Rho kinase inhibitor HA-1077 prevents Rho-mediated myosin phosphatase inhibition in smooth muscle cells

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Nagumo, Hiromitsu, Yasuharu Sasaki, Yoshitaka Ono, Hiroyuki Okamoto, Minoru Seto, and Yoh Takuwa. Rho kinase inhibitor HA-1077 prevents Rho-mediated myosin phosphatase inhibition in smooth muscle cells. Am. J. Physiol. Cell Physiol. 278: C57–C65, 2000.—In smooth muscle, a Rho-regulated system of myosin phosphatase exists; however, its regulation by Rho kinase has not yet been established. Here we demonstrate in permeabilized vascular smooth muscle cells (SMCs) that the vasodilator 1-(5-isoquinolinesulfonyl)-homopiperazine (HA-1077), which we show to be a potent inhibitor of Rho kinase, dose dependently inhibits Rho-mediated enhancement of Ca2+-induced 20-kDa myosin light chain (MLC20) phosphorylation due to abrogating Rho-mediated inhibition of MLC20 dephosphorylation. By an immune complex phosphatase assay, we found that guanosine 5’-O-(3-thiotriphosphate) (GTPγS) stimulation of permeabilized SMCs caused a decrease in myosin phosphatase activity with an increase in the extent of phosphorylation of the 130-kDa myosin-binding regulatory subunit (MBS) of myosin phosphatase in a Rho-dependent manner. HA-1077 abolished both of the Rho-mediated events. Moreover, we observed that the pleckstrin homology/serine-rich domain protein of Rho kinase, a dominant negative inhibitor of Rho kinase, inhibited GTPγS-induced phosphorylation of MBS. These results provide direct in vivo evidence that Rho kinase mediates inhibition of myosin phosphatase activity with resultant enhancement of MLC20 phosphorylation in smooth muscle and reveal the usefulness of HA-1077 as a Rho kinase inhibitor.

myosin light chain dephosphorylation; small G protein; calcium ion; sensitization; vascular smooth muscle; contraction molecules have now been identified, providing much insight into the molecular mechanisms of the Rho actions (2, 20, 25, 34). These include the serine/threonine kinase Rho kinase/ROKα/ROKβ (19, 22) and its close relative ROKβ/ROK1 (13, 19), protein kinase N (PKN), which is another class of serine/threonine protein kinase (18), p140MDia, rhothekin, rhothophil, citron, and citron kinase (2, 20, 25, 34). Among these, the Rho kinase family is of particular interest.

Recent evidence reveals that receptor activation by excitatory agonists in smooth muscle is coupled to activation of a Rho-dependent signaling pathway that leads to inhibition of myosin phosphatase and resultant enhancement of the 20-kDa myosin light chain (MLC20) phosphorylation and of contraction (7–11, 16, 21, 26, 30). It is also shown that, in nonsmooth muscle cells, the receptor agonist stimulation results in a decrease of phosphatase activity in a myosin-rich fraction in a Rho-dependent manner (6). Rho kinase is implicated in Rho inhibition of smooth muscle myosin phosphatase; Rho kinase is shown to be capable of phosphorylating purified smooth muscle myosin phosphatase and consequently inhibiting its activity in vitro (13), and a Rho kinase inhibitor Y-27632 has been demonstrated to inhibit a receptor agonist-induced smooth muscle contraction (7, 33). Smooth muscle myosin phosphatase consists of the 38-kDa catalytic subunit (the protein phosphatase type 1β isoform), the 130-kDa myosin-binding regulatory subunit (MBS), and the 21-kDa regulatory subunit (M21; see Refs. 1 and 28). MBS, which serves as the targeting subunit of myosin phosphatase to myosin and enhances its activity toward myosin, is a subunit phosphorylated by Rho kinase (15). It has been demonstrated in nonmuscle cells that expression of activated Rho mutant and stimulation with receptor agonists induces an increase in the extent of MBS phosphorylation (15, 24). However, direct evidence that Rho kinase indeed acts downstream of Rho to mediate MBS phosphorylation and myosin phosphatase inhibition in vivo in smooth muscle cells (SMCs) has not yet been found.

The protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-homopiperazine (HA-1077) has been previously shown to act as a vasodilator in vivo when administered...
in animals (4) and is currently used for the treatment of cerebral vasospasm. This compound also inhibits agonist-induced contraction of isolated vascular smooth muscle. Agonist stimulation of smooth muscle induces a rise in the intracellular free Ca\(^{2+}\) concentration and the activation of the Ca\(^{2+}\)/calmodulin-dependent enzyme myosin light chain kinase (MLCK), initiating phosphorylation of MLC\(_{20}\), and the resultant enhancement of MLC\(_{20}\) phosphorylation. We next examined whether this was accompanied by inhibition of Rho-mediated MBS phosphorylation. The present results reveal that HA-1077 effectively reverses Rho-mediated MBS phosphorylation and myosin phosphatase suppression with a reduction in MLC\(_{20}\) phosphorylation, providing evidence that Rho kinase is a downstream effector of Rho to regulate myosin phosphatase in vivo in SMCs.

### MATERIALS AND METHODS

Cell culture, permeabilization, and MLC\(_{20}\) phosphorylation. Pig aortic SMCs were obtained as previously described (26) and were used between the 5th and the 15th passages. Before each experiment, the cells were deprived of serum for 24 h. In the experiments of Fig. 4, SMCs freshly isolated by enzymatic digestion of aortic media were seeded onto a culture dish, attached by incubation with 5% serum-containing medium for 5 h, and, after 12 h of serum deprivation, employed for the experiments. Phosphorylation and dephosphorylation of MLC\(_{20}\) in β-escin-permeabilized SMCs were determined as described in detail previously (26). A percent value of the sum of monophosphorylated and diphosphorylated forms of total MLC\(_{20}\) was calculated.

Rho kinase assay. Rho kinase was purified from bovine brain as described previously (22). To determine the effect of HA-1077 on Rho kinase activity in vitro, the indicated concentrations of HA-1077 were included in 200 µl of the assay mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl\(_2\), 1 mM dithiothreitol (DTT), various concentrations of ATP, purified Rho kinase, and 0.1 mg/ml histone HI. Assays were started by the addition of [γ\(^{32}\)P]ATP. \(^{32}\)P incorporation into histone HI was linear over the initial 5 min. The incubation was performed for 5 min at 30°C and was quenched by the addition of 1 ml of ice-cold 10% TCA followed by the addition of 500 µg of BSA as a carrier protein. After the sample was centrifuged at 3,000 rpm for 15 min, the pellet was resuspended in ice-cold 10% TCA, and the centrifugation-resuspension cycle was repeated three times. The final pellet was dissolved in 1 N NaOH, and the radioactivity was measured by a liquid scintillation counter. The Michaelis-Menten equation was used to calculate the Michaelis constant (K\(_{m}\)) and maximal velocity (V\(_{max}\)) of Rho kinase. Data were further analyzed with a secondary plot (inhibitor concentration vs. \(K_m/V_{max}\)) to calculate the inhibitory constant (K\(_{i}\)) values.

Myosin phosphatase assay. Polyclonal rabbit anti-MBS antibody, anti-PP\(_{1α}\) isofrom antibody, and anti-M\(_{21}\) regulatory subunit of myosin phosphatase antibody were raised against the amino-terminal peptide (MKMDAKQKRNE) of chicken MBS and the carboxy-terminal peptide (SGRPVTOORANTPKKR) of human PP\(_{1α}\), and recombinant chicken M\(_{21}\) was fused to GST as described (31). For the immunoprecipitation of myosin phosphatase, SMCs were lysed with a lysis buffer containing 60 mM β-glycerophosphate, 0.5% Nonidet P-40, 0.2% SDS, 100 mM NaF, 1 mM NaVO\(_4\), 2 mM EGTA, 80 µg/ml each of aprotinin and leupeptin, 0.6 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 50 mM Tris-HCl (pH 8.0) and were passed through a 26-G needle five times. After centrifugation at 10,000 g for 5 min, the supernatant was recovered and incubated with anti-MBS antibody at 4°C for 3 h. Immunoprecipitates recovered on protein A-Sepharose (Amersham-Pharmaica Biotechnology) were washed, and then the associated phosphatase activity toward \(^{32}\)P-labeled chicken gizzard MLC\(_{20}\) (1) was measured in vitro in the reaction mixture (100 µl) containing 50 mM Tris (pH 7.5), 4 mM EDTA, 2 mM EGTA, 2 mM DTT, and 10 µM of \(^{32}\)P-labeled chicken gizzard MLC\(_{20}\) at 30°C for 20 min. The reaction was quenched by the addition of 100 µl of ice-cold 20% TCA and 7 µl of 3% BSA. The tubes were left on ice for 15 min and then clarified by centrifugation. The amount of \(^{32}\)P radioactivity released was determined by counting the radioactivity in the supernatant. In preliminary experiments, we found that the amount of \(^{32}\)P radioactivity released showed a linear increase for the first 20 min of the reaction. The amounts of \(^{32}\)P radioactivity released were corrected for the amounts of immunoprecipitated MBS and were expressed as a percentage of the control value.

