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Agonist-induced changes in the phosphorylation of the myosin-binding subunit of myosin light chain phosphatase and CPI17, two regulatory factors of myosin light chain phosphatase, in smooth muscle

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Agonist-induced changes in the phosphorylation of the myosin-binding subunit of myosin light chain phosphatase and CPI17, two regulatory factors of myosin light chain phosphatase, in smooth muscle

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The inhibition of myosin light chain phosphatase (MLCP) enhances smooth muscle contraction at a constant [Ca^{2+}]. There are two components, myosin-binding subunit of MLCP (MBS) and CPI17, thought to be responsible for the inhibition of MLCP by external stimuli. The phosphorylation of MBS at Thr-641 and of CPI17 at Thr-38 inhibits the MLCP activity *in vitro*. Here we determined the changes in the phosphorylation of MBS and CPI17 after agonist stimulation in intact as well as permeabilized smooth muscle strips using phosphorylation-site-specific antibodies as probes. The CPI17 phosphorylation transiently increased after agonist stimulation in both α-toxin skinned and intact fibres. The time course of the increase in CPI17 phosphorylation after stimulation correlated with the increase in myosin regulatory light chain (MLC) phosphorylation. The increase in CPI17 phosphorylation was significantly diminished by Y27632, a Rho kinase inhibitor, and GF109203x, a protein kinase C inhibitor, suggesting that both the protein kinase C and Rho kinase pathways influence the change in CPI17 phosphorylation. On the other hand, a significant level of MBS phosphorylation at Thr-641, an inhibitory site, was observed in the resting state for both skinned and intact fibres and the agonist stimulation did not significantly alter the MBS phosphorylation level at Thr-641. While the removal of the agonist markedly decreased MLC phosphorylation and induced relaxation, the phosphorylation of MBS was unchanged, while CPI17 phosphorylation markedly diminished. These results strongly suggest that the phosphorylation of CPI17 plays a more significant role in the agonist-induced increase in myosin phosphorylation and contraction of smooth muscle than MBS phosphorylation in the Ca^{2+}-independent activation mechanism of smooth muscle contraction.

Key words: histamine, myosin phosphorylation, protein kinase C, Rho-associated kinase, smooth muscle contraction.

INTRODUCTION

It is known that phosphorylation of the regulatory light chain of smooth muscle and non-muscle myosin II [myosin regulatory light chain (MLC)] at Ser-19 is required for the activation of actomyosin contractile activity. Therefore the regulation of myosin II phosphorylation is a critical determinant of contraction of diverse cell contractile events. The best-characterized cell type is the smooth muscle cell, and evidence indicates that myosin phosphorylation in smooth muscle is regulated by two processes, i.e. a Ca^{2+}-dependent process and a Ca^{2+}-independent process.

While several protein kinases can phosphorylate smooth muscle myosin [1–4], it is thought that myosin light chain kinase (MLCK), a Ca^{2+}/calmodulin-dependent MLC-specific protein kinase, is primarily responsible for the phosphorylation of myosin in smooth muscle [1–4], since an increase in cytosolic Ca^{2+} concentration induces MLC phosphorylation in smooth muscle tissues [1,2,4]. On the other hand, it was realized that the force development of smooth muscle is not simply determined by cytoplasmic [Ca^{2+}] [5–8]. For a given [Ca^{2+}], a higher force is achieved by agonist-induced stimulation than by depolarization-induced contraction. Using permeabilized smooth muscle strips having intact receptor-coupled systems, it was shown that an agonist can increase the force developed by smooth muscle even when cytoplasmic [Ca^{2+}] was clamped [9,10]. This agonist-induced 'Ca^{2+} sensitization' indicates that there is an additional mechanism that can regulate smooth muscle contractile machinery. Subsequently, it was shown that the increase in force elicited by an agonist at a given Ca^{2+} is due to the inhibition of myosin light chain phosphatase (MLCP) [10,11]. The possible mechanism by which agonists inhibit MLCP activity appears to be G-protein coupled, because guanosine 5′-[γ-thio]triphosphate (GTP[S]), an activator of G-protein, inhibits myosin dephosphorylating activity in permeabilized smooth muscle strips [9,10,12].

Activation of heterotrimeric G-proteins would activate the phosphatidylinositol cascade, thus producing diacylglycerol and Ins(1,4,5)P_3. Protein kinase C (PKC), which is activated by diacylglycerol, has been suggested to play a role in the regulation of myosin dephosphorylation [13,14]. Recently, it was suggested that PKC mediated inhibition of MLCP via activation of the MLCP inhibitor, CPI17 (where CPI is PKC-activated protein phosphatase inhibitor) [15], whose inhibitory activity requires the phosphorylation at Thr-38 by PKC [16]. It was shown that the addition of the phosphorylated CPI17 enhances the force of the permeabilized smooth muscle fibre [17]. Recently, it was shown that the phosphorylation of CPI17 is actually increased in smooth muscle fibres after agonist stimulation [18].

The Ras-family small G-protein, Rho, has been found to be involved in smooth muscle stimulation–contraction coupling, as

Abbreviations used: DTT, dithiothreitol; ET-1, endothelin-1; GDP[β-S], guanosine 5′-[β-thio]diphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; MBS, myosin-binding subunit of myosin light chain phosphatase; MLC, myosin regulatory light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; β-ME, β-mercaptoethanol; PSS, physiological saline solution.

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first reported by Takai and his collaborators [19]. They found that epidermal differentiation inhibitor (EDIN) and exoenzyme C3 from *Clostridium botulinum*, both of which are known to ADP-ribosylate the Rho p21 family thus inactivating Rho activity, abolish GTP[S]-enhanced Ca^{2+} sensitization of skinned mesenteric arterial smooth muscle. Similar results have been reported by other investigators [20–22]. Supporting this hypothesis, Otto et al. [23] reported that the addition of the constitutively active mutant of RhoA to β-escin-permeabilized smooth muscle enhanced the carbachol-induced increase in Ca^{2+} sensitivity of force production.

MLCP consists of three subunits, a large myosin-binding subunit of MLCP (MBS), a 20 kDa small subunit and a catalytic subunit of the type 1 protein serine/threonine phosphatase family [24–26]. It has been shown that MBS can be phosphorylated by Rho-dependent kinase, resulting in a decrease in MLCP activity *in vitro* [27]. Rho kinase phosphorylates MBS at two sites *in vitro*, i.e. Thr-641 and Thr-799 (in the rat MBS sequence, corresponding to Thr-695 and Thr-850 in chicken M133, respectively), of which Thr-641 is responsible for the inhibition of MLCP activity [28]. This raises the hypothesis that activation of the Rho signalling pathway could phosphorylate MBS by Rho kinase, thus down-regulating MLCP and increasing contraction. Consistent with this idea, it was shown recently that GTP[S] increased the phosphorylation of MBS in permeabilized strips [29] or cultured smooth muscle cells [30]. However, since MBS can be phosphorylated at multiple sites by protein kinases and the site responsible for the inhibition of MLCP activity is Thr-641, an important question is whether the phosphorylation site critical for the regulation of MLCP significantly changes during agonist-induced stimulation of smooth muscle contraction.

In the present study, we examined the changes in MBS phosphorylation at Thr-641 and CPI17 phosphorylation at Thr-38 along with the change in MLC phosphorylation at Ser-19 in intact as well as α-toxin-permeabilized smooth muscle strips during the contraction–relaxation process by using phosphorylation-site-specific antibodies as probes.

EXPERIMENTAL

Proteins

Smooth muscle MLC was expressed in *Escherichia coli* and purified as described [31,32]. The cDNA clone of human Rho kinase (ROCKII) was kindly supplied by Dr T. Leung (University of Singapore). Rat MBS cDNA was supplied by Professor P. Cohen (University of Dundee, Dundee, U.K.). The cDNAs of rabbit PKCα and ϵ were supplied by Dr S. Ohno (Yokohama City University, Yokohama, Japan). The cDNAs containing entire coding region were subcloned into pFastbacHT (Invitrogen, Carlsbad, CA, U.S.A.) baculovirus transfer vector at the poly-linker region downstream of the hexa-histidine tag. Recombinant viruses were produced according to manufacturer's procedures.

To express recombinant PKCs and Rho kinase, Sf9 cells were infected with each viral stock and cultured at 28 °C for 72 h. Harvested Sf9 cells were lysed by sonication in 15 ml of buffer A [0.15 M NaCl, 30 mM Tris/HCl, pH 8.0, 2 mM β-mercaptoethanol (β-ME), 2 mM PMSF, 10% (v/v) glycerol, 0.05% Triton X-100, 10 μg/ml leupeptin and 0.1 mg/ml trypsin inhibitor] for PKC purification, or buffer B [0.5 M NaCl, 30 mM Tris/HCl, pH 8.0, 2 mM β-ME, 2 mM PMSF, 10% glycerol, 0.5% Triton X-100 and 10 μg/ml leupeptin] for Rho kinase purification. After centrifugation at 10000 ***g*** for 20 min at 4 °C, the supernatants were mixed with Ni^{2+}-nitrilotriacetic acid–agarose (Qiagen) on a rotating wheel for 30 min at 4 °C. The resin suspensions were loaded on a column (1 cm × 10 cm) and washed with 10 bed volumes of washing buffer (0.15 M NaCl, 5 mM imidazole/HCl, pH 8.0, 10% glycerol and 2 mM β-ME). PKCs or Rho kinase was eluted with elution buffer (0.15 M NaCl, 0.25 M imidazole/HCl, pH 8.0, 10% glycerol and 2 mM β-ME). After SDS/PAGE analysis, the fractions containing PKCs or Rho kinase were pooled and dialysed against 150 mM NaCl, 30 mM Tris/HCl, pH 7.5 and 1 mM dithiothreitol (DTT). Rat MBS was purified by the same procedure as described above.

