cGMP-Dependent Relaxation of Smooth Muscle Is Coupled With the Change in the Phosphorylation of Myosin Phosphatase

Kensei Nakamura,* Yasuhiko Koga,* Hiroyasu Sakai, Kazuaki Homma, Mitsuo Ikebe

Abstract—Nitric oxide/cGMP pathway induces vasodilatation, yet the underlying mechanism is obscure. In the present study, we studied the mechanism of cGMP-induced relaxation of the smooth muscle contractile apparatus using permeabilized rabbit femoral arterial smooth muscle. 8-Br-cGMP–induced relaxation was accompanied with a decrease in myosin light chain (MLC) phosphorylation. MLC phosphatase (MLCP) activity, once decreased by agonist-stimulation, recovered to the resting level on addition of 8-Br-cGMP. Because MLCP activity is regulated by the phosphorylation of a MLCP-specific inhibitor, CPI17 at Thr38 and MBS (myosin binding subunit of MLCP) at Thr696, we examined the effect of 8-Br-cGMP on the phosphorylation of these MLCP modulators. Whereas CPI17 phosphorylation was unchanged after addition of 8-Br-cGMP, MBS phosphorylation at Thr696 was significantly decreased by 8-Br-cGMP. We found that 8-Br-cGMP markedly increased MBS phosphorylation at Ser695 in the fiber pretreated with phenylephrine. MBS phosphorylation of Thr696 phosphorylated MBS at Ser695 partially resumed MLCP activity inhibited by Thr696 phosphorylation. Whereas Ser695 phosphorylation was markedly increased, the extent of diphasphorylated MBS at Ser695 and Thr696 in fibers was unchanged after cGMP-stimulation. We found that MBS phosphatase activity in arteries for both diphasphorylated MBS and monophosphorylated MBS at Thr696 significantly increased by 8-Br-cGMP, whereas MBS kinase activity was unchanged. These results suggest that the phosphorylation at Ser640 induced by cGMP shifted the equilibrium of the Thr641 phosphorylation toward dephosphorylation, thus increasing MLCP activity. This results in the decrease in MLC phosphorylation and smooth muscle relaxation. (Circ Res. 2007;101:712-722.)

Key Words: cGMP • myosin light chain phosphatase • vasodilation • phosphorylation • smooth muscle

It has been known that endothelial-derived nitric oxide (NO) acts as a vasodilator,1 and the pharmacological NO producing drugs have been used to prevent acute heart failure. NO has been defined as the activator of soluble guanylate cyclase,2 thus increasing cGMP. A number of studies have indicated that cGMP induces relaxation of various smooth muscles contracted either by receptor-coupling agonists or depolarization; therefore, NO-induced vasodilation is thought to be attributable to the cGMP-induced relaxation of vascular smooth muscle. The key question is how cGMP triggers the vascular smooth muscle relaxation. Smooth muscle contraction is controlled by the phosphorylation of the regulatory light chain (RLC) of myosin at Ser194,5 by Ca2+/calmodulin-dependent protein kinase, called myosin light chain kinase (MLCK).4–6 On the other hand, MLC phosphatase (MLCP) activity is also regulated during the agonist-induced contraction of smooth muscle, thus contributing to the increase in RLC phosphorylation, but in contrast to the regulation of MLCK, the mechanism is Ca2+ independent.6

MLCP consists of 3 subunits, a myosin binding large subunit (MBS),7,8 a 20-kDa small subunit (M20), and a catalytic subunit of the type 1 protein serine/threonine phosphatase family.8–10 It has been shown that Rho-kinase11,12 phosphorylates MBS which results in a decrease in MLCP activity in vitro.13 Rho-kinase phosphorylates MBS at 2 sites in vitro, ie, Thr696 and Thr853, among which Thr696 is responsible for the inhibition of MLCP activity.14 On the other hand, the inhibitory activity of a MLCP specific inhibitor protein, CPI17, which is specifically expressed in smooth muscle with high expression in arterial smooth muscle,15 is increased more than 100-fold by phosphorylation at Thr38.16 Both protein kinase C15,16 and Rho-kinase can phosphorlyate CPI17 in vitro.17 Early studies have revealed that cGMP decreases cytosolic Ca2+.7 It was reported that cGMP induces the decrease in Ca2+ sensitivity18 and subsequently, it was found that cGMP induces the relaxation of smooth muscle with constant Ca2+19. These results indicate that cGMP-signaling can directly influence the contractile machinery in addition to the effect on Ca2+ homeosta-
sis. A critical question is how cGMP can regulate MLC phosphorylation, thus smooth muscle contraction.

Wooldridge et al20 reported that cGMP increases Ser695 phosphorylation in the β-scnned skinned ileum smooth muscle and this phosphorylation prevents the Thr696 phosphorylation induced by the addition of ZIP kinase like kinase into the fiber. However, it is obscure how cGMP induces the decrease in MLC phosphorylation in smooth muscle and thus relaxation.

