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SMOOTH MUSCLE CONTRACTION BY SMALL GTPASE RHO

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ABSTRACT

Abnormal contraction of vascular smooth muscle contributes to a variety of diseases such as hypertension and vasospasm in coronary and cerebral arteries. An increment in a cytoplasmic Ca^{2+} concentration is the key event in smooth muscle contraction. However, smooth muscle contraction is modified upon the stimulation by agonists as well as in some pathophysiological situations in Ca^{2+}-independent mechanism. The molecular mechanism underlying this modulation was not elucidated. Recent studies have shown the important role of small GTPase Rho and its effector, Rho-associated kinase (Rho-kinase)/ROK/ROCK in Ca^{2+}-independent regulation of smooth muscle contraction. The Rho/Rho-kinase pathway modulates the phosphorylation level of myosin light chain (MLC) of myosin II, mainly through the inhibition of myosin phosphatase, and contributes to the agonist-induced Ca^{2+}-sensitization in smooth muscle contraction. The Rho/Rho-kinase pathway is involved in the pathogenesis of hypertension, vasospasm and arteriosclerosis, and is a potent target of new therapies for these diseases.

Key Words: Rho, Rho-kinase, myosin light chain (MLC), myosin phosphatase, myosin-binding subunit (MBS), Ca^{2+}-sensitization

INTRODUCTION

Agonists such as serotonin and phenylephrine, acting on receptors coupled to trimeric GTP-binding proteins (trimeric G-protein), activate the phosphatidylinositol cascade. Activation of the phosphatidylinositol cascade results in the release of Ca^{2+} from the sarcoplasmic reticulum. The increment in the concentration of intracellular Ca^{2+} activates Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) to phosphorylate the myosin light chain (MLC) of myosin II. The phosphorylation of MLC activates myosin ATPase, thereby inducing the contraction of smooth muscle.[1-3] Using Ca^{2+} indicators to measure an intracellular Ca^{2+} concentration, it has been shown that the phosphorylation levels of MLC and contraction are not always proportional to the Ca^{2+} concentration.[4] The ratio between contraction force or MLC phosphorylation and intracellular Ca^{2+} concentration due to agonist stimulation is higher than that due to depolarization of the membrane, the so-called Ca^{2+}-sensitization effect of the agonist.[5] These findings raised the possibility that an additional mechanism controls the regulation of MLC phosphorylation and contraction in a Ca^{2+}-independent manner. Subsequent studies have revealed that small GTPase Rho, a member of the Rho subfamily of the Ras superfamily, is responsible for the

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Ca^{2+}-sensitization induced by agonist stimulation.[6,7] Until recently, the molecular mechanism by which Rho regulates smooth muscle contraction was largely unelucidated. Evidence has revealed a signaling pathway in which the downstream effectors of Rho, Rho-associated kinase (Rho-kinase)/ROK/ROCK and the myosin-binding subunit (MBS) of myosin phosphatase play crucial roles in the Ca^{2+}-sensitization of smooth muscle contraction.[8]

REGULATION OF RHO ACTIVITY

Rho belongs to the Rho subfamily of the Ras superfamily of monomeric GTP-binding proteins (monomeric G-proteins).[9-11] Like other G-proteins, Rho exhibits both GDP/GTP-binding activity and GTPase activity. Rho works as a molecular switch by cycling between GDP-bound inactive (GDP·Rho) and GTP-bound active (GTP·Rho) forms. The ratio of these two forms of Rho is dependent of the activity of regulating factors. GTPase-activating proteins (GAPs) act as negative regulators by accelerating the intrinsic GTPase activity of Rho and reconverting it to the inactive GDP·Rho. Guanine nucleotide dissociation inhibitors (GDIs) inhibit the exchange of GDP for GTP. Guanine nucleotide exchange factors (GEFs) facilitate the release of GDP from Rho, thereby promoting the binding of GTP. GTP·Rho interacts with effectors and then triggers various cellular responses.

EFFECTORS OF RHO

Rho is involved in the regulation of various cellular functions such as stress fiber and focal adhesion formation, cell morphology, cell aggregation, cadherin-mediated cell-cell adhesion, cell motility, cytokinesis, membrane ruffling, neurite retraction, microvilli formation, and smooth muscle contraction.[9-11] Rho exerts its functions through specific effector proteins. A number of proteins have been identified through various approaches to be effectors of Rho. These effectors include Rho-associated kinase (Rho-kinase)/ROK/ROCK, the myosin-binding subunit (MBS) of myosin phosphatase, protein kinase N/PRK1, rhophilin, rhotekin, citron, p140 mDia, and citron-kinase.[9-11] Among these effectors, Rho-kinase and MBS are involved in smooth muscle contraction downstream of Rho.

