Identification of Trimeric Myosin Phosphatase (PP1M) as a Target for a Novel PKC-Potentiated Protein Phosphatase-1 Inhibitory Protein (CPI17) in Porcine Aorta Smooth Muscle[1]

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CPI17, a phosphorylation-dependent inhibitory protein of protein phosphatase-1 (PP1), is dominantly expressed in smooth muscle, and the inhibitory activity is potentiated by protein kinase C and its related enzymes [Eto, M. *et al.* (1997) *FEBS Lett.* 410, 356-360]. In order to identify its physiological target in smooth muscle, the myofibrillar extract from porcine aorta media was analyzed by affinity chromatography on CPI17-conjugated Sepharose. The binding of phosphatases to the resin depended on thiophosphorylation of CPI17, and about 90% of the phosphatase activities toward phosphorylated myosin (p-myosin) and phosphorylase-a were bound to the resin and could be eluted with 0.5 M NaCl. The IC_{50} values of thiophosphorylated CPI17 toward phosphatases bound to the resin were in the range of 0.5-3 nM, as expected for the PP1 holoenzymes sensitive to CPI17. The CPI17-sensitive fraction was further separated into several peaks of phosphatase activity by column chromatography on Mono Q, which suggested multiple functions of CPI17 as a mediator of the protein kinase C-related signal transduction pathway in aorta smooth muscle. The major activity toward p-myosin was identified as the myofibril-bound PP1 (PP1M), and its subunit composition (140, 37, and 20 kDa) was consistent with that of PP1M from chicken gizzard and porcine bladder. The purified PP1M was completely inhibited by phosphorylated and thiophosphorylated CPI17. Kinetic analysis showed mixed inhibition of PP1M by CPI17 (K_i = 1.9 nM and K_i' = 5.1 nM). The concentration of CPI17 in aorta smooth muscle cells was estimated to be at least 0.3 μM from the result of Western analysis. This concentration appears to be sufficient to suppress the *in situ* PP1M in aorta smooth muscle, and PP1M is thus identified as a target of CPI17 in vascular smooth muscle.

Key words: affinity chromatography, myosin light chain phosphatase, protein kinase C, protein phosphatase 1, smooth muscle regulation.

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Protein phosphatase-1 (PP1) participates in the regulation of many cellular functions through Ser/Thr dephosphorylation. In contrast to a wealth of results on the regulatory mechanisms of phosphorylation, recent studies on PP1 have presented no more than possible regulatory mechanisms for dephosphorylation (*1*). The intracellular localization and substrate specificity of PP1 can be determined from the interaction between the catalytic subunit (PP1c, 37-38 kDa) and specific regulatory proteins, which are considered to be targeting subunits (*1*). Further, the activity of PP1c is modulated by binding to the specific inhibitory proteins, inhibitor-1 and inhibitor-2, which were originally found as heat-stable inhibitory proteins (*2*). Inhibitory activity of inhibitor-1 is potentiated by phosphorylation with cAMP-dependent protein kinase (*2*). So far, many targeting subunits and inhibitory proteins other than inhibitor-2 have been shown to share a consensus Phe residue that plays a critical role in the interaction with PP1c (*3-5*).

We have previously identified and isolated a novel phosphorylation-dependent inhibitory protein, termed CPI17, from porcine aorta smooth muscle (*6, 7*). CPI17 is similar to inhibitor-1 in its heat stability and phosphorylation dependence. However, its inhibitory activity is potentiated by phosphorylation with protein kinase C (PKC), and its amino acid sequence shows no significant homology to other inhibitory proteins containing the consensus Phe (*7*). Molecular cloning revealed its specific expression in smooth muscle tissues (*7*), in which CPI17 may participate in a novel signal transduction pathway from PKC to PP1s.

Smooth muscle contraction is regulated through phosphorylation of the myosin regulatory light chain (LC20) on Ser19 by Ca^{2+}-calmodulin-dependent myosin light chain kinase. Since the myofibril-bound PP1 holoenzyme (PP1M)

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Abbreviations: ATPγS, adenosine-5'-*O*-(3-thiotriphosphate); CPI17, PKC-potentiated inhibitory protein of PP1; HtCPI17, hexahistidine-tagged CPI17; LC20, myosin regulatory light chain; MOPS, 3-morpholinopropanesulfonic acid; p-, phosphorylated; PKC, protein kinase C; PP1, protein phosphatase 1; PP1c, catalytic subunit of PP1; tp-, thiophosphorylated; u-, unphosphorylated.

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is responsible for dephosphorylating phospho-Ser19 (*8, 9*), the inhibitory activity of CPI17 may be involved in the regulation of smooth muscle contraction. PP1M has been isolated from chicken gizzard (*10, 11*) and porcine bladder (*12*) and consists of a catalytic subunit and two noncatalytic subunits, M110 (110 kDa) and M21 (21 kDa). The latter two subunits associate to form the regulatory M complex, and the M complex associates with the catalytic subunit through M110 (*10*). Although the isolated PP1M was not inhibited by inhibitor-1 and inhibitor-2 (*10*), possible PKC-related regulatory mechanisms for PP1M have been suggested. First, the phosphorylation of M110 reduced the activity of PP1M without dissociation of the subunits (*13-15*). Second, the physiological concentration of arachidonic acid, which activates atypical PKC, reduced the phosphatase activity toward p-myosin (*16, 17*). Similarly, phorbol ester, an activator of PKC, has been reported to suppress PP1M activity (*18, 19*). The suppression of PP1M activity is thought to be involved in the Ca^{2+}-sensitization of smooth muscle contraction mediated by G-protein (reviewed in Ref. *20*), and these results are consistent with our results on the PKC-dependent inhibitory activity of CPI17 (*6, 7*).