The DNA fragment corresponding to the coding region of mouse CPI17 cDNA was supplied by Dr M. Eto (University of Virginia, Charlotteville, VA, U.S.A.) and amplified by PCR using the primers 5′-GAAGGAGATATACccatggCTGCGC-AG-3′ and 5′-GGAGCTCgaattcTCAGGGTGGGGCAGTG-3′, to introduce restriction sites for *Nco*I and *Eco*RI shown by lower-case letters (the initiation and termination codons are indicated by underlines). The 0.45 kb *Nco*I–*Eco*RI fragment was cloned into pET-30 plasmid (Novagen). The constructed plasmid encoded the 44 residues of tag sequence containing $(His)_6$ segment at the N-terminal side of CPI17. *Escherichia coli* BL21(DE3) cells were transformed with this plasmid and the transformed cells were grown in LB media at 37 °C until the attenuance at 600 nm reached 0.6. Protein expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactoside for 3 h at 37 °C. Cells were collected and lysed in 0.5 M NaCl, 20 mM potassium phosphate, pH 7.0, 1 mM benzamidine, 10 mM imidazole/HCl and 0.1 mM PMSF. The lysate was heated at 90 °C for 5 min and clarified by centrifugation at 22000 ***g*** for 20 min at 4 °C. Hexahistidine-tagged CPI17 was absorbed to Ni^{2+}-nitrilotriacetic acid–agarose, and bound proteins were eluted with 500 mM imidazole/HCl, pH 7.0. Protein concentrations were determined by densitometric tracing of the protein bands stained with Coomassie Brilliant Blue on an SDS/polyacrylamide gel with BSA as a standard.

Production of antibodies

Peptides Cys-Ser-Lys-Leu-Gln-Ser-Pro-Ser-Arg-Ala-Arg-Gly-Pro-Gly-Gly-Ser-Pro-Gly-Gly-Leu-Gln-Lys-Arg-His-Ala-Arg (corresponding to amino acids 12–36 of CPI17), Cys-Arg-His-Ala-Arg-Val-phosphoThr-Val-Lys-Tyr-Asp-Arg (amino acids 33–43 of CPI17), Cys-Gln-Ser-Arg-Arg-Ser-phosphoThr-Gln-Gly-Val-Thr-Leu (amino acids 636–646 of rat MBS) and Cys-Glu-Lys-Arg-Arg-Ser-phosphoThr-Gly-Val-Ser-Phe-Trp (amino acids 694–704 of rat MBS) were chemically synthesized as antigens by Genemed Synthesis (South San Francisco, CA, U.S.A.), as well as the dephosphorylated forms of the peptides necessary for the antibody purification. An N-terminal cysteine was added to the peptide for the purpose of conjugation with keyhole limpet haemocyanin. Antibodies were prepared by injecting two rabbits with keyhole limpet haemocyanin-coupled peptide. Phosphorylation-site-specific antibodies against MBS and CPI17 were purified by two-step chromatography: affinity chromatography on phosphopeptide-coupled resin and then absorption in dephosphorylated peptide-coupled resin. Anti-CPI17 antibody, raised against the peptide corresponding to amino acids 12–36 of CPI17, recognized CPI17 regardless of the phosphorylation state and was affinity purified by peptide-coupled affinity chromatography.

Affinity resins were prepared by cross-linking the synthesized peptides to epoxyamino hexyl ('EAH')-Sepharose 4B (Amersham Biosciences) in the presence of sulpho-SMCC (Pierce). Anti-phosphorylated MLC mouse monoclonal antibody was produced as described previously [33]. Anti-MBS antibodies

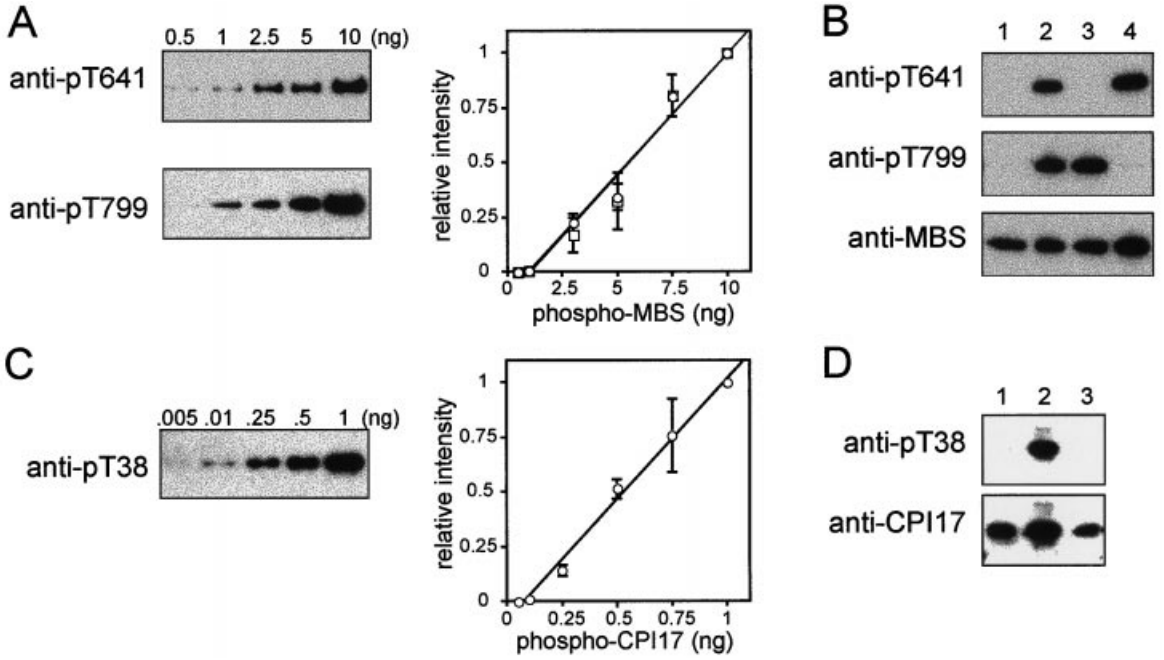

Figure 1 Specificity of phosphorylation-site-specific antibodies

(**A**) Relationship between the amounts of samples and density of Western blot for MBS. MBS was phosphorylated by Rho kinase as described in the Experimental section. Different amounts of phosphorylated MBS indicated above the panel were subjected to SDS/PAGE followed by Western blot using anti-pThr-641 and anti-pThr-799 antibodies as probes. Blots were quantitated by densitometry and the relative intensities were plotted as a function of the amount of MBS. All values are expressed as means ± S.E.M. ($n = 4$). ○, anti-pThr-641; □, anti-pThr-799. (**B**) Dephosphorylated (lane 1) and phosphorylated (lane 2) wild-type MBS, and Thr-641Ala (lane 3), and Thr-799Ala (lane 4) mutant MBS phosphorylated by Rho kinase were subjected to Western blot using anti-pThr-641, anti-pThr-799 and anti-MBS antibodies. 20 ng of MBS was loaded into each lane. (**C**) Relationship between the amount of samples and density of Western blot for CPI17. Mouse CPI17 was phosphorylated by PKCα as described in the text. Different amounts of phosphorylated CPI17 indicated above the panel were subjected to SDS/PAGE followed by Western blot using anti-pThr-38 antibodies as probes. Blots were quantitated by densitometry and the relative intensities were plotted as a function of the amount of CPI17. All values are expressed as means ± S.E.M. ($n = 4$). (**D**) Dephosphorylated (lane 1) and phosphorylated (lane 2) wild-type CPI17 and Thr-38Ala mutant CPI17 (lane 3) phosphorylated by PKCα were subjected to Western blot using anti-pThr-38 antibodies. 1 ng of CPI17 was loaded into each lane.

that recognize both phosphorylated and dephosphorylated forms were obtained from BABCO.

Biochemical procedures

MBS was phosphorylated by Rho kinase in 20 mM Tris/HCl, pH 7.5, 4 mM $MgCl_2$, 35 mM NaCl, 1 mM EGTA, 0.5 mM DTT, 0.2 μM microcystin-LR, 3 μg/ml Rho kinase, 25 μg/ml MBS and 0.1 mM ATP at 25 °C for a period indicated. CPI17 was phosphorylated by PKCα in 30 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl, 0.2 mM $CaCl_2$, 1 mM DTT, 1 μM microcystin-LR, 50 ng/ml phorbol 12,13-dibutyrate (PDBu), 0.1 mg/ml phosphatidylserine, 3 μg/ml PKC and 0.1 mM ATP at 25 °C. SDS/PAGE was carried out on a 5–20% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli [34]. Western blotting was carried out as described previously [35].