In the present study, we attempted to clarify the mechanism of cGMP-induced downregulation of smooth muscle contractile machinery. As a result, we found that MLCP activity downregulated by agonist-stimulation is reactivated by cGMP to the resting level, thus decreasing RLC phosphorylation. Our results indicated that MBS phosphorylation at the inhibitory Thr696 site but not CPI17 phosphorylation is decreased during the cGMP-induced relaxation, thus reactivating MLCP activity and reducing RLC phosphorylation. By analyzing the change in the amount of phosphorylated MBS at various sites after cGMP-stimulation, we concluded that MBS phosphatase activity is activated by cGMP stimulation in arterial ring, thus decreasing the MBS phosphorylation at the inhibitory site.

Materials and Methods

An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org. In brief, the rabbit femoral arteries were prepared and arterial rings were permeabilized with α-toxin.21 The permeabilized arteries were stimulated by agonists at constant [Ca2+] and then 8-Br-cGMP–induced relaxations were monitored and the change in force was compared with the changes in the MLCK, MLCP, MBS kinase, and MBS phosphatase activities. MBS was purified and used for MBS kinase and phosphatase assay as a substrate. The agonist and cGMP-dependent changes in the phosphorylation levels of MBS, CPI-17, and MLC of the arteries were analyzed by Western blotting using the phosphorylation site-specific antibodies.

Results

Effects of cGMP on Tension Development and MLC Phosphorylation in the α-Toxin-Permeabilized Artery

Figure 1a shows the typical force trace of α-toxin–permeabilized arterial ring. The administration of phenylephrine at pCa 6.5 increased the tension to 44.1±11.6% of the maximum contraction achieved at pCa 5.0. The subsequent addition of 8-Br-cGMP, significantly decreased the tension to 3.5±3.5% (n=4, Figure 1b). MLC phosphorylation at Ser19 at pCa 6.5 was significantly increased by phenylephrine in parallel with the increased tension development (Figure 1c).

An additional application of 8-Br-cGMP decreased the level of phosphorylated MLC to 35.5±7.2% of that obtained after the phenylephrine-stimulation but before the addition of 8-Br-cGMP (n=4; Figure 1c and 1d). This phosphorylation level was the same as before phenylephrine-stimulation. These results suggest that cGMP-induced relaxation is caused by the decrease in the MLC phosphorylation.

Effect of cGMP on the Activity of MLCK and MLCP in Fiber

The effects of cGMP on MLCK and MLCP activities in the fibers were examined. The effect of cGMP on MLCK activity was examined by comparing the tension development between 8-Br-cGMP–treated arterial ring and the control in the presence of calyculin A, a potent MLCP inhibitor. The application of calyculin A increased tension development in both 8-Br-cGMP–treated ring and the control (Figure 2a), which is attributable to the increase in MLC phosphorylation induced by MBS kinases including Ca2+-independent MLC kinases in the presence of MLCP inhibitor. Therefore, the rate of tension development is thought to reflect the increase in MLC phosphorylation. The rate of tension development was virtually the same between these 2 groups, suggesting that the effect of cGMP on MLC kinases is minimal (Figure 2b). Furthermore, we directly determined the effect of cGMP on MLC kinase activity. cGMP-treated and -untreated arterial rings were homogenized in the presence of calyculin A and the phosphorylation of isolated smooth muscle myosin was measured. As a result, cGMP treatment did not decrease the MLC kinase activity (not shown).

The effect of cGMP on the MLCP activity was examined by measuring MLCP activity in the rings. MLCP activity after phenylephrine-stimulation was significantly lower than that before phenylephrine-stimulation (Figure 2c). The result is consistent with earlier studies,22 suggesting that agonist-induced Ca2+ independent activation of tension is attributable to the decrease in MLCP activity. On the other hand, MLCP activity after the treatment of 8-Br-cGMP was significantly higher than before the addition of cGMP. These results suggest that cGMP-induced decrease in MLC phosphorylation in the strips is mainly attributable to the increased activity of MLCP. The important finding is that MLCP activity after cGMP-administration was not virtually increased, but resumed to the activity level before the agonist-stimulation.

CGRP-Induced Change in Phosphorylation of MBS and CPI17

We examined whether the addition of 8-Br-cGMP changes MBS phosphorylation at Thr696 or the CPI17 phosphorylation at Thr38. The MBS phosphorylation at T696 was unchanged after phenylephrine-stimulation. However, the additional application of 8-Br-cGMP decreased the signal to 66.2±8.4% of the value obtained from the phenylephrine-stimulated arterial ring (n=5). On the other hand, CPI17 phosphorylation at Thr38 increased by phenylephrine remained practically unchanged after cGMP-stimulation (Figure 3a and 3b).

Specificity of the Antibodies Recognizing Ser695 and Thr696 Phosphorylation of MBS

Previous studies have shown that PKG phosphorylates MBS at 3 sites in vitro, ie, Ser692, Ser695 and Ser852 in human MBS.20 Among these sites, we focused our attention to Ser695, which resides right next to the inhibitory phosphorylation site, Thr696. To monitor the change in MBS phosphorylation at Ser695 and Thr696 in arterial rings, we produced specific antibodies that recognize phospho-Ser695 and diphospho-Ser695/Thr696 of MBS, respectively. As shown in Figure 4a, pSer695Ab recognized WT-MBS phosphorylated by PKG but failed to recognize either phosphorylated Ser695AlaMBS by PKG or unphosphorylated MBS, indicating...
that the antibody specifically recognizes phosphorylated MBS at Ser695. To evaluate the specificity of the pS695/T696Ab, we produced phosphorylated MBS at both Ser695 and Thr696 by sequential phosphorylation by Rho-kinase and PKG.

