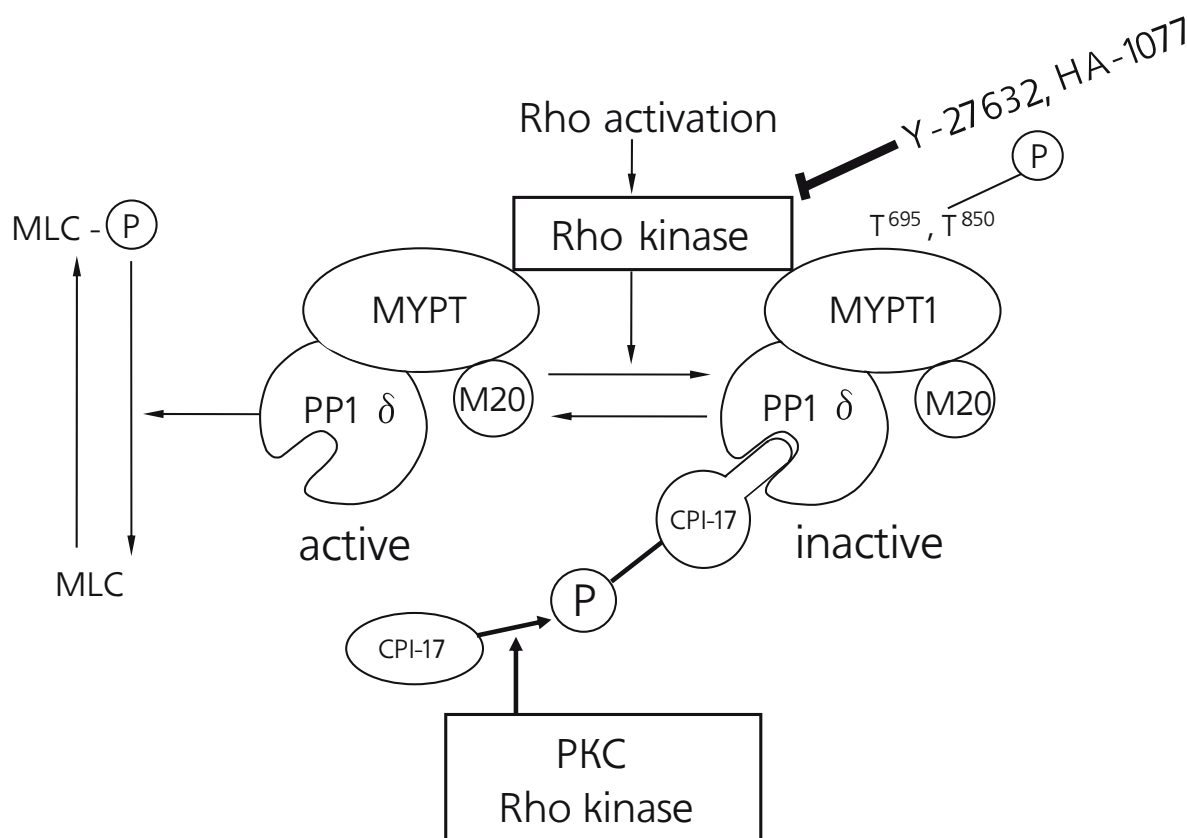


Figure 2. Molecular mechanism of negative regulation of myosin phosphatase (MP). The GTP-bound, active form of Rho induces Rho kinase activation, which leads to phosphorylation of the MP regulatory subunit MYPT1 at Thr⁸⁵⁰ and probably Thr⁶⁹⁵ as well as the MP inhibitor protein CPI-17 at Thr³⁸. It is not totally clear whether Rho kinase directly phosphorylates MYPT1 or indirectly stimulates phosphorylation through activation of other kinases and/or inhibition of phosphatase. The other protein kinases including ZIP/MYPT1 kinase, ILK and protein kinase C may also be involved in phosphorylation of MYPT1 at Thr⁶⁹⁵ and CPI-17. The phosphorylation of both MYPT1 and CPI-17 leads to MP inhibition, which enhances MLC phosphorylation.