Force measurement and myosin light chain phosphorylation of rabbit femoral artery

New Zealand White rabbits (2.5–3.0 kg) were killed by the injection of pentobarbital (100 mg/kg). Smooth muscle strips were dissected from the femoral arteries and placed in physiological saline solution (PSS) consisting of the following: 137 mM NaCl, 5.4 mM KCl, 23.8 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 5.5 mM D-glucose. Fat and adventitia were removed mechanically under a binocular microscope, and endothelium was removed by gentle rubbing with a cotton swab. Arteries were then cut into strips (5 μm thick, 750 μm wide and 3 mm long), and the smooth muscle strips were permeabilized with α-toxin (5000 units/ml) in relaxing solution for 60 min at room temperature and then treated with A23187 for 15 min to deplete the sarcoplasmic reticulum Ca^{2+}. The strips were tied with silk filaments and suspended between the fine tips of two tungsten needles, one of which was connected to a force transducer (FORT-10; World Precision Instruments, Sarasota, FL, U.S.A.). The standard relaxing solution used for resting states of the permeabilized strips contained the following: 100 mM potassium methanesulphonate, 5.9 mM $MgCl_2$, 5.0 mM Na_2ATP, 10 mM EGTA, 10 mM creatine phosphate and 50 mM Tris/maleate, pH 6.8. In the activating solution, a calculated amount of calcium methanesulphonate was added to give the final desired concentration of free Ca^{2+} ions. All solutions were adjusted to pH 6.8 at 25 °C and the developed tension was expressed as a percentage, assigning the respective values in relaxing solution and in the steady state of 10 μM Ca^{2+}-induced contraction, recorded at the end of experiment, to be 0% and 100%.

For the contractility measurement of intact smooth muscle, each strip was mounted vertically in normal PSS, which was maintained at 37 °C and pH 7.4 bubbled with 95% O_2/5% CO_2. The lower end of the strip was fixed by a small clip, and the upper end of the strip was attached by a small clip and thread to a force transducer to record the isometric tension. The strips were stimulated with 118 mM K^+ PSS, which was identical with normal PSS except for an equimolar substitution of KCl for

NaCl, at 15 min intervals until the developed tension reached a maximum. After recording the responsiveness to 118 mM K^+ PSS, each strip was stimulated with a contractile agonist [histamine or endothelin-1 (ET-1)] in normal PSS.

The phosphorylation of MLC, MBS and CPI17 in smooth muscle strips was determined as follows. The contractile responses were terminated by immersing the strips in dry-ice-cold acetone containing 10% trichloroacetic acid and 10 mM DTT. Smooth muscle strips were homogenized in ice-cold acetone containing 10% trichloroacetic acid and 10 mM DTT using a hand-operated glass-to-glass homogenizer. The homogenate was centrifuged at 10000 ***g*** and 4 °C for 5 min and the supernatent was carefully removed. The pellet was washed with ice-cold acetone containing 10 mM DTT three times, and after the third wash the remaining acetone was allowed to completely evaporate for 15 min on ice. Proteins were solubilized by sonication in buffer containing 100 mM Tris/HCl, pH 6.8, 1% SDS, 50 mM DTT, 15% glycerol and 0.002% Bromophenol Blue and then boiled for 5 min. The sample was subjected to SDS/PAGE, followed by immunoblot using various antibodies.

Statistical analysis

The results are expressed as means ± S.E.M. ANOVA was performed to evaluate the global statistical significance, and when a significant F value was found, the Scheffe *post hoc* test was performed to identify the difference among the groups. Values were considered significantly different at $P < 0.05$.

RESULTS

Specificity of the phosphorylation-site-specific antibodies of MBS and CPI17

To monitor the change in the phosphorylation of MBS of MLCP, CPI17 and the MLC in smooth muscle fibre, we produced various phosphorylation-site-specific antibodies. It has been shown that MBS is phosphorylated by Rho kinase and that the major phosphorylation sites are Thr-641 and Thr-791 [28], of which the former site is responsible for the inhibition of MLCP *in vitro*. On the other hand, Thr-38 is the major phosphorylation site of CPI17, and the phosphorylation at this site significantly enhances the inhibitory activity of CPI17 against MLCP [16]. Rat MBS was expressed in Sf9 cells and purified as described in the Experimental section. The purified MBS was phosphorylated by Rho kinase to a stoichiometric ratio of approx. 2 mol of phosphate/mol of MBS. Figures 1(A) and 1(B) shows the Western blot of the phosphorylated MBS. Both phosphorylation-specific antibodies, anti-pThr-641 and anti-pThr-799, recognized MBS phosphorylated by Rho kinase but not its un-phosphorylated form, indicating that these antibodies are specific to the phosphorylated MBS (Figure 1B). To further ensure the specificity of the antibodies for the responsible epitopes, we produced mutant MBS in which the phosphorylatable Thr residues are mutated to Ala (Thr-641Ala and Thr-799Ala). Anti-pThr-641 antibodies failed to recognize Thr-641Ala MBS even after phosphorylation by Rho kinase, while it recognized the phosphorylated Thr-799Ala mutant. On the other hand, anti-pThr-799 antibodies did not react with the phosphorylated Thr-799Ala mutant, but reacted with Thr-641Ala mutant (Figure 1B). These results clearly demonstrated that these antibodies specifically recognize the phosphorylated MBS at Thr-641 and Thr-799, respectively. On the other hand, anti-MBS antibodies recognized MBS regardless of its phosphorylation state (Figure 1B).

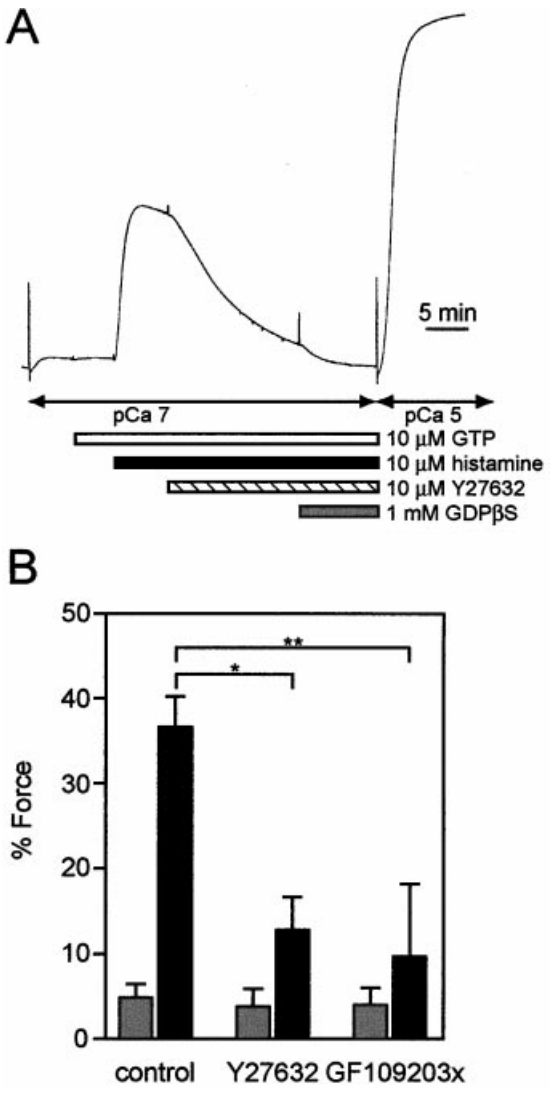

Figure 2 Histamine-induced activation of the contraction of an α-toxin-permeabilized strip of rabbit femoral artery

(**A**) Representative trace of histamine-induced activation of contraction at constant [Ca^{2+}] in α-toxin-permeabilized rabbit femoral artery. Histamine (10 μM) was added at the steady state of the contraction at pCa 7. A Rho kinase inhibitor, Y27632 (10 μM), was added when the histamine-induced sensitization reached steady state. The addition of GDP-[β-S] further diminished the contraction to a level similar to that before the addition of histamine. (**B**) Effect of Y27632 (10 μM) and a PKC inhibitor, GF109203x (5 μM), on the histamine-stimulated activation of contraction at pCa 7 in α-toxin-permeabilized rabbit femoral artery. Inhibitors were applied 5 min before the addition of histamine. Grey bars and black bars indicate Ca^{2+} (pCa 7)-induced contraction and histamine-stimulated activation, respectively. Values are means ± S.E.M. from more than four independent experiments and are expressed as percentages of the maximum contraction obtained at pCa 5. $^*P < 0.05$, $^{**}P < 0.01$.