For Western blotting, immunoprecipitates or cells were solubilized in Laemmli's SDS-sample buffer and resolved by SDS-PAGE (31). Proteins in the gel were electrophoretically transferred to an Immobilon-P membrane (Millipore). After incubation with 3% BSA in Tris-buffered saline (137 mM NaCl and 20 mM Tris-HCl (pH 7.6)) for blocking nonspecific binding of the antibody, the membrane was probed with the respective antibodies, followed by treatment with alkaline phosphatase-conjugated secondary antibody (Zymed).

Phosphorylation of MBS in permeabilized SMCs. Permeabilized SMCs were incubated in the phosphorylation buffer containing 100 µM of [γ\(^{32}\)P]ATP (50 µCi/ml) for 10 min and were lysed in the lysis buffer. MBS protein in cell lysates was immunoprecipitated as described above and was separated on an 8% SDS-PAGE. The gel was dried and subjected to autoradiography. The radioactivity of the band corresponding to MBS was determined by a Fuji BAS-200 Bio-image Analyzer (Fuji, Tokyo, Japan) and was corrected for amounts of MBS.

Plasmids. The cDNA of chicken M\(_{21}\) subunit of myosin phosphatase was cloned by reverse transcription and PCR using total RNA prepared from chicken gizzard. M\(_{21}\) cDNA was ligated into pGEX-2T vector (Amersham-Pharmaica Biotechnology) at the BamHI site, and recombinant GST-M\(_{21}\) fusion protein was produced in the DH5α strain of Escherichia coli as described. The cDNA of the pleckstrin homology (PH)/cystein-rich region (amino acids 1124–1388) of the Rho kinase was subcloned into pQE 30 vector (Qiagen) at the BamHI and HindIII sites, and a recombinant hexahistidine-tagged PH/cystein-rich domain was produced in the M15 strain of E. coli. The nucleotide sequences of the cDNAs obtained by the PCR method were further analyzed with a secondary plot (inhibitor concentration vs. \(K_m/V_{max}\)) to calculate the inhibitory constant (K\(_{i}\)) values.
were confirmed by sequencing with an ALFred DNA sequencer (Amersham-Pharmacia Biotechnology).

Materials. HA-1077 was synthesized by Asahi Chemical Industries. C3 toxin was purchased from Wako (Osaka, Japan). Mouse monoclonal anti-Rho A antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal specific anti-Rho kinase antibody and anti-M21 antibody were raised against the amino-terminal peptide (MSRPPTGKMPGAP) of bovine Rho kinase and the recombinant GST-M21 fusion protein, as described in Ref. 31. Other chemicals were of reagent grade purity.

RESULTS

We first examined whether or not the protein kinase inhibitor HA-1077 inhibits the activity of purified Rho kinase and found that this was the case. The kinetic analysis (Fig. 1) reveals that HA-1077 acts as a competitive inhibitor versus ATP. The \( K_m \) value of Rho kinase for ATP is calculated to be 1.2 mM. The \( K_i \) values of HA-1077 for Rho kinase and several other serine/threonine protein kinases are compared in Table 1. HA-1077 displays the highest affinity for Rho kinase among the protein kinases examined; its affinity for Rho kinase is 2.5 times higher than for another class of Rho-associated protein kinase (PKN), 5 times higher than for cAMP-dependent protein kinase and cGMP-dependent protein kinase, and 10 times higher than for protein kinase C purified from rat brain. Notably, the affinity of HA-1077 for MLCK is ~100 times lower than that for Rho kinase.