We monitored the extent of phosphorylation of unphosphorylated and Rho-kinase–phosphorylated MBS by PKG. The result showed that the extent of phosphate incorporation (γ-32P) by PKG was unaffected by prior incorporation of nonradioactive phosphate to MBS by Rho-kinase (Figure 1S), suggesting that Thr696 MBS phosphorylation does not interfere the incorporation of Pi into Ser695. As shown in Figure 4b, pS695/T696Ab reacted with wMBS sequentially phosphorylated by Rho-kinase and PKG but not with unphosphorylated MBS. Furthermore, it did not recognize the Thr696MBS and Ser695AlaMBS phosphorylated by the above 2 kinases. These results indicate that pS695/T696Ab can only recognize MBS phosphorylated at both Ser695 and Thr696. On the other hand, pT696Ab recognized S695AMBS phosphorylated by these kinases, consistent with our previous report. As expected, pS695Ab recognized T696A MBS phosphorylated by Rho-kinase and PKG, but not wMBS phosphorylated at both Ser695 and Thr696, indicating that pS695Ab specifically recognizes phospho-Ser695/unphospho-Thr696 MBS. It should be mentioned that the signal intensities of both pSer695Ab and pS695/T696Ab were linearly increased with the concentration of phosphorylated MBS at the corresponding sites (Figure 2S).

**Phosphorylation Site-Dependent Differential Dephosphorylation Rate of MBS**

Using these phosphorylation site-specific antibodies, we studied the dephosphorylation rate of MBS at Ser695 and Thr696 by MBS phosphatase in arterial rings. MBS phosphorylated at Ser695/Thr696, Thr696, and Ser695, respectively, was dephosphorylated with arterial homogenates in the conditions eliminating the protein kinase activity described in Material.
and Methods, then the decrease in the MBS phosphorylation was examined by Western blot. As shown in Figure 5a, the signal intensity probed by pS695/T696Ab was rapidly decreased indicating the rapid dephosphorylation of diphosphorylated MBS. The dephosphorylation of phospho-Thr696 of MBS was monitored with pThr696Ab. The rate of dephosphorylation was fast and comparable to that of dephosphorylation of diphosphorylated MBS. On the other hand, the dephosphorylation of phospho-Ser695 MBS was significantly slower that that of phospho-Thr696.

These results suggest that diphosphorylated MBS is primarily converted to monophosphorylated MBS at Ser695 but not Thr696 by MBS phosphatase. To evaluate this notion, we examined the conversion of diphosphorylated MBS to monophosphorylated MBS by using pSer695 Ab (Figure 5b). At time 0, the signal of phosphorylated MBS at Ser695 was weak. The weak signal is likely attributed to the presence of a residual amount of monophosphorylated MBS at Ser695. After adding the arterial homogenates, the signal was increased with time.
Because the experiment was done in the conditions eliminating the protein kinase activity including the presence of the protein kinase inhibitors, it is unlikely that the apparent increase in the amount of phosphorylated Ser695 was attributable to the Ser695 phosphorylation of dephosphorylated MBS. Actually, the incorporation of \(^{32}\)P into MBS by arterial homogenates was markedly diminished in the presence of these inhibitors (Figure 3S). The result is explained that diphosphorylated MBS was rapidly dephosphorylated at Thr696 to produce monophosphorylated MBS at Ser695, thus increasing the signal intensity recognized by pS695Ab. The result further supports the notion that dephosphorylation rate of diphosphorylated MBS at Thr696 is much greater than that of monophosphorylated MBS at Thr696 (Figure 5a). Because the rate of decrease in the amount of diphosphorylated MBS was similar to that of monophosphorylated MBS at Thr696 (Figure 5c), the results suggest that dephosphorylation rate at Thr696 is unchanged by the phosphorylation of neighboring residue of Ser695.

**Effect of cGMP on MBS Kinase and MBS Phosphatase Activities in Smooth Muscle Fiber**

We tried to clarify the mechanism of cGMP-induced change in MBS phosphorylation. Because MBS phosphorylation level is determined by the balance of MBS kinase (protein kinase that phosphorylates MBS) activity and MBS phosphatase (protein phosphatase that dephosphorylates MBS) activity, we examined the effect of 8-Br-cGMP on these enzyme activities in the arterial rings. MBS kinase activity was measured by monitoring MBS phosphorylation at Thr696 in the presence of phosphatase inhibitor (Figure 5d). As shown in Figure 5d, cGMP did not change MBS kinase activity.

MBS phosphatase activity was measured using phosphorylated MBS at various sites as a substrate. cGMP significantly increased MBS phosphatase activity (Figure 5a). Both the rates of dephosphorylation of diphosphorylated and monophosphorylated MBS at Thr696 were significantly enhanced by cGMP (Figure 5a). On the other hand, Ser695 dephosphorylation was slightly enhanced by cGMP, although the rate was slow even after cGMP-stimulation.

