Phosphorylation of the myosin phosphatase inhibitors, CPI-17 and PHI-1, by integrin-linked kinase

Jing Ti DENG*, Cindy SUTHERLAND*, David L. BRAUTIGAN†, Masumi ETO† and Michael P. WALSH*[1]

*Smooth Muscle Research Group and Canadian Institutes of Health Research Group in Regulation of Vascular Contractility, University of Calgary Faculty of Medicine, 3330 Hospital Drive N.W., Calgary, Alberta T2N 4N1, Canada, and †Center for Cell Signaling, University of Virginia School of Medicine, Box 800577-MSB 7225, Charlottesville, VA 22908, U.S.A.

Integrin-linked kinase (ILK) has been implicated in Ca^{2+}-independent contraction of smooth muscle via its ability to phosphorylate myosin. We investigated the possibility that this kinase might also phosphorylate and regulate the myosin light-chain phosphatase inhibitor proteins CPI-17 [protein kinase C (PKC)-dependent phosphatase inhibitor of 17 kDa] and PHI-1 (phosphatase holoenzyme inhibitor-1), known substrates of PKC. Both phosphatase inhibitors were phosphorylated by ILK in an in-gel kinase assay and in solution. A Thr → Ala mutation at Thr^{38} of CPI-17 and Thr^{57} of PHI-1 eliminated phosphorylation by ILK. Phosphopeptide mapping, phospho amino acid analysis and immunoblotting using phospho-specific antibodies indicated that ILK predominantly phosphorylated the site critical for potent inhibition, i.e. Thr^{38} of CPI-17 or Thr^{57} of PHI-1. CPI-17 and PHI-1 thiophosphorylated by ILK at Thr^{38} or Thr^{57} respectively inhibited myosin light-chain phosphatase (MLCP) activity bound to myosin, whereas the site-specific mutants CPI-17-Thr^{38}Ala and PHI-1-Thr^{57}Ala, treated with ILK under identical conditions, like the untreated wild-type proteins had no effect on the phosphatase. Consistent with these effects, both thiophospho-CPI-17 and -PHI-1 induced Ca^{2+} sensitization of contraction of Triton X-100-demembranated rat-tail arterial smooth muscle, whereas CPI-17-Thr^{38}Ala and PHI-1-Thr^{57}Ala treated with ILK in the presence of adenosine 5′-[γ-thio]triphosphate failed to evoke a contractile response. We conclude that ILK may activate smooth-muscle contraction both directly, via phosphorylation of myosin, and indirectly, via phosphorylation and activation of CPI-17 and PHI-1, leading to inhibition of MLCP.

Key words: Ca^{2+} sensitization, muscle contraction, smooth muscle.

INTRODUCTION

Myosin phosphorylation is catalysed predominantly by Ca^{2+}/calmodulin-dependent myosin light-chain kinase (MLCK) [1]. In addition, Ca^{2+}-independent MLCK activity is associated with smooth-muscle myofilaments, whereby inhibition of myosin light-chain phosphatase (MLCP) activity causes increased phosphorylation at Ser^{19} of the 20 kDa light chains (LC_{20}s) of myosin II, resulting in activation of actomyosin cross-bridge cycling and contraction of smooth muscle without an increase in cytoplasmic free Ca^{2+} concentration [2]. Integrin-linked kinase (ILK) was isolated as the Ca^{2+}-independent MLCK from a smooth-muscle myofilament fraction [3]. ILK was originally discovered as a binding protein to the cytoplasmic domain of integrin β subunits and was reported to be involved in cytoskeletal regulation via interaction with actin-binding proteins, such as paxillin and affixin, via its N-terminal ankyrin repeat domain (see [4] for a review). Smooth-muscle myofilament-associated ILK phosphorylates LC_{20}, leading to contraction in the absence of Ca^{2+}, suggesting that ILK-mediated phosphorylation of myosin contributes to Ca^{2+} sensitization of smooth-muscle contraction [3].

Inhibition of smooth-muscle MLCP occurs in response to various agonists. Phosphorylation of the myosin-targeting subunit of MLCP (MYPT1), a regulatory subunit of MLCP, reduces phosphatase activity (see [5] for a review). In addition, CPI-17 [protein kinase C (PKC)-dependent phosphatase inhibitor of 17 kDa] is a specific inhibitor protein for MLCP, expressed predominantly in smooth-muscle tissues [6–8] with higher levels in arteries [9]. When phosphorylated at Thr^{38} by PKC, CPI-17 becomes a potent inhibitor of type 1 protein Ser/Thr phosphatases (PP1s), such as MLCP, and is effective on both the isolated MLCP and the MLCP activity in Triton X-100-demembranated vascular-smooth-muscle strips [6,8,10–13]. CPI-17 is also phosphorylated by Rho-associated kinase (ROCK) [14], protein kinase N (PKN) [15] and zipper-interacting protein (ZIP)-like kinase [16]. Phosphorylation at Thr^{38} of CPI-17 occurs in intact rabbit femoral arterial smooth muscle in response to histamine [11,17]. CPI-17 phosphorylation in arteries is partially suppressed by the kinase inhibitors GF109203x (PKC-specific) and Y27632 (PKC/ROCK-specific), or is eliminated by production of nitric oxide [17,18]. CPI-17 has been suggested to have an important physiological function, particularly in vascular smooth muscles, where its expression level compared with that of MLCP is particularly high [9]. This function involves the phosphorylation-dependent inhibition of MLCP leading to Ca^{2+} sensitization of force development, i.e. increased force at a fixed, submaximal $[Ca^{2+}]$ [13]. PHI-1 (phosphatase holoenzyme

Abbreviations used: CPI-17, protein kinase C-dependent phosphatase inhibitor of 17 kDa; DTT, dithiothreitol; H-T buffer, Hepes-Tyrode's buffer; ILK, integrin-linked kinase; LC_{20}, 20 kDa light chain of myosin; MLCK, myosin light-chain kinase; MLCP, myosin light-chain phosphatase; MYPT1, myosin-targeting subunit of MLCP; PHI-1, phosphatase holoenzyme inhibitor-1; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKN, protein kinase N; PP1, type 1 protein serine/threonine phosphatase; ROCK, Rho-associated kinase; ZIP, zipper-interacting protein.

[1] To whom correspondence should be addressed (e-mail walsh@ucalgary.ca).

inhibitor-1) is an orthologue that is structurally and functionally related to CPI-17, and is also a PKC substrate [19]. Phosphorylation occurs at both serine and threonine residues, but the functionally important site is Thr^{57}. In contrast with CPI-17, PHI-1 is expressed in most cell types [19].

Since myofilament-associated ILK has been implicated in the regulation of smooth-muscle contraction via its ability to phosphorylate myosin, and the phosphatase inhibitors CPI-17 and PHI-1 can be regulated by various protein kinases, we investigated the possibility that ILK could phosphorylate and regulate CPI-17 and PHI-1.

