Dephosphorylation of the two regulatory components of myosin phosphatase, MBS and CPI17

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Abstract Dephosphorylation of the two key regulatory factors of myosin light chain phosphatase (MLCP), CPI17 and MBS (myosin binding subunit) of MLCP was studied. While Thr38 phosphorylated CPI17 is quite susceptible to protein phosphatases, phosphorylated MBS was highly resistant to dephosphorylation. Type 2A, 2B and 2C protein phosphatases (PP2A, PP2B and PP2C), but not type 1 (PP1), dephosphorylated CPI17. The majority of the CPI17 phosphatase activity in smooth muscle was attributed to PP2A and PP2C. Phospholipids inhibited dephosphorylation of MBS and arachidonic acid (AA) inhibited PP2A activity against both MBS and CPI17, raising the possibility that AA favors the preservation of active MLCP. Consistently, while the phosphorylation of CPI17 was promptly decreased when the agonist was removed, the phosphorylation of MBS was unchanged in intact smooth muscle fiber. The results suggest that MBS phosphorylation mediated regulation of MLCP is not suitable for regulating rapid change in myosin phosphorylation. On the other hand, phosphorylated CPI17 is readily dephosphorylated thus likely to play a role in regulating fast phosphorylation–dephosphorylation cycle in cells.

Key words: Smooth muscle; Myosin phosphatase; CPI17; Rho-kinase; Protein kinase C; Phospholipid

1. Introduction

Motor activity of myosin II is activated by phosphorylation at Ser19 of its regulatory light chain [1–3]. The protein kinase responsible for the phosphorylation at this site is myosin light chain kinase (MLCK) [1–4] but other protein kinases [5–7] might also contribute to myosin II phosphorylation.

On the other hand, myosin light chain phosphatase (MLCP) is regulated by various signaling pathways [8]. MLCP consists of three subunits: myosin binding subunit (MBS), a 20 kDa small subunit (M20), and a catalytic subunit of type 1 protein phosphatase (PP1c) [9]. MBS can be phosphorylated by Rho-dependent kinase [10] that results in a decrease in MLCP activity. Therefore, it has been postulated that an activation of the Rho signaling pathway would down-regulate MLCP and, thus, increase myosin II activity. The phosphorylation site responsible for the inhibition of MLCP is Thr641 (rat sequence) [11]. MBS can also be phosphorylated by ZIP kinase [12] and myotonic dystrophy protein kinase [13] at the inhibitory site. Activation of protein kinase C (PKC) inhibits MLCP, thus inducing smooth muscle contraction [14], which is due to an activation of MLCP-specific inhibitor protein, CPI17, by phosphorylation at Thr38 [15,16]. CPI17 can be phosphorylated by Rho-kinase [17] and protein kinase N [18] at the same site; therefore the Rho pathway may regulate MLCP via two different mechanisms, i.e. CPI17 phosphorylation and MBS phosphorylation. However, not much is known about the significance of these two regulatory components of MLCP in rapid change in myosin phosphorylation during the cell activation induced by external stimuli. A key question is whether or not the phosphorylated states of these factors are subjected to fast phosphorylation–dephosphorylation cycling.

Nothing is known about the protein phosphatases responsible for the dephosphorylation of MBS and CPI17. Since the phosphorylation level of MBS and/or CPI17 is determined by the relative activity of the responsible protein kinases and phosphatases, it is critical to determine the susceptibility of MBS and CPI17 against protein phosphatases. It is also critical to identify the MBS and/or CPI17 phosphatases for understanding the regulatory mechanism of myosin II phosphorylation. Four major cellular serine/threonine-specific protein phosphatases have been reported and are classified into four types termed PP1, PP2A, PP2B (calcineurin), and PP2C [19–21], based upon their substrate specificity, sensitivity to inhibitors, and the requirement of divalent cations for their activity [19–21]. While PP1 and PP2A have no divalent cation requirement for their activity, PP2B is a Ca^{2+}-dependent phosphatase that contains a calmodulin binding subunit. PP2C is a Mg^{2+}-dependent phosphatase. In the present study, we studied the susceptibility of MBS and CPI17 in their phosphorylated form against protein phosphatases and attempted to determine the protein phosphatases responsible for the dephosphorylation of MBS and CPI17. The results clearly showed that MBS is highly resistant to dephosphorylation while CPI17 is readily dephosphorylated by protein phosphatases.