We then examined how HA-1077 affected the Rho-mediated regulation of MLC20 phosphorylation in vascular SMCs (Fig. 2). In \( \beta \)-escin-permeabilized SMCs, increasing the ambient free Ca\(^{2+} \) concentration caused a dose-dependent increase in the extent of MLC20 phosphorylation. As we previously reported (26), the addition of guanosine 5\(^{\gamma}\)-O-(3-thiotriphosphate) (GTP\( \gamma \)S) enhances Ca\(^{2+} \)-induced MLC20 phosphorylation in a Rho-dependent manner. HA-1077 (10 \( \mu \)M) totally inhibits GTP\( \gamma \)S-induced enhancement of MLC20 phosphorylation (Fig. 2A), suggesting the involvement of Rho kinase in this process. The inhibition of GTP\( \gamma \)S-induced enhancement of MLC20 phosphorylation is dose dependent, with an \( IC_{50} \) value of ~2 \( \mu \)M (Fig. 2B). Importantly, the addition of HA-1077 up to 10 \( \mu \)M does not significantly inhibit Ca\(^{2+} \)-induced MLC20 phosphorylation (Fig. 2A and B). HA-1077 at 30 \( \mu \)M

![Fig. 1. Kinetic analysis of Rho kinase inhibition by 1-(5-isoquinoline-sulfonyl)-homopiperazine (HA-1077). Enzyme activity of Rho kinase purified from bovine brain was measured in the absence and presence of the indicated concentrations of HA-1077 and various amounts of ATP. Competitive inhibition was observed with respect to ATP.](http://ajpcell.physiology.org/)

![Table 1. \( K_i \) values of HA1077 for various serine/threonine protein kinases](http://ajpcell.physiology.org/)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>( K_i, \mu )M</th>
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<tbody>
<tr>
<td>Rho kinase</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>PKN</td>
<td>1.10</td>
</tr>
<tr>
<td>cAMP-dependent protein kinase</td>
<td>1.6</td>
</tr>
<tr>
<td>cGMP-dependent protein kinase</td>
<td>1.6</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>Myosin light chain kinase</td>
<td>32</td>
</tr>
</tbody>
</table>

Values for Rho kinase and protein kinase C are means ± SE for 3 and 5 determinations, respectively. Inhibitory constant (\( K_i \)) values were obtained from Dixon plots using 4 concentrations of ATP and 3–5 concentrations of HA-1077. PKN, protein kinase N (recombinant enzymes produced in Sf9 cells; Ono, unpublished observation). Value for PKN is mean of duplicate determinations. \( K_i \) values for cAMP- and cGMP-dependent protein kinases and myosin light chain kinase are from Ref. 4. Protein kinase C enzymes were purified from rat brain (12).
partially inhibits Ca2+-induced MLC20 phosphorylation. These observations indicate that HA-1077 at the effective concentrations does not inhibit MLCK in SMCs, which is consistent with the results shown in Table 1.

We next studied the mechanism for Rho kinase-mediated enhancement of MLC20 phosphorylation by using HA-1077; we examined whether Rho kinase mediates inhibition of MLC20 dephosphorylation or potentiation of MLC20 phosphorylation in permeabilized SMCs. As we reported previously (26), GTPγS has a profound inhibitory effect on the dephosphorylation of MLC20, although MLC20 is gradually dephosphorylated over 10 min in the absence of GTPγS, in the presence of GTPγS the extent of MLC20 phosphorylation initially declines but stops decreasing at a level of ~0.45 at 5 min (Fig. 3A). In contrast, in the presence of GTPγS plus HA-1077 (10 µM), the MLC20 phosphorylation level falls down almost to zero within 5 min. Thus HA-1077 abolishes the inhibitory effect of GTPγS on MLC20 dephosphorylation. When adenosine 5'-O-(3-thiotriphosphate) is used as substrate instead of ATP, MLC20 is thiophosphorylated. Ca2+-induced thiophosphorylation of MLC20 continues to increase for up to 10 min (Fig. 3B), since thiophosphorylated MLC20 is resistant to the action of phosphatase (5). GTPγS did not enhance thiophosphorylation of MLC20 at any time point examined. Further addition of HA-1077 does not affect the levels of MLC20 thiophosphorylation. Thus HA-1077 reduces the extent of MLC20 phosphorylation by accelerating dephosphorylation of MLC20.