EXPERIMENTAL

Materials

[γ-^{32}P]ATP (> 5000 Ci/mmol) was purchased from ICN, and Triton X-100 and Tween 80 were from Fisher Scientific. ILK was partially purified from chicken gizzard smooth muscle, as described by Deng et al. [3]. Constitutively active PKCδ was prepared from pig aorta, as described by Eto et al. [11]. ROCK2 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, U.S.A.). cDNAs encoding murine PHI-1 and porcine CPI-17 were inserted into pET30 vectors (Novagen, Madison, WI, U.S.A.) to produce His_6-tagged proteins with a 44-residue N-terminal tail after expression in *Escherichia coli* strain BL21(DE3), as described previously [7]. Site-specific mutations, CPI-17-Thr^{38}Ala [10] and PHI-1-Thr^{57}Ala [19], were introduced by the QuikChange protocol (Stratagene) according to the manufacturer's directions. Sequences of all cDNA inserts were verified by dideoxy sequencing in the Biomolecular Core Facility at the University of Virginia. Tagged proteins were purified on Ni^{2+}-nitrilotriacetate resin (Qiagen), as described previously [7]. Calmodulin [20] and myosin II [21] were isolated from chicken gizzard smooth muscle, as described previously. The myosin preparation was found to contain MLCP activity, and so this enzyme/substrate combination was used to examine the effects of CPI-17 and PHI-1 on MLCP activity. Phospho-specific antibody for CPI-17 phosphorylated at Thr^{38}, and antibodies for total CPI-17 and total PHI-1 were prepared as described previously [9,17,19]. Rabbit phospho-specific antibody for PHI-1, phosphorylated at Thr^{57}, was produced using the phosphopeptide CGKV(pT)VKYDRKE conjugated with haemocyanin via the N-terminal cysteine as an antigen. The specific IgG fraction was obtained using Protein A–agarose resin (Sigma), following pre-absorption with unphosphorylated peptide on beads. No cross reaction against unphosphorylated PHI-1 was detected using the purified anti-[P-PHI-1(Thr^{57})] antibody (results not shown). Monoclonal anti-ILK (clone 65.1.9) was purchased from Upstate Biotechnology. Immunoprecipitation was performed as described by the manufacturer. A monoclonal antibody against chicken lamin A was produced as described for anti-human high-molecular-mass alkaline phosphatase by Deng and Parsons [22], with some modifications. Briefly, 6-week-old female Balb/c mice were immunized with three intraperitoneal injections of 50 μg of chicken lamin A at 3-week intervals: antigen (100 μl) was mixed with 100 μl of adjuvant (MPL + TDM emulsion; R-700 from RIBI ImmunoChem Research Inc., Hamilton, MT, U.S.A.). At 1 week after the last injection, antigen (100 μg) was injected intraperitoneally, and 5 days after this booster injection, spleen cells were fused with Sp_2/mIL-6 myeloma cells. Fused cells were distributed into 96-well plates and cultured in RPMI 1640 medium containing 20 % (v/v) fetal bovine serum. Monoclonal anti-(lamin A) was purified on Protein A–Sepharose CL-4B (Amersham Biosciences). The antibody immunoprecipitated lamin A specifically, as demonstrated by MS.

In-gel kinase assay

ILK was mixed with an equal volume of 2 × SDS gel sample buffer [50 mM Tris/HCl (pH 6.8)/1 % (w/v) SDS/30 % (v/v) glycerol/0.01 % Bromophenol Blue], and incubated at 20 °C for 2 h before electrophoresis in SDS gels with a 10–20 % acrylamide gradient [23] with CPI-17 or PHI-1 incorporated throughout the 0.75 mm-thick gel (6 μg/ml running gel solution). Gels were washed at room temperature with a solution comprising 25 mM Tris/HCl, pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 10 mM EGTA and 2.5 % Triton X-100 for 2 h to remove the SDS, and then washed for a further 2 h in a solution containing 20 mM Tris/HCl, pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 10 mM EGTA and 0.1 % Tween 80 (kinase assay buffer). The buffer was replaced with 25 ml of fresh kinase assay buffer, and protein phosphorylation was initiated by addition of 60 μM ATP containing 100 μCi of [γ-^{32}P]ATP. After incubation at 20 °C for 3 h, the gel was washed extensively with 5 % (w/v) trichloroacetic acid/1 % sodium pyrophosphate, until the radioactivity in the wash solution was negligible. The gel was stained, destained, dried and exposed to X-ray film [23].

Determination of protein concentration

Duplicate samples of stock solutions of CPI-17 and PHI-1 (40 μl each) were dried and hydrolysed in 100 μl of 5.7 M HCl/0.1 % phenol *in vacuo* for 1 h at 160 °C. Of each hydrolysate, 25 % was subjected to amino acid analysis at the Alberta Peptide Institute (University of Alberta, Canada). Protein concentrations were calculated from the measured amounts of alanine, glycine, leucine, aspartate and glutamate, and the known amino acid compositions of the proteins.

Phosphorylation of CPI-17 and PHI-1 by ILK

CPI-17 (2.9 μM) or PHI-1 (1.8 μM) was incubated at 30 °C with ILK (20 % v/v) in buffer comprising 25 mM Tris/HCl, pH 7.5, 10 mM EGTA, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.1 % (v/v) Tween 80 and 10 μM microcystin-LR. Reactions were started by addition of [γ-^{32}P]ATP ($\approx$ 200 c.p.m./pmol) to a final concentration of 0.4 mM. Samples (10 μl) were withdrawn at selected times, and spotted on to P81 phosphocellulose paper for quantification of ^{32}P incorporation, as previously described [2].

Thiophosphorylation of CPI-17 and PHI-1 by ILK

CPI-17 (2.9 μM) or PHI-1 (1.8 μM) was incubated at 30 °C for 90 min with ILK (10 %, v/v) in 25 mM Tris/HCl, pH 7.5, 10 mM EGTA, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT and 0.1 % (v/v) Tween 80. Reactions were started by addition of adenosine 5′-[γ-thio]triphosphate to a final concentration of 1 mM. Thiophosphorylation was used in some instances, since thiophosphorylated proteins are generally resistant to the action of protein phosphatases [24].

Assay of MLCP activity

Myosin (1.1 μM) containing MLCP activity was phosphorylated by incubation for 10 min at 30 °C with MLCK (10 μg/ml) and

calmodulin (10 μg/ml) in 25 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM $CaCl_2$ and 1 mM [γ-^{32}P]ATP ($\approx$ 100 c.p.m./pmol). An equal volume of CPI-17 or PHI-1 thiophosphorylation reaction mixture (or control reaction mixture) was added, and samples (10 μl) were withdrawn at specified times and spotted on to P81 phosphocellulose paper for quantification of ^{32}P incorporation, as described previously [23].