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Abbreviations: MLC, myosin light chain; MLCK, myosin light chain kinase; PP1, type 1 protein phosphatase; MLCP, myosin light chain phosphatase; MBS, myosin binding subunit of PP1; PP1c, catalytic subunit of PP1; PP2A, type 2A protein phosphatase; PP2Ac, catalytic subunit of PP2A; PP2B, type 2B protein phosphatase; PP2C, type 2C protein phosphatase; PKC, protein kinase C; 2-ME, 2-mercaptoethanol; PS, phosphatidylserine; PI, phosphatidylinositol; AA, arachidonic acid; DAG, diacylglycerol; LPA, lysophosphatidic acid

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2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Du Pont-NEN, Boston, MA, USA; bovine serum albumin (BSA), okadaic acid, and phospholipids were from Sigma, St. Louis, MO, USA. All other chemicals were of reagent grade.

2.2. Proteins

The MLC was prepared as described [22]. MLCK was purified from frozen turkey gizzard [23]. Calmodulin was purified from bovine testis [24]. The phosphorylated MLC by MLCK was prepared as described [25]. The catalytic subunits of PP1 (PP1c) and PP2A (PP2Ac) were purified from rabbit skeletal muscle [26]. PP2B was purchased from Sigma RBI (St. Louis, MO, USA). PP2C was purified from turkey gizzard [27]. Purification and phosphorylation of the recombinant CPI17 were according to Eto et al. [28] and Kitazawa et al. [16], respectively. Recombinant rabbit PKCα, Rho-kinase, and MBS were expressed with baculovirus expression system and purified with Ni^{2+}-NTA agarose affinity chromatography [29].

2.3. Antibodies

Rabbit polyclonal antibodies against porcine CPI17, phospho-T38 porcine CPI17, phospho-T641-rat1 MBS or anti-phospho-T799-rat1 MBS were prepared as described [30,31]. The peptides, CSKLQSPSRARGPGGSPGGLQKRGAR for CPI17, CRHARV[-phospho-T]VKYDR for phospho-T38 CPI17, CQSRRS[phospho-T]QGVTL for phospho-T641-rat1 MBS, or CEKRRS[phospho-T]GVSFW for phospho-T799-rat1 MBS was chemically synthesized and conjugated with keyhole limpet hemocyanin at the NH_2-terminal cysteine residue. The obtained anti-serum was subjected to affinity-purification with the peptide-conjugated affinity column. Anti-monoclonal MBS antibody that recognizes the MBS regardless its phosphorylation states was purchased from BAbCO (Richmond, CA, USA). Anti-phosphorylated myosin regulatory light chain antibodies were made as described [32].

2.4. Phosphatase assays

The phosphatase assay for PP1 and PP2A was performed at 30°C in buffer containing 50 mM Tris–HCl, pH 7.0, 0.1 mM EGTA, 0.3% BSA, 0.03% Brig-35, 0.1% 2-mercaptoethanol (2-ME) and a substrate. The assay for PP2B was carried out at 30°C in buffer containing 50 mM Tris–HCl, pH 7.0, 0.2 mM $CaCl_2$, 1 mM $MnCl_2$, 0.3% BSA, 0.03% Brig-35, 0.1% 2-ME and a substrate. The assay for PP2C was performed at 30°C in buffer containing 50 mM Tris–HCl, pH 7.0, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EGTA, 0.3% BSA, 0.03% Brig-35, 0.1% 2-ME and a substrate. The reaction was terminated by boiling with one-third volume of the stop buffer containing 4% sodium dodecyl sulfate (SDS), 200 mM NaF, 4 mM dithioerythritol, 40 mM EDTA, and 40 mM EGTA, 0.04% BPB, and 60% glycerol. The samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The phosphorylation levels were determined by the incorporation of radioactive phosphate. The protein bands in the gels were excised and the radioactivity was counted by a scintillation counter (Beckman LS6500, Fullerton, CA, USA). The phosphorylation levels at the specific sites were determined by Western blot using the phosphorylation site-specific antibodies as described [32] followed by densitometry.