Rho-kinase

Rho-kinase is a serine/threonine protein kinase, which was identified as a GTP·Rho-binding protein using bovine brain extract with affinity column chromatography on matrix-bound GTP·Rho. Rho-kinase was also identified as ROKα and ROCK2. ROKβ/ROCK1 is an isoform of Rho-kinase/ROKα/ROCK2.[11] We hereafter refer to both Rho-kinase/ROKα/ROCK2 and ROKβ/ROCK1 as Rho-kinase in this review. The kinase domain of Rho-kinase is situated at NH_2 (N)-terminal end, and has 72% sequence homology with the kinase domain of myotonic dystrophy kinase.[12] Rho-kinase has a coiled-coil domain in its middle portion, and a putative pleckstrin-homology domain that is split by the insertion of a cystein-rich region in its COOH (C)-terminal end. GTP·Rho interacts with the C-terminal portion of coiled-coil domain, and activates the phosphotransferase activity of Rho-kinase. The loss of the C-terminal portion of Rho-kinase makes it constitutively active. In contrast, the kinase activity-deficient form and various C-terminal fragments lacking the kinase domain function as the dominant negative form of Rho-kinase in cultured cells.[13]

Myosin-binding subunit (MBS) of myosin phosphatase

The phosphorylation of MLC of myosin II plays a crucial role in regulating the contractile activity of smooth muscle. Myosin phosphatase, which is physiologically responsible for the dephosphorylation of MLC, is composed of three subunits: a 37-kDa type 1 phosphatase catalytic subunit (PP1c); a 130-kDa myosin-binding subunit (MBS); and a 20-kDa subunit (M20: its function remains unknown).[14] MBS has a series of ankyrin repeats at the N-terminal end, and interacts with both PP1c and a number of substrates, such as MLC and adducin.[11] MBS is thought to target the enzyme to the particular location and to control the phosphatase activity.

CALCIUM-DEPENDENT CONTRACTION IN SMOOTH MUSCLE CELLS

A rise in the concentration of cytoplasmic free Ca^{2+} is the major trigger of contraction in both smooth and striated muscles. In striated muscle, the contraction is produced by actin-