In a previous paper, collaborating with Kitazawa's group, we further revealed that phosphorylated CPI17 could induce the contraction of permeabilized vascular smooth muscle under the sub-maximal Ca^{2+} concentration with increase in the extent of myosin phosphorylation (*21*). In the present paper, we fractionated several CPI17-sensitive PP1 holoenzymes in the myofibrillar extract from porcine aorta smooth muscle. The results demonstrated extensive roles of CPI17 in the PKC-dependent signal transduction pathway in smooth muscle, and PP1M comprises a major fraction of the highly sensitive PP1 holoenzymes, which is potently inhibited by CPI17.

MATERIALS AND METHODS

Materials—Myosin was prepared from porcine aorta by the method of Hasegawa *et al.* (*22*). LC20, myosin light chain kinase (MLCK) and calmodulin were prepared from frozen chicken gizzard (*7*). Phosphorylase-*b* was prepared as described by Fischer and Krebs (*23*). Phosphorylase kinase was purchased from Sigma. Phosphorylated LC20 (p-LC20) and phosphorylated myosin (p-myosin) were prepared according to Yoshida and Yagi (*24*), and phosphorylase-a was prepared as described by Cohen *et al.* (*25*). CPI17 was prepared from porcine aorta (*6*). Hexahistidine-tagged CPI17 (HtCPI17) was expressed in *Escherichia coli* and purified by successive chromatographies on Ni-bound Chelating Sepharose and Ultrogel AcA 54 (*7*). Phosphorylated (p-) and thiophosphorylated (tp-) HtCPI17 were prepared by phosphorylation and thiophosphorylation of the unphosphorylated HtCPI17 (u-HtCPI17) with porcine brain protein kinase C (PKC) in the presence of 0.1 mM ATP and 1 mM ATPγS, respectively (*7*). Thiophosphorylated HtCPI17 was further purified by gel filtration on Ultrogel AcA 54 (2.6×55 cm). Thiophosphorylated Ht-CPI17 and u-HtCPI17 were conjugated to Sepharose 4B (tp- or u-HtCPI17-Sepharose) using CNBr-activated Sepharose 4B (Pharmacia). About 1 mg of each protein was immobilized in 1 ml of the resin.

The antiserum against CPI17 was produced in rabbit using u-HtCPI17 as an antigen. The antibody against the catalytic subunit of PP1, which cross-reacted with protein phosphatase 2A, 2B, and X, was purchased from Santa Cruz. Microcystin-LR and okadaic acid were purchased from Research Biochemicals International. Inhibitor-2 and ATPγS were purchased from New England Biolabs and Boehringer Mannheim, respectively.

Preparation of the 0-12% Polyethylene Glycol (PEG) Fraction from the Myofibrillar Extract of Porcine Aorta Media—The myofibrillar extract was obtained by the method of Alessi *et al.* (*10*) with minor modifications. All procedures were carried out at 4°C. Minced porcine aorta media was homogenized with 5 volumes of buffer A [5 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, and 20 mM Tris-HCl, pH 8.0] with the protease inhibitors cocktail (1 mM phenylmethanesulfonyl fluoride, 4 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.1 mM diisopropylfluorophosphate, 1 mM benzamidine). Myofibrils were precipitated by centrifugation for 20 min at 4,400×g. After another washing with 5 volumes of buffer A, the pellet was homogenized with 3 volumes of buffer B [0.6 M NaCl, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, and 20 mM Tris-HCl, pH 8.0, with protease inhibitors cocktail]. The homogenate was stirred for 30 min and centrifuged for 30 min at 4,400×g. The supernatant (myofibrillar extract) was fractionated by the addition of 50% polyethylene glycol #6,000 (M_r=7,300-9,000, Nacalai Tesque) solution to 12% (w/v). After standing for 1 h, the pellet was collected by centrifugation at 4,400×g for 15 min and suspended in 100 ml of buffer C [0.1 mM EGTA, 0.02% (w/v) Brij-35, 10% glycerol, 0.1% (v/v) 2-mercaptoethanol, and 20 mM Tris-HCl, pH 7.5] containing 0.1 M NaCl and the protease inhibitors cocktail. The suspension was centrifuged at 31,000×g for 15 min and the supernatant was used as the 0-12% PEG fraction of myofibrillar extract.

Purification of Protein Phosphatase 1M of Porcine Aorta Smooth Muscle—The 0-12% PEG fraction of myofibrillar extract was prepared from 500 g of porcine aorta media as described above. The fraction was loaded onto a column (1.9×3.5 cm) of tp-HtCPI17-Sepharose equilibrated with buffer C containing 0.1 M NaCl and the protease inhibitors cocktail. After washing the column with the same buffer, the bound proteins were eluted with 50 ml of buffer C containing 0.5 M NaCl and the protease inhibitors cocktail, and 2-ml fractions of the effluent were collected with continuous monitoring of proteins by absorbance at 280 nm. The effluent was diluted with buffer C to decrease the concentration of NaCl to 0.1 M and loaded onto a Mono Q column (0.5×5.0 cm, Pharmacia) equilibrated with buffer C containing 0.1 M NaCl. The column was washed with the same buffer, then bound proteins were eluted with a linear gradient (40 ml) of the concentration of NaCl from 0.1 to 0.5 M at a flow rate of 0.5 ml/min, and the effluent (0.5 ml/fraction) was collected. The fractions containing the major p-myosin phosphatase activity were pooled and diluted with buffer C. The resulting solution was loaded onto a Mono S column (0.5×5.0 cm, Pharmacia) equilibrated with buffer C containing 0.1 M NaCl. Bound proteins were desorbed by a linear gradient elution (0.1 to 0.4 M NaCl, 60 ml) at a flow rate of 0.5 ml/min, and the effluent (0.5 ml/fraction) was collected. The p-myosin phosphatase activities were eluted as two peaks, as the breakthrough fraction and the fraction eluted at about 0.25 M NaCl. The second

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half of the latter peak consisted of three polypeptides with estimated molecular masses of 140, 37, and 20 kDa on SDS-gel, and these fractions were combined. The combined fraction was loaded onto a DEAE-Toyopearl 650M column (0.1 ml) equilibrated with buffer C containing 0.1 M NaCl, and the trimeric phosphatase (PP1M) was eluted with 0.2 ml of buffer C containing 1.0 M NaCl. The concentrated PP1M (about 10 μg) with the specific activity of around 1 unit/mg was stored at −20°C after addition of an equal volume of glycerol.