2.5. Isometric force measurement of rabbit femoral artery and Western blot

New Zealand white rabbits (2.5–3.0 kg) were sacrificed by the injection of pentobarbital (100 mg/kg). Smooth muscle strips (70 μm thick, 750 μm wide and 3 mm long) were dissected from femoral arteries and freed of connective tissue under binocular microscope in normal physiological saline solution (PSS) containing (in mM) NaCl 123, KCl 4.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 15.5 and D-glucose 5 gassed with 95% O_2 and 5% CO_2. The endothelium was removed by gentle rubbing with a cotton swab. Then strips were tied with silk filament 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, USA). Before starting experiments, strips were stimulated with 118 mM K^+ PSS, which was made by an equimolar substitution of KCl for NaCl, every 15 min until the maximal response was obtained. The contractile responses were terminated by the immersion of dry ice-cold acetone containing 10% (w/v) trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). Proteins were extracted in SDS-sample buffer containing 100 mM Tris–HCl pH 6.8, 50 mM DTT, 1% SDS, 15% glycerol and 0.002% bromophenol blue and then subjected to SDS–PAGE, followed by Western blot. For determination of MBS and MLC, proteins separated on 5–20% gradient slab gel were transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). For CPI-17, proteins were blotted onto polyvinyldifluoride membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked in Tris-buffered saline containing 5% non-fat milk and then incubated with primary antibodies. The immune complexes of the primary antibodies were

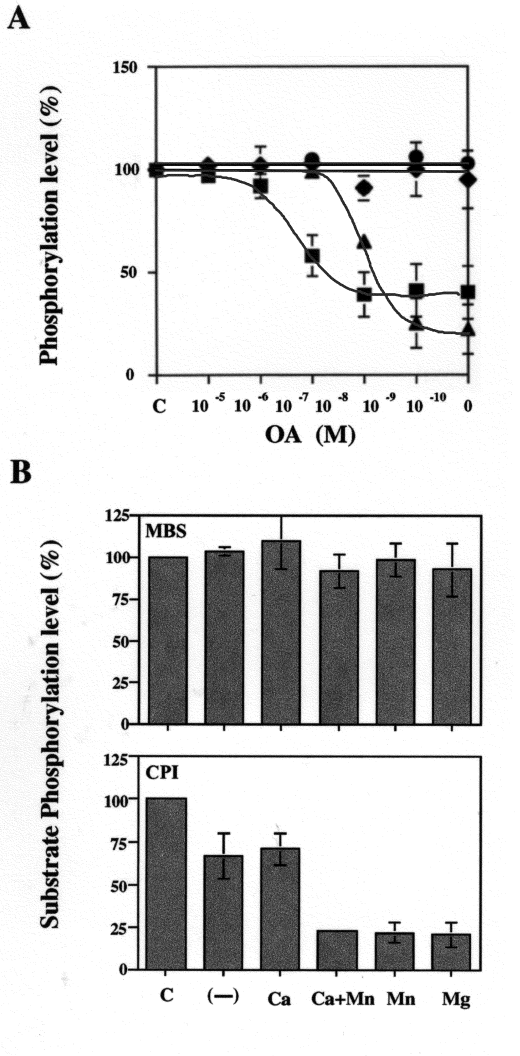

Fig. 1. Dephosphorylation of phospho-CPI17 and phospho-MBS by smooth muscle extract. Turkey gizzard was sonicated for 1 min with buffer containing 50 mM Tris–Cl, pH 7.5, 250 mM sucrose, 60 mM KCl, 1% Triton X-100, 0.5% nonidate P40, 2 mM EGTA, 2 mM EDTA, 10 mg/l leupeptin, 0.1% 2-ME, and 0.1 mM phenylmethylsulfonyl fluoride. The resulting extract was used for the phosphatase assay. C, before dephosphorylation. A: Effect of okadaic acid on dephosphorylation. 0.193 mg/ml gizzard extract containing 50 mM Tris–Cl, pH 7.0, 0.1 mM EGTA, 0.1% 2-ME, 0.3 mg/ml BSA, 0.03% Brig-35, and various concentrations of okadaic acid was incubated with each substrate for 10 min at 30°C. MBS (3.3 μg/ml, ●), MBS phosphorylated at T641 (3.3 μg/ml, ♦), CPI17 at Thr38 (66 μg/ml, ▲), and MLC (83 μg/ml, ■). Error bars indicate S.E.M. of three independent experiments. B: Effect of divalent cations on dephosphorylation. (−), without divalent cations; Ca, 0.2 mM $CaCl_2$; Ca+Mn, 0.2 mM $CaCl_2$ and 10 mM $MnCl_2$; Mn, 10 mM $MnCl_2$; Mg, 10 mM $MgCl_2$. Error bars indicate S.D. ($n=3$).

detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (Pierce, Rockford, IL, USA).