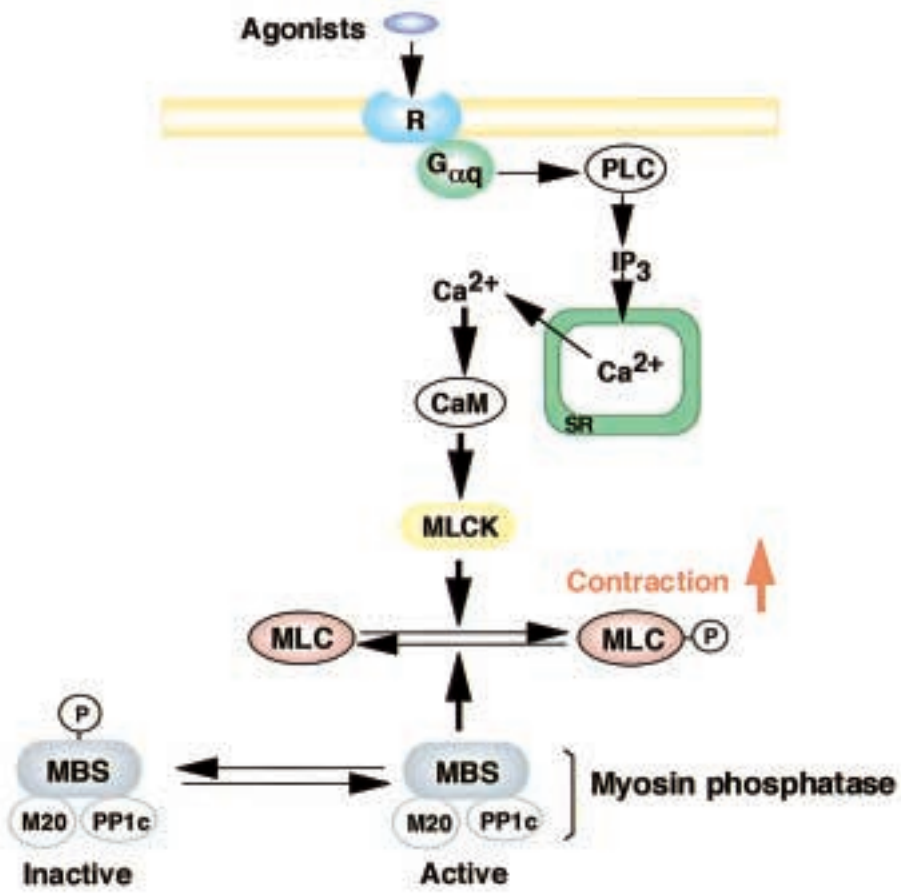

Figure 1. Calcium-dependent contraction in smooth muscle cells. R, Receptor for agonists; G, heterotrimeric G-protein; PLC, phospholipase C; IP_3, inositol 1,4,5-triphosphate; SR, sarcoplasmic reticulum; CaM, calmodulin; MLCK, myosin light chain kinase; MLC, myosin light chain; PP1c, catalytic subunit of myosin phosphatase; M20, 20-kDa subunit of myosin phosphatase.

associated proteins, troponin and tropomyosin, under control of the membrane potential. On the other hand, in smooth muscle, the phosphorylation/dephosphorylation of MLC at Ser-19 is the essential determinant of the extent of smooth muscle contraction under the membrane potential-independent mechanism. Agonists activate phospholipase C via trimeric G-protein. This activation of the phosphatidylinositol cascade induces the production of inositol-1,4,5-triphosphate (IP_3), resulting in the release of Ca^{2+} from the sarcoplasmic reticulum. The increment of intracellular Ca^{2+} enhances the binding of Ca^{2+} to calmodulin (CaM). A Ca^{2+}-calmodulin complex activates MLCK to phosphorylate MLC at Ser-19. The phosphorylation of MLC promotes the interaction between actin and myosin II, and thereby induces the contraction of smooth muscle.[1-3] (Fig. 1)

CALCIUM SENSITIZATION

Intracellular Ca^{2+} plays a critical role in the contraction of smooth muscle. However, early observations using Ca^{2+} indicators revealed that the degree of contraction is not always proportional to the Ca^{2+} concentration.[4] In smooth muscle, a high concentration of K^+ evokes a membrane depolarization-dependent increment in the Ca^{2+} concentration. The force of contraction and the phosphorylation of MLC induced by agonist stimulation are higher than those induced by a high concentration of K^+ at an equal intracellular Ca^{2+}. This phenomenon, in which a higher force is developed at an equal concentration of intracellular Ca^{2+}, is called Ca^{2+}-sensitization.[5] These findings raised the question of what molecules are involved in Ca^{2+}-sensitization. A nonhydrolyzable GTP analogue (GTPγS) mimics the Ca^{2+}-sensitization effect of agonists, and GDPβS inhibits the agonist-induced Ca^{2+}-sensitization.[15] These studies indicate that G-protein is involved in the pathway of Ca^{2+}-sensitization.

Rho has been identified as a molecule implicated in the Ca^{2+}-sensitization by agonists in smooth muscle. The Ca^{2+}-sensitizing effect of agonists or GTPγS is completely blocked by inhibitory toxins specific for Rho.[6,7] The introduction of GTPγS·Rho or the constitutively active form of Rho into permeabilized smooth muscle cells induces Ca^{2+}-sensitization. Furthermore, it was reported that the increment in the phosphorylation of MLC by the activated Rho at a constant Ca^{2+} concentration is due to a reduction in the dephosphorylation rate, and not to an increment in the phosphorylation rate.[16] Thus, Rho supposedly regulates Ca^{2+}-sensitivity via the regulation of myosin phosphatase activity.

REGULATION OF SMOOTH MUSCLE CONTRACTION BY RHO-KINASE AND MYOSIN PHOSPHATASE DOWNSTREAM OF RHO

How does Rho mediate the inhibition of myosin phosphatase activity in the Ca^{2+}-sensitization of smooth muscle? Evidence is accumulating that Rho regulates MLC phosphorylation through its effectors, Rho-kinase and MBS. Agonists activate Rho through the activation of certain heterotrimeric G-protein-coupled receptors, and the activated Rho interacts with Rho-kinase, leading to the activation of Rho-kinase. The activated Rho-kinase subsequently phosphorylates MBS, and inhibits the activity of myosin phosphatase.[17] Concomitantly, Rho-kinase directly phosphorylates MLC at the same site that is phosphorylated by MLCK.[18] Thus, Rho-kinase may be able to regulate smooth muscle contraction via two processes, the inactivation of myosin phosphatase and direct MLC phosphorylation.[8,17,18] (Fig. 2)