Figures 1(C) and 1(D) show the Western blot of CPI17 with anti-CPI17 antibodies. CPI17 phosphorylated by PKC (1.0 mol of phosphate/mol of CPI17) as well as its unphosphorylated form were subjected to Western blot analysis. Anti-phospho-CPI17 (anti-pThr-38) antibodies recognized the phosphorylated CPI17, but not its unphosphorylated form. Furthermore, anti-pThr-38 did not recognize Thr-38Ala CPI17 incubated with PKC for phosphorylation. These results demonstrated that anti-pThr-38 is specific to the phosphorylated Thr-38 of CPI17. For all three phosphorylation-site-specific antibodies tested, the signal

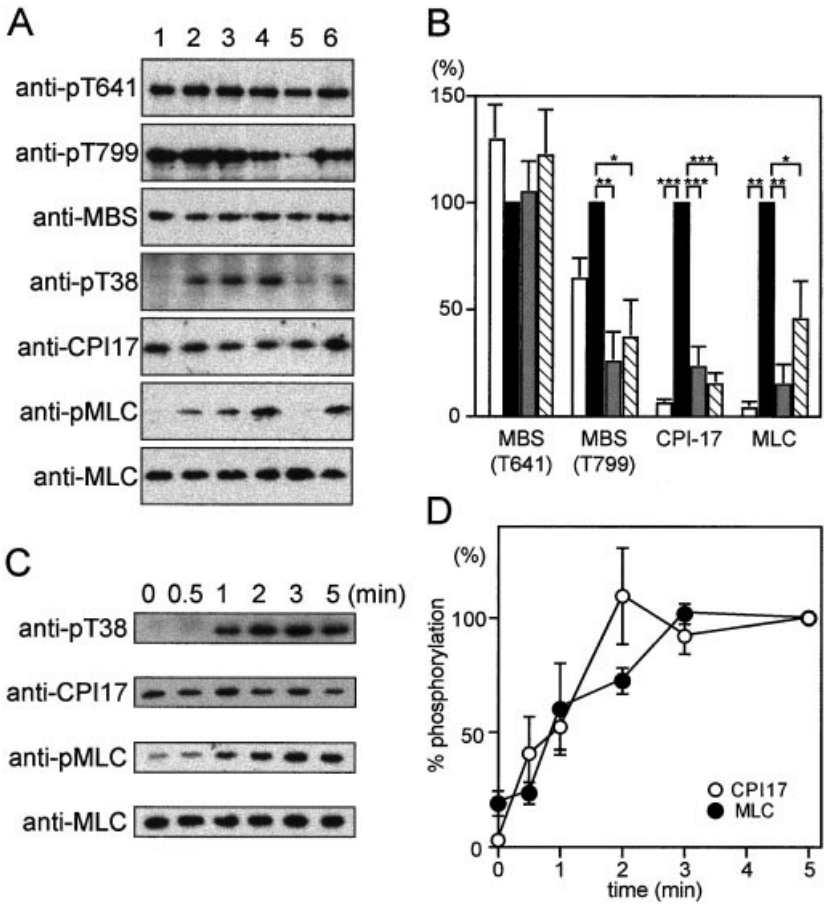

Figure 3 Change in the phosphorylation of MLC, MBS and CPI17 during the histamine-induced contraction of α-toxin-permeabilized rabbit femoral artery

(**A**) Smooth muscle strips incubated for 5 min in pCa 7 alone (lane 1) or with 10 μM histamine (lanes 2–4, 1, 3 and 5 min after histamine stimulation, respectively), 10 μM histamine plus 10 μM Y27632 (lane 5) or 10 μM histamine plus 5 μM GF109203x (lane 6) were homogenized and subjected to Western blot using anti-pThr-641, anti-pThr-799, anti-MBS, anti-pThr-38, anti-CPI17, anti-pMLC and anti-MLC antibodies. (**B**) Quantitative analysis of Western blot. Bands of the Western blot were compared densitometrically and expressed as percentages of the value from the histamine-stimulated rabbit femoral artery. White bars, before histamine stimulation; black bars, 5 min after histamine stimulation; grey bars, 5 min after histamine stimulation in the presence of 10 μM Y27632; hatched bars, 5 min after histamine stimulation in the presence of 5 μM GF109203x. Data represent the means $\pm$ S.E.M. from more than four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**C**) Representative images of Western blot showing temporal changes in the phosphorylation level of CPI17 and MLC. Permeabilized smooth muscle strips were stimulated with 10 μM histamine at pCa 7. Contractile responses were terminated at the various times indicated and then homogenates of the muscle strips were subjected to SDS/PAGE and Western blot analysis. (**D**) Quantitative analysis of temporal changes in the phosphorylation of CPI17 at Thr-38 and MLC. Relative phosphorylation levels of CPI17 at Thr-38 (○) and MLC (●) are indicated as ratios (anti-pThr-38/anti-CPI17 and anti-pMLC/anti-MLC, respectively) of the bands in the Western blot. Each value represents the mean $\pm$ S.E.M. from more than three independent experiments and indicated as a percentage of the values at 5 min after histamine stimulation.

intensity of the blots showed a linear relationship with the amount of phosphorylated proteins loaded on to the gel (Figures 1A and 1C). The intensities of Western blot bands obtained from the smooth muscle strips fell in the linear portion of the curves.

Agonist-induced change in the phosphorylation of MBS and CPI17 in permeabilized smooth muscle strips

To investigate the role of CPI17 and MBS phosphorylation in the agonist-induced changes in myosin phosphorylation and smooth muscle contraction, we utilized α-toxin-permeabilized smooth muscle strips. It is known that α-toxin treatment produces small holes in the plasma membrane, thus allowing passage of only small-molecular-mass substances, not large-molecular-mass cellular components such as proteins. Furthermore, α-toxin treatment does not disturb agonist-induced signalling systems [9,10]. Figure 2 shows the typical force traces of α-toxin-permeabilized rabbit femoral artery smooth muscle strips. At pCa 7, a minimum level of force was developed that was markedly augmented by 10 μM histamine at constant Ca^{2+} (Figures 2A and 2B). The phosphorylation level of MLC was monitored by using anti-pMLC specific antibody that recognizes the phosphorylated Ser-19 of the regulatory light chain [33]. The strips were quickly immersed in trichloroacetic acid/acetone at various times after histamine stimulation to quench the reaction. The strips were then subjected to Western blot analysis. Phosphorylation of MLC probed by anti-pMLC-specific antibody at pCa 7 was significantly increased by histamine (Figures 3A and 3B). CPI17 phosphorylation was negligible before histamine stimulation at pCa 7, but the phosphorylation markedly increased after histamine treatment in α-toxin-permeabilized fibres (Figures 3A and 3B). On the other hand, MBS phosphorylation at Thr-641 was practically unchanged after histamine stimulation, although Thr-799 phosphorylation of MBS increased slightly (Figures 3A and 3B). Phosphorylation levels of Thr-641 and Thr-799 on MBS and Thr-38 on CPI17

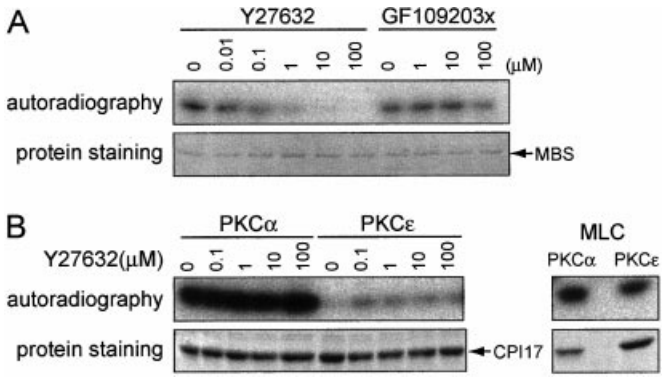

Figure 4 Specificity of the inhibitors against PKC and Rho kinase

(**A**) Effect of Y27632 and GF109203x on Rho kinase. MBS was phosphorylated by Rho kinase in the buffer described in the Experimental section, except that [γ-^{32}P]ATP was used as a substrate in the absence or presence of various concentrations of Y27632 or GF109203x. Phosphorylated MBS was subjected to SDS/PAGE, followed by autoradiography. (**B**) Effect of Y27632 on PKCα and ϵ. CPI17 was phosphorylated by PKCα or ϵ in the buffer described in the Experimental section, except that [γ-^{32}P]ATP was used in the presence of various amounts of Y27632. Phosphorylated CPI17 was subjected to SDS/PAGE, followed by autoradiography. MLC (0.2 mg/ml) was also phosphorylated by PKCα or ϵ in the presence of 30 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl, 0.2 mM $CaCl_2$, 1 mM DTT, 1 μM microcystin-LR, 50 ng/ml PDBu, 0.1 mg/ml phosphatidylserine, 3 μg/ml PKC and 0.1 mM [γ-^{32}P]ATP at 25 °C for 30 min.

after histamine stimulation, estimated from the relationship between the signal strength of the Western blot and the protein amount loaded shown in Figures 1(A) and 1(B), were $62.5 \pm 8.9\,\%$ ($n = 9$), $45.9 \pm 3.8\,\%$ ($n = 7$) and $65.0 \pm 5.8\,\%$ ($n = 10$), respectively. The time course of the increase in CPI17 phosphorylation was closely correlated with the increase in MLC phosphorylation (Figures 3C and 3D), suggesting that the histamine-induced Ca^{2+}-independent increase in MLC phosphorylation is due to the CPI17 phosphorylation-induced inhibition of MLCP.

It has been suggested that the Rho/Rho kinase pathway and/or heterotrimeric G-protein/PKC pathway are involved in the agonist-induced Ca^{2+}-independent activation of smooth muscle contraction [13,14,19–21,23]. To clarify the pathways responsible for the regulation of MLCP modulators, a Rho kinase-specific inhibitor and a PKC-specific inhibitor were tested for their effect on Ca^{2+}-independent contraction and phosphorylation of MLCP modulators. Histamine-induced Ca^{2+}-independent activation of force was significantly diminished by 10 μM Y27632, a Rho kinase-specific inhibitor (Figure 2), suggesting that Rho/Rho kinase cascade is involved in the Ca^{2+}-independent activation of force. The addition of guanosine 5′-[β-thio]diphosphate (GDP[β-S]) further diminished the contraction to the level similar to that before addition of histamine.

Consistently, the phosphorylation of MLC in the fibre was significantly diminished by Y27632 treatment (Figures 3A and 3B). Phosphorylation of CPI17 was also markedly attenuated by Y27632 (Figures 3A and 3B). We checked whether Y27632 can inhibit PKCs, since it has been recently reported that Y27632 can inhibit the proteolytic fragment of PKCδ [36]. Neither PKCα nor PKCϵ was inhibited by Y27632 up to 100 μM (Figure 4B). The results indicate that Rho kinase plays a role in histamine-induced Ca^{2+}-independent activation of α-toxin-permeabilized smooth muscle, and that Rho kinase is involved in CPI17 phosphorylation at Thr-38. This is consistent with the recent report that Rho kinase can phosphorylate CPI17 *in vitro* [37].