2.6. Other procedures

SDS–PAGE was performed according to Laemmli [33]. The Western blot was carried out as described previously [31]. Protein concentrations were determined with the Bradford method [34] using BSA as a standard.

3. Results

3.1. Dephosphorylation of MBS, CPI17 or MLC by smooth muscle extracts

MBS of MLCP and CPI17 were phosphorylated by Rho-kinase or PKCα, to the extent of 2.3 and 1.35 mol/mol, respectively. These phosphorylated MLCP regulatory proteins were used for the dephosphorylation experiment. In order to identify the type of phosphatase responsible for the dephosphorylation of MBS and CPI17 in smooth muscle cells, we examined the effect of okadaic acid in the dephosphorylation of these MLCP modulators by total smooth muscle extract that contains all four types of the phosphatases [35,36]. The IC_{50} of the dephosphorylation of MLC phosphorylated by MLCK was 10 nM (Fig. 1), which is consistent with the previous results [19,20]. The IC_{50} for CPI17 phosphorylated at Thr38 was 1 nM, much lower than that of phosphorylated MLC, suggesting that PP2A is a predominantly responsible phosphatase for CPI17. While the majority of the CPI17 phosphatase activity was inhibited by okadaic acid, there were residual phosphatase activities insensitive to okadaic acid. To identify the okadaic acid insensitive phosphatases, we examined the divalent cation sensitivity of the CPI17 phosphatases. The activity was insensitive to 0.2 mM Ca^{2+} but increased with 10 mM Mg^{2+} (Fig. 1B), suggesting that PP2C but not PP2B can dephosphorylate CPI17 at Thr38. On the other hand, phosphorylated MBS was not significantly dephosphorylated by smooth muscle extract (Fig. 1), suggesting that MBS dephosphorylation is not a fast event in smooth muscle. The lack of dephosphorylation of MBS was also confirmed by using phospho-Thr641-specific antibodies (Fig. 1A). The result indicated that the inhibitory phosphorylation site of MBS, i.e. Thr641, is resistant to dephosphorylation by protein phosphatases.

3.2. Dephosphorylation of MBS, and CPI17 by purified PP1c, PP2Ac, PP2B and PP2C

To further study which types of phosphatases can dephosphorylate MBS and CPI17, purified PP1c, PP2Ac, PP2B or PP2C were used for the dephosphorylation experiments. As shown in Fig. 2A, MLC, but not CPI17 or MBS, is a good substrate for PP1c. Recombinant PP1δ, the catalytic subunit of MLCP [9], also dephosphorylated MLC, but not CPI17 and MBS (not shown).

On the other hand, PP2Ac dephosphorylated CPI17 very well and the dephosphorylation rate of CPI17 by PP2Ac was about 10 times greater than that of MLC. MBS was highly resistant to the dephosphorylation by PP2Ac, and the rate was at least 100–500 times and 300–1000 times lower than that of MLC and CPI17, respectively. To determine whether M20 and/or PP1δ affect the dephosphorylation of MBS by PP2Ac, we reconstituted MBS with recombinant PP1δ and

