Ca\(^{2+}\)-Dependent Activation of Rho and Rho Kinase in Membrane Depolarization–Induced and Receptor Stimulation–Induced Vascular Smooth Muscle Contraction

Sotaro Sakurada, Noriko Takuwa, Naotoshi Sugimoto, Yu Wang, Minoru Seto, Yasuharu Sasaki, Yoh Takuwa

Abstract—Ca\(^{2+}\) sensitization of vascular smooth muscle (VSM) contraction involves Rho-dependent and Rho-kinase–dependent suppression of myosin phosphatase activity. We previously demonstrated that excitatory agonists in fact induce activation of RhoA in VSM. In this study, we demonstrate a novel Ca\(^{2+}\)-dependent mechanism for activating RhoA in rabbit aortic VSM. High KCl-induced membrane depolarization as well as noradrenalin stimulation induced similar extents of sustained contraction in rabbit VSM. Both stimuli also induced similar extents of time-dependent, sustained increases in the amount of an active GTP-bound form of RhoA. Consistent with this, the Rho kinase inhibitors HA1077 and Y27632 inhibited both contraction and the 20-kDa myosin light chain phosphorylation induced by KCl as well as noradrenalin, with similar dose-response relations. Either removal of extracellular Ca\(^{2+}\) or the addition of a dihydropyridine Ca\(^{2+}\) channel antagonist totally abolished KCl-induced Rho stimulation and contraction. The calmodulin inhibitor W7 suppressed KCl-induced Rho activation and contraction. Ionomycin mimicked W7-sensitive Rho activation. The expression of dominant-negative N\(^{19}\)RhoA suppressed Ca\(^{2+}\)-induced Thr\(^{695}\) phosphorylation of the 110-kDa regulatory subunit of myosin phosphatase and phosphorylation of myosin light chain in VSM cells. Finally, either the combination of extracellular Ca\(^{2+}\) removal and depletion of the intracellular Ca\(^{2+}\) store or the addition of W7 greatly reduced noradrenalin-induced and the thromboxane A\(_2\) analogue–induced Rho stimulation and contraction. Taken together, these results indicate the existence of the thus-far unrecognized Ca\(^{2+}\)-dependent mechanism in VSM. Excitatory receptor agonists are suggested to use this pathway for simulating Rho. (Circ Res. 2003; 93:548-556.)

Key Words: contraction ■ smooth muscle ■ Rho ■ Rho kinase ■ calcium

Calcium ions play a primary role in regulating vascular smooth muscle (VSM) contraction.\(^1\) Excitatory receptor agonists, many of which act via heptahelical G protein–coupled receptors (GPCRs) to stimulate phospholipase C, induce a rise in the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and resultant activation of the calmodulin-dependent enzyme myosin light chain kinase (MLCK).\(^2\) MLCK phosphorlates the 20-kDa myosin light chain (MLC), leading to the initiation of contraction.\(^1\) Accumulating evidence\(^4\)-\(^9\) demonstrates that excitatory agonists also downregulate myosin phosphatase activity by mechanisms involving the small GTPase Rho and the Rho effector Rho kinase, resulting in sensitization of MLC phosphorylation to Ca\(^{2+}\). Rho kinase was demonstrated to phosphorylate the 110-kDa myosin–targeting subunit MYPT1/MBS of smooth muscle myosin phosphatase at Thr\(^{695}\) (numbering of chicken M133 isoform) to inhibit myosin phosphatase activity.\(^7\) In addition, Rho kinase, as well as protein kinase C, was shown to phosphor-ylate and activate a smooth muscle–specific myosin phosphatase inhibitor phosphoprotein, CPI-17,\(^10\),\(^11\) suggesting that CPI-17 also probably participates in Rho-mediated and Rho–mediated Ca\(^{2+}\) sensitization.