phosphorylated state and becomes 1000-fold stimulated when phosphorylated at Thr³⁸ *in vitro*. Excitatory receptor agonists increased Thr³⁸ phosphorylation of CPI-17 in smooth muscle (25), suggesting that CPI-17 participates in Ca²⁺-sensitization. CPI-17 was shown to be phosphorylated *in vitro* by several protein kinases, including protein kinase C (24), Rho kinase, PKN, ZIP/MYPT1-kinase and ILK. Protein kinase C inhibitors partially inhibited agonist-induced phosphorylation of CPI-17 at Thr³⁸ with inhibition of contraction (25). Rho kinase inhibitors also reduced agonist-induced phosphorylation of CPI-17 (26). These observations suggest that CPI-17 becomes active as a MP inhibitor through its phosphorylation by protein kinases including Rho kinase and protein kinase C, participating in MP inhibition (Fig. 2).

REGULATION OF RHO BY EXCITATORY AGONISTS

Despite accumulation of the observations showing the importance of the Rho-signaling pathway in the regulation of smooth muscle contraction, no direct effect of excitatory agonists on Rho activity in smooth muscle was yet demonstrated. Rho cycles between a GDP-bound inactive state and a GTP-bound active state, like Ras (9). We recently developed a new biochemical assay which employs a recombinant RhoA-binding protein, Rhotekin, for determining amounts of active GTP-bound form of RhoA (GTP-Rho) in smooth muscle tissues (27). In rabbit aortic smooth muscle, the thromboxane A₂ mimetic U46619, which induced a sustained contractile response, caused a sustained rise in the amount of GTP-Rho in a dose-dependent manner with an EC₅₀ value

similar to that for the contractile response. U46619-induced Rho activation was thromboxane A₂ receptor-mediated and reversible. Other agonists including noradrenaline, serotonin, histamine and endothelin-1 also stimulated Rho, albeit to a lesser extent than U46619. In contrast, angiotensin II and phorbol 12,13-dibutyrate failed to increase GTP-Rho. The tyrosine kinase inhibitor genistein substantially inhibited Rho activation by these agonists, except for endothelin-1. Thus, the magnitude and mode of agonist-induced Rho activation does not appear to be uniform among agonists.

The cycling of Rho between the GTP-bound active and GDP-bound inactive states is under the tight regulation by the two major groups of proteins, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (9,28). Previous studies, largely on non-muscle cells, demonstrated that stimulation with G protein-coupled receptor agonists including lysophosphatidic acid, endothelin-1 and thrombin induced Rho activation, which was mediated through the receptor coupling to the G_{12/13} family of the heterotrimeric G proteins (29). Indeed, direct physical and functional interaction of G_{α_{12/13}} with a group of structurally related GEFs that act on Rho (RhoGEFs) was demonstrated (30). More recent studies (30,31) showed that G_q also had the ability to mediate stimulation of Rho, but the signaling pathway that mediates G_q-mediated Rho stimulation is still incompletely understood although a G_{α_q}-RhoGEFs interaction was observed. In cultured vascular smooth muscle cells, the expression of activated forms of G_{α₁₂} and G_{α₁₃} but not G_{α_q} was shown to induce a contraction that was inhibited by C3 toxin and a Rho kinase inhibitor, whereas the expression of dominant negative forms of G_{α₁₂} and G_{α₁₃} inhibited receptor agonist-induced, C3 toxin- and Rho kinase inhibitor-sensitive contraction (10). Thus, these observations indicated that G_{12/13} mediates Rho- and Rho kinase-dependent contraction in receptor agonist-stimulated cultured vascular smooth muscle cells. However, our observations described above suggest that more than a single mechanism for activating Rho might be operating in smooth muscle.

CALCIUM-DEPENDENT RHO ACTIVATION AND MYOSIN PHOSPHATASE INHIBITION

We recently found a novel, Ca²⁺-dependent mechanism for Rho activation in vascular smooth muscle. High KCl-induced membrane depolarization caused sustained increases in GTP-Rho with stimulation of MYPT1 phosphorylation in rabbit aortic smooth muscle, as well as the receptor agonist noradrenaline (11). Membrane depolarization-induced Rho activation was totally Ca²⁺-dependent. Thus, KCl-induced

Rho activation was inhibited by either removal of extracellular Ca²⁺ or addition of a dihydropyridine Ca²⁺ channel antagonist. In vascular smooth muscle cells, ionomycin-induced Ca²⁺ mobilization produced Rho activation with stimulation of MYPT1 phosphorylation and MLC phosphorylation, which was suppressed by the expression of a dominant negative RhoA mutant, N¹⁹RhoA. The Rho kinase inhibitors HA1077 and Y27632 inhibited both contraction and MLC phosphorylation induced by KCl as well as noradrenaline in rabbit aortic smooth muscle, with similar dose-response relationships. Thus, Ca²⁺-dependent Rho activation contributed to MP inhibition and resultant MLC phosphorylation in a Rho kinase-dependent manner.