Phosphatase Assay—Phosphatase assay was carried out as described previously (7). The conditions used were 50 mM MOPS-NaOH (pH 7.2), 0.1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mg/ml BSA, and 0.02% Brij-35, at 25°C for 10 min, in the presence of 10 μM phosphorylase-a or 2 μM p-myosin as a substrate. Caffeine was added to 5 mM in the phosphorylase phosphatase assay. The inhibitory effect of the inhibitors was assayed in the presence of 50 mM NaCl. One unit of the activity was defined as the activity releasing 1 μmol of phosphate from the substrate in 1 min.

Determination of the Amino Acid Sequence—Partial amino acid sequences of the peptides derived from PP1M were determined as follows. The major p-myosin phosphatase fraction eluted from the Mono Q column was concentrated by ultrafiltration using Ultrafree C3 (Millipore). The concentrated sample was subjected to SDS-PAGE. The individual bands visualized by Coomassie Blue staining were excised from the gel. The gel pieces were placed in a microtube and washed three times for 10 min each with 200 μl of 25% isopropanol using a vortex mixer, and another three times with 45% ethanol, then lyophilized. Five microliters of 0.1 mg/ml lysylendopeptidase (Wako Pure Chemical Industries) in buffer D (8 M urea, 50 mM Tris-HCl, pH 9.0) was added and allowed to permeate into the dried gel, then 195 μl of buffer D was added. After incubation at 37°C for 6 h, another 5 μl of 0.1 mg/ml lysylendopeptidase was added, and the mixture was left at 37°C for 12 to 18 h. The peptide solution was collected by centrifugation and saved (1st extract). The residual peptides in the gel were further extracted with 0.1% trifluoroacetic acid by stirring for 1 h with a vortex mixer. The supernatant was collected and combined with the 1st extract. The peptides in the extract were separated by reverse-phase HPLC using a TSK-gel ODS-80Ts column (Tosoh, 4.6×150 mm), applying a linear gradient of 0–60% acetonitrile in the presence of 0.1% trifluoroacetic acid. The amino acid sequence of the purified peptides was analyzed using a Perkin Elmer Applied Biosystems 492 Protein Sequencer.

Others—Protein concentration was determined according to the improved method of Bradford (26) using BSA as a standard. SDS-PAGE was carried out according to Porzio and Pearson (27). Proteins in gel were visualized by staining with Coomassie Blue G-250 or by the silver staining (28). Marker proteins used were the rabbit skeletal myosin heavy chain (210 kDa), phosphorylase-*b* (92.5 kDa), BSA (68 kDa), ovalbumin (43 kDa), chymotrypsinogen (25.7 kDa), and lysozyme (14.3 kDa). Western blotting was carried out as follows. Proteins in the SDS-gel were transferred onto a sheet of nitrocellulose filter (Schleicher & Schuell, BA79) using a semi-dry blotting apparatus (Biocraft). After blocking nonspecific sites on the filter with 2% (w/v) gelatin in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% (v/v) Tween 20, CPI17 was detected with the rabbit anti-CPI17 antiserum, peroxidase-conjugated anti-rabbit IgG (BioRad) and SuperSignal Substrate Western Blotting (Pierce). The catalytic subunit of PP1 on the blot was visualized with anti-PP1 antibody, alkaline phosphatase-conjugated anti-rabbit antibody (BioRad), and alkaline phosphatase conjugate substrate kit (BioRad). For quantitative analysis of the content of CPI17 in the smooth muscle extract, the intensity of the visualized band was measured with use of a densitometer (ATTO, AE6920W), and the amount of CPI17 in the extract was determined by comparison with the intensity of the standard CPI17 isolated from porcine aorta on the same blot. Gel filtration of PP1M was carried out as follows. The isolated PP1M (10 μg) was loaded onto a Superdex 200 HR column (1.0×30 cm, Pharmacia) equilibrated with buffer C containing 0.15 M NaCl, and the column was eluted with the same buffer at a flow rate of 0.25 ml/min at 4°C. The effluent fractions (0.25 ml/tube) were collected. The molecular weight was estimated from the elution volume by comparison with those of the marker proteins: apoferritin (450 kDa), immunoglobulin (160 kDa), BSA (68 kDa), ovalbumin (43 kDa), and myoglobin (17.6 kDa).

Kinetic analysis of the inhibitory activity of CPI17 was performed as described by Dixon *et al.* (29). The initial velocities of the phosphatase reaction at 0.22–10 μM p-LC20 were measured in the presence of 0, 1.3, 2.5, 5.0, 10, and 20 nM tp-HtCPI17. A Lineweaver-Burk plot, 1/velocity *vs.* 1/[p-LC20], indicated a mixed-type inhibition (data not shown). The K_i and K_i' values were determined from a secondary plot of the slopes and the intercepts from the Lineweaver-Burk plot against the concentration of tp-HtCPI17. The secondary plot showed a mixed inhibition (29), and the results were analyzed by the linear curve-fitting using KaleidaGraph (Synergy Software).