The above experiments were conducted using passaged SMCs derived from pig aorta. We examined whether the Rho kinase inhibitor HA-1077 inhibited sensitization of MLC20 phosphorylation in freshly isolated primary SMCs, as it does in passaged SMCs. The myosin content and expression of Rho A, Rho kinase, MBS, and PP1δ proteins are shown in Fig. 4A. Quantitation of densities of the proteins by densitometry shows that the amounts of the proteins in passaged SMCs are slightly smaller (78–96% of those in primary SMCs; Table 2). As shown in Fig. 4B, GTPγS enhanced Ca2+-induced MLC20 phosphorylation in both permeabilized primary SMCs and passaged SMCs. HA-1077 (10 µM) completely abolishes GTPγS enhancement of MLC20 phosphorylation in both cell types.

We also determined the effect of HA-1077 on intact vascular SMCs. Stimulation of intact SMCs with PGF2α (30 µM) induced time-dependent increases in both the monophosphorylated and diphosphorylated forms of MLC20 (Fig. 5). Treatment of SMCs with HA-1077 (10 µM) lowered the resting level of the monophosphorylated form of MLC20 and partially inhibited PGF2α-induced increases in monophosphorylated MLC20. HA-1077 exerted a profound inhibitory effect on levels of the diphosphorylated form of MLC20, totally abolishing the PGF2α-induced increase.

We then determined how HA-1077 affects the myosin phosphatase activity of SMCs. Western blot analysis of the anti-MBS immunoprecipitate revealed the presence of 130-, 38-, and 21-kDa proteins, which were reactive with anti-MBS antibody, anti-PP1δ antibody, and anti-M21 antibody, respectively (Fig. 6A). We measured the phosphatase activity associated with the anti-MBS immunoprecipitate obtained from permeabilized SMCs. The amounts of immunoprecipitated MBS from the cells treated variously are shown in Fig. 6B. GTPγS stimulation of SMCs causes a 55% decrease in the phosphatase activity toward MLC20, and pretreatment of SMCs with C3 toxin abolishes this decrease (Fig. 6C). The addition of HA-1077 to cells reversed the GTPγS inhibition of the phosphatase activity dose dependently. These results are consistent with the notion that GTPγS-induced inhibition of myosin phosphatase is mediated through Rho and Rho kinase.

We examined the effect of HA-1077 on the phosphorylation state of MBS in SMCs under the same conditions as described in Fig. 6, B and C. In permeabilized SMCs, the addition of GTPγS induced a threefold increase in

Fig. 3. A: HA-1077 prevents GTPγS-induced inhibition of MLC20 dephosphorylation. Permeabilized SMCs were first incubated in the phosphorylation buffer (0.3 µM Ca2+) with or without GTPγS (30 µM) and/or HA-1077 (10 µM) for 15 min and then were switched to the dephosphorylation buffer (the Ca2+-free, 2 mM EGTA, and 50 µM wortmannin-containing phosphorylation buffer) with or without GTPγS and/or HA-1077. Each value represents the mean ± SE of 3 determinations. B: neither GTPγS nor HA-1077 has any effect on thiophosphorylation of MLC20. Permeabilized SMCs were incubated in Ca2+ (0.1 µM) alone, Ca2+ + GTPγS (30 µM), or Ca2+ + GTPγS and HA-1077 (10 µM) in the presence of adenosine 5'-O-(3-thiotriphosphate) for the indicated time periods.
the extent of phosphorylation of MBS that was nearly totally abolished by pretreatment with C3 toxin (Fig. 7). The addition of HA-1077 (10 µM) to cells also totally abolished the stimulatory effect of GTPγS. We further examined the involvement of Rho kinase in GTPγS-induced myosin phosphatase inhibition by studying the effect of the PH/cystein-rich domain of Rho kinase, a dominant inhibitor for Rho kinase (2). The addition of the recombinant PH/cystein-rich domain of Rho kinase (3 µM) to permeabilized SMCs inhibited GTPγS-induced enhancement of MLC20 phosphorylation by 75% (Fig. 8A), whereas the PH/cystein-rich domain of Rho kinase at 3 µM had no effect on MLC20 phosphorylation induced by Ca2+ alone. The addition of the Rho kinase PH/cystein-rich domain dose dependently inhibi-