Force measurements

Male Sprague–Dawley rats (300–350 g) were killed by halothane inhalation and decapitation, as approved by the Animal Care Committee (Faculty of Medicine, University of Calgary, Canada). Excess adventitia and adipose tissue were dissected free from the tail artery, and placed in Ca^{2+}-free Hepes–Tyrode's buffer [H-T buffer; 140.6 mM NaCl/2.7 mM KCl/1.0 mM $MgCl_2$/10 mM Hepes (pH 7.4)/5.6 mM glucose]. Rat-tail artery segments were placed over a 0.31 mm needle and moved back and forth 40 times to remove the endothelium, before cutting into 6 mm $\times$ 1.5 mm helical strips and mounting on a Grass isometric force-transducer (model FTO3C) connected to a PowerLab (ADInstruments) eight-channel recording device. Strips were mounted on the transducer with a resting tension of 0.45 g, and incubated for 20 min in H-T buffer (this time of composition 137.0 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 7.4, and 5.6 mM glucose) in a bath volume of 0.8 ml. Muscle strips were then incubated for 5 min in Ca^{2+}-free H-T buffer, and for 5 min in 30 mM Tes, 0.5 mM DTT, 50 mM KCl, 5 mM K_2EGTA, 150 mM sucrose, pH 7.4 (buffer A) and subsequently skinned by incubation for 2 h in buffer A containing 1 % Triton X-100. Skinned tissues were washed 3 times for 5 min each wash in solution containing 20 mM Tes, pH 6.9, 4 mM K_2EGTA, 5.83 mM $MgCl_2$, 7.56 mM potassium propionate, 1 mM NaN_3, 3.9 mM Na_2ATP, 0.5 mM dithioerythritol, 16.2 mM phosphocreatine and 15 units/ml creatine kinase (pCa 8.1 solution), followed by incubation for 15 min in solution comprising 20 mM Tes, pH 6.9, 4 mM CaEGTA, 5.66 mM $MgCl_2$, 7.53 mM potassium propionate, 1 mM NaN_3, 3.9 mM Na_2ATP, 0.5 mM dithioerythritol, 16.2 mM phosphocreatine and 15 units/ml creatine kinase (pCa 4.4 solution), to elicit a Ca^{2+}-induced contraction of force comparable with that elicited in the intact tissue by K^+ [25]. Skinned muscle strips were then relaxed by incubation in pCa 8.1 solution for 10 min, and washed for 2 periods of 5 min in pCa 8.1 solution. Tissues were transferred to pCa 6.2 solution (a sub-threshold Ca^{2+} concentration in the Triton X-100-skinned preparation), and washed in pCa 6.2 solution prior to the addition of thiophosphorylated protein (CPI-17, CPI-17-Thr^{38}Ala, PHI-1 or PHI-1-Thr^{57}A) at pCa 6.2. Once a stable response was achieved, muscle strips were transferred to pCa 4.4 solution to elicit a maximal contraction. Finally, tissues were relaxed by transfer to pCa 8.1 solution. The free [Ca^{2+}] of each bathing solution was determined using fura-2 (Molecular Probes) as described by the manufacturer.

RESULTS

We first tested the possibility that ILK is capable of phosphorylating the protein phosphatase inhibitors CPI-17 and PHI-1 using an in-gel kinase assay (Figure 1). Kinase activity corresponding to ILK of 59 kDa (Figure 1, upper panel, lane 1) was detected in gels containing either CPI-17 (Figure 1, upper panel, lane 2) or PHI-1 (Figure 1, upper panel, lane 3), demonstrating direct phosphorylation of CPI-17 and PHI-1 by ILK. In control gels without phosphatase inhibitor proteins, or with site-specific mutants CPI-17-Thr^{38}Ala or PHI-1-Thr^{57}Ala, no phosphorylation was detected (results not shown). To confirm that phosphorylation was effected by ILK and not a kinase of identical molecular mass, ILK was immunoprecipitated and shown to phosphorylate CPI-17 and PHI-1 in an in-gel kinase assay (Figure 1, lower panel). In-gel kinase activity of the ILK preparation used for immunoprecipitation is shown in Figure 1, lower panel (lanes 1 and 4). Lanes 2 and 5 demonstrate in-gel kinase activity of immunoprecipitated ILK with CPI-17 (lane 2) or PHI-1 (lane 5) as substrate. As a negative control, a monoclonal antibody against lamin A failed to immunoprecipitate kinase activity towards CPI-17 (lane 3) or PHI-1 (lane 6).

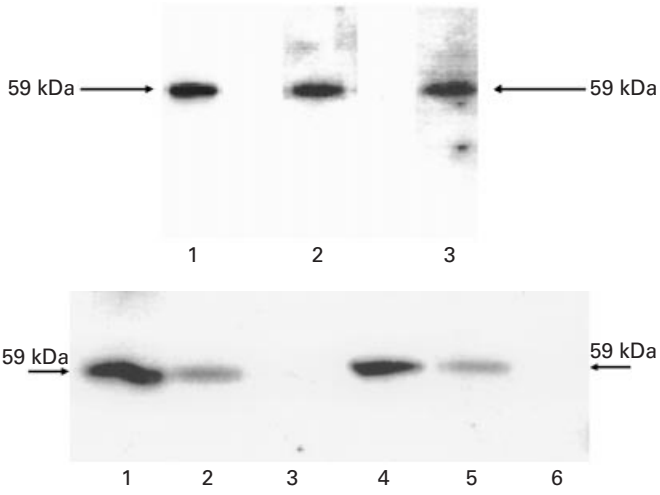

Figure 1 Phosphorylation of CPI-17 and PHI-1 by ILK: in-gel kinase assay

Upper panel: ILK purified through Mono Q column chromatography [3] was subjected to SDS/PAGE and Western blotting with anti-ILK (lane 1). Specific labelling of the 59 kDa ILK was observed. In-gel kinase assays were performed with this ILK preparation, as described in the Experimental section. ILK was electrophoresed in gels containing CPI-17 (lane 2) or PHI-1 (lane 3). Following protein renaturation, gels were soaked in kinase assay buffer containing [γ-^{32}P]ATP, excess radiolabelled ATP was washed out and gels were exposed to X-ray film. The autoradiograms demonstrate specific phosphorylation of CPI-17 (lane 2) and PHI-1 (lane 3) by ILK (59 kDa). Lower panel: ILK purified through Mono Q column chromatography [3] was immunoprecipitated with anti-ILK, as described in the Experimental section. The immunoprecipitate was subjected to SDS/PAGE and in-gel kinase assay with CPI-17 (lane 2) or PHI-1 (lane 5) as substrate. ILK purified through Mono Q column chromatography [3] was used as a positive control (lanes 1 and 4). As a negative control, the ILK purified through Mono Q column chromatography [3] was immunoprecipitated with a monoclonal antibody against lamin A and the immunoprecipitate was subjected to SDS/PAGE and in-gel kinase assay with CPI-17 (lane 3) or PHI-1 (lane 6) as substrate.