RESULTS

Type 1 Protein Phosphatases as the Target of CPI17 in the Aorta Smooth Muscle—Since CPI17 is specifically expressed in smooth muscle cells in which the myofibril is well developed (7), we searched for the target phosphatase(s) of CPI17 in the myofibrillar extract from porcine aorta media. A fraction of the myofibrillar extract precipitated at 12% PEG (the 0–12% PEG fraction) was prepared and subjected to affinity chromatography on tp-HtCPI17 Sepharose (Fig. 1). Almost all of the phosphorylase-*a* phosphatase activity (593 mU) in the 0–12% PEG fraction was bound to the resin, and only 51 mU of the phosphatase activity was detected in the breakthrough fraction. About 190 mU of phosphorylase-*a* phosphatase was eluted by increasing the concentration of NaCl to 0.5 M. The p-myosin phosphatase activity was co-eluted with phosphorylase-*a* phosphatase. Further activity was not eluted with increase in the concentration of NaCl to 1 M or by 3 M KSCN. In contrast, none of the phosphatase activity bound to the u-HtCPI17-conjugated resin (data not shown). Therefore, both phosphorylase-*a* phosphatase and p-myosin phosphatase in the myofibrillar extract were bound to the CPI17-conjugated resin depending on thiophosphorylation of CPI17.

Effects of tp-HtCPI17 on the phosphatase activities are

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shown in Fig. 2. The phosphorylase-*a* phosphatase activity bound to the tp-HtCPI17 resin was completely inhibited by tp-HtCPI17 with IC_{50} of 0.7 nM. Similarly, p-myosin phosphatase activity bound to the resin was also completely suppressed by tp-HtCPI17 with IC_{50} of 3 nM. On the other hand, the phosphatase activities in the breakthrough fraction, although minor in amounts, required higher concentrations of tp-HtCPI17 for inhibition. IC_{50} was about 20 nM for p-myosin phosphatase, and phosphorylase-*a* phosphatase activity was suppressed only to 20% in the presence of 10 μM tp-HtCPI17.

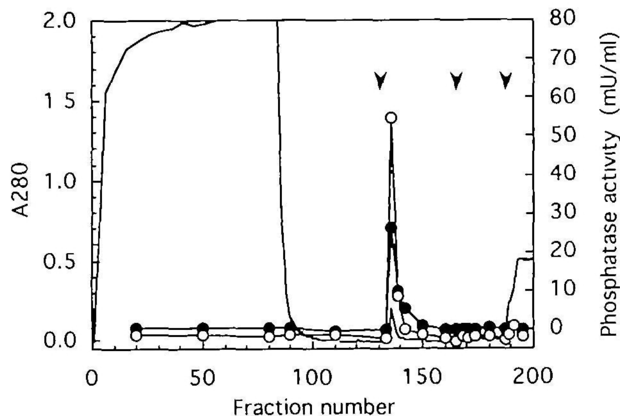

Fig. 1. **Thiophosphorylated-HtCPI17-Sepharose 4B affinity chromatography of the myofibrillar extract from porcine aorta smooth muscle.** The 0-12% PEG fraction of the myofibrillar extract was loaded onto a tp-HtCPI17 Sepharose column (1.5×3.0 cm). After washing with buffer C containing 0.1 M NaCl, the bound proteins were successively eluted with 0.5 M NaCl, 1.0 M NaCl, and 3 M KSCN at the positions indicated by arrowheads. Two-milliliter fractions were collected. Phosphorylase-*a* and p-myosin phosphatase activities are indicated by closed and open circles, respectively. Solid line indicates the absorbance at 280 nm.

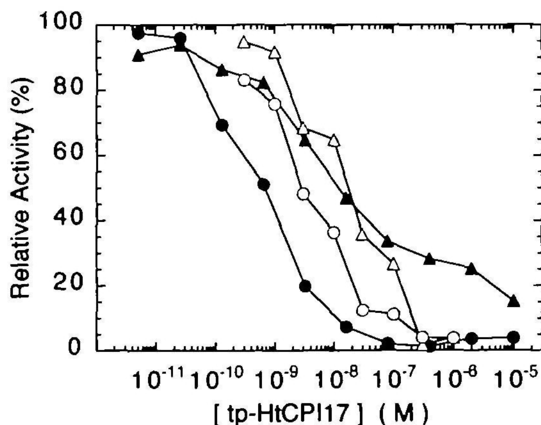

Fig. 2. **Effects of tp-HtCPI17 on the phosphatase activities separated by the affinity chromatography.** The phosphatase fractions bound to the tp-HtCPI17 resin (circles), and in the breakthrough fraction (triangles) were compared. Open and closed symbols indicate the activities for p-myosin (2 μM) and phosphorylase-*a* (10 μM), respectively. Reactions were started by the addition of phosphatase preparations and continued for 10 min (for myosin phosphatase) or 30 min (for phosphorylase phosphatase) at 25°C. The 100% value indicates the activity without tp-HtCPI17. The indicated data are the average of triplicate assays.