To examine whether Ser695 MBS phosphorylation affects Thr696 MBS phosphorylation, dephosphorylated MBS and prephosphorylated MBS at Thr696 by PKG were phosphorylated by arterial homogenates with protein phosphatase inhibitor, and Thr696 phosphorylation was measured by using pT696Ab and pS695Ab, respectively (Figure 5e). The results clearly indicate that MBS phosphorylation rate at Thr696 is unchanged by the phosphorylation of neighboring residue of Ser695.

**Effect of MBS Phosphorylation at Ser695 on MLCP Activity**

We examined the effect of Ser695 MBS phosphorylation on MLCP activity. Thr696AlaMBS was phosphorylated with PKG and ATP-γ-S and subjected to MLCP assay. The dephosphorylation rate of phosphorylated myosin was unchanged by Ser695 MBS phosphorylation (Figure 6a). We also examined the effect of Ser695 phosphorylation of MBS prephosphorylated at Thr696 on the MLCP activity. As shown in Figure 6b, Thr696 MBS phosphorylation by Rho-kinase decreased MLCP activity, and the subsequent phosphorylation at Ser695 resumed the activity, suggesting that Ser695 phosphorylation cancels the inhibitory effect of Thr696 phosphorylation. Supporting this notion, this effect was diminished with Ser695Ala mutation. On the other hand, the inhibitory
Figure 5. Phosphorylation and dephosphorylation of MBS at Ser695 and Thr696 by arterial ring homogenates. a, Effect of cGMP on the dephosphorylation rate of the phosphorylated MBS by arterial homogenates. Permeabilized arteries with or without cGMP-stimulation were used. The diphospho-Ser695/Thr696, mono-phospho-MBS at T696 or S695 was dephosphorylated by arterial homogenates as described in Material and Methods. The changes in MBS phosphorylation were monitored by Western blot. The y axis is the fractional value of MBS phosphorylation with the value 1.0 at 0 minutes. The data represent mean±SEM (n=3). b, Dephosphorylation of diphospho-Ser695/Thr696 MBS by arterial homogenates. The diphosphorylated MBS produced by Rho-kinase and PKG was dephosphorylated by arterial homogenates and the change in MBS phosphorylation was monitored by Western blot. c, MBS dephosphorylation at Ser695 or Thr696 by arterial homogenates. The diphospho-Ser695/Thr696 MBS, mono-phospho-MBS at T696 or S695 was dephosphorylated by arterial homogenates. The y axis is the fractional value of MBS phosphorylation with the value 1.0 at 0 minutes. The data represent mean±SEM (n=2 to 4). d, Effects of cGMP on MBS kinase activity. Phenylephrine-stimulated arterial ring with or without cGMP-stimulation was used for MBS kinase assay as described in Material and Methods. The y axis is an arbitrary unit. The data represent mean±SEM (n=3). e, Effect of Ser695 prephosphorylation on Thr696 MBS phosphorylation. Unphosphorylated MBS or phosphorylated MBS by PKG was incubated with Rho-kinase (5 μg/mL) and 0.2 μmol/L microcystin LR, and the change in the Thr696 phosphorylation was monitored. As for unphosphorylated MBS, phosphorylation at Thr696 was monitored using pT696Ab. Phosphorylation at Thr696 of pre-phosphorylated MBS at Ser695 was monitored using pS695/T696Ab.
cGMP-Induced Changes in MBS Phosphorylation at Ser695 and Thr696 in the \(\alpha\)-Toxin–Permeabilized Artery

We examined the effect of agonist and cGMP on MBS phosphorylation in \(\alpha\)-toxin-permeabilized femoral arteries. Although Thr696 MBS phosphorylation level was unchanged after Phenylephrine-stimulation, the extent of Thr696 phosphorylation was significantly decreased by cGMP-stimulation from 0.29 mol/mol to 0.15 mol/mol (Figure 7a). The time course of the decrease in MBS phosphorylation at Thr696 after cGMP-stimulation was comparable to the decrease in MLC phosphorylation (Figure 7b). On the other hand, MBS phosphorylation at Ser695 markedly increased after cGMP-stimulation from 0.17 mol/mol to 0.43 mol/mol. Of interest is that the level of diphospho-Ser695/Thr696 MBS (0.27 mol/mol) was not significantly changed even after cGMP-stimulation (Figure 7a). The detected signal represents diphosphorylated MBS in the fiber because the addition of calcineurin A to the ring markedly increased the signal intensity recognized by diphosphorylated MBS specific-antibody (Figure 7c).

The amount of pSer695 increased significantly after cGMP stimulation, presumably because of the activation of PKG phosphorylating Ser695. It is expected that the increase in the concentration of pSer695 results in the increase in the diphosphorylated MBS if the equilibrium between pSer695 and pThr696/pSer695 are unchanged by cGMP-stimulation. As described above, the dephosphorylation rate of diphosphorylated MBS at Thr696 was significantly increased by cGMP stimulation. We think that the increased dephosphorylation rate of diphosphorylated MBS at Thr696 by cGMP counteracts the effect of cGMP-induced increase in pSer695 concentration in smooth muscle.