The major sites of phosphorylation of MBS by Rho-kinase are identified as Thr-697/695 and Ser-854/849 (in rat3 MBS isoform / chicken M133 MBS isoform).[19,20] The substitution of Thr-

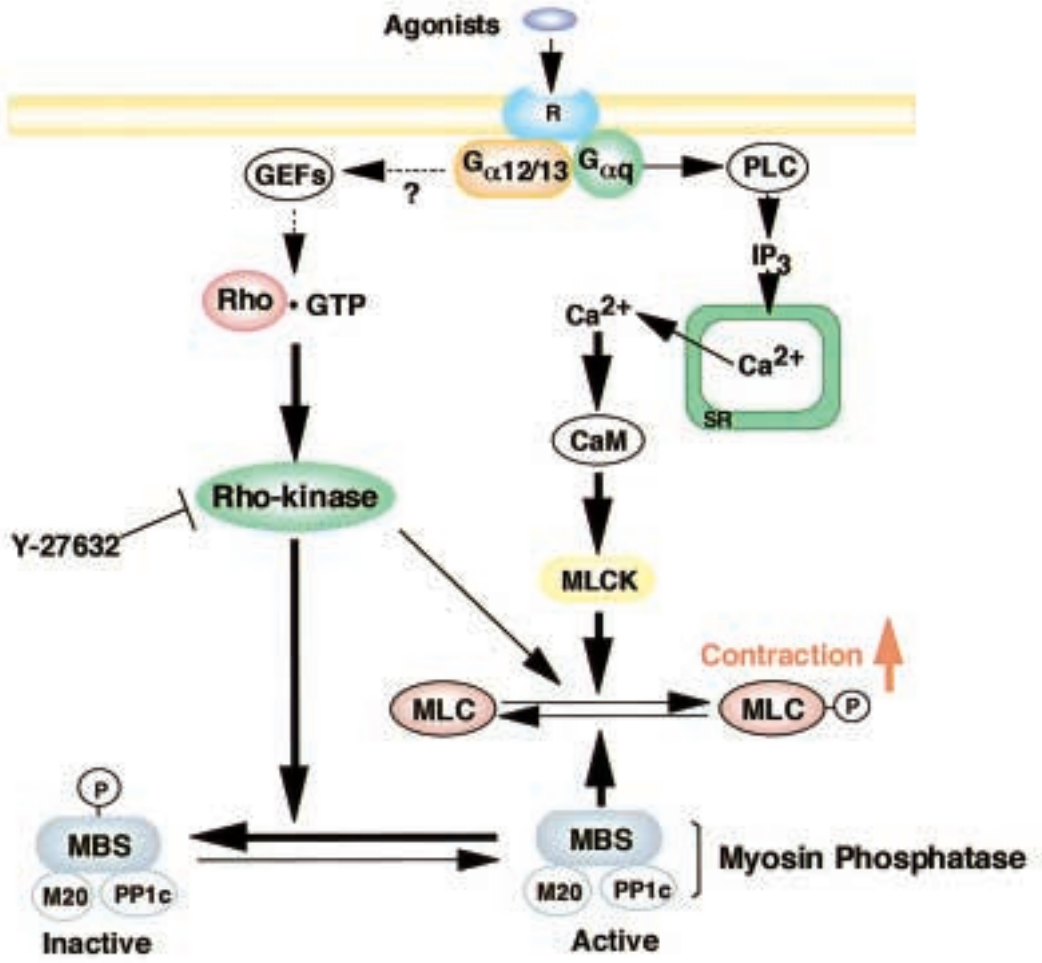

Figure 2. Regulation of smooth muscle contraction by Rho-kinase and myosin phosphatase downstream of Rho. The activated Rho activates Rho-kinase, which then phosphorylates MBS of myosin phosphatase and inhibits the myosin phosphatase activity. Rho-kinase also phosphorylates MLC directly. As a result, the increased phosphorylation of MLC induces greater contraction. GEFs, guanine nucleotide exchange factors. Y-27632 indicates the representative inhibitory probes that specifically inhibit Rho-kinase activity.

