Rho-associated Kinase Directly Induces Smooth Muscle Contraction through Myosin Light Chain Phosphorylation*

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From the First Department of Internal Medicine, Mie University School of Medicine, Tsu, Mie 514, Japan, the First Department of Physiology, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan, and the Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma, Nara 630-01, Japan, and the Division of Molecular Cardiology, Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

Small GTPase Rho plays pivotal roles in the Ca\textsuperscript{2+} sensitization of smooth muscle. However, the GTP-bound active form of Rho failed to exert Ca\textsuperscript{2+}-sensitizing effects in extensively Triton X-100-permeabilized smooth muscle preparations, due to the loss of the important diffusible cofactor (Gong, M. C., Iizuka, K., Nixon, G., Browne, R. P., Hall, A., Ecleston, J. F., Sugai, M., Kobayashi, S., Somlyo, A. V., and Somlyo, A. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1340–1345). Here we demonstrate the contractile effects of Rho kinase (Rho-kinase), recently identified as a putative target of the activated Rho (10–12) could exert Ca\textsuperscript{2+}-sensitizing effects on saponin- or β-escin-permeabilized smooth muscle. However, the activated Rho failed to induce Ca\textsuperscript{2+} sensitization on extensively Triton X-100-permeabilized smooth muscle (11). Considering that extensive Triton X-100-permeabilization allows higher molecular weight compounds to diffuse from the cytosol of smooth muscle of the rabbit portal vein (13), important diffusible factor(s) for the Ca\textsuperscript{2+} sensitization of smooth muscle might be lost during extensive permeabilization by Triton X-100, an event that would result in no response to activated Rho.

We have recently reported that Rho-kinase, which is activated by GTP-bound active form of Rho (14–16), phosphorylates not only MLC, thereby activating myosin ATPase (17), but also myosin phosphatase, thus inactivating it in vitro (18). These findings in a cell-free system, plus the previous reports of G-protein-mediating Ca\textsuperscript{2+} sensitization as described above, suggest that Rho-kinase may induce contraction and concomitant MLC phosphorylation of the smooth muscle. We examined the effects of the constitutively active form of Rho-kinase on smooth muscle extensively permeabilized by Triton X-100 and attempted to determine if Rho-kinase would be the factor lost during extensive Triton X-100 permeabilization.

**EXPERIMENTAL PROCEDURES**

Materials and Chemicals—The catalytic subunit of recombinant Rho-kinase (CAT; molecular mass is about 80 kDa) was expressed as a glutathione S-transferase fusion protein and purified using a baculovirus system and a glutathione-Sepharose column (17). The kinase activity of the elute was determined by phosphorylation assay using 56 peptide as a substrate (15) in buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 100 μM ATP (0.5–20 Gbq/mmol). CaM was purified from bovine brain by previously described method (19). Other materials and chemicals were obtained from commercial sources.

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1 The abbreviations used are: MLC, myosin light chain; GTPγS, guanosine 5′-[(y-thio)triphosphate; Rho-kinase, Rho-associated serine/threonine kinase; CaM, calmodulin; CAT, the catalytic subunit of recombinant Rho-kinase; WM, wortmannin; DTT, dithiothreitol.

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permeabilized and intact rabbit portal veins were homogenized in extraction buffer containing 50 mM Tris-HCl, pH 7.2, 400 mM NaCl, 2 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 µM p-aminophenylmethanesulfonyl fluoride hydrochloride, 10 µg/ml leupeptin, and 1 mM benzamidine. Each extract was centrifuged at 41,000 × g for 30 min at 4 °C, and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (20) followed by immunoblotting (21). Anti-Rho-kinase rabbit polyclonal antibodies were provided by Dr. J. T. Stull (University of Texas Southwestern Medical Center, Dallas, TX). Immunostained proteins were visualized by Supersignal (Pierce). Densities of bands of immunoblotting using anti-MLC antibodies as

**RESULTS AND DISCUSSION**

Introduction of CAT, which is not only the constitutively active form of Rho-kinase but also the highly homologous domain among rat (14), bovine (15), human (16), and mouse Rho-associated kinases (23), into the cytosol of the extensively Triton X-100-permeabilized rabbit portal vein smooth muscle provoked a contraction both at a constant cytosolic Ca2+ (pCa 6.5; Fig. 1a) and at a nominally zero cytosolic Ca2+ buffered with 10 mM EGTA (pCa ≈ 8.0; Fig. 1c). CAT exerted contraction, whereas the vehicle had no effect on the force (Fig. 1, a and c). These contractions were completely reversed by wash out of CAT, contrary to those induced by 10 µM microcystin-LR (24, 25) (Fig. 1b). In the absence of cytosolic Ca2+ at pCa ≈ 8.0, the CAT-induced contraction was also reversible (data not shown). In neither intact nor α-toxin-permeabilized strips of the portal vein did CAT exert the contractile effects (data not shown). These observations are interpreted to mean that constitutively active CAT could be introduced into the cytosol of the smooth muscle only by extensive membrane permeabilization to induce a reversible contraction.