Excitatory receptor agonists induce an increase in the [Ca²⁺]_i (32). This suggests the possibility that receptor agonist-induced Rho activation might also be dependent on Ca²⁺ although G protein-coupled receptors were shown to mediate Rho activation *via* G_{12/13} in vascular smooth muscle cells (10) and non-muscle cells (29). Excitatory receptor agonists mobilize Ca²⁺ from both the intracellular and extracellular pools. The combination of extracellular Ca²⁺ removal and depletion of the intracellular Ca²⁺ store by using caffeine pretreatment almost completely abolished agonist-induced Ca²⁺ mobilization in smooth muscle (11). Rho stimulation and contraction induced by noradrenaline and thromboxane A₂ analogue U46619 was inhibited by this procedure, indicating that receptor agonist-induced Rho activation was Ca²⁺-dependent. Intriguingly, noradrenaline- and U46619-induced Rho activation was Ca²⁺-dependent to a varying degree: the Ca²⁺-depletion procedure totally abolished noradrenaline-induced Rho activation whereas it only partially inhibited U46619-induced Rho activation. Thus, U46619-induced Rho activation was less dependent on Ca²⁺. The results, together with the previous observation that U46619 is less effective in mobilizing Ca²⁺ (33), suggest that U46619-induced Rho activation is more strongly dependent on G_{12/13} than on noradrenaline. Most likely, the Ca²⁺-dependent mechanism for activating Rho operates in cooperation with a G_{12/13}-mediated mechanism. Because G_q mediates Ca²⁺-mobilization *via* activation of phospholipase C, multitudes of heterotrimeric G protein-mediated signaling pathways likely regulate Rho activity in excitatory receptor agonist-stimulated vascular smooth muscle (Fig. 3).

It was unknown how Ca²⁺ is involved in Rho regulation in smooth muscle. During our investigation into the molecular mechanisms of Ca²⁺-dependent Rho activation, we found that phosphoinositide 3-kinase (PI3K) inhibitors suppressed membrane depolarization-induced Rho activation and contraction with suppression of MYPT1 and MLC