Rho cycles between the GTP-bound active and GDP-bound inactive states, which are under tight regulation by the two major proteins, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins.\(^12\) Previous studies\(^13\),\(^14\) on nonmuscle cells demonstrated that stimulation of GPCRs with receptor agonists including lysophosphatic acid, endothelin-1, and thrombin induced Rho activation through receptor coupling to the G\(_{12/13}\) family of the heterotrimeric G proteins. Direct physical and functional interaction of G\(_{12/13}\) with a group of structurally related GEFs acting on Rho (RhoGEF) was demonstrated.\(^14\) More recent studies\(^14\),\(^15\) 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 yet incompletely understood. In cultured...
VSM cells, the expression of activated forms of \(G_{\alpha_{12}}\) and \(G_{\alpha_{13}}\), but not \(G_{\alpha_q}\), was shown to induce contraction that was inhibited by \(C_3\) toxin and a Rho kinase inhibitor. However, the expression of dominant-negative forms of \(G_{\alpha_{12}}\) and \(G_{\alpha_{13}}\) inhibited receptor agonist–induced, \(C_3\) toxin–sensitive, and Rho kinase inhibitor–sensitive contraction. Thus, these observations suggested that \(G_{12/13}\) mediates Rho-dependent and Rho kinase–dependent contraction in receptor agonist–stimulated cultured VSM cells.

We recently demonstrated in rabbit aortic VSM that various excitatory receptor agonists indeed activated Rho. The magnitude and mode of agonist-induced Rho activation did not seem to be uniform among various agonists, suggesting the existence of more than a single mechanism for receptor agonist–triggered Rho activation in VSM. In the present study, we demonstrate in rabbit aortic VSM that depolarization with high KCl induces substantial Rho activation and Rho kinase–dependent contraction, which are both totally \(Ca^{2+}\)-dependent. The \(Ca^{2+}\)-dependent Rho activation mechanism also operates in VSM stimulated with noradrenaline and a thromboxane \(A_2\) mimetic, which mobilizes \(Ca^{2+}\) via \(G_{\alpha}\) and phospholipase C. Thus, Rho activity is likely dually regulated by the \(G_{12/13}\) and the \(G_{\alpha}/Ca^{2+}\) pathways in excitatory agonist-induced contraction of VSM.

**Materials and Methods**

Noradrenalin, nitrindipine, ionomycin, diphenylhydramine, and caffeine were purchased from Sigma. W7, W5, KN93, and KN92 were bought from Seikagaku. U46619 was purchased from Calbiochem. A mouse monoclonal anti-RhoA antibody (clone 26C4) and mouse monoclonal anti-MLC antibody were purchased from Santa Cruz Biotechnology and Sigma, respectively. CV-11974, ONO3708, and Y27632 were donated by Takeda Pharmaceuticals (Osaka, Japan), Ono and Pharmaceuticals (Kyoto, Japan), and WelFide corporation (Osaka, Japan), respectively.

**Tissue Preparation and Tension Measurements**

The animals were maintained in compliance with the Guidelines of the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University. The descending portion of the thoracic aorta of New Zealand White rabbits supplied by Sankyo Laboratories (Toyama, Japan) was removed and dissected. Deendothelialized aortic rings were equilibrated under the resting tension of \(1 \text{ g}\) in the modified Krebs-Henseleit buffer (in mmol/L: NaCl 119, KCl 4.7, KH2PO4 1.2, MgSO4 1.5, CaCl2 1.5, NaHCO3 25, and glucose 11) at 37°C and gassed with 95% O2 and 5% CO2, and isometric tension was measured as described previously. Before test stimulation, the rings were several times precontracted, each time for 5 minutes by replacing the normal Krebs-Henseleit buffer with the 60 mmol/L KCl-containing buffer followed by washing with the normal Krebs-Henseleit buffer. This precontraction-relaxation procedure was repeated with 1-hour intervals (4 times on average) until a stable contraction was obtained. The tension during the measurement was expressed as the percent of the maximum force during the last KCl preconstriction. In the 60-mmol/L KCl buffer, an \(\alpha_2\)-adrenergic receptor blocker phentolamine (10 \(\mu\)mol/L), a \(\beta\)-adrenergic receptor blocker propranolol (10 \(\mu\)mol/L), histamine \(H_1\) receptor antagonist diphenhydramine (10 \(\mu\)mol/L), an angiotensin AT1 receptor antagonist ONO-3708 (1 \(\mu\)mol/L), and a thromboxane \(A_2\) receptor antagonist ONO-11974 (1 \(\mu\)mol/L) were included to prevent inappropriate effects of excitatory agonists released from the nerve ends and other cell types of the vascular tissues in the aortic preparation during high \(KCl\)-induced membrane depolarization.