Simulation of the Change in MBS Phosphorylation at Thr696 and Ser695 After cGMP-Stimulation

Based on the results obtained in the present study, we performed computer simulation of the change in MBS phosphorylation after cGMP stimulation (Figure 8a). The following conditions were applied for the calculation. (1) Thr696 dephosphorylation is increased 2-fold by cGMP-stimulation. (2) Thr696 dephosphorylation of diphosphorylated MBS is increased 2-fold by cGMP-stimulation. (3) Thr696 phosphorylation rate is unchanged by cGMP-stimulation. (4) Thr696 phosphorylation rate is unchanged by cGMP-stimulation. (5) Ser695 phosphorylation rate is significantly decreased when Ser695 is phosphorylated. (6) Ser695 phosphorylation rate is markedly increased after cGMP-stimulation. As shown in Figure 8a, cGMP significantly decreases Thr696 monophosphorylated MBS, increases Ser695 monophosphorylated MBS, and only slightly increases diphosphorylated MBS. Quite interestingly, when we calculated the distribution of MBS species without considering the inhibition of MBS phosphorylation at Thr696 by Ser695 phosphorylation, the level of Thr696 monophosphorylated MBS after cGMP-stimulation was calculated to be 0.14 mol/mol, and was not significantly changed.

Discussion

The present study addressed the mechanism by which cGMP induces the relaxation of vascular smooth muscle contractile machinery. cGMP induced decrease the relaxation of \(\alpha\)-toxin-permeabilized femoral artery, and this was accompanied with a decrease in MLC phosphorylation. The result is consistent with the previous report.\(^1\) Because PKG does not phosphorylate Ser19 MLC, it is anticipated that cGMP indirectly changes MLC phosphorylation either by activating MLC phosphatase activity or by inactivating MLC kinase activity. We found that MLC activity but not MLCK activity in smooth muscle was affected by cGMP. A critical finding is that MLC activity is decreased by agonist stimulation, and the subsequent addition of cGMP resumed MLC activity to the resting level. Previously, Lee et al (1997)\(^2\) reported that cGMP enhanced the dephosphorylation of MLC phosphorylation. They compared the rate of MLC dephosphorylation in smooth muscle at PCa5 with or without cGMP and reported that cGMP significantly increased MLC dephosphorylation rate. It is plausible that MLC activity is partially inhibited at high Ca\(^{2+}\). Supporting this notion, it has been reported that high K\(^+\) also induces Ca\(^{2+}\) sensitization suggesting that high Ca\(^{2+}\) may influence the signaling pathway affecting MLCP activity.\(^24\,25\)

It was shown previously\(^21\,26\) that agonist-induced downregulation of MLC activity in arterial smooth muscle is accompanied with the increase in CPI17 phosphorylation but not MBS phosphorylation at the inhibitory site (Thr696). Therefore, we anticipate that CPI17 phosphorylation level at Thr38 but not MBS at Thr696 changes by cGMP. However, to our surprise, we found that Thr696 MBS phosphorylation but not CPI17 phosphorylation changed after cGMP stimulation, although Thr696 MBS phosphorylation was unchanged by agonist which is consistent with earlier studies.\(^21\,26\) The result is different from

- **Figure 6.** Effect of MBS phosphorylation at Ser695 on MLCP activity. a, Effects of PKG-induced MBS phosphorylation on MLCP activity. Open circles represent unphosphorylated control and closed circles represent PKG-treated MLCP. b, PKG-induced disinhibition of MLCP activity. MLCP with WT MBS or Ser695Ala MBS was thiophosphorylated by Rho-kinase\(^2\) alone (open square) or by Rho-kinase followed by PKG (closed square) with 0.1 mmol/L ATP=γS. Then, the thiophosphorylated MLCP was subjected to MLCP assay.

Open circles represent unphosphorylated MLCP (control). Data represent mean±SEM (n=3). \(^*\)<0.05 vs Rho-kinase treated MLCP.
that of Bonnevier and Arner (2004) who reported that PDBu induced Thr38 CPI17 phosphorylation in permeabilized small intestinal smooth muscle decreases after cGMP-stimulation. In the present study, we used arterial smooth muscle expressing significant amount of CPI17, much greater than gastrointestinal smooth muscle. The apparent contradictory results may be attributable to the difference in the type of smooth muscle having different expression level of CPI17. The other