It has been shown that Rho kinase phosphorylates MBS at two sites *in vitro*, Thr-641 and Thr-799, and that phosphorylation at the former site but not the latter decreases the MLCP enzymic activity *in vitro* [28]. Thr-799 phosphorylation of MBS, a Rho kinase-specific phosphorylation site, was decreased by Y27632 (Figures 3A and 3B), and this suggests that Rho kinase cascade is activated by histamine stimulation. However, the phosphorylation at Thr-641, a site responsible for the inhibition of MLCP, was changed by neither histamine stimulation nor Y27632 in smooth muscle fibre, indicating that MBS phosphorylation at Thr-641 is not significantly influenced by

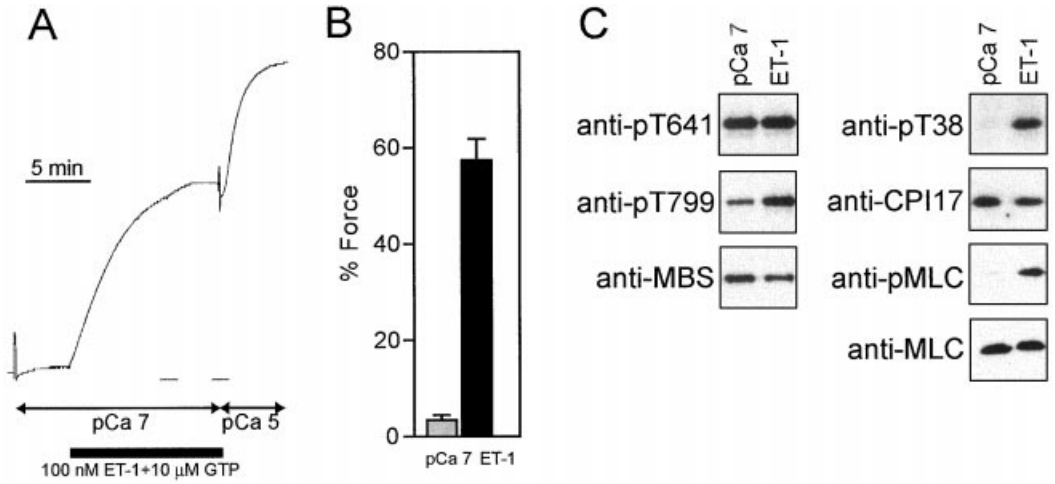

Figure 5 ET-1-induced activation of the contraction of an α-toxin-permeabilized strip of rabbit femoral artery

(**A**) Representative trace of ET-1-induced activation of contraction at constant Ca^{2+} in α-toxin-permeabilized rabbit femoral artery. ET-1 (100 nM) and 10 μM GTP were added at the steady state of the contraction at pCa 7. (**B**) Effect of the ET-1 on the force of the α-toxin-permeabilized strip of rabbit femoral artery at pCa 7. The grey and black bars indicate Ca^{2+} (pCa 7)-induced contraction and ET-1-induced sensitization, respectively. Values are means $\pm$ S.E.M. from four independent experiments and expressed as percentages of the maximum contraction obtained at pCa 5. (**C**) Change in the phosphorylation of MLC, MBS and CPI17 during the ET-1-induced contraction of the α-toxin-permeabilized rabbit femoral artery. Smooth muscle strips were incubated for 5 min in Ca^{2+} solution (pCa 7), then stimulated by 100 nM ET-1. Smooth muscle strips were homogenized and subjected to Western blot using antibodies against pThr-641 of MBS, pThr-799 of MBS, MBS, pThr-38 of CPI17, CPI17, pSer-19 of MLC and MLC.

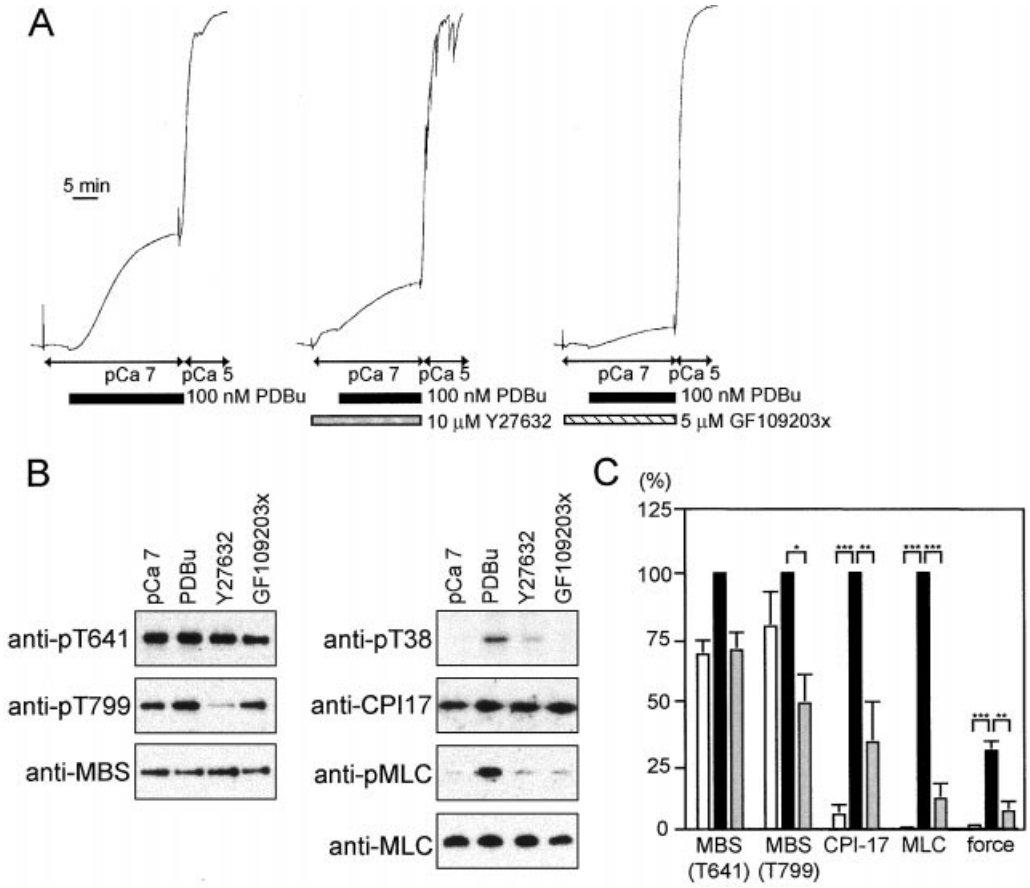

Figure 6 Change in the phosphorylation of MLC, MBS and CPI17 during phorbol ester-induced contraction of α-toxin-permeabilized strips of rabbit femoral artery

(**A**) Representative trace of PDBu-induced activation of contraction at pCa 7. PDBu (100 nM) was added at the steady state of the contraction at pCa 7 in the absence (left trace) and presence of 10 μM Y27632 (middle trace) or 5 μM GF109203x (right trace). Arteries were pretreated with inhibitors for 5 min prior to the addition of PDBu. (**B**) Changes in the phosphorylation of MLC, MBS and CPI17 during the PDBu-induced contraction. Smooth muscle strips were incubated for 5 min in pCa 7, then stimulated for 15 min by 100 nM PDBu in the absence or presence of 10 μM Y27632 or 5 μM GF109203x. Smooth muscle strips were homogenized and subjected to Western blot using antibodies against pThr-641 of MBS, pThr-799 of MBS, MBS, pThr-38 of CPI17, CPI17, pSer-19 of MLC and MLC. (**C**) Effect of Y27632 on the PDBu-induced changes in force development and phosphorylation of MBS, CPI17 and MLC. Inhibitors were applied 5 min before the addition of PDBu. White, black and grey bars indicate levels of phosphorylation or contractions induced by Ca^{2+} alone (pCa 7), Ca^{2+} and PDBu, and PDBu at pCa 7 in the presence of Y27632, respectively. Relative levels of phosphorylation are indicated as a percentage of the value obtained after PDBu stimulation. Values of force are indicated as percentages of the force at pCa 5. All data are means $\pm$ S.E.M. from more than four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Rho kinase cascade (Figures 3A and 3B). Similar effects were observed for treatment with GF109203x, a PKC-specific inhibitor (Figures 2 and 3). To assure the specificity of GF109203x, the inhibitory effects of the inhibitors were tested against MBS phosphorylation by Rho kinase (Figure 4). While Y27632 potently inhibited Rho kinase and the activity was almost completely inhibited at 10 μM, GF109203x showed little inhibitory activity against Rho kinase. Therefore, the change in the phosphorylation level of the proteins tested in the fibre should be attributed to PKC but not Rho kinase. GF109203x significantly inhibited the histamine-induced activation of contraction (Figure 2) and MLC phosphorylation (Figures 3A and 3B). While CPI17 phosphorylation was decreased by GF109203x, MBS phosphorylation at Thr-641 was unchanged by GF109203x. These results suggest that PKC pathway is also involved in the Ca^{2+}-independent activation of contraction and the regulation of CPI17 phosphorylation, an MLCP modulator. The results also suggest that CPI17 phosphorylation is predominantly responsible for the regulation of MLCP and, therefore, the increase in MLC phosphorylation during histamine stimulation. It is interesting that the PKC inhibitor, GF109203x, diminished the phosphorylation at Thr-799 of MBS in the fibre. Because Thr-799 cannot be phosphorylated by PKC *in vitro*, and GF109203x does not inhibit Rho kinase (Figure 4), the results suggest that PKC influences the Rho kinase pathway.

