Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase

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Abstract Phosphorylation of CPI-17 by Rho-associated kinase (Rho-kinase) and its effect on myosin phosphatase (MP) activity were investigated. CPI-17 was phosphorylated by Rho-kinase to 0.92 mol of P/mol of CPI-17 in vitro. The inhibitory phosphorylation site was Thr^{38} (as reported previously) and was identified using a point mutant of CPI-17 and a phosphorylation state-specific antibody. Phosphorylation by Rho-kinase dramatically increased the inhibitory effect of CPI-17 on MP activity. Thus, CPI-17 as a substrate of Rho-kinase could be involved in the Ca^{2+} sensitization of smooth muscle contraction as a downstream effector of Rho-kinase.

Key words: Rho-kinase; Myosin phosphatase; CPI-17; Smooth muscle; Ca^{2+} sensitization

1. Introduction

It is accepted that phosphorylation of the regulatory light chain of myosin is an essential mechanism controlling contractile activity in smooth muscle [1,2]. Increased Ca^{2+} sensitization is a well-known phenomenon observed in smooth muscle contraction stimulated by agonists [2,3]. One explanation for this effect is via inhibition of myosin phosphatase (MP) [4], which increases the level of myosin phosphorylation and reinforces contraction at constant cytoplasmic calcium [2,3]. The inhibition of MP following agonist stimulation is transmitted through G-protein-coupled mechanisms and the small GTPase RhoA plays a pivotal role in this process [3,5].

MP is composed of three subunits: a catalytic subunit, the δ isoform of the catalytic subunit of type 1 protein phosphatase (PP1cδ), and two putative regulatory subunits, termed MYPT1 and M20 [4]. A plausible mechanism to account for the RhoA-induced inhibition of MP is by phosphorylation of MYPT1 by Rho-associated kinase (Rho-kinase), a downstream target of RhoA [3–7]. Arachidonic acid also can induce Ca^{2+} sensitization and, at least in part, this is due to activation of Rho-kinase [3,8]. Another potential pathway to increase Ca^{2+} sensitization is by protein kinase C (PKC) activation and this is independent of the RhoA/Rho-kinase links [3]. It was suggested that PKC inhibited MP by phosphorylation of CPI-17 [9].

CPI-17 is a phosphorylation-dependent inhibitory protein for MP specifically expressed in smooth muscle [9]. Phosphorylation of CPI-17 at Thr^{38} by PKC enhances its inhibitory potency about 1000-fold in vitro and the phosphorylated CPI-17 could induce contraction in permeabilized smooth muscle [10,11]. In an earlier report on CPI-17 [9], the kinase involved showed some inconsistencies with PKC and thus raised the possibility that another kinase could be implicated. Recently, the phosphorylation of CPI-17 was demonstrated in permeabilized vascular smooth muscle following agonist stimulation. The phosphorylation was catalyzed by PKC and also by a kinase sensitive to Y-27632, i.e. Rho-kinase or PKN [12].

Here we will show that CPI-17 is a substrate of Rho-kinase in vitro and that the phosphorylation of Thr^{38} by Rho-kinase converts CPI-17 into a more potent inhibitor of MP.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Du Pont-NEN; phorbol 12-myristate 13-acetate and L-α-phosphatidyl-L-serine were from Sigma Chemical Co.; and microcystin-LR was from Wako Pure Chemical Co. All other chemicals were of reagent grade.

2.2. Protein preparations

Rho-kinase and kinase-free MP were prepared from chicken gizzard [8]. CPI-17 was purified from porcine aorta as described previously [9]. Other preparations were as follows: 20 kDa myosin light chain (MLC20) [13] and myosin light chain kinase (MLCK) [14] from chicken gizzard; calmodulin from bovine brain [15]; PKC from human platelets [16]; glutathione *S*-transferase (GST)-RhoA [8]; ^{32}P-labelled MLC20 phosphorylated by MLCK (^{32}P-MLC20) [17].