The phosphatase fraction bound to tp-HtCPI17 resin was separated into five peaks of p-myosin phosphatase activity by column chromatography on Mono Q (Fig. 3). As shown in Fig. 3, the ratio of p-myosin phosphatase activity to phosphorylase-*a* phosphatase activity differed for each peak. SDS-PAGE showed that each phosphatase fraction consisted of a 37-kDa polypeptide that cross-reacted with anti-PP1 antibody, and several polypeptides of various sizes (data not shown). Therefore, several types of PP1 holoenzyme with high sensitivity to CPI17 may play different roles in myofibrils of vascular smooth muscle. Since the fraction eluted at around 0.35 M NaCl showed the highest content of p-myosin phosphatase activity, the

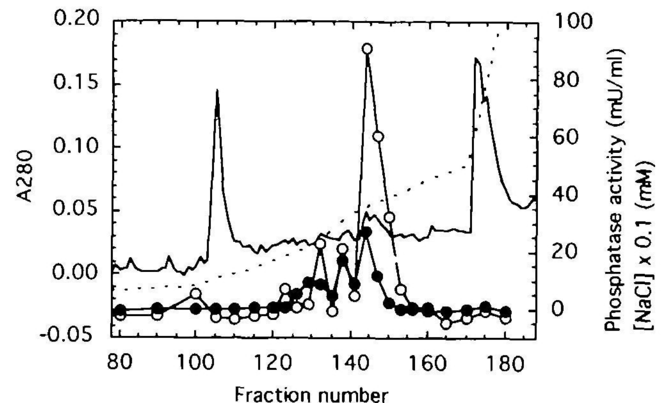

Fig. 3. **Mono Q column chromatography of the phosphatase fraction bound to the tp-HtCPI17 resin.** The fractions eluted with 0.5 M NaCl from tp-HtCPI17-Sepharose were loaded onto a Mono Q HR 5/5 column, and the bound proteins were eluted with a linear gradient of [NaCl] from 0.1 to 0.5 M (40 ml). Fractions of 0.5 ml were collected. Phosphorylase-*a* (solid circles) and p-myosin (open circles) phosphatase activities were measured for 10 min at 25°C. The absorbance at 280 nm and the concentration of NaCl are indicated by the solid line and the dotted line, respectively.

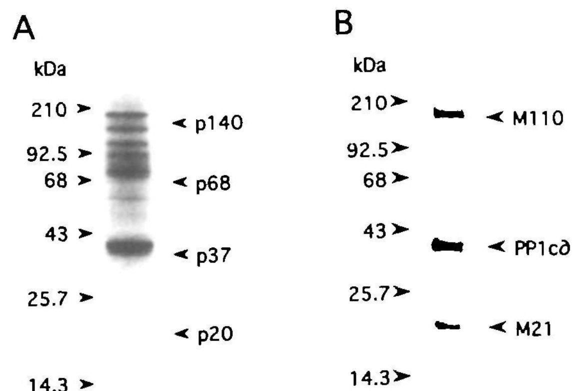

Fig. 4. **SDS-PAGE of the CPI17-sensitive p-myosin phosphatase fractions.** The crude p-myosin phosphatase fraction loaded onto the Mono S column (A), and the purified enzyme (10 ng) (B). Proteins in gel were stained with Coomassie Blue (A) or silver (B). Molecular weights of marker proteins are indicated at the left side. In panel A, bands of 140, 68, 37, and 20 kDa are indicated as p140, p68, p37, and p20, respectively. In panel B, three subunits of PP1M are indicated.

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enzyme in this fraction was characterized further.

Purification of a Myofibrillar Trimeric Myosin Phosphatase (PP1M)—As shown by SDS-PAGE (Fig. 4A), the major p-myosin phosphatase fraction from the Mono Q column consisted of many polypeptides, including those with estimated molecular masses of 140, 110, 90, 68, 54, 37, and 20 kDa. After storage on ice for a few days, the 140-, 110-, and 90-kDa polypeptide bands disappeared, while the intensity of 68-kDa band (p68) increased (data not shown). Since p68 was suggested to be a proteolytic fragment of these polypeptides, this possibility was examined by *micro*-sequence analysis. After excising the corresponding band, p68 within the gel piece was digested with lysylendopeptidase, and the resulting peptide fragments extracted from the gel were purified by HPLC. The amino acid sequences of the two peptides derived from p68 are shown in Table I. Homology search using the FASTA program (*30*) revealed that the two sequences were nearly identical to those of M110 subunit of human PP1M at the region of 429-437 and 228-238 (*31*). Therefore, p68 seems to be a proteolytic fragment of M110. Then, the amino acid sequence of one of the peptides derived from 140-kDa polypeptide (p140) was determined. The resulting sequence showed 90% identity to the region of 596-605 of M110 subunit of human PP1M (Table I). Since M_r of p140 is the highest in this fraction and is similar to that of M110 subunit of chicken gizzard and porcine bladder PP1M, p140 seems to be the intact M110 subunit of aorta PP1M. Similarly, peptides from a 20-kDa polypeptide (p20) band were isolated and their sequences were found homologous to both chicken M21 (*32*) and human M110 (*31*) of PP1M (Table I). The overall sequence of four fragments derived from p20 showed 91 and 85% identities to those of M21 and M110, respectively. The amino acid sequence of peptides in the digest of 37-kDa polypeptide (p37) identified p37 as a delta isoform of PP1c (data not shown) (*33*). Therefore, the major p-myosin phosphatase fraction from the Mono Q column is most probably assigned as PP1M, a heterotrimeric protein consisting of p140, p37, and p20.

To verify the subunit composition of PP1M in mammalian aorta smooth muscle, the major p-myosin phosphatase fraction was further purified by chromatography on Mono S, and the trimeric PP1M was isolated ("MATERIALS AND METHODS"). Figure 4B shows a result of the SDS-PAGE of the isolated PP1M, in which three polypeptide

TABLE I. **Amino acid sequences of peptides derived from p140, p68, and p20.** The partial amino acid sequences of p140, p68, and p20 were determined as described in "MATERIALS AND METHODS." Corresponding sequences were sought by use of the FASTA program (*30*), and the identity to sequences of M110 from human (*31*) and M21 from chicken (*32*), respectively, is indicated in the right column. x denotes a residue that could not be identified.