**Cells**

Rabbit aortic smooth muscle cells were obtained as previously described and were used between the 5th and 10th passages. Cells were grown in DMEM supplemented with 10% FCS (JRH Biosciences), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin (Wako Pure Chemicals). Before each experiment, the cells were deprived of serum for 48 hours. Adenoviruses encoding myc-tagged \(N^\varepsilon\)RhoA and LacZ were described and amplified. Cells were infected with adenoviruses at a multiplicity of infection of \(\sim 100\) and allowed to recover in the respective medium with 10% FCS for 3 hours and then serum-deprived before experiments. This condition conferred expression of LacZ as a marker gene in nearly 100% of transfected cells.

**Determination of Tissue GTP-RhoA**

Determination of a GTP-bound active form of RhoA in aortic VSM tissues was described previously. Briefly, aortic rings that were contracted isometrically were quickly frozen by immersing in liquid nitrogen. Frozen tissues were homogenized in a homogenizing buffer and supernatants were recovered by centrifugation, and after protein concentrations were determined by Lowry’s method, the supernatants (450 \(\mu\)g of proteins) were incubated with recombinant glutathione-S-transferase mouse rhotekin (7-89) fusion protein immobilized onto glutathione-Sepharose 4B beads (Amersham Biosciences) at 4°C for 30 minutes. RhoA bound to beads was analyzed using SDS 15% polyacrylamide gel electrophoresis (PAGE) followed by Western blotting using a specific mouse monoclonal anti-RhoA antibody and an alkaline phosphatase–conjugated rabbit anti-mouse IgG antibody (Zymed). A portion (1/27) of supernatants was analyzed for total RhoA. Densities of bands corresponding to RhoA were quantitated, and the quantitative data of normalized amounts of GTP–RhoA in figures were expressed as multiples over a value in unstimulated tissues, which was expressed as 1.0.

**Determination of MLC Phosphorylation**

Arterial strips mounted for isometric studies were frozen by immersion in dry-ice acetone trichloroacetic acid and determined as described previously. In experiments using cultured cells, the cells were fixed with ice-cold reaction stop buffer containing 10% trichloroacetic acid, as described previously. Myosin was extracted and analyzed by urea-glycerol PAGE followed by Western blotting using specific mouse monoclonal anti-RhoA antibody (Sigma). Densities of bands corresponding to MLC were quantitated, and the ratio of nonphosphorylated, monophosphorylated, and diphosphorylated forms of MLC were calculated as described previously.

**Preparation of Antibodies Against MYPT1**

Phosphorylated at Thr\(^{695}\) and the N-terminus of MYPT1 and Determination of Thr\(^{695}\) Phosphorylation of MYPT1

A polyclonal antibody (antibody pMYPT\(^{695}\)) against MYPT1 phosphorylated at Thr\(^{695}\) was raised in New Zealand white rabbits using a synthetic peptide corresponding to residues 690 to 703, including phosphorylated Thr\(^{695}\) and the N-terminus of MYPT1. The ECL system was used for the visualization (Amersham Biosciences).

**Statistics**

Each set of data were expressed as a mean±SE of the determinations done in triplicate or more. In each figure, experiments were repeated at least twice with similar results. One-way or 2-way ANOVA
followed by Dunnett’s test or unpaired t test were performed to determine the statistical significance of differences between mean values.

Results

KCl Stimulates Rho in VSM and the Receptor Agonist Noradrenalin
Stimulation of deendothelialized rabbit aortic VSM with either noradrenalin (3 μmol/L) or KCl (60 mmol/L) induced rapid and sustained contractile responses of similar amplitudes to reach peaks within 4 to 6 minutes (Figure 1A). We determined the amounts of an active, GTP-bound form of RhoA (GTP-Rho) in VSM using a pull-down assay. The amount of GTP-Rho in unstimulated muscle was at a detectable level and ≈0.5% of total cellular amount of RhoA. The amount of GTP-Rho significantly increased within 1 minute of noradrenalin stimulation to reach a nearly maximal level within 2 minutes and was sustained for the observation period of time (Figure 1B). The relative values of GTP-Rho normalized for the amounts of total Rho are shown in Figure 1C. Unexpectedly, KCl stimulation also induced increases in the amount of GTP-Rho (Figure 1B, right). The time course and amplitude of the KCl-induced Rho stimulation were similar to those of noradrenalin (Figure 1C). Like KCl-induced contraction, KCl stimulation of Rho was concentration-dependent; the half-maximally effective concentration values of KCl for contraction and Rho activation were ≈30 and 40 mmol/L, respectively (Figures 2A and 2B). These results clearly indicate that membrane depolarization as well as receptor stimulation induce Rho activation in VSM.