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Table 2. Expression levels of myosin heavy chains, Rho A, Rho kinase, MBS, and PP1δ

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Primary SMCs, %</th>
<th>Passaged SMCs, %</th>
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<tbody>
<tr>
<td>Myosin heavy chain</td>
<td>100</td>
<td>81</td>
</tr>
<tr>
<td>Rho A</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>Rho kinase</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td>MBS</td>
<td>100</td>
<td>88</td>
</tr>
<tr>
<td>PP1δ</td>
<td>100</td>
<td>78</td>
</tr>
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</table>

Values are means of duplicate determinations. MBS, myosin-binding regulatory subunit; SMCs, smooth muscle cells. Expression of the proteins in primary SMCs and passaged (passage 5) SMCs were examined as described in Fig. 4, and expression levels of each protein were quantitated by densitometry and expressed as a percentage of values in primary SMCs.
mented GTPγS-induced MBS phosphorylation, with the complete inhibition obtained at 5 µM (Fig. 8B).

DISCUSSION

Accumulating evidence shows that Rho kinase is one of the major Rho effectors that are implicated in the regulation of various cell functions (2, 20, 25, 34). The reported substrate proteins for Rho kinase include MBS (15), MLC20 (3), vimentin and glial fibrillary acidic protein (17), the ezrin/radixin/moesin family proteins (23), and adducin (25, 34). A specific Rho kinase inhibitor would be beneficial to see how Rho kinase-catalyzed phosphorylation of these proteins affects cell functions. In the present study, we demonstrated that HA-1077 acts as a potent in vivo Rho kinase inhibitor. We next analyzed a role for Rho kinase in Rho-dependent myosin phosphatase inhibition in vascular SMCs by using this compound, demonstrating that in vivo Rho kinase acts downstream of Rho to induce phosphorylation of MBS, resulting in inhibition of myosin phosphatase and consequent enhancement of MLC20 phosphorylation.