The time course and stoichiometry of phosphorylation of CPI-17 and PHI-1 by ILK are shown in Figure 2 (upper panel). At the end of the phosphorylation reactions, samples were subjected to SDS/PAGE and autoradiography to verify the specific phosphorylation of CPI-17 and PHI-1 (Figure 2, lower panel, lanes 7–9 and 13–15 respectively). No significant phosphorylation of CPI-17-Thr^{38}Ala or PHI-1-Thr^{57}Ala was detected [Figure 2, upper panel and lower panel (lanes 4–6 and 10–12 respectively), and Figure 3].

As reported previously [11,14], PKC and ROCK2 phosphorylate predominantly Thr^{38} of CPI-17 (Figure 3A). ILK-phosphorylated, ^{32}P-labelled CPI-17 gave the same phosphopeptide mapping pattern as PKC- or ROCK2-phosphorylated [^{32}P]CPI-17, indicating predominant phosphorylation at Thr^{38} of CPI-17 by ILK. Phosphorylation at Thr^{38} of CPI-17 was confirmed by immunoblotting using anti-[P-CPI-17(Thr^{38})] antibody. A minor phosphopeptide was detected in each lane of the alkaline gel (Site 2 in Figure 3A). The minor PKC phosphorylation site was

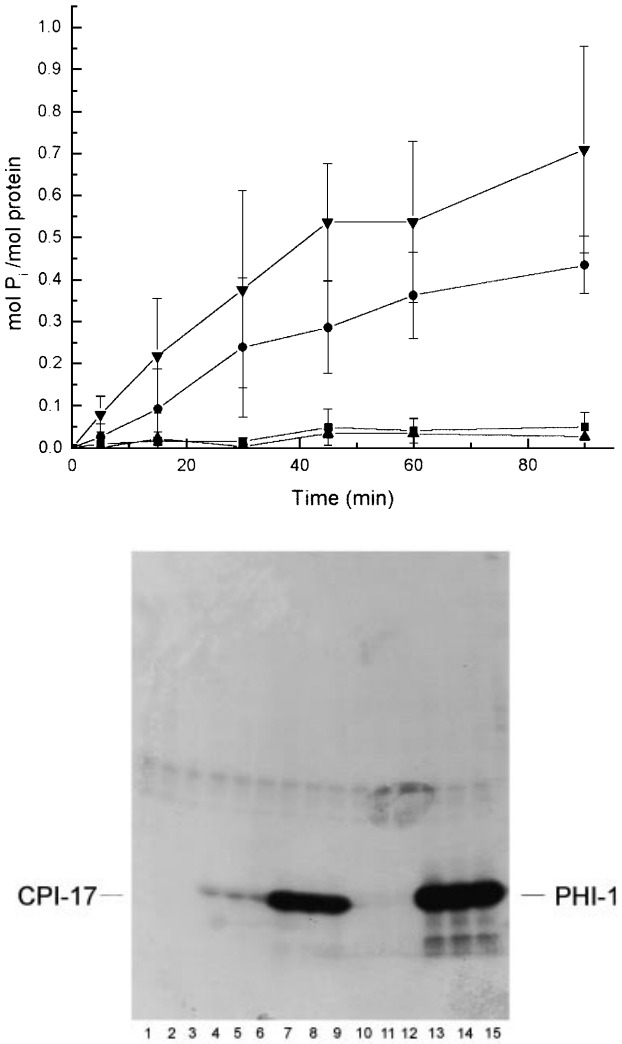

Figure 2 Time courses of phosphorylation of CPI-17 and PHI-1 by ILK and identification of the sites of phosphorylation

Upper panel: wild-type CPI-17 (●), CPI-17-Thr^{38}Ala (■), wild-type PHI-1 (▼) and PHI-1-Thr^{57}Ala (▲) were phosphorylated with ILK as described in the Experimental section. Phosphorylation stoichiometry was determined following removal of samples at the indicated times after addition of radiolabelled ATP. Values represent the means ± S.E.M. ($n = 6$, each experiment in duplicate), and were corrected for background determined from similar reaction mixtures without ILK and a very low level of autophosphorylation of ILK, where appropriate. Lower panel: at the end of the reactions, samples (50 μl each) were mixed with an equal volume of 2 × SDS gel buffer, boiled and subjected to SDS/PAGE and autoradiography. Results of triplicate analyses are shown. Lanes 1–3, ILK alone; lanes 4–6, CPI-17-Thr^{38}Ala + ILK; lanes 7–9: wild-type CPI-17 + ILK; lanes 10–12, PHI-1-Thr^{57}Ala + ILK; lanes 13–15, wild-type PHI-1 + ILK.

identified as Ser^{12} of CPI-17 [6], suggesting that Ser^{12} might be a second site phosphorylated by both ILK and ROCK2.

Because the amino acid sequence around the inhibitory phosphorylation site is highly conserved between CPI-17 and PHI-1, PKC was used for phosphorylation of recombinant PHI-1 [19]. We showed that ILK phosphorylated PHI-1 as well as CPI-17. The phosphorylation of PHI-1 by ILK was completely eliminated by a single Thr^{57} mutation (Figures 2 and 3B). Only phosphothreonine was detected in ILK-phosphorylated PHI-1 by phospho amino acid analysis (Figure 3B). Thr^{57} phosphorylation was detected by immunoblotting using anti-[P-PHI-1(Thr^{57})] anti-

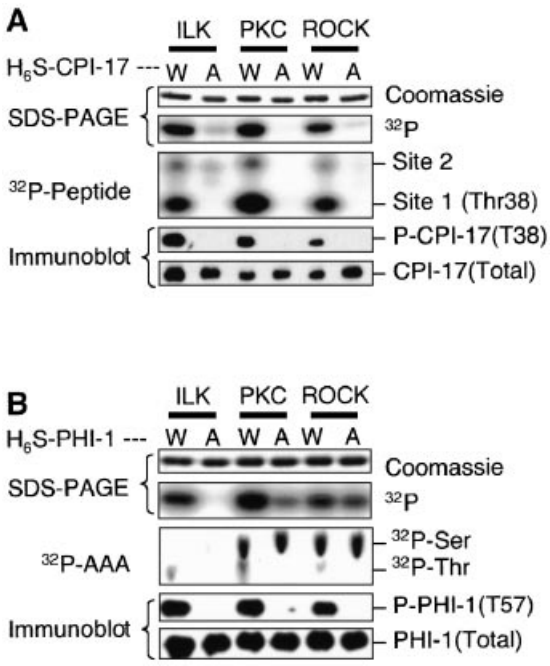

Figure 3 Identification of the sites in CPI-17 (A) and PHI-1 (B) phosphorylated by ILK, PKCδ and ROCK2