Excised band	Determined sequence	Identity (%)	
		M110	M21
p140	ITTGSxSAGT	90	–
p68	xESPASxR	83	–
	DYDGxTxLxA	100	–
p20	xDNQRLK	100	100
	TERRALERK	78	78
	LYESALAENQ	70	90
	xENGALIRVI	100	100

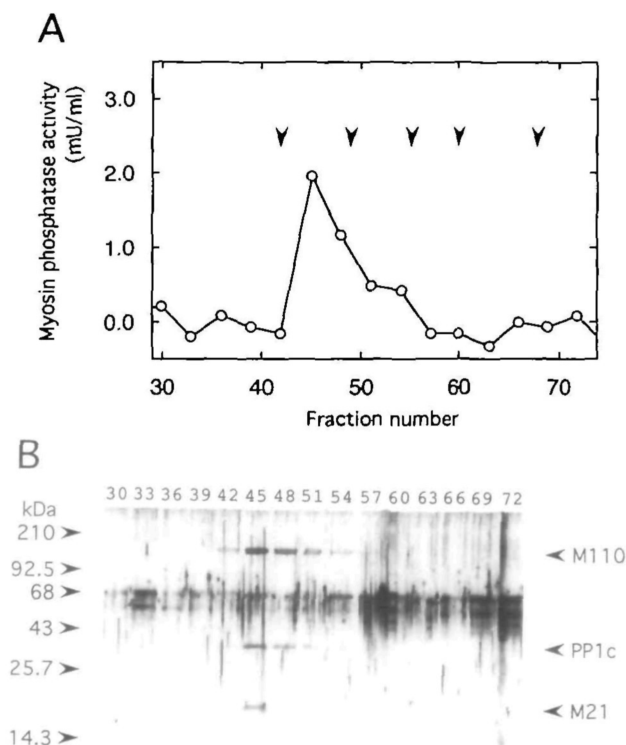

Fig. 5. **Gel filtration of the purified trimeric p-myosin phosphatase.** A: Elution profile of the phosphatase activity. Ten micrograms of the trimeric phosphatase was loaded onto a Superdex 200 HR column. Flow rate was 0.25 ml/min, and 0.25-ml fractions were collected. Open circles indicate the p-myosin phosphatase activity, and the arrowheads, from left to right, indicate the elution positions of apoferritin (450 kDa), immunoglobulin (160 kDa), BSA (68 kDa), ovalbumin (43 kDa), and myoglobin (17.6 kDa), respectively. B: Silver-stained SDS-gel of the fractions in panel A. The figures on the top of each well correspond to the fraction numbers in panel A. The molecular weight of marker proteins are indicated at the left side. Three subunits of PP1M are indicated.

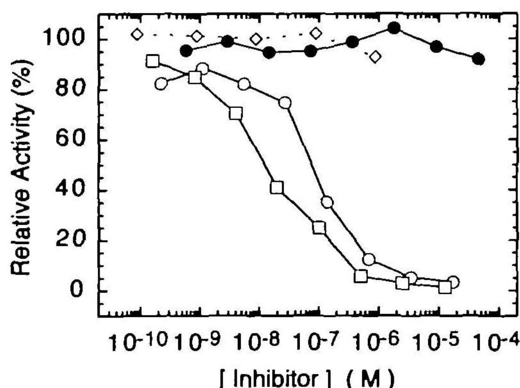

Fig. 6. **Effects of HtCPI17 and inhibitor-2 on the PP1M activity.** The p-myosin phosphatase activity of the isolated PP1M was measured in the presence of u- (solid circles), p- (open circles), and tp-HtCPI17 (squares). Phosphatase reaction was started by addition of the enzyme. Details are described in "MATERIALS AND METHODS." The activity in the presence of inhibitor-2 (diamonds) was measured after preincubation of the enzyme with inhibitor-2 for 15 min at 25°C.

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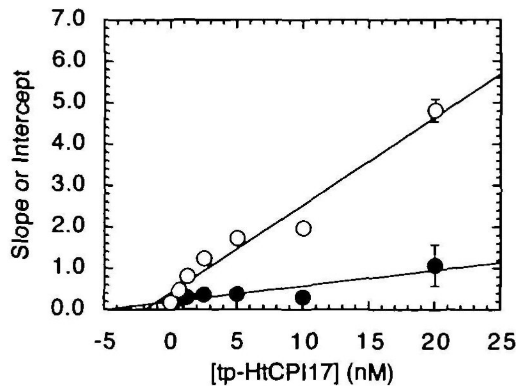

Fig. 7. **Inhibition of PP1M by tp-HtCPI17.** The phosphatase activities at 0.22–10 μM p-LC20 were measured in the presence of various concentrations of tp-HtCPI17. The values of slope (open circles) and intercept of Y-axis (closed circles) in the Lineweaver-Burk plot were plotted against the concentration of tp-HtCPI17. The solid lines show the best-fit curves drawn according to the equations for mixed inhibition (*29*). Assays were carried out in the presence of 50 mM NaCl for 5-30 min at 25°C. The concentration of PP1M (73 pM) is low enough that the amount of tp-HtCPI17 bound to the enzyme can be neglected. The values of K_i and K_i' giving the best-fit curves were 1.9 and 5.1 nM, respectively.

bands of 140-, 37-, and 20-kDa corresponding to subunit M110, PP1cδ, and M21, respectively, can be identified. The isolated PP1M was analyzed by gel filtration on Superdex 200HR 10/30 (Fig. 5A). A single peak of the p-myosin phosphatase activity with an apparent molecular mass of 340 kDa was observed. Results of SDS-PAGE showed simultaneous elution of the three bands corresponding to 140-, 37-, and 20-kDa polypeptides that coincided with the single peak of the p-myosin phosphatase activity (Fig. 5B). Thus, the PP1M in mammalian vascular smooth muscle is the trimeric holoenzyme.