MLC phosphorylation mediated by Ca2+-CaM-dependent MLC kinase pathway plays a primary role in smooth muscle contraction through myosin-actin-interaction and the consequent activation of myosin ATPase (2–4). To investigate involvement of Ca2+-CaM-dependent MLC kinase pathway in the CAT-induced contraction, we examined the effects of wortmannin (WM), a potent MLC kinase inhibitor (26) on force development induced by cumulative application of CAT (Fig. 2). In the presence of cytosolic Ca2+ at pCa 6.5, which Ca2+-CaM-dependent MLC kinase should be active, 10 µM WM did not affect CAT-induced force development. In the absence of CAT, treatment of 10 µM WM completely inhibited the cytosolic Ca2+-provoked contraction at pCa 6.5, in the Triton X-100-permeabilized fibers. Considering our finding that WM did not affect the activity of CAT up to 100 µM in vitro (data not shown), this WM-sensitive component of CAT-induced contraction at pCa 6.5 seemed to be due to inhibition of the Ca2+-provoked contraction through the Ca2+-CaM-dependent MLC kinase pathway but not related to the CAT-mediated pathway. All these observa-
To clarify whether CAT induces contraction with a concomitant increase in levels of MLC phosphorylation, we examined the effects of CAT on MLC phosphorylation, using immunoblotting with anti-MLC polyclonal antibody (Fig. 3). As shown in lanes 1–3 of Fig. 3a, at pCa < 8.0, monophosphorylation of MLC was detected only in the presence of CAT and was insensitive to 10 μM WM (42.77 ± 9.22% of the total amount of immunostained MLC (n = 4) in the absence of WM, 35.95 ± 3.39% (n = 4, p > 0.05) in the presence of WM, respectively). At pCa 6.5, shown in lanes 4–6 of Fig. 3a, CAT potentiated the level of monophosphorylation of MLC (60.33 ± 1.42%, n = 4), which was partially inhibited by 10 μM WM (26.05 ± 5.18%, n = 4, p < 0.01). Based on the statistical analysis and the results in Fig. 2, this WM-sensitive component of CAT-induced MLC phosphorylation at pCa 6.5 also seemed to be due to inhibition of Ca²⁺-CaM-dependent MLC kinase activity. These results are consistent with that of counterparts of the effects of CAT on the contractile responses (Fig. 3b). It was concluded that CAT potentiates the contractile response by increasing the extent of monophosphorylation of MLC.

To determine if native Rho-kinase is one of the cofactors diffusible during permeabilization by Triton X-100, we examined the amounts of native Rho-kinase in intact and permeabilized fibers by immunoblot analysis using rabbit polyclonal antibodies against Rho-kinase. To standardize the densitometrical value, the ratio of densitometrical quantification of immunostaining of Rho-kinase to that of MLC was calculated in both intact and permeabilized fibers. As shown in Fig. 4, the amounts of native Rho-kinase in the Triton X-100-permeabilized rabbit portal vein were markedly lower than those in intact tissue (0.06 ± 0.01 (n = 4) for permeabilized sample, 0.95 ± 0.02 (n = 4) for intact sample, respectively), whereas the amounts of the possible cytoskeletal proteins, such as MLC and myosin heavy chain in permeabilized fibers were similar to the counterparts of intact fibers. These results confirm that extensive permeabilization by Triton X-100 allows for the loss of cytosolic proteins, including Rho-kinase, whereas cytoskeletal proteins such as myosin are stable. Based on all of these findings taken together plus evidence that the direct activation of G-proteins did not exert contractile effects on the extensively Triton X-100-permeabilized smooth muscle (11), we consider that Rho-kinase may be a valid candidate for the key molecule in G-protein-mediated smooth muscle contraction and may be
the molecule lost during extensive permeabilization by Triton X-100.

We demonstrate here what seems to be the first evidence that Rho-kinase is a direct effector on the contractile apparatus of smooth muscle, independently of the Ca\(^{2+}\)-CaM-dependent MLC kinase pathway. Except for Ca\(^{2+}\)-independent MLC kinase (9, 13), we find no documentation that the exogenous addition of kinases to the cytosol of permeabilized smooth muscle directly exerts contractile responses comparable with findings with CAT. Because the inhibition of myosin phosphatase may possibly be the main mechanism of the G-protein-mediated Ca\(^{2+}\) sensitization of smooth muscle contraction (1, 9, 24, 25, 27), the CAT-induced contraction of G-protein-uncoupled smooth muscle permeabilized by Triton X-100 (Fig. 1) may be also mediated by the inhibition of myosin phosphatase. This notion is supported by our previous finding that Rho-kinase inhibited the activity of myosin phosphatase through thiophosphorylation of its myosin-binding subunit in vitro (18). However, at cytosolic zero Ca\(^{2+}\), microcystin-LR-induced contraction of the permeabilized smooth muscle was reduced by an MLC kinase inhibitor (25), whereas the CAT-induced contraction was insensitive to it (Fig. 2). Such differential sensitivities of the MLC kinase inhibitor to CAT- and myosin phosphatase inhibitor-induced contractions support the idea that myosin phosphatase inhibition alone cannot account for the CAT-induced contraction at the cytosolic zero Ca\(^{2+}\). Taking this together with our report that Rho-kinase directly provokes the phosphorylation of MLC and activates myosin in vitro (17), we suggest that the mechanism(s) of CAT-induced contraction of Triton X-100-permeabilized rabbit portal vein might be a concomitant monophosphorylation of MLC directly induced by CAT independently of a Ca\(^{2+}\)-CaM-dependent MLC kinase pathway. We propose that Rho-kinase is considered a valid key molecule in G-protein-mediating Ca\(^{2+}\) sensitization of smooth muscle contraction.

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