Figure 7. A change in MBS phosphorylation at Thr696, Ser695, and Ser695/Thr696 in the artery after cGMP-stimulation. a, cGMP-induced changes in phospho-Thr696, phospho-Ser695, and diphospho-Ser695/Thr696 MBS in the α-toxin-permeabilized arteries. Arterial rings were stimulated and the samples were used for Western blotting. The calibration curves of the antibodies were obtained by using isolated phospho-MBS. The amounts of phospho-MBS in the samples were within the linear range of the corresponding standard curves. Phosphorylation level was estimated based on the obtained standard curves. The phosphorylation level of MBS phosphorylated by recombinant protein kinase to the maximum level was assigned to be a 100%. The stoichiometry of the phosphorylation was \(0.28\pm0.1\) mol/mol of mono-phosphorylated MBS at Thr696 and \(0.26\pm0.04\) mol/mol of diphosphorylated MBS at rest, and \(0.17\pm0.02\) mol/mol of Ser695 was phosphorylated after cGMP-stimulation. The data represent mean±SEM (n=3) b, Time course of cGMP-induced dephosphorylation of MBS at Thr696 and MLC in the arteries. cGMP-treated arterial rings were quickly frozen at the indicated times, then the samples were subjected to Western blot. The y axis is the fractional value of MBS phosphorylation with the value 1.0 and 0 for before and 30 minutes after cGMP-stimulation, respectively. Data represent mean±SEM (n=3). c, The effect of calyculin A on the diphosphorylation of MBS at Ser695/Thr696 in the artery. The diphosphorylation in the artery was measured using calyculin A–treated arterial ring. Calyculin A (1 μmol/L) was applied to the artery for 15 minutes before cGMP stimulation. The samples were obtained 20 minutes after cGMP-stimulation.
The possibility is the difference in the stimulant between the 2 studies. Bonnevier and Arner (2004) used PDBu, a strong pharmacological activator of PKC, for the stimulation of CPI17 phosphorylation, whereas we used phenylephrine, a G protein–coupled receptor agonist activating RhoA/Rho-kinase signaling in addition to PKC pathway. Because both PKC and Rho-kinase can phosphorylate CPI17, it is plausible that cGMP diminishes CPI17 phosphorylation solely induced by PKC pathway but not CPI17 phosphorylation induced by G protein–coupled agonist-stimulation which significantly activates RhoA/Rho-kinase pathway. It was reported that Thr853 MBS phosphorylation, a Rho-kinase specific site, decreases the affinity of MBS to myosin in vitro, and it has been thought that this phosphorylation may play a role in Rho-kinase–dependent downregulation of MLCP. However, our result showed that CPI117 phosphorylation, in which Rho-kinase plays a significant role, is unaffected by cGMP, suggesting that cGMP does not diminish RhoA/Rho-kinase activity because CPI17 phosphorylation is markedly inhibited by a Rho-kinase inhibitor after agonist stimulation, which activates RhoA/Rho-kinase pathway. Therefore, it is less likely that Thr853 MBS phosphorylation has a predominant role in cGMP-induced decrease in MLC phosphorylation, although further study may be required for the possible involvement of Thr853 phosphorylation in cGMP-induced relaxation.

Because PKG phosphorylates Ser695, right next to the inhibitory site, we examined whether Ser695 phosphorylation affects MLCP activity. Although Ser695 MBS phosphorylation by itself did not alter MLCP activity, Ser695 MBS phosphorylation prephosphorylated at Thr696 reversed the inhibition of MLCP activity. We thought that the production
of diphosphorylated MBS explains cGMP-induced decrease in MLC phosphorylation in smooth muscle. However, the amount of diphosphorylated MBS at Ser695 and Thr696 in arterial ring was unchanged after cGMP-stimulation. The result suggests that the increase in MLCP activity by cGMP in the arterial ring (Figure 2c) is not predominantly attributable to the change in the formation of diphosphorylated MBS at both Thr696 and Ser695. However, it is plausible that the change in the diphosphorylation of MBS might be operating in certain cell types thus contributing to the regulation of MLC phosphorylation.

One of the most important findings of the present study is that cGMP decreases MBS phosphorylation at the inhibitory site, thus releasing the inhibition of MLCP leading MLC dephosphorylation and smooth muscle relaxation. Because MBS phosphorylation should be determined by the relative activity of MBS kinases and MBS phosphatases (the kinase and the phosphatase using MBS as a substrate), we examined the effect of cGMP on these enzyme activities. We found that MBS phosphatase is significantly activated by cGMP-stimulation. The result is consistent with the finding that cGMP stimulation decreases MBS phosphorylation at the inhibitory site, i.e., Thr696.

Because cGMP-stimulation significantly increased Ser695 MBS phosphorylation, it is expected that cGMP-stimulation produces the multiple species of phosphorylated MBS, i.e., pThr696, pSer695 and pThr696/pSer695 MBS. Based on the parameters obtained in the present study, we simulated the effect of cGMP on the MBS phosphorylation. Based on the computer simulation, we found that the 2 factors contribute to cGMP-induced decrease in the amount of phosphorylated MBS at Thr696. As described above, one of them is the activation of MBS phosphatase at Thr696 of MBS. The other is the phosphorylation of MBS at Ser695. Because Ser695 phosphorylation reduces unphosphorylated MBS otherwise increased by the activation of MBS phosphatase by cGMP, it contributes to reduce the concentration of pThr696 MBS. If Ser695 phosphorylation does not occur, the decrease in pThr696 MBS was calculated to be half of the one shown in the Figure 8a.

We also found that the phosphorylation rate of phospho-Ser695 MBS at Thr696 by MBS kinases in smooth muscle is significantly lower than that of unphosphorylated MBS at Thr696. The result is consistent with previous report that Ser695 phosphorylation interferes with the subsequent Thr696 MBS phosphorylation by isolated ZIP-like kinase.20 We calculated whether this affects the change in pThr696 MBS concentration after cGMP-stimulation. As a result, the decrease in Thr696 phosphorylation rate by Ser695 phosphorylation does not significantly affect the concentration of pThr696 MBS.