Characterization of Porcine Aortic PP1M as a Target of CPI17—As shown in Fig. 6, the PP1M activity was completely inhibited by tp-HtCPI17 as well as by p-HtCPI17 without any preincubation steps, while u-HtCPI17 could not suppress the activity. The IC_{50} values of tp- and p-HtCPI17 were 12 and 80 nM, respectively. In contrast to CPI17, inhibitor-2, which is a well-known inhibitor protein of PP1, did not inhibit the activity of PP1M even after 30 min of preincubation (Fig. 6). The sensitivities of porcine aorta PP1M to okadaic acid (IC_{50} = 11 nM) and to microcystin LR (IC_{50} = 0.14 nM) were consistent with the previous report for chicken gizzard PP1M (*10*).

Results of kinetic analysis of the inhibitory effect of HtCPI17 are shown in Fig. 7. In these experiments, the isolated chicken gizzard LC20 was used as a substrate to avoid contamination of any p-myosin phosphatase activities. Effect of the substrate concentration (0.22–10 μM) on the initial velocity was studied at various concentrations (0-20 nM) of tp-HtCPI17 and the results were analyzed by Lineweaver-Burk plotting. The set of linear relationships in the Lineweaver-Burk plots revealed a mixed inhibition by tp-HtCPI17 (data not shown). As shown in Fig. 7, the secondary plots on values of the slope and the intercept from Lineweaver-Burk plot against the concentration of HtCPI17 yielded a nearly linear relationship (Fig. 7). The results in Fig. 7 were analyzed by curve-fitting to the

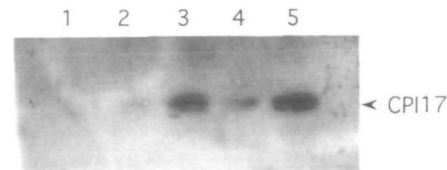

Fig. 8. **Detection of CPI17 in the extracts from porcine aorta smooth muscle.** Porcine aorta media was successively extracted with 3 vol each of buffer A (lanes 1 and 2), buffer B (lane 3), and buffer A plus 0.5% Triton X100 (lane 4). The samples corresponding to the whole extract from 1 mg of the starting material (aorta media) were loaded on the SDS-gel, and the resolved CPI17 was visualized by Western analysis using the anti-CPI antiserum. Isolated CPI17 (4 ng) was used as a marker (lane 5).

equation derived for mixed inhibition (*29*), which gave K_i and K_i' values of 1.9 and 5.1 nM, respectively. These results show that CPI17 is a potent inhibitory protein of trimeric PP1M in porcine aorta. The K_i and K_i' values of CPI17 are in a similar range to the values of inhibitor-1 towards PP1c, using phosphorylase-*a* as a substrate (*34*).

Estimation of CPI17 in Porcine Aortic Smooth Muscle—The concentration of CPI17 in smooth muscle cells was estimated by Western blotting. As shown in Fig. 8, the signal of CPI17 around 20 kDa was dominantly detected in the extract with buffer B containing 0.6 M NaCl. By comparing the band intensity with that of an internal standard, the amount of CPI17 was estimated as about 2 ng per mg of porcine aorta smooth muscle tissue. The value indicates the concentration of CPI17 to be at least submicromolar in smooth muscle cells (see "DISCUSSION").

DISCUSSION

Smooth muscle contraction is regulated through the Ca^{2+}-dependent phosphorylation of myosin. CPI17 was found in porcine aorta smooth muscle as a phosphorylation-dependent inhibitory protein of the p-myosin phosphatase preparation consisting of the 37-kDa delta isoform of PP1c and the 69-kDa noncatalytic subunit (*6*): the latter subunit was identified in the present work as the proteolytic derivative from M110 subunit of PP1M. Recent results of molecular cloning revealed the specific expression of CPI17 in smooth muscle tissues (*7*). Physiological function of CPI17 was confirmed by experiments on permeabilized vascular smooth muscle in which phosphorylated CPI17 could induce contraction with increase in the extent of myosin phosphorylation (*21*).

Since the inhibitory activity of CPI17 is potentiated by PKC, CPI17 can be expected to work as a mediator of PKC-related signal transduction pathways through the regulation of specific PP1s. At least five PP1 activities in porcine aorta smooth muscle were fractionated as the CPI17-sensitive PP1 by tp-CPI17 affinity chromatography (Fig. 3), which is a powerful tool to search for the target protein of an immobilized ligand (*35, 36*). Although the concentration of HtCPI17 in the affinity column (47 μM, or 1 mg CPI17/1 ml of resin) was much higher than IC_{50} for u-HtCPI17 [8 μM against the degraded PP1M (*21*)], the phosphatases were bound to the resin depending on thiophosphorylation of CPI17 on Thr-38. Since the activity of unbound phosphatases was suppressed to 50% by the

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addition of 1 μM inhibitor-2, a well-known inhibitory protein of PP1, a part of unbound phosphatases is classified as type 1 (data not shown). Therefore, the tp-HtCPI17 on the affinity resin specifically recognized type 1 phosphatases that are highly sensitive to CPI17, and the PP1 activities bound to the resin can be considered as the possible physiological target of CPI17 in aorta smooth muscle. These results may imply novel PKC-dependent signal transduction pathways through the regulation of PP1s mediated by CPI17 or related inhibitory proteins in other tissues.