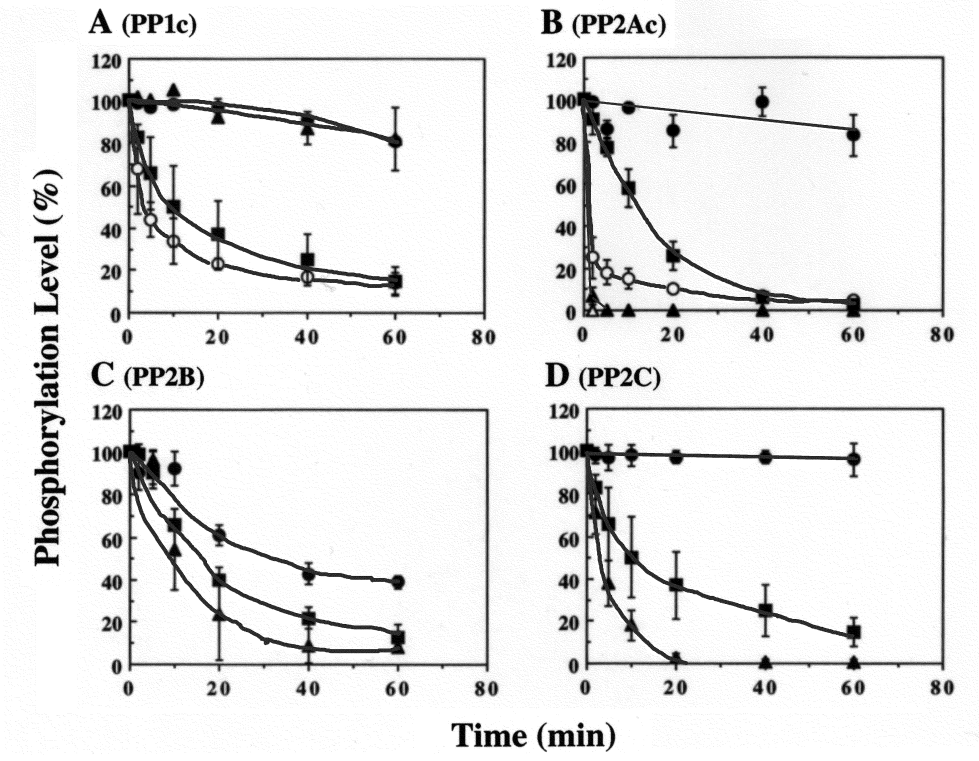

Fig. 2. Dephosphorylation of phosphorylated MBS and CPI17 by various types of protein phosphatases. The assay was done as described in Section 2. 1 U of the activity is defined as the amount of enzyme that catalyzes the release of 1 μmol of phosphate of MLC in 1 min. MBS (3.3 μg/ml, ●, ○), CPI17 (66 μg/ml, ▲, △), MLC (83 μg/ml, ■). A: PP1c: 0.2 mU/ml (closed symbol) or 3 mU/ml (open symbol) of PP1c was used. B: PP2Ac: 0.4 mU/ml (closed symbol) or 20 mU/ml (open symbol) of PP2Ac was used. C: PP2B: 0.15 mU/ml of PP2B was used. D: PP2C: 0.6 mU/ml of PP2C was used. Error bars indicate S.E.M. of three independent experiments.

M20 to form holoenzyme. M20 and PP1δ had almost no effect on the dephosphorylation at the Thr641 site of MBS (not shown).

PP2B could dephosphorylate CPI17 and MBS (Fig. 2C). However, PP2B would not be a dominant phosphatase for MBS and CPI17, since Ca^{2+} had no effect on dephosphorylation of CPI17 and MBS by total cell extract. PP2C dephosphorylated CPI17 well but failed to dephosphorylate MBS (Fig. 2D). Dephosphorylation of MBS could not be detected even with a 250-fold higher concentration of PP2C (not shown). The result is consistent with that of Fig. 1 and suggests that PP2C play a role in dephosphorylating CPI17.

Taken together, our results showed that MBS is highly resistant to protein phosphatases. CPI17 is predominantly dephosphorylated by PP2Ac and PP2C. However, it is anticipated that PP2A is the most likely physiological phosphatase since the majority of the CPI17 phosphatase activity in total tissue extract was inhibited by okadaic acid with nanomolar range.