2.3. Plasmid constructs and site-directed mutagenesis

cDNA encoding the open reading frame of CPI-17 was amplified by nested PCR from a λgt10 porcine aorta cDNA library (Clontech). The primers used were based on the published sequence [18]. These were 5′-GCCGGGCCAGGACCGCGATGG-3′ and 5′-CGGAGGGGGGTCACAGAGGCC-3′ in the first PCR for 5′ and 3′, respectively, and 5′-GCCAGGGGATCCATGGCAGCTCAG-3′ (underlined residues show a *Bam*HI site) and 5′-CCGGGGAATTCGTCAGGGCGGCGC-3′ (underlined residues show an *Eco*RI site) in the second PCR for 5′ and 3′, respectively. PCR was carried using Takara LA Taq™ with GC buffer I (Takara Shuzou) and the conditions used were 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 30 cycles. The obtained PCR product was inserted into pCR2.1 vector (Invitrogen) and verified by DNA sequencing. The full length CPI-17

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Abbreviations: Rho-kinase, Rho-associated kinase; MP, myosin phosphatase; PP1, type 1 protein phosphatase; PP1cδ, the δ isoform of the catalytic subunit of PP1; MLCK, myosin light chain kinase; MLC20, 20 kDa myosin light chain; PKC, protein kinase C; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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cDNA was retrieved from the vector as a *Bam*HI–*Eco*RI fragment, then subcloned into pGEX4T-1 vector (Amersham Pharmacia Biotech) for the GST fusion protein (termed rG-CPI-17). To obtain the plasmid for the His-tagged CPI-17 recombinant, cDNA for CPI-17 was re-amplified using the above plasmid (pGEX4T-1 vector ligated with CPI-17 cDNA) as a template, Takara Ex Taq™ with EX buffer (Takara Shuzou) and the following set of primers: 5′-GCCAGGGGATCCATGGCAGCTCAG-3′ (underlined residues show a *Bam*HI site) for 5′ and 5′-CCGGGAAGCTTGTCAGGGCGGCGC-3′ (underlined residues show a *Hin*dIII site) for 3′. The conditions used were 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min for 30 cycles. The obtained PCR product was digested as a *Bam*HI–*Hin*dIII fragment, then subcloned into the pQE30 vector (Qiagen) for the His-tagged protein (termed rH-CPI-17). The ligated PCR product was verified by DNA sequencing. The point mutant of CPI-17 at the inhibitory phosphorylation site (substitution of Thr^{38} to Ala; termed rH-CPI-17^{T38A}) was created on a CPI-17 cDNA-ligated pQE30 vector using a site-directed mutagenesis kit according to the manufacturer's instructions (Clontech). The desired mutation was incorporated into one pair of oligonucleotide primers, each complementary to opposite strands of the parental DNA template. The selection primer used was 5′-GAGTGCACGATATCCGGTGTGAAA-3′ which was designed by incorporating a two base change within a *Nde*I restriction site in the original plasmid (underlined). The mutagenic primer was 5′-GCGCGTGTCGCCGTCAAGTACGATCG-3′ which was designed by incorporating a one base change in CPI-17 cDNA (underlined, original sequence was A). The mutant was selected by *Nde*I digestion before the synthesized DNA was transformed into *Escherichia coli* BMH 71-18 *mutS*. The sequence of the mutant was verified by DNA sequencing.

2.4. Preparation of recombinant proteins and antibodies

rG-CPI-17 was expressed in *E. coli* BL21(DE3) strain and purified on glutathione-Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech). rH-CPI-17 and rH-CPI-17^{T38A} were expressed in *E. coli* M15 strain and purified on Ni-NTA agarose according to the manufacturer's instructions (Qiagen).

Rabbit polyclonal anti-CPI-17 antibody was raised against rG-CPI-17. This antibody was affinity-purified using a CNBr-activated Sepharose 4B column (Amersham Pharmacia Biotech) coupled with rH-CPI-17. For the site- and phosphorylation state-specific antibody against Thr^{38} in CPI-17, two peptides (Cys-Arg^{33}-His-Ala-Arg-Val-Thr^{38}-Val-Lys-Tyr-Asp-Arg^{43} and Cys-Arg^{33}-His-Ala-Arg-Val-phospho-Thr^{38}-Val-Lys-Tyr-Asp-Arg^{43}) were synthesized and coupled with keyhole limpet hemocyanin (Sawaday Co). The rabbit polyclonal anti-phosphopeptide antibody was prepared and affinity-purified as described [7]. This antibody is referred to as pCPI-17^{T38}.