ET-1 also induced a Ca^{2+}-independent contraction of the permeabilized smooth muscle strips (Figure 5). Consistently, MLC phosphorylation was significantly increased by ET-1 stimulation at constant [Ca^{2+}]. During ET-1-induced contraction, CPI17 phosphorylation at Thr-38 significantly increased while MBS phosphorylation at Thr-641 was unchanged. On the other hand, MBS phosphorylation at Thr-799 increased upon ET-1 stimulation. These results suggest that during ET-1 stimulation the phosphorylation of CPI17 increases. This is reflected by the increase in Ca^{2+}-independent MLC phosphorylation, whereas the phosphorylation of MBS is unchanged. This result suggests that CPI17 phosphorylation plays a more significant role in the regulation of MLCP than does MBS phosphorylation, thus affecting MLC phosphorylation in smooth muscle strips. Since the phosphorylation of MBS at Thr-799 increased upon ET-1

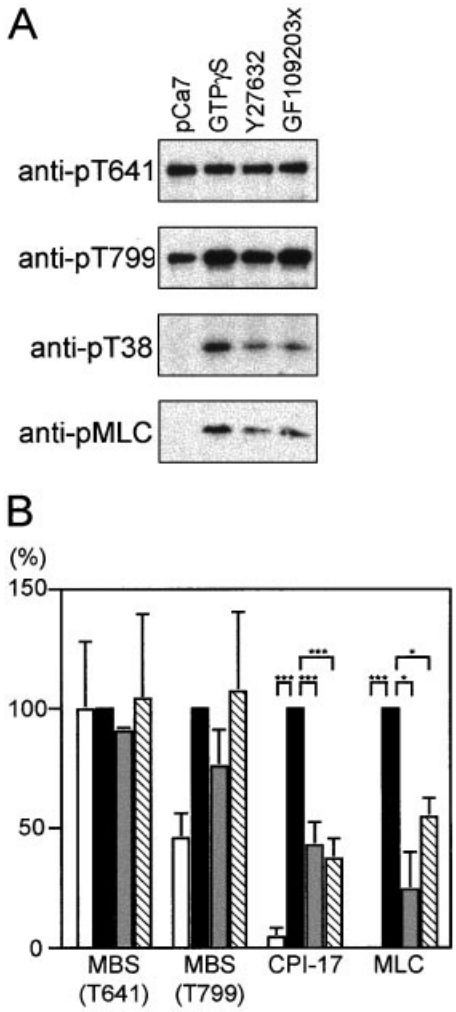

Figure 7 Effect of GTP[S] on the change in the phosphorylation of MLC, MBS and CPI17 in α-toxin-permeabilized strips of rabbit femoral artery

(**A**) Representative Western blot analysis of phospho-MBS, CPI17 and MLC during the GTP[S] (GTPγS)-induced contraction in the absence and presence of 10 μM Y27632 or 5 μM GF109203x. Smooth muscle strips were incubated for 5 min in pCa 7, then stimulated for 10 min with 10 μM GTP[S] in the absence or presence of 10 μM Y27632 or 5 μM GF109203x. Smooth muscle strips were homogenized and subjected to Western blot using anti-pThr-641, anti-pThr-799, anti-pThr-38 and anti-pMLC, respectively. (**B**) Effect of Y27632 and GF109203x on the GTP[S]-induced changes in the phosphorylation of MBS, CPI17 and MLC. Inhibitors were applied 5 min before the addition of GTP[S]. White, black, grey and hatched bars indicate the phosphorylation levels induced by pCa 7 alone, GTP[S] at pCa 7, GTP[S] at pCa 7 in the presence of 10 μM Y27632, and GTP[S] at pCa 7 in the presence of 5 μM GF109203x, respectively. Relative levels of phosphorylation are indicated as a percentage of the value obtained after GTP[S] stimulation. All data are means $\pm$ S.E.M. from more than four independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$.

stimulation, it is anticipated that ET-1 can activate the Rho/Rho kinase cascade, whereas Thr-641 phosphorylation of MBS remains unchanged.

The above results suggest that PKC signalling plays a role in the Ca^{2+}-independent increase in MLC phosphorylation and the contraction of smooth muscle, and that CPI17 phosphorylation is involved in the PKC-induced activation of contraction. We therefore examined the effect of the activation of PKC on CPI17 phosphorylation and MLC phosphorylation more directly. Figure 6 shows the effect of PDBu on force and the phosphorylation of MLC as well as MLCP modulators. PDBu increased force at constant free [Ca^{2+}] (pCa 7) and the increase in MLC phosphorylation at Ser-19. During the PDBu-induced Ca^{2+}-independent increase in force, CPI17 phosphorylation significantly increased. However, no significant increase in MBS phosphorylation was observed for Thr-641 site. Interestingly, PDBu increased the phosphorylation of MBS at Thr-799 and this increase in the phosphorylation at Thr-799 was inhibited by Y27632. In addition, not only GF109203x but also Y23632 significantly diminished PDBu-induced activation of force development and MLC phosphorylation. CPI17 phosphorylation was also significantly attenuated by both inhibitors, although the extent of inhibition by Y27632 was less than by GF109203x (Figure 6). In contrast, MBS phosphorylation at Thr-641 was not affected by these inhibitors. The results further support the idea that CPI17 phosphorylation, but not MBS phosphorylation at Thr-641, is predominantly responsible for the regulation of MLCP upon external stimuli of smooth muscle contraction. It should be noted that the phosphorylation of MBS at Thr-799 was significantly reduced by Y27632. The results suggest that PKC pathway influences the Rho kinase activity in smooth muscle fibre, since PDBu does not directly activate Rho/Rho kinase and PKC cannot directly phosphorylate MBS at Thr-799. The results also support the notion that both PKC and Rho kinase contribute to the phosphorylation of CPI17.

It is known that GTP[S] activates both the membrane-associated heterotrimeric G-protein cascade and the small G-protein cascade and, therefore, most prominently activates Ca^{2+}-independent contraction of smooth muscle. Actually, GTP[S] at pCa 7 induced $66.5 \pm 4.2\,\%$ of maximum contraction obtained at pCa 5, higher than the histamine- or PDBu-induced Ca^{2+}-independent activation at pCa 7 (results not shown). As shown in Figure 7, even in GTP[S]-induced stimulation, MBS phosphorylation at Thr-641 did not change. This result suggests that MBS phosphorylation at Thr-641 does not significantly change in smooth muscle fibre even with GTP[S], a most potent stimulant. Consistently, neither Y27632 nor GF109203x changed the Thr-641 phosphorylation of MBS. In contrast, MLC and CPI17 phosphorylation markedly increased by GTP[S] stimulation, and the both inhibitors diminished the increase in the phosphorylation of MLC and CPI17 (Figure 7). All the above results showed that the phosphorylation of MBS at Thr-641 is not changed during stimulation by various agonists that trigger the Ca^{2+}-independent activation of MLC phosphorylation and muscle contraction.

Agonist-induced change in the phosphorylation of MBS and CPI17 in intact smooth muscle strips

Figure 8 shows histamine-induced contraction of intact rabbit femoral artery. The addition of histamine promptly increased force. Phosphorylation of MLC was significantly increased within 30 s of histamine stimulation and stayed constant (Figures 8B and 8C). As shown in Figure 8(B), CPI17 phosphorylation at Thr-38 was undetectable before stimulation and markedly increased upon histamine stimulation with similar kinetics to the increase in MLC phosphorylation (Figure 8C). The phosphorylation of CPI17 at Thr-38 reached a maximum in 30 s of histamine stimulation and stayed constant. On the other hand, a significant level of MBS phosphorylation was detected at both Thr-641 and Thr-799 under resting conditions (Figure 8B). Histamine stimulation hardly changed the phosphorylation of MBS at Thr-641. Removal of histamine immediately diminished force that is accompanied by a decrease in MLC phosphorylation (Figure 8). During the relaxation CPI17 phosphorylation significantly decreased, whereas the phosphorylation of MBS at Thr-641 was