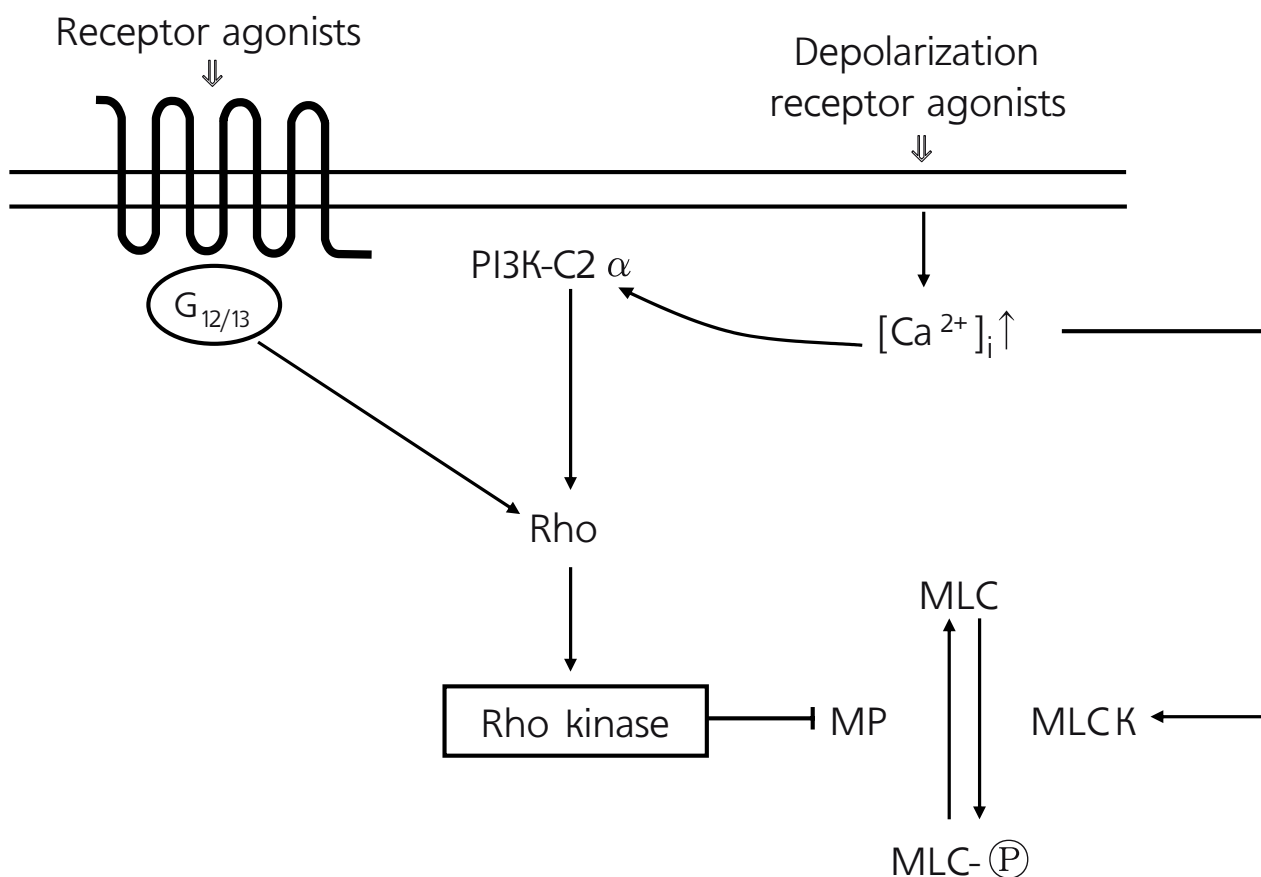


Figure 3. The dual roles of Ca^{2+} in MLC phosphorylation. Ca^{2+} not only activates MLCK but also induces Rho activation through a mechanism involving PI3K-C2 α . Rho activation induces MP inhibition, which in cooperation with MLCK causes effective MLC phosphorylation. Ca^{2+} - and PI3K-C2 α -dependent Rho activation presumably cooperates with a $G_{12/13}$ -mediated mechanism.

phosphorylation (34). The structurally different two PI3K inhibitors wortmannin and LY294002 exerted similar extents of maximal inhibition for contraction and Rho activation, suggesting that inhibition of a PI3K is a mechanism for Rho suppression by the inhibitors. Notably, the EC_{50} values of these inhibitors for suppression of contraction and Rho were higher than those previously reported for inhibition of PI3K-mediated effects including Akt stimulation and cell survival (35). PI3Ks are divided into three main classes based on their structure and substrate preference (35,36). Class I enzymes are heterodimers of the catalytic subunits including p110 α , p110 β , p110 γ and p110 δ and the regulatory subunits including p85 and p101. Class I enzymes are rapidly activated by a variety of receptor tyrosine kinases and/or G protein-coupled receptors and exert such diverse biological activities as cell proliferation, survival, motility and control of carbohydrate metabolism. Previous studies identified Akt as an important downstream effector of

class I PI3Ks (36). Class II enzymes include PI3K-C2 α , - β , and - γ isoforms. It is poorly understood how their activities are regulated and what physiological functions they serve. Class III enzyme, a homologue of yeast Vps34p, plays an important role in vesicular trafficking. LY294002 and wortmannin inhibit these PI3K enzymes with similar potencies, except PI3K-C2 α that is at least one order less sensitive to either of PI3K inhibitors (35,37). In vascular smooth muscle, at least 4 PI3K isoforms, class I p110 α and p110 β , class II PI3K-C2 α , and PI3K-C2 β , were detected. We immunoprecipitated each of p110 α , p110 β and PI3K-C2 α from vascular smooth muscle, and determined their sensitivities to wortmannin. We found that PI3K-C2 α was less sensitive to wortmannin than p110 α and p110 β . This observation suggested that PI3K-C2 α might mediate Rho activation and contraction. To directly test this possibility, we examined the effect of small interfering RNA (siRNA)-mediated PI3K-C2 α down-regulation on contraction