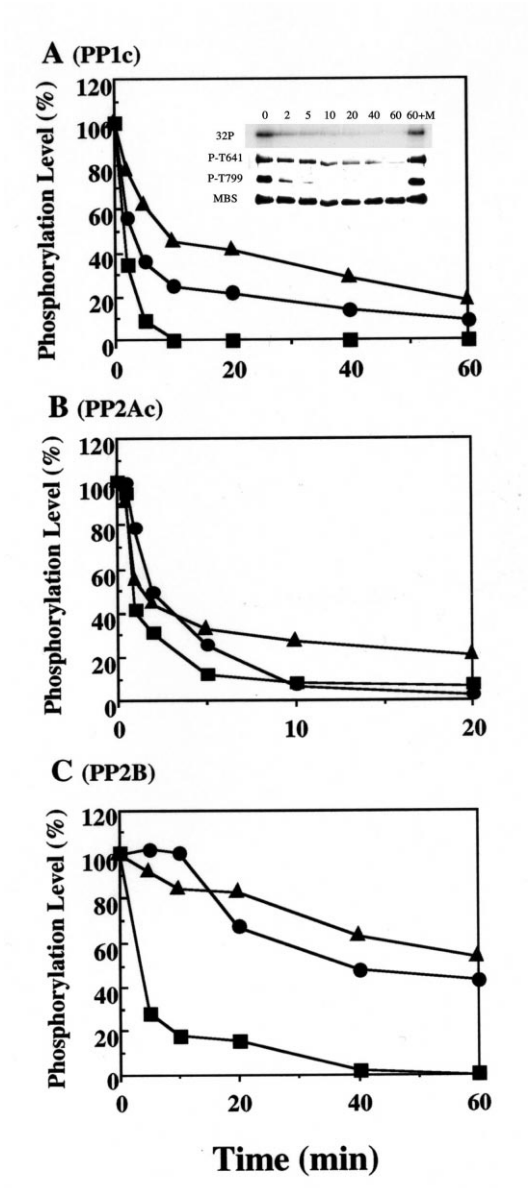

Fig. 3. Dephosphorylation of MBS at Thr641 and Thr799 by various types of protein phosphatases. The assay was done as described in Section 2. PP1c (3 mU/ml, 30°C), PP2Ac (20 mU/ml, 20°C), and PP2B (0.15 mU/ml, 30°C) were used. MBS phosphorylation (●), phospho-Thr641 of MBS (▲) and phospho-Thr799 of MBS (■). Total extent of phosphorylation was estimated by ^{32}P-incorporation. The phosphorylation of the Thr641 and Thr799 are determined by Western blot using the phosphorylation site-specific antibodies. Similar results have been obtained from three independent experiments. Inset: Time course of the dephosphorylation of MBS determined by Western blot and autoradiography. Times (min) after adding the phosphatases were indicated on the top of the panel. The relative amount of the protein (MBS) loaded onto the gel was determined by Western blot using anti-MBS monoclonal antibody. M indicates the presence of 1 μM microcystin LR.

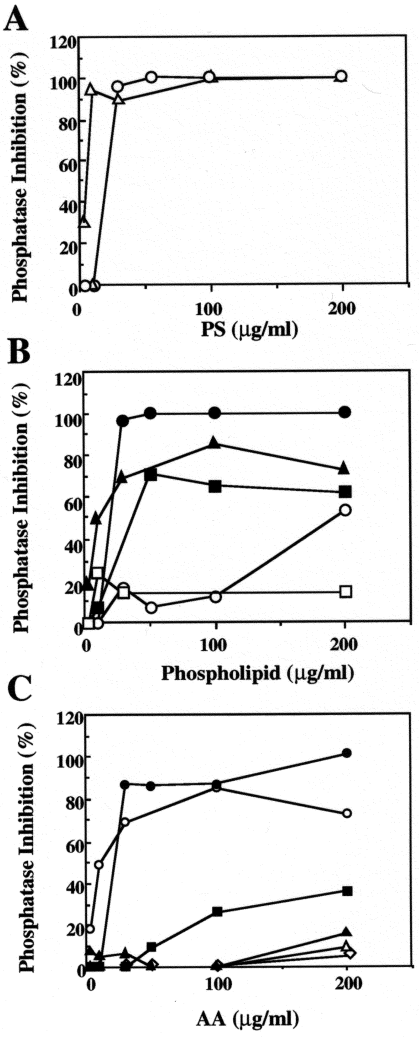

Fig. 4. Effect of phospholipids on the dephosphorylation at Thr641 of MBS and Thr38 of CPI17. The assay was done as described in Section 2. PP1c (3 mU/ml, 30°C, 20 min), PP2Ac (20 mU/ml, 20°C, 3 min), PP2B (0.15 mU/ml, 30°C, 30 min) or PP2C (0.6 mU/ml, 30°C, 30 min) was used for the assay. A: Effect of PS on dephosphorylation of MBS. PP2Ac (○), PP2B (△). B: Effect of phospholipids on the dephosphorylation at MBS. The assay was performed with PS (●), AA (▲), LPA (■), diacylglycerol (DAG; ○), or PI (□). C: Effect of AA on dephosphorylation of MBS (open symbols) and CPI17 (closed symbols). The assay was performed with PP1c (◇), PP2Ac (●, ○), PP2B (▲, △), or PP2C (■).