KCl-Induced Contraction Is Rho Kinase–Dependent
To explore whether KCl-induced contraction is dependent on Rho kinase, we determined the effects of the two structurally unrelated Rho kinase inhibitors, Y27632 and HA1077, on contraction induced by KCl and also noradrenalin. The preincubation of VSM with various concentrations of Y27632 reduced not only contraction induced by noradrenalin (3 μmol/L) but also contraction induced by KCl (60 mmol/L) with the similar dose-effect relations (Figure 3A). HA1077 also exerted inhibitory actions on both noradrenalin- and KCl-induced contraction with the similar dose-effect relations (Figure 3B). Y27632 and HA1077 inhibited the initial and sustained phases of contraction similarly (data not shown). Y27632 (3 μmol/L) inhibited contraction induced by different concentrations of KCl with relatively larger inhibition of contraction by lower concentrations of KCl (Figure 3C). Either of the Rho kinase inhibitors at the doses used induced the maximal 60% to 70% inhibition of both noradrenalin- and KCl-induced contraction. The contraction remaining in the presence of the Rho kinase inhibitor was markedly inhibited by MLCK inhibitor ML-9 (data not shown). The two Rho kinase inhibitors also suppressed KCl- and noradrenalin-induced MLC phosphorylation in dose-dependent manners (Figures 3D and 3E). Thus, Rho kinase is involved in not only noradrenalin- but also KCl-induced MLC phosphorylation and contraction. These observations were in agreement with the findings reported previously by Mita et al. Consistent with these, KCl stimulated Thr695 phosphorylation of the myosin binding subunit MYPT1 of myosin phosphatase (Figure 3F).

KCl-Induced Rho Activation Is Dependent on Ca2+ and Calmodulin
Membrane depolarization with KCl activates the L-type of voltage-dependent Ca2+ channels (VDCCs) and stimulates entry of extracellular Ca2+ into the cell interior. We determined the involvement of extracellular Ca2+ and the L-type VDCCs in KCl-induced Rho activation. Removal of extracellular Ca2+ abolished both KCl-induced contraction and Rho stimulation (Figures 4A and 4B). The L-type VDCC blocker nitrendipine (1 μmol/L) substantially inhibited KCl-
induced force generation and increases in the amount of GTP-Rho. On the other hand, a Ca\(^{2+}\)/H\textsuperscript{11001}\textsuperscript{11001}\textsuperscript{11001} ionophore ionomycin (3 \textmu mol/L), which stimulates Ca\(^{2+}\)/H\textsuperscript{11001}\textsuperscript{11001}\textsuperscript{11001} entry into the cell interior, induced a contractile response and a 3-fold increase in the amount of GTP-Rho. These observations strongly suggest that an increase in [Ca\(^{2+}\)]\textsubscript{i} attributable to stimulated Ca\(^{2+}\)/H\textsuperscript{11001}\textsuperscript{11001}\textsuperscript{11001} influx across the plasma membrane mediates Rho activation.