Wild-type CPI-17 or PHI-1 (W) and CPI-17-Thr^{38}Ala or PHI-1-Thr^{57}Ala (A) were phosphorylated with ILK, PKCδ or ROCK2 in the presence of [γ-^{32}P]ATP and subjected to SDS/PAGE. The top two panels show the resultant autoradiogram below the corresponding region of the Coomassie Brilliant Blue-stained gel containing CPI-17 (**A**) or PHI-1 (**B**). The CPI-17 band was excised, trypsin-digested and subjected to phosphopeptide mapping using alkaline PAGE [33]. Phosphorylated tryptic peptides were detected by autoradiography (**A**, middle panel). Aliquots of phospho-CPI-17 were subjected to immunoblotting using anti-[P-CPI-17(Thr^{38})] or anti-[CPI-17(total)] antibodies (**A**, lowest two panels). The PHI-1 band was cut out of the gel, acid-hydrolysed and subjected to phospho amino acid analysis, with detection of phospho amino acids (^{32}P-AAA) by autoradiography (**B**, middle panel). The positions of phosphothreonine and phosphoserine standards were identified by ninhydrin staining. Specific phosphorylation of PHI-1 at Thr^{57} was confirmed by immunoblotting using anti-[P-PHI-1(Thr^{57})] and anti-[PHI-1(total)] antibodies (**B**, lowest two panels).

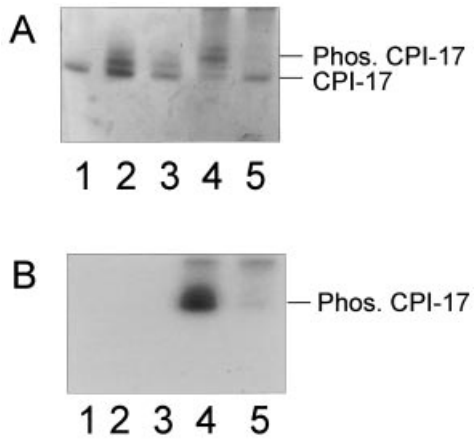

Figure 4 Confirmation of the phosphorylation of CPI-17 at Thr^{38}

Wild-type and mutant CPI-17 were phosphorylated as described in the Experimental section, and subjected to urea/Mops gel electrophoresis, as previously described [13]. The gel was stained with Coomassie Brilliant Blue (**A**) and exposed to X-ray film (**B**). Key to lanes: 1, untreated CPI-17-Thr^{38}Ala; 2, untreated wild-type CPI-17; 3, wild-type CPI-17 − ILK; 4, wild-type CPI-17 + ILK; 5, CPI-17-Thr^{38}Ala + ILK.

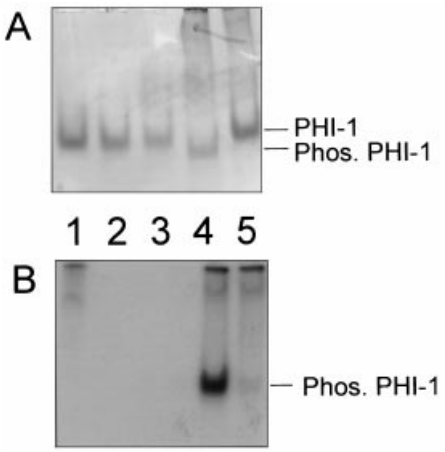

Figure 5 Confirmation of the phosphorylation of PHI-1 at Thr[57]

Wild-type and mutant PHI-1 were phosphorylated as described in the Experimental section, and subjected to urea/Tris/glycine gel electrophoresis, as described previously [34]. The gel was stained with Coomassie Brilliant Blue (**A**) and exposed to X-ray film (**B**). Key to lanes: 1, PHI-1-Thr[57]Ala − ILK; 2, untreated wild-type PHI-1; 3, wild-type PHI-1 − ILK; 4, wild-type PHI-1 + ILK; 5, PHI-1-Thr[57]Ala + ILK.

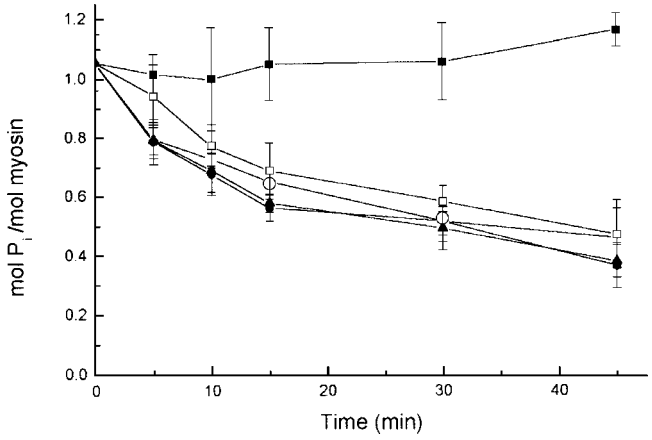

Figure 6 CPI-17 thiophosphorylated at Thr[38] by ILK inhibits MLCP

MLCP activity was assayed as described in the Experimental section with no additions (▲), ILK alone (□), wild-type CPI-17 alone (●), wild-type CPI-17 + ILK (■) or CPI-17-Thr[38]Ala + ILK (○). Values represent the means ± S.E.M. ($n = 4$, each in duplicate).

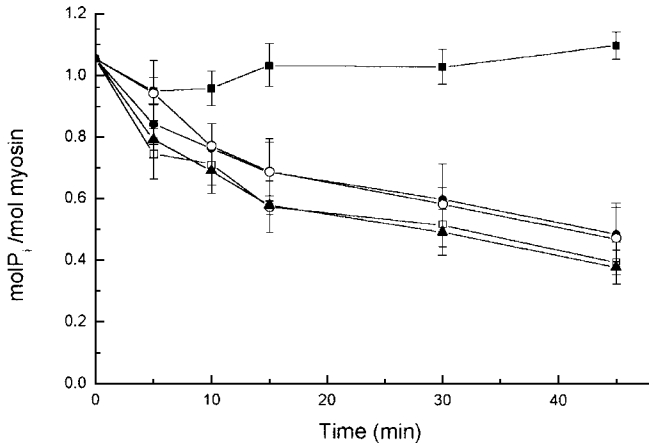

Figure 7 PHI-1 thiophosphorylated at Thr[57] by ILK inhibits MLCP

MLCP activity was assayed as described in the Experimental section with no additions (▲), ILK alone (○), wild-type PHI-1 alone (□), wild-type PHI-1 + ILK (■) or PHI-1-Thr[57]Ala + ILK (●). Values represent the means ± S.E.M. ($n = 4$, each in duplicate).

body. These results indicate that ILK exclusively phosphorylates Thr[57] of PHI-1. On the other hand, PKC significantly phosphorylated the PHI-1-Thr[57]Ala mutant (Figure 3B) [19]. The second phosphorylation site has not been identified. We found that ROCK2 could phosphorylate Thr[57] of PHI-1, although this phosphorylation of PHI-1 by ROCK2 was not as specific as the phosphorylation by ILK (Figure 3B).