The schematic model explaining the effect of cGMP and agonist on the MLCP activity is shown in Figure 8b. At rest, the significant fraction of MBS is phosphorylated at Thr696 whereas CPI17 is dephosphorylated. This means that MLCP activity is partially inhibited at rest because of MBS phosphorylation at the inhibitory site i.e., Thr696. The increase in the inhibitory activity of CPI17 after agonist stimulation further decreases MLCP activity, thus increasing MLC phosphorylation. After cGMP stimulation, Thr696 MBS phosphorylation decreases whereas CPI17 phosphorylation is unchanged. The decrease in Thr696 MBS phosphorylation is attributable to the cGMP-induced activation of MBS phosphatase and Ser695 phosphorylation by PKG that shifts the equilibrium toward decreasing the amount of pThr696 MBS. The inhibition of MLCP caused by MBS phosphorylation at the inhibitory site is reversed and MLCP activity is resumed, although CPI17-dependent inhibition of MLCP is not reversed.

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Disclosures
None.

References


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**Materials and Methods**

**Measurement of tension**

The rabbit femoral artery was prepared and each arterial ring was permeabilized with α-toxin as described.\(^1\) The levels of tension obtained with \(\text{Ca}^{2+}\)-free and 10 \(\mu\text{mol/L}\) \(\text{Ca}^{2+}\) (8.689 ± 0.645 mN) were assigned to be 0% and 100%, respectively.

**Antibodies**

Mouse anti-MLC antibody was purchased from Sigma and mouse anti-phospho-Ser19 of MLC antibody was prepared as described.\(^2\) Rabbit anti-phospho-Ser695 MBS antibody \([\text{pS695Ab}]\) raised against ARQSRRpSTQG and anti-diphospho-Ser695/Thr696 MBS antibody \([\text{pS695/pT696Ab}]\) raised against RQSRRpSpTQG were prepared by Genemed Synthesis. All antibodies were affinity purified and other antibodies were prepared as described.\(^1\)

**Western blotting**

The phosphorylation of MLC, MBS or CPI17 in arterial strips was determined as described.\(^1\)

**Purification of proteins**

The cDNA clone of human Rho-kinase, rat MBS, and bovine cGMP-dependent protein kinase (PKG) were kindly supplied by Dr T. Leung (University of Singapore), Dr. P. Cohen (University of Dundee, U.K.), and Dr. F. Hofmann (Technische Universitat, Germany), respectively. The mutagenesis was performed according to the manufacturer's protocol (Stratagene). The cDNAs were subcloned into pFastbacHT baculovirus transfer vector, and the recombinant proteins were expressed in Sf9 cells and purified according to the
manufacturer’s protocol (QIAGEN). Smooth muscle myosin and MLCK were prepared as described.\textsuperscript{3,4}

**MLCPassay**

The MLCP assay was carried out at 25 °C using $^{32}$P-labeled smooth muscle myosin as a substrate.\textsuperscript{5} It should be noted that no detectable phosphatase activity was present in the $^{32}$P-labeled myosin. The arterial rings were immersed in liquid nitrogen at indicated times and homogenized in 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L KCl, 40 mmol/L MgCl\textsubscript{2}, 1 mmol/L EGTA, 1 mmol/L DTT, 10 µg/ml leupeptin and 1 mmol/L PMSF. Then, the homogenates (4 mg/ml) were incubated with $^{32}$P-labeled 3 µmol/L myosin in the presence of 100 mmol/L NaCl, 30 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L EGTA, 0.2 mg/ml bovine serum albumin. The reactions were terminated by the addition of 10% trichloroacetic acid. The liberated $^{32}$P was determined by Cerenkov counting.

**MBS kinase assay**

The MBS kinase assay was performed at 25 °C using full length Ser\textsuperscript{695}AlaMBS as a substrate. The frozen rings described above were homogenized in 50 mmol/L HEPES (pH 7.5), 100 mmol/L NaCl, 1% Triton X-100, 10 µg/ml leupeptin (buffer A), 1 mmol/L PMSF, 1 mmol/L DTT and 3 µmol/L calyculin A. Then, the homogenates (2 mg/ml) were incubated with 20 µg/ml Ser\textsuperscript{695}AlaMBS in the presence of 5 mmol/L MgCl\textsubscript{2}, 1 mmol/L CaCl\textsubscript{2} and 0.1 mmol/L ATP at 25 °C. The MBS phosphorylation was monitored by Western blotting using pThr\textsuperscript{696}Ab.

**MBS phosphatase assay**

The MBS phosphatase assay was performed at 25 °C using full length wild type(WT)MBS
phosphorylated by Rho-kinase and/or PKG as a substrate. MBS (30 µg/ml) was phosphorylated by Rho-kinase (5 µg/ml) or PKG (5 µg/ml) in the buffer containing 20 mmol/L Tris-Cl, pH 7.5, 4 mmol/L MgCl₂, 25 mmol/L NaCl, 1 mmol/L PMSF, 30 µg/ml Leupeptine, 2 mM DTT, 0.1 mmol/L ATP with or without 3 µmol/L cGMP (for activating PKG). Phosphorylated MBS by Rho-kinase was sequentially phosphorylated by adding 3 µmol/L cGMP and PKG (5 µg/ml). The frozen rings were homogenized in buffer A with 1 µmol/L staurosporine, 30 µmol/L Y-27632 and 30 µmol/L DT-3. Then, the homogenates (2 mg/ml) were incubated with WT-MBS phosphorylated by Rho-kinase at 25 °C. The MBS phosphorylation was monitored by using pThr696Ab. It should be noted that similar results were obtained when the frozen rings were homogenized with hexokinase and glucose in the above buffer to deplete endogenous ATP in the homogenates, thus quenching the kinase activity.