We next explored the involvement of calmodulin and the calmodulin-dependent protein kinase CAMKII by examining the effects of their specific inhibitors on KCl- and ionomycin-induced responses. The calmodulin antagonist W-726 (200 \textmu mol/L) substantially inhibited KCl-induced contraction and Rho stimulation (Figure 5). However, the structurally related but inactive analogue W5\textsuperscript{27} (200 \textmu mol/L) did not affect either contraction or Rho stimulation. CAMKII is a widespread calmodulin effector and is activated when the [Ca\(^{2+}\)]\textsubscript{i} is elevated. The CAMKII-specific inhibitor KN93\textsuperscript{27} (30 \textmu mol/L), but not its inactive analogue KN92 (30 \textmu mol/L), induced considerable inhibition of contraction and Rho stimulation (Figure 5). Ionomycin-induced contraction and Rho activation, like KCl responses, were inhibited by W7, but not by KN93 (Figure 6). Thus, these observations suggest the involvement of calmodulin in Ca\(^{2+}\)/H\textsuperscript{11001}\textsuperscript{11001}\textsuperscript{11001} dependent Rho activation in KCl-and ionomycin-contracted muscle. The results also suggest that CAMKII is involved specifically in KCl-induced responses.

Ionomycin-Induced Phosphorylation of MYPT1 and MLC Is Dependent on Rho

Stimulation of cultured rabbit aortic VSM cells with ionomycin (0.5 \textmu mol/L) increased the amount of GTP-Rho by \approx 2.5-fold above the background level (Figure 7A). We determined whether ionomycin induced Thr\textsuperscript{695} phosphorylation of MYPT1 and, if so, whether ionomycin-induced phosphorylation of MYPT1 was dependent on Rho. We infected VSM cells with adenoviruses carrying a dominant-negative RhoA mutant, myc-tagged N\textsuperscript{19}RhoA, or \beta-galactosidase (LacZ) as a control 48 hours before measurements. The expression of N\textsuperscript{19}RhoA was confirmed by Western analysis (Figure 7B). In control LacZ-expressing VSM cells, ionomycin stimulated Thr\textsuperscript{695} phosphorylation of MYPT1 by \times 40\% (Figure 7C). In myc-N\textsuperscript{19}RhoA–expressing cells, the basal level of MYPT1 Thr\textsuperscript{695} phosphorylation was reduced and ionomycin-induced stimulation of MBS Thr\textsuperscript{695} phosphorylation was abolished. Ionomycin stimulated MLC phosphorylation by \approx 3-fold in LacZ-infected cells (Figure 7D). In the cells expressing myc-N\textsuperscript{19}RhoA, ionomycin-stimulated MLC phosphorylation was reduced by \approx 50\% compared with that in LacZ-infected cells.

Receptor Agonist–Induced Rho Activation Is Ca\(^{2+}\)- and Calmodulin-Dependent

We finally examined the dependence on Ca\(^{2+}\) and calmodulin of receptor agonist–induced Rho activation. The combination of depletion of the intracellular Ca\(^{2+}\) stores with caffeine and removal of extracellular Ca\(^{2+}\) (see the legend for Figure 8 for details) abolished contraction induced by either the stable thromboxane A\(_2\) receptor agonist U46619 (100 nmol/L) or noradrenalin (3 \textmu mol/L) (Figure 8A). The same procedure for Ca\(^{2+}\) depletion substantially reduced the basal amount of GTP-Rho and totally abolished noradrenalin-induced Rho stimulation. This procedure also inhibited U46619-induced Rho stimulation greatly but not completely (Figure 8B). The Ca\(^{2+}\) depletion did not change NA- and U46619-induced c-Jun N-terminal kinase activation, suggesting that this procedure did not affect receptor activation itself. Noradrenaline-induced contraction and Rho activation were reduced by W7 but not W5 (Figures 8C and 8D).

Discussion

Accumulated evidence demonstrates\textsuperscript{3–9} that Rho serves as a molecular switch that regulates the Ca\(^{2+}\) sensitivity of the
contractile proteins largely by regulating myosin phosphatase in various types of smooth muscle, including VSM. We developed the sensitive assays to estimate the levels of active Rho in VSM tissues and previously showed that excitatory receptor agonists stimulate Rho in agonist-specific manners. In the present study we demonstrated for the first time that depolarization with KCl stimulates Rho activity in VSM. The experiments with the Ca\(^{2+}\) channel blocker and the Ca\(^{2+}\) ionophore suggested that a stimulated Ca\(^{2+}\) influx across the plasma membrane and a resultant [Ca\(^{2+}\)]cyt increase mediate Rho activation. We found that Rho activation induced by physiological receptor agonists is also strongly dependent on Ca\(^{2+}\). This Ca\(^{2+}\)-dependent Rho activation was suggested to involve calmodulin. The CAMKII inhibitor reduced KCl-but not ionomycin- or noradrenalin-induced responses, suggesting that the involvement of CAMKII downstream of Ca\(^{2+}\) and calmodulin is unlikely. Thus, these results reveal the existence of the novel, Ca\(^{2+}\)-dependent mechanism for activating Rho, which promotes understanding of the molecular mechanisms of the Rho-dependent regulation of MLC phosphorylation and contraction. Consistent with the present results, it was previously shown that in permeabilized VSM, a high free Ca\(^{2+}\) (30 \(\mu\)mol/L) induced the translocation of RhoA to the membrane fraction and that in intact VSM the Rho-inactivating chimeric toxin DC3B partially inhibited KCl-induced contraction.