Phosphorylation of CPI-17 at Thr[38] and of PHI-1 at Thr[57] by ILK was confirmed by separating the phosphorylated and unphosphorylated forms of CPI-17 and PHI-1 by using, respectively, Mops and Tris/glycine gel electrophoresis in urea (Figures 4 and 5). Again, the site-specific mutants, CPI-17-Thr[38]Ala and PHI-1-Thr[57]Ala, were not phosphorylated by ILK.

Figure 6 shows that wild-type CPI-17 thiophosphorylated by ILK inhibited MLCP activity, whereas wild-type CPI-17 incubated under identical conditions without ILK had no effect on the phosphatase activity. On the other hand, the site-specific mutant CPI-17-Thr[38]Ala, treated with or without ILK, had no effect on MLCP activity. These results are consistent with the earlier finding that CPI-17 phosphorylated at Thr[38] by PKC is a potent inhibitor of MLCP [12].

Similar results were obtained with PHI-1 (Figure 7). Wild-type PHI-1 thiophosphorylated by ILK inhibited MLCP, whereas non-thiophosphorylated, control PHI-1 did not. PHI-1-Thr[57]Ala, treated with or without ILK, had no effect on the phosphatase. Again, these results are in agreement with the earlier observation that PHI-1 phosphorylated at Thr[57] by PKC inhibits MLCP [19].

Consistent with the inhibitory effects of phosphorylated wild-type CPI-17 and PHI-1 on MLCP (Figures 6 and 7), the wild-type proteins thiophosphorylated by ILK induced Ca^{2+} sensitization of contraction of Triton-X-100-skinned rat-tail arterial smooth muscle (Figures 8A and 8C): when added to skinned smooth-muscle strips at a sub-threshold [Ca^{2+}], thiophosphorylated wild-type CPI-17 and PHI-1 induced a contractile response. On the other hand, the site-specific mutants treated in an identical fashion had no Ca^{2+}-sensitizing effect (Figures 8B and 8D).

DISCUSSION

In the present study, we show for the first time that ILK phosphorylates and regulates the two phosphatase inhibitory proteins CPI-17 and PHI-1. We found that, in smooth muscle, there is a distinct subpopulation of ILK that is tightly bound to the myofilaments, and this ILK is capable of phosphorylating myosin in a Ca^{2+}-independent manner to elicit a Ca^{2+}-independent contractile response [2,3]. Furthermore, we have shown recently [26] that ILK is also capable of phosphorylating MYPT1, the myosin-targeting subunit of MLCP, with phosphorylation occurring at three sites, including Thr[695], which results in inhibition of phosphatase activity. Our discovery that ILK also phosphorylates CPI-17 and PHI-1 suggests that this enzyme may trigger an increase in myosin phosphorylation and elicit a contractile response both directly, via phosphorylation of myosin, and indirectly, via phosphorylation and activation of

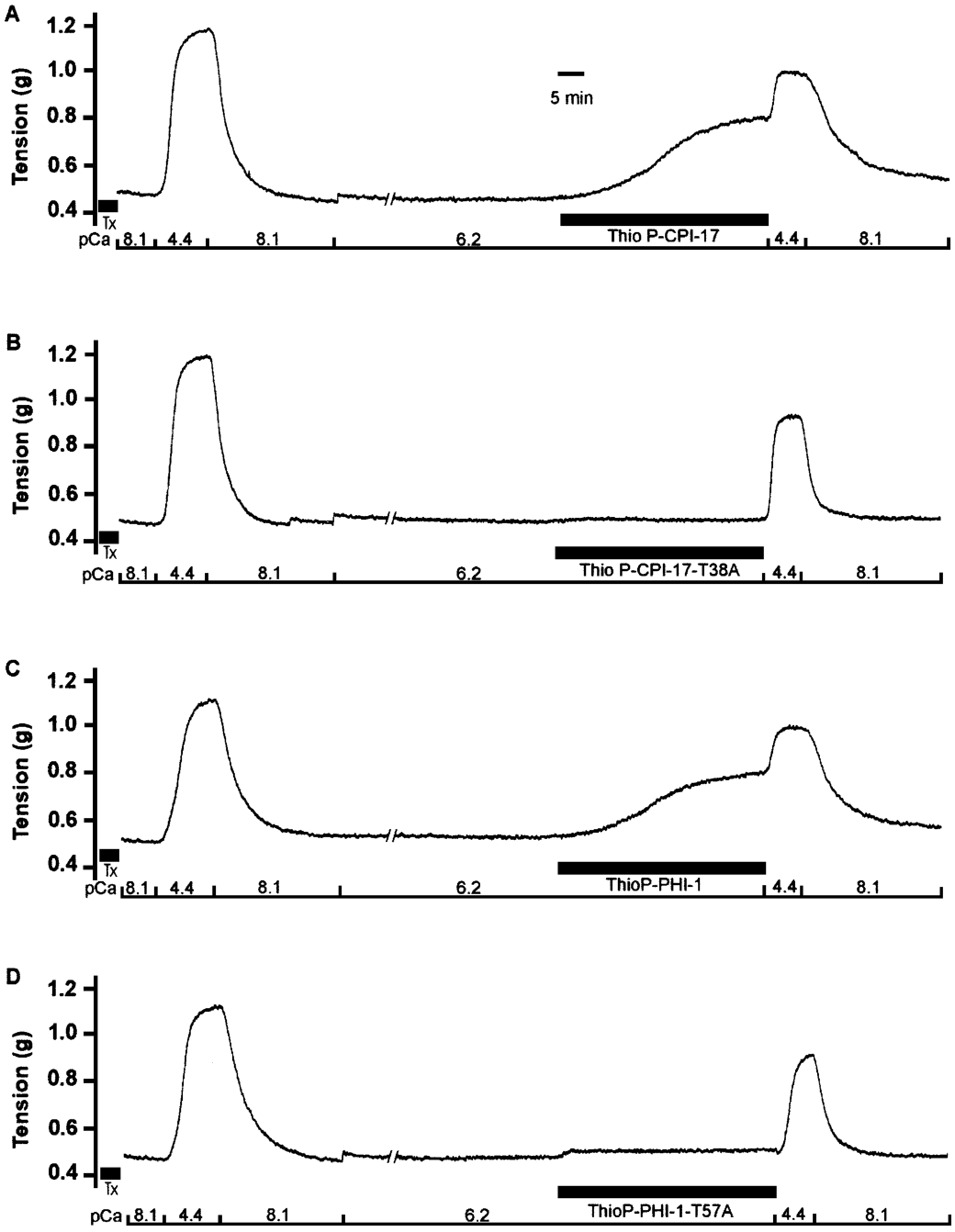

Figure 8 CPI-17 and PHI-1 thiophosphorylated by ILK induce Ca^{2+} sensitization of smooth-muscle contraction