In the present work, PP1M was purified from porcine aorta smooth muscle, which showed similar properties to PP1Ms from chicken gizzard and porcine bladder in sensitivities to okadaic acid, microcystin LR, and inhibitor-2 (*10, 12*). The isolated PP1M with an estimated molecular mass of 340 kDa was composed of three subunits with estimated molecular masses of 140, 37, and 20 kDa (Figs. 4 and 5). The partial amino acid sequences of these subunits were highly homologous to those of M110, PP1cδ, and M21 subunit from chicken and human PP1Ms (*31, 32*). No information on M21 subunit has yet been presented on mammalian vascular smooth muscle myosin phosphatase; this is the first such report, and the findings suggest the general trimeric nature of PP1M. It is noteworthy that PP1M holoenzyme binds directly to tp-HtCPI17 resin, which indicates that p-CPI17 associates with PP1M without dissociation of its regulatory subunits. Therefore, the notable feature of CPI17 was also confirmed in this work.

Use of the isolated PP1M in detailed kinetic analysis on the inhibitory effects of CPI17 provided an insight into the interaction of CPI17 with PP1M. As reported for chicken gizzard PP1M (*10*), the binding of M complex to the catalytic subunit may raise the catalytic activity of aorta PP1M toward p-myosin due to the direct binding of M complex to myosin (*1, 37-39*). The binding of M complex may also cause a conformational change in the active site for favorable fitting to the structure around phosphorylated Ser19 of LC20 (*1, 4, 9*). Figure 9 shows the amino acid sequences of LC20 and CPI17 around each phosphorylation site, of which Thr38 of CPI17, a phosphorylation site for PKC, overlaps with Thr18 of LC20 (*6, 7, 40*). PP1M participates in dephosphorylation of the major (Ser19) and the secondary (Thr18) phosphorylation sites of LC20 (*37, 41*). In the N-terminal region adjacent to the phosphorylation sites is a basic cluster consisting of three basic residues (black box). According to the crystal structure of PP1c, an acidic groove is located near its catalytic center (*42*), and the acidic residues within it are possibly involved in the recognition of the basic residues in the N-terminal region adjacent to phosphorylated Ser or Thr (*43*). As shown in Fig. 9, the alignment of the phosphorylatable Thr38 with

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                   *
CPI17 (30-46)   LQKRHARVTVKYDRREL
LC20 (10-26)    TKKRPQRATSNVFAMFD
                   **
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Fig. 9. **Alignment of the amino acid sequences around phosphorylatable threonine residues of CPI17 and LC20.** The numbers in parentheses indicate the positions of each peptide segment in the original sequence (*7, 40*). Asterisks and black boxes indicate the phosphorylatable residues and the conserved basic residues, respectively.

the adjacent basic residues (black box in Fig. 9) in CPI17 is similar to that in LC20. Since the kinetic analysis showed the mixed-type inhibition of CPI17, CPI17 may work as a pseudosubstrate, and the direct binding of CPI17 to the catalytic center of PP1 may be possible. Therefore, M complex may change the structure around the catalytic center to increase the affinity for p-LC20, which is also favorable for the binding of p-CPI17, leading to the potent inhibition of PP1M.

As confirmed in the present work, tp-CPI17 can directly interact with PP1M, and the isolated PP1M from aorta smooth muscle was potently inhibited by tp-CPI17 with inhibition constants of 1.9 and 5.1 nM (Fig. 7). Further, the results in Fig. 8 yielded an estimated myofibrillar concentration of CPI17 that is consistent with its function as a physiological regulator of PP1M. Since 1 mg of aorta media contains about 2 ng of CPI17 (Fig. 8), the concentration of CPI17 in smooth muscle cells is estimated to be at least 0.3 μM by assuming the fractional volume of smooth muscle cells in aorta media to be 39%, as in femoral artery (*44*). Since the *in situ* concentration of PP1M was estimated to be sub-micromolar (*10*), the concentration of CPI17 is sufficient to for it act as a regulator of PP1M in aorta smooth muscle. The result is consistent with the result of our previous physiological experiments in which inhibition of the *in situ* p-myosin phosphatase activity by tp-CPI17 accompanied contraction of the permeabilized smooth muscle strip under sub-maximal conditions for contraction depending on phosphorylation of LC20 (*21*). Therefore, the biochemical properties of CPI17 strongly support the putative physiological role of CPI17 as a mediator of the PKC-dependent signal transduction pathway in smooth muscle contraction.

Since inhibitor-1 and -2 do not inhibit gizzard PP1M, studies on regulation of PP1M activity have focused on the role of M110. Gong *et al.* reported that arachidonic acid releases the catalytic subunit of PP1M from the holoenzyme with reduction of activity toward p-myosin (*16*). PP1M activity is also regulated by the phosphorylation of M110 subunit by an unidentified endogenous kinase (*13, 14*) and by rho-kinase (*15*). It is noteworthy that the phosphorylated residue in M110 is very labile because of auto-dephosphorylation, which suggests that the phosphorylation of M110 works for the temporary regulation of the PP1M activity. The activation of PKCs in smooth muscle tissues by phorbol ester led to contraction *via* the inhibition of myosin phosphatase activity *in situ* (*18, 19*). Gailly *et al.* also reported that phenylephrine induces Ca^{2+} sensitization of smooth muscle contraction through the activation of atypical PKC, in which the myosin phosphatase activity was suppressed (*17*). These results indicated the presence of signal pathways to control the activity of PP1M through PKC activation in smooth muscle. In the present work, aortic trimeric PP1M was identified as a target protein for CPI17. Since phosphorylation of Thr-38 by PKC caused the maximum potentiation of CPI17 (*19, 21*), and p-CPI17 was scarcely dephosphorylated by PP1M (Eto, M., unpublished observations), CPI17 is expected to work as a stable regulator for PP1M in the PKC-mediated pathway in smooth muscle. The relationship between phosphorylation of CPI17 and myosin LC20 after stimulation of smooth muscle should be clarified.

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