It was recently shown that treatment of intact platelets with a thromboxane A2 analog induced a decrease in the activity of myosin phosphatase isolated by immunoprecipitation using anti-MBS antibody (24). The association of Rho and Rho kinase with anti-MBS immunoprecipitate was observed; however, no functional involvement of Rho and Rho kinase in the agonist-induced myosin phosphatase inhibition was shown (24). It was also recently shown in vascular endothelial cells that thrombin inhibited phosphatase activity toward MLC20 in myosin-enriched cell fractions in a Rho-dependent manner (6). However, it was not directly demonstrated that Rho kinase mediated the thrombin-induced inhibition of phosphatase activity. Also, the effect of thrombin on the phosphorylation status of MBS was not shown (6). In the present study, we demonstrated that GTPγS stimulation indeed leads to inhibition of myosin phosphatase activity in a Rho-dependent manner in SMCs (Fig. 6). We further found that the Rho kinase inhibitor HA-1077 totally abolishes
GTP\textsubscript{S} inhibition of myosin phosphatase activity and consequent enhancement of MLC\textsubscript{20} phosphorylation (Figs. 2, 3, and 6). In agreement with this, HA-1077 strongly inhibits the receptor agonist-induced increase in phosphorylation, especially diphosphorylation, of MLC\textsubscript{20} in intact SMCs (Fig. 5). The observations indicate that, among Rho targets, Rho kinase is responsible for mediating myosin phosphatase suppression in smooth muscle. Moreover, the present study shows that GTP\textsubscript{S}-induced, Rho-dependent myosin phosphatase inhibition is accompanied by a concomitant increase in phosphorylation of MBS, which is also blocked by HA-1077 (Fig. 7). It was reported recently (18) that the addition of the constitutively active catalytic fragment of Rho kinase to permeabilized vascular smooth muscle preparations caused a Ca\textsuperscript{2+}/calmodulin-independent phosphorylation of MLC\textsubscript{20} and contraction. It was also demonstrated previously (3) that Rho kinase phosphorylates purified myosin in vitro at the MLCK phosphorylation site (Ser\textsuperscript{19}) of MLC\textsubscript{20} and increases actin-activated myosin ATPase activity. These observations may sug-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig7}
\caption{HA-1077 and C3 inhibit GTP\textsubscript{S}-induced phosphorylation of the 130-kDa MBS. Permeabilized SMCs were left unpretreated or were pretreated with HA-1077 (10 \textmu M) for 10 min or C3 (1 \mu g/ml) for 20 min and then were stimulated with GTP\gamma S (30 \mu M) for 10 min in the presence of [gamma-\textsuperscript{32}P]ATP. MBS was immunoprecipitated and separated on 8% SDS-PAGE, followed by autoradiography. Autoradiographs showing \textsuperscript{32}P phosphorylation of MBS and anti-MBS blots are displayed on top. Radioactivity of the band corresponding to the MBS was determined by a Fuji BAS 2000 Bio-Image Analyzer and was expressed as the degree of increase over the radioactivity of a nonstimulated control. Each value represents the mean \pm SE of 4 determinations. ** and \dagger\dagger P < 0.01 (by Dunnett's test) compared with no stimulation and GTP\gamma S stimulation without pretreatment, respectively.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig8}
\caption{Pleckstrin homology (PH)/cystein-rich domain of Rho kinase (PH domain) inhibits GTP\gamma S-induced enhancement of MLC\textsubscript{20} phosphorylation and MBS phosphorylation in permeabilized SMCs. A: cells were incubated with Ca\textsuperscript{2+} (0.3 \mu M) alone or Ca\textsuperscript{2+} + GTP\gamma S (30 \mu M) in the presence of various concentrations of the recombinant PH domain of Rho kinase. B: permeabilized SMCs were incubated with the recombinant PH/cystein-rich domain at the indicated concentrations for 10 min and then were stimulated with GTP\gamma S (30 \mu M) for a further 10 min in the presence of [gamma-\textsuperscript{32}P]ATP. Phosphorylation of MBS was analyzed as described in the legend for Fig. 7. Each value represents the mean \pm SE of 3 determinations. ** P < 0.01 by Dunnett's test compared with no stimulation; \dagger P < 0.05 and \dagger\dagger P < 0.01 compared with GTP\gamma S stimulation in the absence of PH domain. Representative autoradiographs and anti-MBS Western blots are shown on top in B.}
\end{figure}
gest that direct phosphorylation of MLC20 by Rho kinase could contribute to a Rho kinase-mediated increase in MLC20 phosphorylation under certain experimental conditions. However, we (26) and others (16) previously observed in permeabilized SMCs and vascular strips that GTPγS stimulation did not increase Ca2+-induced thiophosphorylation of MLC20. Because thiophosphorylated MLC20 is resistant to the action of myosin phosphatase (5), these observations indicate that the myosin kinase activity is not enhanced in GTPγS-stimulated smooth muscle. Furthermore, in the present study, the Rho kinase inhibitor HA-1077 does not inhibit Ca2+-induced thiophosphorylation of MLC20 in the presence of GTPγS but does reverse GTPγS-induced inhibition of MLC20 dephosphorylation (Fig. 3). These results indicate that in SMCs, at least under the present conditions, Rho-dependent enhancement of MLC20 phosphorylation is mediated largely through the downregulation of myosin phosphatase activity.

It was recently reported that a new compound, Y-27632 (33), which has a totally different molecular structure from HA-1077, potently inhibits Rho kinase and its isoform ROCK1/ROKβ. The K1 value of Y-27632 for ROCK1 was calculated to be 0.14 µM, which was based upon the measured K1 value (0.1 µM) for ROCK1 for ATP. Consistent with our results, Y-27632 was shown to inhibit GTPγS enhancement of Ca2+-induced smooth muscle contraction (7, 33). However, the effects of Y-27632 on MBS phosphorylation and myosin phosphatase activity were not reported. It was also shown that Y-27632 lowers blood pressure in rat hypertension models. The Rho kinase inhibitors would serve as useful tools for dissecting the roles for Rho kinase in the regulation of functions of smooth muscle and non-muscle cells.

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