Triton X-100-skinned rat-tail arterial smooth-muscle strips were induced to contract by transfer from low [Ca^{2+}] solution (pCa 8.1) to high [Ca^{2+}] solution (pCa 4.4). Relaxation followed return to the pCa 8.1 solution. Strips were then equilibrated with pCa 6.2 solution prior to addition of thiophosphorylated CPI-17 (**A**), CPI-17-Thr^{38}Ala (**B**), PHI-1 (**C**) or PHI-1-Thr^{57}Ala (**D**). Once a stable response was achieved, maximal contractions were elicited by transfer to the pCa 4.4 solution. Muscle strips relaxed following return to the pCa 8.1 solution. Similar results were obtained in six independent experiments.

CPI-17 and PHI-1, and also via phosphorylation of MYPT1 at Thr^{695}, leading to inhibition of MLCP.

Several protein kinases have been shown to phosphorylate recombinant CPI-17:PKC [6], ROCK [14], PKN [15], ZIP-like kinase [16], and now ILK. Also multiple kinases, such as PKC [19], ZIP-like kinase [16] and ROCK2 (Figure 3), phosphorylate recombinant PHI-1. However, these kinases show relatively low activities towards PHI-1, compared with CPI-17 as a substrate. ILK is unique in that it phosphorylates Thr^{57} of PHI-1 preferentially and specifically (Figures 2 and 3B). Even though the amino acid sequences of CPI-17 and PHI-1 around the phosphorylation sites are highly conserved (eight out of ten residues are identical; [19]), different kinases might recognize these minor differences in sequence and/or local structure around the phosphorylation site. Selective phosphorylation of CPI-17 or PHI-1 may, therefore, mediate signalling via different agonists.

Protein inhibitors of protein serine/threonine phosphatases have been studied for many years, and the best known are inhibitor-1, inhibitor-2 and DARPP-32 ('dopamine- and cAMP-regulated phosphoprotein of 32 kDa') [27]. All three proteins act specifically on PP1s, and their activities are regulated by phosphorylation. However, these inhibitors act only on the free catalytic subunit, not the phosphatase holoenzymes. Since all the PP1 catalytic subunits ('PP1c') in cells are likely to be bound to targeting or regulatory subunits, it appears that these inhibitor proteins could only function as phosphatase inhibitors when the catalytic subunit dissociates from its interacting partners. CPI-17 and PHI-1, on the other hand, are effective inhibitors of phosphatase holoenzymes including MLCP. Phospho-CPI-17 is more potent towards MLCP compared with phospho-PHI-1 [8,19]. Since CPI-17 has a restricted tissue distribution, it may be specific to smooth-muscle MLCP, and have a specific function in agonist-induced Ca^{2+} sensitization of smooth-muscle contraction. Delcommenne et al. [28] showed that ILK is stimulated and regulates protein kinase B in response to phosphatidylinositol 3,4,5-trisphosphate production by phosphoinositide 3-kinase (PI3K) activation. In smooth-muscle cells, PI3K activity is elevated in response to angiotension II stimulation [29]. Furthermore, PI3K mediates angiotensin II-induced activation of protein kinase B in smooth-muscle cells [30]. Thus it is quite possible that angiotensin II induces activation of ILK via PI3K stimulation. Therefore, in addition to eliciting an increase in intracellular [Ca^{2+}], angiotensin II may induce activation of smooth-muscle ILK, leading to increased phosphorylation of myosin via two pathways: direct phosphorylation and MLCP inhibition by activation of CPI-17/PHI-1.

PHI-1, on the other hand, with a broad tissue distribution and the ability to inhibit both MLCP and glycogen-bound PP1, probably has a wider range of physiological functions. ILK also has a broad tissue distribution and, therefore, has the capacity to regulate diverse cellular functions via its phosphorylation of PHI-1. ILK activity is also enhanced in response to focal adhesion formation, suggesting that ILK signalling is involved in integrin-mediated signal transduction at the level of the plasma membrane, such as cell migration [31]. The catalytic subunit of PP1 localizes to focal adhesion complexes in cultured smooth-muscle cells and fibroblasts [31], and focal adhesion kinase is a candidate for a focal adhesion-targeting subunit of PP1 [32]. We postulate, therefore, that PHI-1 phosphorylated by ILK inhibits PP1 activity in focal adhesions to mediate integrin signalling.

This work was supported in part by a grant to M. P. W. from the Canadian Institutes of Health Research, by grants to D. L. B. from the National Institutes of Health (CA40042 and GM56362) and by a postdoctoral fellowship to M. E. from the American Heart Association Mid-Atlantic Affiliate. M. P. W. is an Alberta Heritage Foundation for Medical Research Senior Scientist and recipient of a Canada Research Chair (Tier I) in Biochemistry. The authors are grateful to Dr David Wilson (University of Calgary) for helpful discussions and assistance with the force measurements and to Dr Meg Kargacin (University of Calgary) for measurements of free [Ca^{2+}].