2.5. Protein phosphorylation

Phosphorylation of native CPI-17 and recombinant rH-CPI-17 by Rho-kinase was carried out at 30°C for different times in 30 mM Tris–HCl, pH 7.5, 30 mM KCl, 0.1 mM dithiothreitol, 1 mM $MgCl_2$, 2 μg/ml Rho-kinase, 2 μM guanosine 5′-3-*O*-(thio)triphosphate (GTPγS)·GST-RhoA, 250 μM [γ-^{32}P]ATP and 10 μM substrate as indicated in a final volume of 30 μl. The reactions were initiated by addition of [γ-^{32}P]ATP and terminated by addition of trichloroacetic acid to 10%, followed by filtration with 0.45 μm nitrocellulose filters (Toyo Roshi). The filters were extensively washed with 5% trichloroacetic acid and ^{32}P incorporation was determined by Cerenkov counting. To determine the effects of phosphorylation of CPI-17 on MP activity, phosphorylation of CPI-17 was carried out for 120 min under similar conditions using non-radioactive ATP. The reactions were boiled at 90°C for 5 min, dialyzed against 30 mM Tris–HCl, pH 7.5, 30 mM KCl and 1 mM EDTA and assayed for phosphatase activity. Phosphorylation of CPI-17 by PKC was carried out as follows: 10 μM native CPI-17 or recombinant rH-CPI-17 was incubated at 30°C for 120 min with 10 μg/ml PKC in 30 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 30 mM KCl, 1 mM EGTA, 100 ng/ml phorbol 12-myristate 13-acetate, 100 μg/ml L-α-phosphatidyl-L-serine and 250 μM ATP.

2.6. Phosphatase assays

Phosphatase assays were carried out at 30°C using ^{32}P-MLC20 as substrate (final concentration 5 μM). Assay conditions were: 30 mM Tris–HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.2 mg/ml bovine serum albumin and 0.5 μg/ml MP in the presence of various concentrations of CPI-17 as indicated. The reactions were started by the addition of substrate and terminated by the addition of trichloroacetic acid to 5%. After sedimentation at $5000 \times g$ for 5 min, the radioactivity of the supernatant was determined by Cerenkov counting.

2.7. Other procedures

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out with the discontinuous buffer system of Laemmli [19]. Western blotting was carried out as described previously [20]. Protein concentrations were determined with either the BCA (Pierce) or Bradford (Bio-Rad) procedures, using bovine serum albumin as a standard.

3. Results

To test if CPI-17 could be phosphorylated by Rho-kinase, the time course and stoichiometry of phosphorylation by Rho-kinase for native and recombinant CPI-17 were determined (Fig. 1). As shown in Fig. 1A, native CPI-17 was phosphorylated by Rho-kinase to 0.92 ± 0.03 mol of P/mol of CPI-17. K_m, V_{max} and k_{cat} values of Rho-kinase for native CPI-17 in the presence of the GTPγS-bound active form of GST-RhoA and 200 μM ATP were 1.42 ± 0.25 μM, 146.7 ± 14.1 nmol/mg/min and 23.6 ± 2.7 min^{-1} ($n = 3$; the molecular mass used for Rho-kinase was 160.8 kDa), respectively. Wild-type His-tagged CPI-17 (rH-CPI-17) was phosphorylated by Rho-kinase to 1.29 ± 0.33 mol of P/mol of recombinant and substitution of Thr^{38} to Ala (rH-CPI-17^{T38A}) resulted in decreased phosphorylation to 0.32 ± 0.01 mol of P/mol of mutant (Fig. 1B). These results indicate that Rho-kinase can phosphorylate Thr^{38} on CPI-17 which is the inhibitory phosphorylation site for PKC [9].

To confirm that Rho-kinase phosphorylates Thr^{38} on CPI-17, a rabbit polyclonal antibody which recognizes phosphorylation of CPI-17 at Thr^{38} (pCPI-17^{T38}) was prepared. As shown in Fig. 2, native CPI-17 phosphorylated by PKC (lane 2) or Rho-kinase (lane 3) was recognized by pCPI-17^{T38}. Wild-type rH-CPI-17 phosphorylated by Rho-kinase was detected (lane 4), while rH-CPI-17^{T38A} phosphorylated by Rho-kinase was not (lane 5). These results are consistent with the phosphorylation of CPI-17 at Thr^{38} by Rho-kinase.