in vascular smooth muscle cells. The vascular smooth muscle cells, which are enzymatically dispersed from aortic media and cultured in the serum-free chemically defined medium, maintain contractile responses to various vasoactive substances including noradrenaline (38). Down-regulation of PI3K-C2 α by approximately 70% inhibited noradrenaline-induced contraction and MYPT1 phosphorylation (34). In contrast, down-regulation of p110 α did not inhibit noradrenaline-induced contraction or MYPT1 phosphorylation. These observations indicate that PI3K-C2 α is involved in Ca²⁺-dependent Rho activation and resultant negative regulation of MP, thus contributing to MLC phosphorylation and contraction (Fig. 3). This is the first demonstration of the novel role of class II PI3K in the regulation of vascular system

The discovery of the Ca²⁺-dependent Rho activation suggests that membrane depolarization induces Rho-dependent MP inhibition *in vivo*. Smooth muscle is depolarized by excitatory receptor agonists and mechanical strains (39). Vascular smooth muscle is constantly exposed to stretch derived from the intravascular pressure. Stretch of vascular smooth muscle induces gating of cation channels, resulting in membrane depolarization and stimulation of Ca²⁺ influx through voltage-dependent Ca²⁺ channels. Likely, the resultant increase of [Ca²⁺]_i activates not only MLCK but also Rho, the latter of which leads to MP inhibition and potentiation of MLC phosphorylation and thereby contraction. We observed that intravenous wortmannin infusion induced a decrease in the arterial blood pressure with inhibition of arterial PI3K-C2 α activity, Rho activity and MYPT1 phosphorylation (34), suggesting that PI3K-C2 α -dependent Rho and MP regulation is operating in the blood vessel *in vivo*. Thus, Ca²⁺-dependent Rho activation likely contributes to vascular tone regulation.

CONCLUSION

Since we first reported in 1995 that agonist-induced Ca²⁺ sensitization of smooth muscle MLC phosphorylation and contraction involves Rho-dependent inhibition of myosin phosphatase (8), great progress has been made in understanding the mechanisms of Rho-dependent Ca²⁺ sensitization. Excitatory receptor agonists, most of which act on heptahelical G protein-coupled receptors, stimulate Rho through multitudes of mechanisms involving G_{12/13} and G_q-Ca²⁺ (11). The Ca²⁺-dependent mechanism has thus far been unrecognized in non-muscle cells and may be unique to smooth muscle. Activation of Rho phosphorylates the regulatory subunit MYPT1 of MP *via* Rho kinase (6). Rho kinase may also phosphorylate to activate the MP inhibitor phosphoprotein CPI-17 (5). Recent studies demonstrated the possibilities that several other serine/

threonine kinases may also be involved in phosphorylation of both MYPT1 and CPI-17, and thus in the regulation of MP activity (5,6). Consistent with the roles for Rho kinase in agonist-induced contraction, Rho kinase inhibitors reduce agonist-induced contraction. These observations support the model for the signaling of agonist-induced contraction, in which activation of receptors by excitatory agonists triggers Ca²⁺ mobilization and consequent activation of MLCK *via* G_q, as well as Rho-Rho kinase-dependent inhibition of MP *via* G_{12/13} and G_q. The activation of MLCK and inhibition of MP operate in concert to result in efficient phosphorylation of MLC and contraction (Fig. 3). Protein kinase C was recently shown to be involved in inhibition of MP through phosphorylation of CPI-17. However, a number of previous studies also suggested that protein kinase C contributes to contraction in a manner independent of MLC phosphorylation (1,3). Thus, the protein kinase C branch of the G_q-signaling may regulate both MLC phosphorylation-dependent and -independent mechanisms of contraction.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Japan Society for the Promotion of Science, the Space Utilization Program by Japan Space Forum, Mitsubishi Pharma Research Foundation, and Novartis Pharma. We thank Ms Chiemi Hirose and Ms Yumiko Ohta for assistance in the experiments and preparing the manuscript.

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