697 for Ala completely abolishes the inhibitory effect of Rho-kinase on the myosin phosphatase activity *in vitro*. Therefore, the major site involved in the inhibition of myosin phosphatase activity is Thr-697.[19)]

It has been reported that an unidentified endogenous kinase and Zip-like kinase were copurified with myosin phosphatase from chicken gizzard and myofibrillar pellet of cow bladder, respectively.[21,22)] Endogenous kinase phosphorylated MBS at Thr-697, and thereby inactivated the myosin phosphatase activity.[21)] Zip-like kinase closely resembles ZIPK, which is related to the death-associated protein kinase, and is involved in apoptosis. Interestingly, Zip-like kinase phosphorylated not only the inhibitory site of myosin phosphatase, but also MLC at Thr-18 and Ser-19 in a Ca^{2+}-independent manner.[22,23)] The physiological contribution of endogenous kinase and ZIP-like kinase to *in vivo* Ca^{2+}-sensitization remains to be elucidated. Judging from

the data independently reported, endogenous kinase is distinct from Rho-kinase and Zip-like kinase, because the sensitivities against kinase inhibitors and the sites of phosphorylation in MLC by each kinase are different.

UPSTREAM REGULATION OF RHO/RHO-KINASE PATHWAY IN SMOOTH MUSCLE

Recently, the signaling pathway linking agonists to the Rho activation has been clarified in non-muscle cells.[24,25] Upon stimulation with certain agonists, activated $G_{\alpha 13}$ binds to p115 RhoGEF, one of the specific GEFs for Rho, and stimulates the activity of p115 RhoGEF to catalyze nucleotide exchange on Rho. However, it has not been clarified how Rho is activated downstream of vasocontractive agonists in smooth muscle.

A potential problem with Rho is its localization of active enzyme in cells. Activated Rho and Rho-kinase are thought to be associated with the plasma membrane. On the other hand, myosin phosphatase is associated with the contractile apparatus. How does Rho/Rho-kinase transmit the signal to the contractile apparatus? Katoh *et al.* have provided a clue to the answer of this question.[26] In stress fibers (non-muscle contractile apparatus) isolated with glycerol, Rho, Rho-kinase and MBS were co-localized on the stress fibers. The addition of ATP induced contraction in a Rho-kinase-dependent manner in the absence of Ca^{2+}, and contraction was accompanied by the enhanced phosphorylation of MLC and MBS. These results suggest that some activated Rho and Rho-kinase are closely associated with the contractile apparatus.

PATHOLOGICAL ROLES OF RHO/RHO-KINASE IN VARIOUS DISEASES

Pathological as well as physiological roles of Rho-kinase have been evaluated using specific Rho-kinase inhibitors such as Y-27632. Y-27632 reduced the high blood pressure of several hypertensive rats, whereas the comparable concentration of Y-27632 had no effect on normal blood pressure.[27] This finding revealed that the Ca^{2+}-sensitization of smooth muscle contraction could cause hypertension via the Rho/Rho-kinase pathway.

The physiological role of the Rho/Rho-kinase pathway has been investigated in more detail using a spastic model of porcine coronary artery, in which the adventitia of the artery is locally treated with an inflammatory cytokine, interleukin-1β (IL-1β), thereby inducing an the adventitial inflammatory lesion and sequential arteriosclerotic remodeling.[28] In the local segment treated with IL-1β, serotonin induces coronary hyperconstriction and coronary artery spasm. In the serotonin-induced spastic site, the phosphorylation of MLC and MBS were enhanced, and there were positive correlations between the extent of vasocontraction and the level of MLC and MBS phosphorylations.[29,30] A consistent result was reported in canine cerebral vasospasm after experimental subarachnoid hemorrhage.[31] These results indicate that the enhanced MLC phosphorylation via the Rho/Rho-kinase pathway plays a crucial role in the pathogenesis of vasospasm. Interestingly, in the spastic site, the expression of ROKβ mRNA was significantly increased.[30] This suggests that Rho-kinase is up-regulated at the spastic site and causes hypercontraction by the inhibition of myosin phosphatase activity through the phosphorylation of MBS. More recent evidence revealed that the Rho/Rho-kinase pathway plays roles in not only vasocontraction, but also in the formation of arteriosclerotic lesions.[32-35]

CONCLUDING REMARKS

Abnormal contraction of vascular smooth muscle is a major cause of certain diseases, such as hypertension and vasospasm in coronary and cerebral arteries. Various lines of evidence have shown important roles of small GTPase Rho and its effector, Rho-kinase in Ca^{2+}-independent regulation of smooth muscle contraction. This mechanism also participates in pathogenesis of hypertension, vasospasm, and arteriosclerosis, and is a potent target of new therapies for these diseases.

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