The effects of unphosphorylated CPI-17 and CPI-17 phosphorylated by Rho-kinase on the activity of MP were investigated. Native CPI-17 phosphorylated by Rho-kinase showed a dose-dependent inhibition of MP holoenzyme activity with an IC_{50} value of 36.9 ± 5.4 nM (Fig. 3A). The un-

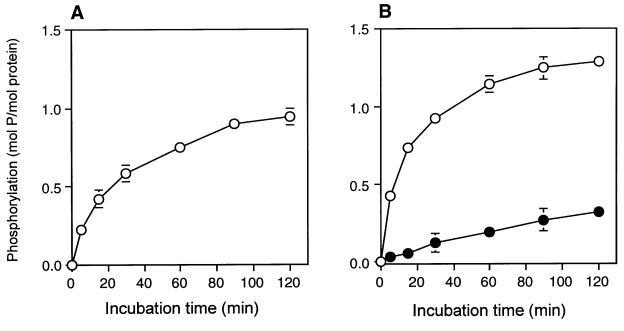

Fig. 1. Phosphorylation of CPI-17 by Rho-kinase. (A) Native CPI-17; (B) wild-type rH-CPI-17 (○) and rH-CPI-17^{T38A} (●). Each protein (10 μM) was phosphorylated by Rho-kinase for the indicated time under the conditions as described in Section 2. Error bars indicate means ± S.E.M. ($n = 3$).

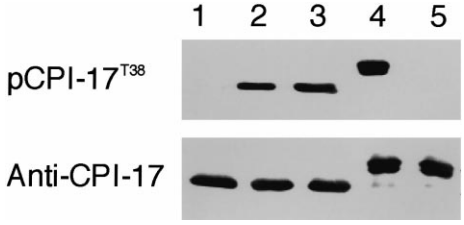

Fig. 2. Immunoblot analysis of CPI-17 phosphorylated by Rho-kinase and PKC using site- and phosphorylation state-specific antibody. Lane 1, unphosphorylated native CPI-17; lane 2, native CPI-17 phosphorylated by PKC (1.2 mol P/mol protein); lane 3, native CPI-17 phosphorylated by Rho-kinase (0.9 mol P/mol protein); lane 4, wild-type rH-CPI-17 phosphorylated by Rho-kinase (1.3 mol P/mol protein); lane 5, rH-CPI-17^{T38A} phosphorylated by Rho-kinase (0.27 mol P/mol protein). Samples were subjected to SDS–PAGE followed by Western blot with pCPI-17^{T38A} (upper panel, 500 ng of protein in each lane) and with anti-CPI-17 antibody (lower panel, 100 ng of protein in each lane).

phosphorylated CPI-17 showed a considerably weaker inhibitory effect and MP activity was reduced to 51.3 ± 2.9% at 1 μM unphosphorylated CPI-17 (Fig. 3A). As shown in Fig. 3B, wild-type recombinant His-tagged CPI-17 (rH-CPI-17) showed a similar phosphorylation-dependent inhibition of MP activity (IC_{50} = 46.0 ± 8.4 nM). Inhibition of MP activity by the Thr^{38} to Ala mutant (rH-CPI-17^{T38A}) was similar to unphosphorylated CPI-17 and inhibitory potency was not increased following phosphorylation by Rho-kinase (Fig. 3B).

4. Discussion

CPI-17 was discovered as a heat-stable inhibitory protein of PP1 and it was shown that its inhibitory potency was increased by phosphorylation with PKC, or an unidentified endogenous kinase [9]. It was subsequent shown that CPI-17 phosphorylated by PKC induced contraction of permeabilized vascular smooth muscle at submaximal Ca^{2+} concentrations with an accompanying increase of myosin phosphorylation [10,11]. An unusual feature of CPI-17, compared to inhibitor 1 and inhibitor 2, is that it can inhibit both the free catalytic subunit and holoenzyme of PP1 [9,21]. The inhibitory phosphorylation site on CPI-17 was identified as Thr^{38} [9]. From our studies, using the point mutant of CPI-17 (Thr^{38} to Ala) and the phosphorylation-state-specific antibody, it was found that Rho-kinase also phosphorylates this site and induces a more potent inhibitory form of CPI-17. Potency of inhibition following phosphorylation by Rho-kinase increased about 100 fold, whereas a slightly higher increase was reported for PKC [9,18,21]. Some differences in sensitivity for various reports could partly be due to various assay conditions. CPI-17 is expressed specifically in smooth muscle. It is relatively high in aorta and bladder and the expression of CPI-17 in other smooth muscles may vary [12,18]. In aorta, the concentration of CPI-17 was estimated to be at least 0.3 μM [21], which is within the range of its K_m for Rho-kinase (1.4 μM). An inhibitor similar to CPI-17, but found in many tissues, was reported recently and was termed phosphatase holoenzyme inhibitor (PHI) with two isoforms, 1 and 2 [22]. Phosphorylation of PHI-1 by PKC enhances the inhibitory potency about 50-fold. It is not known if PHI-1 or -2 can be phosphorylated by Rho-kinase.