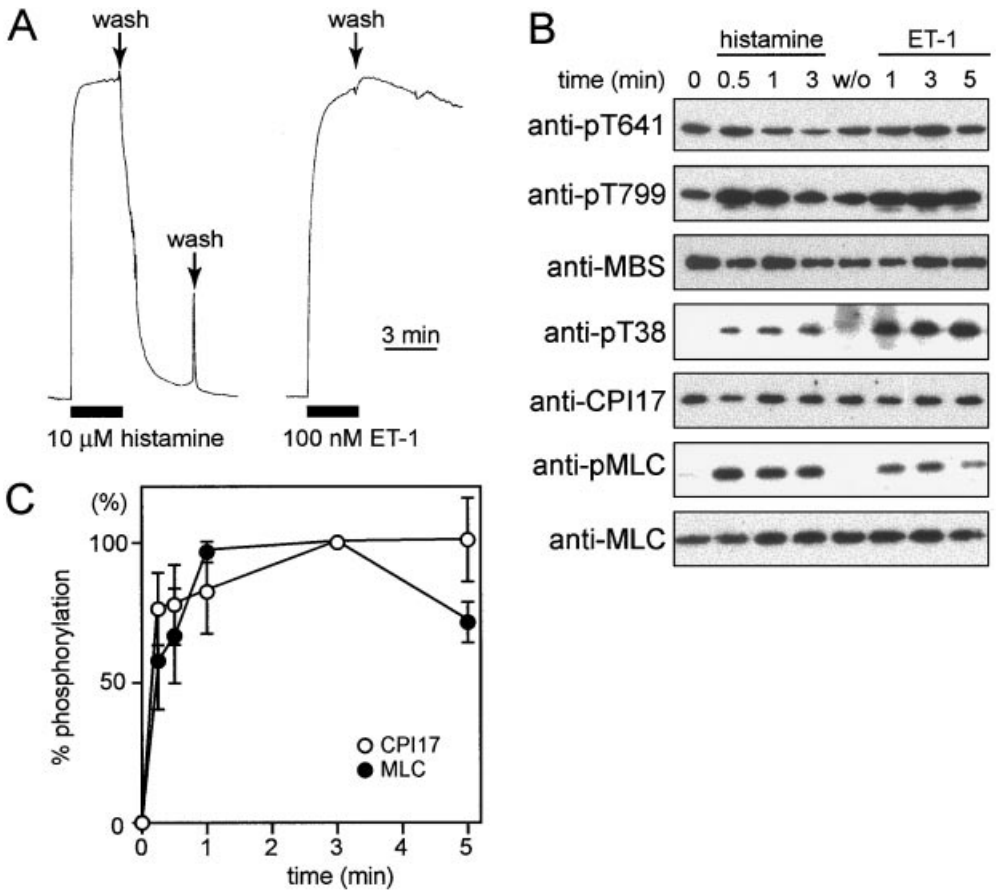

Figure 8 Agonist-induced change in the phosphorylation of MLC, MBS and CPI17 in intact rabbit femoral artery

(**A**) Representative trace of histamine and ET-1-induced contraction of intact rabbit femoral artery. Smooth muscle strips were stimulated for 3 min in the presence of 10 μM histamine (left trace) or 100 nM ET-1 (right trace). After stimulation, agonists were washed out with normal PSS. ET-1-induced contraction was irreversible by washing out, presumably due to the incomplete dissociation of ET-1 from its receptor, whereas histamine was successfully washed out. (**B**) Changes in the phosphorylation of MLC, MBS and CPI17 during the histamine- or ET-1-induced contraction of intact rabbit femoral artery. Smooth muscle strips were incubated for various durations in the presence of 10 μM histamine or 100 nM ET-1. Smooth muscle strips were homogenized and subjected to Western blot using antibodies against pThr-641 of MBS, pThr-799 of MBS, MBS, pThr-38 of CPI17, CPI17, pSer-19 of MLC and MLC. Strips before stimulation (time 0) and washed out by PSS (w/o) after histamine stimulation were also examined. (**C**) Temporal changes in the phosphorylation of CPI17 and MLC during histamine-induced contraction in intact rabbit femoral artery. Relative phosphorylation levels of CPI17 and MLC are indicated as ratios (anti-pThr-38/anti-CPI17 and anti-pMLC/anti-MLC, respectively) of the bands in Western blot. Each value represents the mean $\pm$ S.E.M. from more than three independent experiments and are percentages of the values obtained at 3 min after histamine stimulation.

virtually unchanged. These results clearly indicate that while CPI17 phosphorylation significantly changes during the contraction–relaxation cycle after histamine stimulation in intact vascular smooth muscle, MBS phosphorylation at Thr-641 does not change significantly during the contractile response of smooth muscle. On the other hand, Thr-799 phosphorylation increased upon histamine stimulation, suggesting that the Rho/Rho kinase system was activated upon stimulation.

It is known that ET-1 produced by endothelial cells in blood vessels is an important natural agonist for vascular smooth muscle contraction [38]. ET-1 initiated the contraction of intact femoral arterial smooth muscle strips (Figure 8). MLC phosphorylation increased upon ET-1 stimulation along with the increase in force. ET-1 stimulation also enhanced the phosphorylation of CPI17. The phosphorylation level reached a maximum within 1 min and stayed at a high level, which correlates with the high MLC phosphorylation level and force. In contrast, MBS phosphorylation was not significantly changed by ET-1 stimulation at Thr-641 (Figure 8). The results are similar to those for histamine stimulation, and indicate that vasoconstrictor agonist stimulation changes CPI17 phosphorylation, but not MBS at its inhibitory site, in intact vascular smooth muscle. Washing out the strip with normal PSS could not reverse ET-1-induced contraction, presumably due to the incomplete dissociation of ET-1 from its receptor. Phosphorylation of CPI17 and MLC remained at similar levels to those during contraction (results not shown).

DISCUSSION

There are two major factors regulating MLCP. CPI17, a MLCP-specific inhibitor, requires phosphorylation at Thr-38 for its inhibitory activity. PKC phosphorylates CPI17 *in vitro* and it has been shown that Rho kinase can also phosphorylate CPI17 *in vitro* [37]. Therefore, PKC and/or Rho kinase cascades may regulate CPI17 activity, thus controlling MLCP activity (Figure 9). A second regulator is MBS, a myosin-binding subunit of MLCP. It has been shown that MBS phosphorylation by Rho kinase at Thr-641 decreases MLCP holoenzyme activity *in vitro* [28] (Figure 9). A key issue is whether phosphorylation

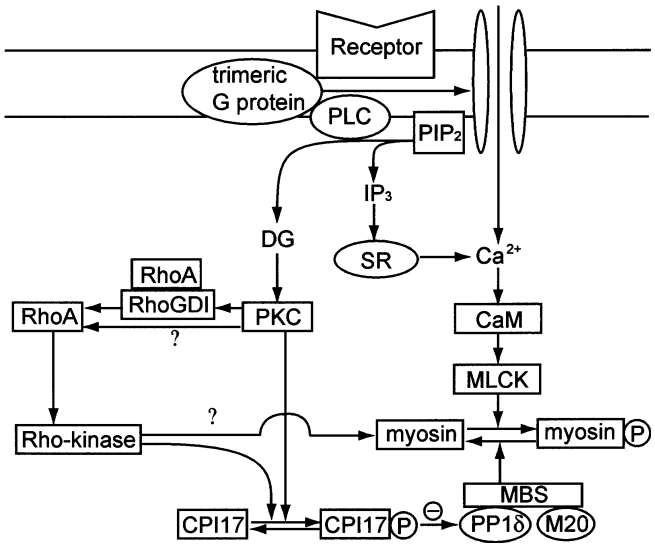

Figure 9 Diagram of the agonist-induced contraction of smooth muscle

Upon the stimulation of specific receptor by vasoconstrictors, both PKC- and Rho kinase-mediated pathways are activated. The PKC pathway may influence the upstream of Rho/Rho kinase pathway via RhoGDI phosphorylation and its dissociation from RhoA. Both Rho kinase and PKC phosphorylate CPI17 thus causing the inhibition of MLCP. The phosphorylation of MBS at its inhibitory site (Thr-641) has a minimal effect on the regulation of MLCP. Rho kinase might phosphorylate myosin, thus activating the contractile machinery directly. PLC, phospholipase C; PIP_2, PtdIns(4,5)P_2; DG, diacylglycerol; IP_3, Ins(1,4,5)P_3; CaM, calmodulin; M20, small non-catalytic subunit; SR, sarcoplasmic reticulum; PP1, protein phosphatase type-1.

of CPI17 and MBS are correlated with the change in MLC phosphorylation.

The present study addressed this question using phosphorylation-site-specific antibodies for Thr-38 of CPI17, Thr-641 and Thr-799 of MBS, and Ser-19 of MLC as probes. This approach enables us to monitor the change in the phosphorylation at the functionally important specific sites of the target proteins in smooth muscle fibres. Since the conventional method using radioactive phosphate incorporation only reports the overall phosphorylation level of the proteins, the present approach is particularly powerful for the proteins phosphorylated at multiple sites. This is particularly important for MBS that is phosphorylated at two or more sites [39], and MLC that can be phosphorylated at Ser-1/Ser-2 and Thr-9 [40] in addition to Ser-19 and Thr-18.

A critical finding of this study is that while CPI17 phosphorylation markedly increased after vasoconstrictor agonist, such as histamine and ET-1, MBS phosphorylation at Thr-641 hardly changed. The results were consistently obtained for both intact and permeabilized fibres. Furthermore, while Y27632, a Rho kinase inhibitor, and GF109203x, a PKC inhibitor, markedly diminished the agonist-induced force activation in permeabilized fibre, these inhibitors did not decrease MBS phosphorylation at Thr-641, the site responsible for the inhibition of MLCP. These results indicate that MBS phosphorylation does not play a significant role in a mechanism for MLCP regulation, at least in the regulation of smooth muscle contraction. On the other hand, CPI17 phosphorylation increased promptly after agonist stimulation. The importance of CPI17 in the Ca^{2+} sensitization mechanism has been reported. Kitazawa et al. [15] showed that the addition of the CPI17 with PKC to permeabilized smooth muscle strips results in the activation of force at a constant [Ca^{2+}], suggesting that CPI17 plays a role in a Ca^{2+}-sensitization mechanism in smooth muscle. Quite recently, it was shown that the phosphorylation of CPI17 in smooth muscle fibre is significantly elevated at the peak force after agonist stimulation compared with that in the resting state [18].