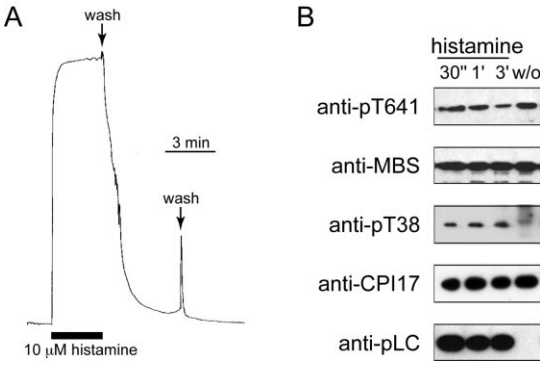

Fig. 5. Changes in the CPI-17 and MBS phosphorylation in intact smooth muscle after removal of the agonist. A: Representative recording of change in the force of intact rabbit femoral artery induced by histamine. Rabbit femoral artery was pre-contracted by histamine (10 μM) and when the force reached steady state (3 min after the application of histamine), histamine was removed by washing out with PSS. B: Phosphorylation of MBS, CPI-17 and MLC in intact smooth muscle. Smooth muscle strips during the contraction by histamine (30 s, 1 min and 3 min after histamine stimulation) and 10 min after the removal of histamine were immersed in TCA/acetone to stop the reaction and then subjected to Western blotting. The same amount of proteins (10 μg) were applied to SDS–PAGE. Note that the signals of anti-MBS and anti-CPI17 that recognize the target proteins regardless of their phosphorylation states were the same among the lanes indicating that the same amount of proteins was loaded.

3.3. Determination of site-specific dephosphorylation of MBS by various protein phosphatases

It is known that MBS has two major phosphorylation sites, i.e. Thr641 and Thr799 [11], among which the former site is responsible for the downregulation of MLCP activity. We compared the rate of dephosphorylation of each site by various phosphatases using site-specific phospho-MBS antibodies. The rate of dephosphorylation of Thr641 was significantly slower than that of Thr799 site for all three types of phosphatases (Fig. 3). The results show that Thr641, the inhibitory phosphorylation site of MBS, is highly resistant to dephosphorylation.

3.4. Effect of phospholipids on the dephosphorylation of MBS by protein phosphatases

It is known that MBS localizes on plasma membrane in the meta-phase of mitotic cells [37], yet the effect of the membrane binding on the phosphorylation/dephosphorylation cycle of MBS is unknown. We examined whether phospholipids affect the dephosphorylation of MBS (Fig. 4). Among various phospholipids tested, lysophosphatidic acid (LPA) and phosphatidylserine (PS) were most effective for the inhibition of the dephosphorylation of MBS by PP2Ac. The inhibition was not observed for MLC as a substrate (not shown) suggesting that the effect is not directly due to the enzyme but rather due to the change in MBS structure, presumably blocking the sites by phospholipid binding. Consistently, the similar inhibition of dephosphorylation of MBS by PS was observed for PP2B. On the other hand, a physiological concentration of arachidonic acid (AA) also inhibited MBS dephosphorylation. However, the inhibition was not specific to MBS but the dephosphorylation of CPI17 and MLC was also inhibited by AA at the similar concentration (not shown), suggesting that AA directly inhibits PP2Ac activity.