It is accepted that a major mechanism responsible for Ca^{2+} sensitization in smooth muscle involves inhibition of MP by a G-coupled mechanism(s). There is strong evidence indicating RhoA [3] and its downstream partner, Rho-kinase [3,5,6]. In support of this, it is known that Rho-kinase inhibitors, such as Y-27632 and HA-1077, block agonist-induced Ca^{2+} sensitization in smooth muscle [23–26]. One mechanism to account for the role of Rho-kinase is that it phosphorylates MYPT1 and inhibits MP activity. It was shown that thiophosphorylation by Rho-kinase of Thr^{695} on MYPT1 (chicken isoform) is critical for inhibition in vitro and that this residue was phosphorylated in cultured fibroblasts following lysophosphatidic acid stimulation [7]. In cultured smooth muscle cells, it was also proposed that RhoA and its downstream target, Rho-kinase, mediated MYPT1 phosphorylation and inhibition of MP activity [27].

This study suggests another potential role for Rho-kinase, namely that it could inhibit MP activity by phosphorylation of CPI-17. Thus, in those smooth muscles in which CPI-17 is expressed, there could be two RhoA/Rho-kinase-dependent mechanisms to inhibit MP, i.e. phosphorylation of either MYPT1 or CPI-17. Recently, it was reported that stimulation of intact and permeabilized smooth muscle by histamine generated higher levels of CPI-17 phosphorylation at Thr^{38} compared to phenylephrine stimulation [12]. Both PKC and Rho-kinase inhibitors reduced force and CPI-17 phosphorylation on stimulation with histamine. The phenylephrine-induced force was more sensitive to the Rho-kinase inhibitor. The possibility of different signaling pathways for histamine and phenylephrine therefore was raised and it was suggested that both PKC and Rho-kinase may function in parallel to phosphorylate CPI-17. Distinct signaling pathways for PKC and Rho-kinase are not unexpected but the situation becomes more complex when proposing two substrates for Rho-kinase. It is not known how inhibition of MP by phosphorylation of MYPT1 and/or CPI-17 is integrated and obviously future studies are required to resolve this issue.

In summary, the results presented above provide evidence for the in vitro phosphorylation of CPI-17 by Rho-kinase and a concurrent increase in inhibitory potency towards MP. Therefore, it is suggested that two RhoA/Rho-kinase-dependent mechanisms may contribute to inhibition of MP activity, i.e. phosphorylation of MYPT1 and phosphorylation of CPI-17. It is not known if the two mechanisms are exclusive or

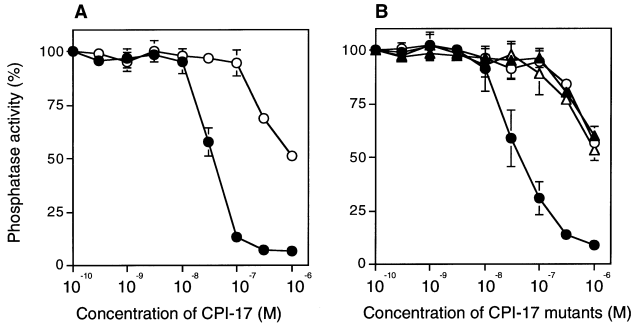

Fig. 3. Effect of CPI-17 on MP activity. (A) Effects of native CPI-17 unphosphorylated (○) and phosphorylated (●) by Rho-kinase. (B) Effect of wild-type rH-CPI-17 (circle) and rH-CPI-17^{T38A} (triangle) unphosphorylated (open symbols) or phosphorylated (closed symbols) by Rho-kinase. Error bars indicate means ± S.E.M. (n = 3). The phosphatase activity in the absence of CPI-17 was expressed as 100%.

operate synergistically and participation of the two mechanisms may vary with the agonist used and the identity of the smooth muscle.

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