The present results are consistent with earlier reports and further revealed that the kinetics of the increase in CPI17 phosphorylation are fast and that the time course of the increase in MLC phosphorylation after agonist stimulation is closely correlated with the increase in CPI17 phosphorylation at Thr-38. The results further suggest that CPI17 phosphorylation and, therefore, down-regulation of MLCP, contribute to the agonist-induced initial increase in MLC phosphorylation in smooth muscle, in addition to the more prolonged sustained phase of MLC phosphorylation. Recently, Miyazaki et al. [41] reported that the Rho/Rho kinase pathway predominantly contributes to the increase in myosin phosphorylation near the plasma membrane, while the Ca^{2+}/MLCK pathway phosphorylates throughout the cytoplasm of smooth muscle cells. It is thought that CPI17 is a soluble protein that is present throughout cytosol. The cytosolic proteins move in cytosol by diffusion. When CPI17 is phosphorylated by the activated PKC or Rho kinase at the plasma membrane, it is plausible that the phosphorylated CPI17 concentration would be higher near the membrane because the phosphorylated CPI17 can be dephosphorylated with time during the diffusion process, thus contributing to the increase in MLC phosphorylation near the plasma membrane by decreasing MLCP activity.

The increase in MBS phosphorylation by GTP[S] in permeabilized smooth muscle strips [29] and cultured smooth muscle cells [30] has been studied previously by monitoring the incorporation of radiolabelled phosphate into MBS. It was reported that the phosphorylation of MBS was increased after agonist stimulation [29,30]. There is an apparent discrepancy between these previous reports and the present study. The previous method of using radioactive ATP monitors the addition of the phosphorylation at MBS, but not the absolute level of phosphorylation. For this reason, the extent of phosphorylation in the resting state cannot be detected. The approach utilized in the present study enables us to determine the extent of MBS phosphorylation in the resting state, hence the difference in MBS phosphorylation between the resting level and the post-stimulation level. We found a significant level of MBS phosphorylation in the resting state. The estimated MBS phosphorylation level at Thr-641 in the resting state was approx. 60%.

Another critical issue is that MBS is phosphorylated at multiple sites, but Thr-641 is the only site responsible for inhibition of the holoenzyme phosphatase activity [28]. The method used in previous studies was the incorporation of radioactive phosphate into MBS [29,30], and therefore only the overall change in phosphorylation at multiple sites of MBS could be observed, not the change in phosphorylation of MBS at specific sites, i.e. Thr-641. The present approach enables us to determine the change in the phosphorylation of MBS at specific sites, i.e. Thr-641 and Thr-799. Actually, our results showed that the phosphorylation at Thr-799 of MBS changed with GTP[S] as well as with agonists. Therefore, the overall extent of MBS phosphorylation is increased in the present study by GTP[S] stimulation. This is consistent with previous reports [29,30]. But, the important point is that Thr-641, not Thr-799, is the site responsible for phosphatase inhibition. The present results showed that the significant level of phosphorylation at Thr-641 is present at rest that does not significantly change after agonist stimulation. There is a possibility that Thr-641 phosphorylation may increase in a

distinct part of the cell and decrease in another compartment after agonist stimulation, which would make the total level of phosphorylation detected by phospho-specific antibody unchanged. The answer to this requires further study.

Nagumo et al. [30] measured MLCP activity of cultured smooth muscle cells in the presence and absence of GTP[S]. Cells were lysed and then MLCP was immunoprecipitated with antibodies against MBS. It was found that MLCP activity was decreased by approx. 50 % with GTP[S] treatment. The present results do not agree with the result by Nagumo et al. [30], since GTP[S] treatment of the fibre did not change Thr-641 phosphorylation of MBS. One possible explanation for this apparent discrepancy is that the immunoprecipitated MLCP may contain CPI17, thus the phosphatase activity is changed by GTP[S]-induced phosphorylation of CPI17, since CPI17 can be co-immunoprecipitated with MLCP [42].

It has been thought that fast transient contraction after agonist stimulation is due to Ca^{2+} mobilization. The resulting activation of the calmodulin/MLCK pathway for MLC phosphorylation and the down-regulation of MLCP then contributes to the increase in MLC phosphorylation during the prolonged phase after stimulation. However, the present study demonstrates that the increase in CPI17 phosphorylation at Thr-38 eliciting the down-regulation of MLCP is not much slower than the initial increase in MLC phosphorylation after histamine stimulation. Therefore, it is anticipated that MLCP down-regulation by CPI17 phosphorylation plays a role in the increase in MLC phosphorylation after agonist stimulation for both the transient phase and the prolonged phase of contraction. However, it would play a more significant role in the latter phase when Ca^{2+}-dependent activation signalling has ceased.

A question is the identity of CPI17 kinase in smooth muscle fibre. Both Y27632, a Rho kinase-specific inhibitor, and GF109203x, a PKC inhibitor, markedly attenuated CPI17 phosphorylation at Thr-38. The results suggest that both Rho kinase and PKC contribute to the phosphorylation of CPI17 *in vivo* (Figure 9). This is consistent with *in vitro* results that both PKC and Rho kinase can phosphorylate CPI17 at Thr-38 [16,37].

The protein kinase that phosphorylates MBS *in vivo* is complex. It has been thought that the predominant protein kinase that phosphorylates MBS is Rho kinase [27]. While Thr-641 of MBS is not phosphorylated during the agonist-induced stimulation of smooth muscle, the results indicate that agonist stimulation activated the Rho/Rho kinase pathway because phosphorylation of the Rho kinase-specific phosphorylation site, Thr-799 of MBS, increased by agonist stimulation and the Rho kinase inhibitor reversed the agonist-induced increase in Thr-799 phosphorylation. Our results also indicate that the increase in the phosphorylation of Thr-799 on MBS by histamine stimulation is mediated by Rho kinase because of its sensitivity to a Rho kinase inhibitor; however, GTP[S] may activate other kinases which can phosphorylate Thr-799 on MBS since the increase in the phosphorylation of Thr-799 by GTP[S] was only partially inhibited by Y27632 (Figure 7). Nevertheless, Thr-641 phosphorylation, another Rho kinase phosphorylation site of MBS *in vitro*, was changed neither by agonist stimulation nor by GTP[S] stimulation. Moreover, a significant level of Thr-641 phosphorylation is observed under resting conditions. These results suggest that Thr-641 can be phosphorylated by protein kinases other than Rho kinase, and Rho kinase does not readily phosphorylate MBS in response to agonist stimulation *in vivo*, presumably because the Thr-641 is already phosphorylated by other kinases in the resting state, while the phosphorylation level of Thr-799 under resting conditions is lower than Thr-641 and can be increased by Rho kinase upon stimulation. Recently, MacDonald et al. [43] reported that a zipper-interacting protein kinase ('ZIP kinase') fragment can associate with MBS and utilize MBS as a substrate. It was also reported that integrin-linked kinase can phosphorylate MBS at Thr-641 *in vitro*, although Thr-641 is not the preferred site [44]. Further studies are required to identify the protein kinase responsible for the phosphorylation of MBS at Thr-641 *in vivo*. While this manuscript was being prepared, Shin et al. [45] reported that prostaglandin $F_{2\alpha}$ increased MBS phosphorylation at Thr-641 in ferret portal vein, although the change in CPI17 phosphorylation was not determined. Therefore, MBS phosphorylation may contribute to MLCP inactivation under certain types of smooth muscle stimulation.

Since a significant level of Thr-641 phosphorylation is observed in the resting state in both intact and permeabilized smooth muscle, it is reasonable to assume that Thr-641 phosphorylation is not subjected to a rapid phosphorylation–dephosphorylation cycle *in vivo* but that it is rather stable. Consistent with this notion, we found that Thr-641 phosphorylation is resistant to dephosphorylation by major protein phosphatases [46]. MBS phosphorylation at Thr-641, hence the regulation of MLCP via MBS, might function in slower cellular processes such as cell migration or gene regulation.

An interesting observation is that GF109203x, a PKC-specific inhibitor, markedly diminished the phosphorylation of MBS at Thr-799, a Rho kinase-specific phosphorylation site (Figure 3). PKC actually does not phosphorylate the Thr-799 site *in vitro* (results not shown) and GF109203x hardly inhibited Rho kinase (Figure 4). Therefore, it is reasonable to assume that PKC is involved in the activation pathway of Rho kinase. Consistent with this notion, Y27632 inhibited MLC phosphorylation during PDBu-induced stimulation (Figure 6). Since PDBu does not directly activate Rho/Rho kinase, the results suggest that PKC activated by PDBu stimulates the Rho/Rho kinase pathway. Recently, it was reported [47] that PKC phosphorylates RhoGDI (where 'GDI' is guanine nucleotide dissociation inhibitor), and this is correlated with the increase in Rho activity, presumably due to the change in the interaction between GDI and Rho. The present finding is consistent with this report, although the mechanism by which PKC modulates the Rho pathway (Figure 9) is not clear at this moment and further studies are required.

The present study demonstrates that the regulation of MLCP during agonist-induced contraction in smooth muscle is primarily mediated via CPI17 phosphorylation at Thr-38. Another pathway of the regulation of MLCP, i.e. the phosphorylation of MBS at Thr-641, would play a more important role in the regulation of myosin light chain phosphorylation in non-muscle cell systems, such as platelets [42] and cultured non-muscle cell lines [48].

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