3.5. Dephosphorylation of MBS and CPI17 in intact smooth muscle fiber

The above results suggest that the phosphorylation of MBS at Thr641, a site responsible for the downregulation of MLCP, is resistant to dephosphorylation by protein phosphatases while the phosphorylation at Thr38 of CPI17, a site critical to the phosphatase inhibitory activity, is readily dephosphorylated in vitro. A critical question is whether this is the case in vivo. To address this question, we examined the effect of removal of agonist on the phosphorylation of MBS and CPI17 in intact smooth muscle fiber. Rabbit femoral artery was stimulated by histamine as an agonist, then the agonist was removed (Fig. 5). The force was promptly decreased after the agonist was removed and this was accompanied with a decrease in MLC phosphorylation (Fig. 5A,B). During the decrease in force due to the removal of the agonist, CPI17 phosphorylation at Thr38 was markedly decreased. In contrast, the phosphorylation of MBS at Thr641 was hardly decreased during the decrease in force in intact smooth muscle fiber. The results are consistent with those obtained under in vitro conditions and support the idea that while CPI17 is susceptible to dephosphorylation by protein phosphatases, phosphorylation of MBS at Thr641 is resistant to the dephosphorylation reaction.

4. Discussion

The present study clearly demonstrates that phosphorylated MBS is highly resistant to dephosphorylation by protein phosphatases, especially at Thr641, which is responsible for the inhibition of MLCP. This implies that MBS, once phosphorylated, remains for a prolonged time in the phosphorylated form. Therefore, it is plausible that MBS phosphorylation-based regulation of MLCP is more suitable for the long-term regulation of MLCP, thus the regulation of MLC phosphorylation, rather than the acute regulation such as smooth muscle contraction/relaxation cycle.

On the other hand, phosphorylated CPI17 at Thr38 is readily dephosphorylated by smooth muscle cell extract containing various protein phosphatases. The present results suggest that predominant CPI17 phosphatases are PP2A and PP2C. Significant portions of CPI17 phosphatase activity in total tissue extract were inhibited by nanomolar concentration of okadaic acid. While PP4 and PP5 activities are also inhibited by the similar concentration range of okadaic acid, they are less likely to be responsible for the dephosphorylation of CPI17 since both PP4 and PP5 are predominantly found in the nucleus [38,39]. The rate of dephosphorylation of CPI17 by these phosphatases is quite fast (even faster than the dephosphorylation of isolated MLC). It was shown recently that CPI17 is phosphorylated in intact and permeabilized smooth muscle strip after agonist stimulation [30]. Although the stability of the phosphorylated CPI17 in cells has not been determined yet, the present result suggests that the phosphorylated CPI17 in cells would be readily dephosphorylated after the kinase activating signaling is ceased. Therefore, the present study supports the notion that CPI17 phosphorylation plays a role in regulating MLCP in MLC phosphorylation during the agonist-induced contraction–relaxation cycle.

Recently, new MBS binding proteins were found in addition to myosin (e.g. moesin, which affects microvilli formation and adducin, which affects membrane ruffling and cell motil-

ity). The localization of these proteins is regulated by their phosphorylation [40,41], raising a possibility that change in the MLCP activity via MBS phosphorylation may play a role in regulating the localization of these MBS binding proteins. MBS is also found in the nucleus in addition to cytoplasm [42]. Distinct MBS phosphatases might be responsible for the dephosphorylation of MBS that binds to different target proteins.

An interesting finding is that MBS dephosphorylation is significantly inhibited by phospholipids. It has been shown that MBS has a high affinity for acidic phospholipids such as PS or PA and others via its C-terminal region [37]. Interestingly, the order of inhibitory potency of MBS dephosphorylation by the various phospholipids (Fig. 4) coincides with the order of the binding affinities between MBS and these phospholipids [37]. The result suggests that the binding of MBS to phospholipids occludes the phosphorylated sites thus preventing the dephosphorylation by the phosphatases. The present results also suggest that the binding of MBS to membrane would preserve the phosphorylated state for a prolonged time. It has been shown that MBS localized at the membrane ruffling sites in MDCK cells has an increased level of phosphorylation after HGF (hepatocyte growth factor) stimulation [42]. Since the membrane ruffling sites represent a highly increased membrane surface, and thereby an increased amount of phospholipids present, this previous finding is consistent with the present finding that phospholipids prevent the dephosphorylation of MBS.

On the other hand, AA inhibits the dephosphorylation of CPI17 by PP2A via direct inhibition of PP2A activity. It has been shown that AA induces the Ca^{2+}-sensitization of smooth muscle contraction [43]. The AA effect in Ca^{2+}-sensitization was originally proposed to be due to the dissociation of MLCP holoenzyme and resulting inactivation of the enzyme. The present study raises another possibility that AA induces Ca^{2+}-sensitization via inhibition of CPI17 dephosphorylation.

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