The present results demonstrated that ionomycin-induced increase in the [Ca\(^{2+}\)]cyt, stimulates Thr\(^{695}\) phosphorylation of the myosin phosphatase regulatory subunit MYPT1 and an increase in the level of phosphorylated MLC in a Rho-dependent manner (Figures 7C and 7D). The observations suggested that Ca\(^{2+}\)-induced Rho activation contributes to inhibition of myosin phosphatase, thus stimulating MLC phosphorylation. It is well-established that an increase in the [Ca\(^{2+}\)]cyt also induces activation of the calmodulin-dependent enzyme MLCK. Thus, the dual regulation by Ca\(^{2+}\) of phosphorylation and dephosphorylation of MLC likely leads to an effective increase in the level of phosphorylated MLC and contraction in VSM. Indeed, Mita et al. recently have shown that Rho kinase inhibitors as well as a MLCK inhibitor effectively inhibited KCl-induced contraction. Consistent with this notion, previous studies demonstrated that...
relatively small increases in the \([\text{Ca}^{2+}]_i\), resulted in large changes in the level of phosphorylated MLC in KCl- or ionomycin-stimulated vascular and nonvascular smooth muscle. However, these investigations showed that receptor activation induces larger increases in the level of phosphorylated MLC than KCl-induced depolarization at a given level of the \([\text{Ca}^{2+}]_i\). These observations suggest that the mechanism for activating Rho may not be exactly the same between receptor agonists and KCl. Most likely, in addition to the \([\text{Ca}^{2+}]_i\)-dependent Rho activation, receptor agonists use the \(G_{12/13}\)-mediated and also probably the non-\([\text{Ca}^{2+}]_i\)-dependent \(G_q\)-mediated pathways for stimulating Rho-guanine nucleotide exchange factors (see below), which are both demonstrated to operate in nonmuscle cells, that several agonists acted on GPCRs to elicit Rho-dependent responses through two major heterotrimeric G protein families, \(G_{12/13}\) and \(G_q\). More recent studies demonstrated that a subfamily of RhoGEFs, which share the conserved structural motif known as the regulator of G protein signaling domain (the RGS box), including p115RhoGEF, PDZ-RhoGEF, and LARG, physically associate with the \(\alpha\)-subunits of the \(G_{12/13}\) and also \(G_q\) families and suggested that the direct interaction brings about stimulation of GEF activity and consequent Rho activation. However, a different study suggested the involvement of the second messengers, protein kinase C and \([\text{Ca}^{2+}]_i\), in \(G_q\) induction of a Rho-dependent response in nonmuscle cells. Because direct measurements of levels of active Rho were not reported in that study, it was possible that protein kinase C and \([\text{Ca}^{2+}]_i\) were required for the observed response at a site distal to Rho activation but not for Rho activation per se. In smooth muscle, there has been so far no study reporting the roles for \(G_q\) and its downstream signaling molecules \([\text{Ca}^{2+}]_i\) and protein kinase C in constrictor regulation of Rho. We demonstrated in the present study that the second messenger \([\text{Ca}^{2+}]_i\) mediates

Figure 5. Inhibitors of calmodulin and CAMKII inhibit KCl-induced contraction and Rho activation in VSM. A, VSM was pretreated with 30 \(\mu\text{mol/L}\) KN93, 30 \(\mu\text{mol/L}\) KN92, 200 \(\mu\text{mol/L}\) W7, or 200 \(\mu\text{mol/L}\) W5 for 60 minutes or not pretreated and challenged with 60 \(\mu\text{mol/L}\) KCl for 5 minutes. B, VSM was treated as in panel A and subjected to the pull-down assay for GTP-Rho. *P<0.05 and **P<0.01 compared with KCl alone; #P<0.05 compared with no stimulation.