REFERENCES

1 Kamm, K. E. and Stull, J. T. (2001) Dedicated myosin light chain kinases with diverse cellular functions. J. Biol. Chem. **276**, 4527–4530
2 Weber, L. P., Van Lierop, J. E. and Walsh, M. P. (1999) Ca^{2+}-independent phosphorylation of myosin in rat caudal artery and chicken gizzard myofilaments. J. Physiol. (Cambridge, U.K.) **516**, 805–824
3 Deng, J. T., Van Lierop, J. E., Sutherland, C. and Walsh, M. P. (2001) Ca^{2+}-independent smooth muscle contraction. A novel function for integrin-linked kinase. J. Biol. Chem. **276**, 16365–16373
4 Wu, C. and Dedhar, S. (2001) Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. J. Cell Biol. **155**, 505–510
5 Hartshorne, D. J., Ito, M. and Erdödi, F. (1998) Myosin light chain phosphatase: subunit composition, interactions and regulation. J. Muscle Res. Cell Motil. **19**, 325–341
6 Eto, M., Ohmori, T., Suzuki, M., Furuya, K. and Morita, F. (1995) A novel protein phosphatase-1 inhibitory protein potentiated by protein kinase C. Isolation from porcine aorta media and characterization. J. Biochem. (Tokyo) **118**, 1104–1107
7 Eto, M., Senba, S., Morita, F. and Yazawa, M. (1997) Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: its specific localization in smooth muscle. FEBS Lett. **410**, 356–360
8 Senba, S., Eto, M. and Yazawa, M. (1999) 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. J. Biochem. (Tokyo) **125**, 354–362
9 Woodsome, T. P., Eto, M., Everett, A., Brautigan, D. L. and Kitazawa, T. (2001) Expression of CPI-17 and myosin phosphatase correlates with Ca^{2+} sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. J. Physiol. (Cambridge, U.K.) **535**, 553–564
10 Hayashi, Y., Senba, S., Yazawa, M., Brautigan, D. L. and Eto, M. (2001) Defining the structural determinants and a potential mechanism for inhibition of myosin phosphatase by the protein kinase C-potentiated inhibitor protein of 17 kDa. J. Biol. Chem. **276**, 39858–39863
11 Eto, M., Kitazawa, T., Yazawa, M., Mukai, H., Ono, Y. and Brautigan, D. L. (2001) Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase C α and δ isoforms. J. Biol. Chem. **276**, 29072–29078
12 Li, L., Eto, M., Lee, M. R., Morita, F., Yazawa, M. and Kitazawa, T. (1998) Possible involvement of the novel CPI-17 protein in protein kinase C signal transduction of rabbit arterial smooth muscle. J. Physiol. (Cambridge, U.K.) **508**, 871–881
13 Kitazawa, T., Takizawa, N., Ikebe, M. and Eto, M. (1999) Reconstitution of protein kinase C-induced contractile Ca^{2+} sensitization in Triton X-100-demembranated rabbit arterial smooth muscle. J. Physiol. (Cambridge, U.K.) **520**, 139–152
14 Koyama, M., Ito, M., Feng, J., Seko, T., Shiraki, K., Takase, K., Hartshorne, D. J. and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. FEBS Lett. **475**, 197–200
15 Hamaguchi, T., Ito, M., Feng, J., Seko, T., Koyama, M., Machida, H., Takase, K., Amano, M., Kaibuchi, K., Hartshorne, D. J. and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein kinase N. Biochem. Biophys. Res. Commun. **274**, 825–830
16 MacDonald, J. A., Eto, M., Borman, M. A., Brautigan, D. L. and Haystead, T. A. J. (2001) Dual Ser and Thr phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by MYPT-associated kinase. FEBS Lett. **493**, 91–94
17 Kitazawa, T., Eto, M., Woodsome, T. P. and Brautigan, D. L. (2000) Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. J. Biol. Chem. **275**, 9897–9900
18 Etter, E. F., Eto, M., Wardle, R. L., Brautigan, D. L. and Murphy, R. A. (2001) Activation of myosin light chain phosphatase in intact arterial smooth muscle during nitric oxide-induced relaxation. J. Biol. Chem. **276**, 34681–34685
19 Eto, M., Karginov, A. and Brautigan, D. L. (1999) A novel phosphoprotein inhibitor of protein type-1 phosphatase holoenzymes. Biochemistry **38**, 16952–16957
20 Walsh, M. P., Valentine, K. A., Ngai, P. K., Carruthers, C. A. and Hollenberg, M. D. (1984) Ca^{2+}-dependent hydrophobic interaction chromatography. Isolation of a novel Ca^{2+}-binding protein and protein kinase C from bovine brain. Biochem. J. **224**, 117–127
21 Persechini, A. and Hartshorne, D. J. (1981) Phosphorylation of smooth muscle myosin: evidence for cooperativity between the myosin heads. Science **213**, 1381–1385
22 Deng, J. T. and Parsons, P. (1988) Solid phase immunoassay for high molecular weight alkaline phosphatase in human sera using a specific monoclonal antibody. Clin. Chim. Acta **176**, 291–302
23 Winder, S. J. and Walsh, M. P. (1990) Smooth muscle calponin. Inhibition of actomyosin MgATPase and regulation by phosphorylation. J. Biol. Chem. **265**, 10148–10155
24 Gratecos, D. and Fischer, E. H. (1974) Adenosine 5′-O(3-thiotriphosphate) in the control of phosphorylase activity. Biochem. Biophys. Res. Commun. **58**, 960–967
25 Wilson, D. P., Sutherland, C. and Walsh, M. P. (2002) Ca^{2+} activation of smooth muscle contraction. Evidence for the involvement of calmodulin that is bound to the Triton-insoluble fraction even in the absence of Ca^{2+}. J. Biol. Chem. **277**, 2186–2192
26 Murányi, A., MacDonald, J. A., Deng, J. T., Wilson, D. P., Haystead, T. A. J., Walsh, M. P., Erdődi, F., Kiss, E., Wu, Y. and Hartshorne, D. J. (2002) Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. Biochem. J. **366**, 211–216

27 Oliver, C. J. and Shenolikar, S. (1998) Physiologic importance of protein phosphatase inhibitors. Frontiers Biosci. **3**, D961–D972
28 Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J. and Dedhar, S. (1998) Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc. Natl. Acad. Sci. U.S.A. **95**, 11211–11216
29 Saward, L. and Zahradka, P. (1997) Angiotensin II activates phosphatidylinositol 3-kinase in vascular smooth muscle ells. Circ. Res. **81**, 249–257
30 Takahashi, T., Taniguchi, T., Konishi, H., Kikkawa, U., Ishikawa, Y. and Yokoyama, M. (1999) Activation of Akt/protein kinase B after stimulation with angiotensin II in vascular smooth muscle cells. Am. J. Physiol. **276**, H1927–H1934
31 Murata, K., Hirano, K., Villa-Moruzzi, E., Hartshorne, D. J. and Brautigan, D. L. (1997) Differential localization of myosin and myosin phosphatase subunits in smooth muscle cells and migrating fibroblasts. Mol. Biol. Cell **8**, 663–673
32 Fresu, M., Bianchi, M., Parsons, J. T. and Villa-Moruzzi, E. (2001) Cell-cycle-dependent association of protein phosphatase 1 and focal adhesion kinase. Biochem. J. **358**, 407–414
33 Dadd, C. A., Cook, R. G. and Allis, C. D. (1993) Fractionation of small tryptic phosphopeptides by alkaline PAGE followed by amino acid sequencing. Bio/Techniques **14**, 266–273
34 Perrie, W. T. and Perry, S. V. (1970) An electrophoretic study of the low molecular-weight components of myosin. Biochem. J. **119**, 31–38

Received 3 April 2002/24 June 2002; accepted 29 July 2002
Published as BJ Immediate Publication 29 July 2002, DOI 10.1042/BJ20020522