Computer simulation

Fraction population of [S₆⁹⁵T₆⁹⁶], [S₆⁹⁵pT₆⁹⁶], [pS₆⁹⁵T₆⁹⁶] and [pS₆⁹⁵pT₆⁹⁶] in the presence and the absence of cGMP at steady state were estimated by computer simulation using STELLA v8.1.1 software (iseesystems, Lebanon, NH). The rate constants (arbitrary) shown in the Fig. 8a in the absence of cGMP were as follow. k⁺₁ = 1, k⁻¹ = 0.6, k⁻₂ = k'⁻₂ = 0.7, k⁺₂ = k’⁺₂ = 1, k₂ = k’₂ = 1.5. The rate constants in the presence of cGMP were as follow. k⁺₁ = 1, k’⁺₁ = 0.6, k⁻₁ = k’⁻₁ = 1.4 (2-fold increase), k⁺₂ = k’⁺₂ = 3 (3-fold increase), k₂ = k’₂ = 1.5. The rate of [S₆⁹⁵T₆⁹⁶] formation was described as k₁ [S₆⁹⁵pT₆⁹⁶] + k₂ [pS₆⁹⁵T₆⁹⁶] – (k⁺₁ + k⁺₂) [S₆⁹⁵T₆⁹⁶]. The rate of [S₆⁹⁵pT₆⁹⁶] formation was described as k⁺₁ [S₆⁹⁵T₆⁹⁶] + k’⁻₂ [pS₆⁹⁵pT₆⁹⁶] – (k⁻₁ + k’⁻₂) [S₆⁹⁵pT₆⁹⁶]. The rate of [pS₆⁹⁵T₆⁹⁶] formation was described as k⁺₂ [S₆⁹⁵T₆⁹⁶] + k⁻₁ [pS₆⁹⁵pT₆⁹⁶] – (k⁻₂ + k’⁻₁) [pS₆⁹⁵T₆⁹⁶]. The rate of [pS₆⁹⁵pT₆⁹⁶] formation was described as k’⁺₂ [S₆⁹⁵pT₆⁹⁶] + k’⁻₁ [pS₆⁹⁵T₆⁹⁶] – (k’⁻₂ + k’⁻₁).
The initial values of \([pS^{695}T^{696}], [S^{695}pT^{696}], [pS^{695}T^{696}]\) and \([pS^{695}pT^{696}]\) at time zero were 100, 0, 0 and 0, respectively. The simultaneous differential equation was solved by Runge-Kutta 4\(^{th}\) approximation with calculation time interval of 0.0025. Halving and doubling the calculation time interval did not affect the result.

**Statistical analysis**

The results are expressed as mean ± s.e.mean. Data were compared by Student’s \(t\) -test. Values were considered significantly different at \(P < 0.05\).

**Supplementary Figure Legends**

**Fig. 1S. The phosphorylation of un-phosphorylated and Rho-kinase-prephosphorylated MBS by PKG.** The pre-phosphorylation of MBS (180 µg/ml) by Rho-kinase (12 µg/ml) to the maximum level was performed in the presence of 0.1 mmol/L cold ATP at 25°C for 60min as described previously.\(^1\) Unphosphorylated MBS or pre-phosphorylated MBS was incubated with 5 µmol/L cGMP and PKG (12 µg/ml) in the presence of 0.1 mmol/L \(\gamma\)-\(^3\)P-ATP and 0.2 µmol/L microcyctin LR, and the increase in \([\text{\(^3\)}\text{P}]\) incorporation to MBS was monitored by autoradiography. The \([\text{\(^3\)}\text{P}]\) incorporation of the unphosphorylated MBS obtained at 30 min by PKG was taken as 100%. Data represent mean±S.E.M. (n=4).

**Fig. 2S. The linear relationship between the amount of MBS and the western blot signal intensities for phosphorylated MBS.**

Various amounts of mono-phosphorylated MBS at Ser695 by PKG and di-phosphorylated MBS at Ser695/Thr696 by Rho-kinase/PKG were subjected to SDS-PAGE, followed by western blot using anti-phosphoSer695 antibody and anti-di-phosphoSer695/Thr696 antibody as probes,
respectively. The signal intensities were quantified by densitometry. All values were expressed as means±S.E.M. (n = 3)

Fig. 3S. The effect of the protein kinase inhibitors on the MBS phosphorylation by the arterial homogenates.

Arterial rings were homogenized in the presence or absence of PKG inhibitor (DT-3), Rho-kinase inhibitor (Y-27632) and a non-specific kinase inhibitor (staurosporine) as described in Material and Methods. Di-phosphorylated MBS by Rho-kinase/PKG with cold ATP (see Materials and Methods) was further phosphorylated by arterial homogenates using $\gamma^{32}$P-ATP and the $[^{32}$P]$^{}$ incorporation into MBS was monitored by autoradiography. It should be noted that MBS can be phosphorylated at various sites in addition to Ser695 and Thr696 in vitro.
References


Fig. 3S

(-) kinase inhibitor

2  5  10  20  30

(+ ) kinase inhibitor

2  5  10  20  30 (min)

MBS phosphorylation

C.B.B. staining