Figure 6. Calmodulin inhibitor W7, but not the CAMKII inhibitor, inhibits ionomycin-induced contraction and Rho activation in VSM. A, VSM was pretreated with the inhibitors as in Figure 5 or not pretreated and challenged with 3 \(\mu\text{mol/L}\) ionomycin for 40 minutes. B, VSM was treated as in panel A and subjected to the pull-down assay for GTP-Rho. *P<0.05 and **P<0.01 compared with ionomycin alone; #P<0.05 compared with no stimulation.
Rho activation in VSM (Figures 1, 2, and 4). On the other hand, phorbol ester, a potent protein kinase C activator, failed to stimulate Rho despite inducing a strong contraction, which precludes protein kinase C as a mediator of receptor agonist–induced Rho stimulation in VSM. Because both receptor agonists, noradrenalin and the thromboxane A2 mimetic U46619, activate phospholipase C to mobilize Ca2+, which is mediated via Gq, the Ca2+-dependent Rho stimulation in the vasoconstrictor-stimulated VSM is suggested to be mediated by Gq.

The present study suggests that vasoconstrictors that act on GPCRs use dual signaling pathways, ie, the G12/13-mediated and the Gq-mediated, Ca2+-dependent pathways, for activating Rho. We observed that U46619-induced Rho stimulation was less dependent on Ca2+ compared with that by noradrenalin (Figure 8B). Concerning this, noteworthy is a previous observation that U46619 is a relatively weaker agonist than noradrenalin in terms of Ca2+ mobilization in intact VSM but is more effective than noradrenalin in inducing Ca2+ sensitization of MLC phosphorylation and contraction in permeabilized VSM. Thus, the relative contributions of the G12/13- and the Gq-Ca2+ pathways in vasoconstrictor-induced Rho stimulation seem to be different among vasoconstrictors. We previously demonstrated that angiotensin II, which is not efficiently coupled to G12/13, is ineffective in stimulating Rho. This observation may suggest the importance of the synergistic cooperation of the G12/13- and Gq-Ca2+ pathways for receptor-mediated Rho activation.

In the present study, the Ca2+-dependent Rho activation was inhibited by the calmodulin inhibitor (Figures 5, 6, and 8). The observations suggest the involvement of Ca2+ and calmodulin in the stimulation process of RhoGEF, although it also remains possible that Ca2+-dependent inhibition of Rho GTPase-activating protein could be responsible for a Ca2+-dependent increase in the level of GTP-Rho. At present, more than 10 different RhoGEFs are known. These are known examples for calmodulin regulation of GEFs of the other small GTPases. For example, the Ras-GEFs Ras-GRF 1 and...
2 which possess an IQ motif that serves as a calmodulin-binding site, were shown to be activated by Ca\(^{2+}\)-influx in an IQ motif-dependent manner.\textsuperscript{35} The Rac-GEF Tiam1 was shown to be directly phosphorylated and activated by CAMKII in vivo.\textsuperscript{36} There is so far no known RhoGEF that possesses an IQ motif. Which of the RhoGEFs mediate the Ca\(^{2+}\)-dependent Rho stimulation in VSM and how the Ca\(^{2+}\)-triggered signal is exactly relayed to a RhoGEF remain to be clarified.

In conclusion, we demonstrated in the present study that there exists a novel Ca\(^{2+}\)-dependent mechanism for activating Rho in VSM. This mechanism probably involves calmodulin. This Ca\(^{2+}\)-dependent mechanism for Rho activation seems to operate on stimulation of G\(_q\)-coupled vasoconstrictor receptors in cooperation with the G\(_{q/11}\)-mediated mechanism. Thus, multitudes of heterotrimeric G protein–mediated signaling pathways regulate Rho activity in VSM, directing the Ca\(^{2+}\)-sensitivity regulation of MLC phosphorylation and contraction.

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