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

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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\(^{39}\) 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\(^{37}\) of PHI-1. CPI-17 and PHI-1 throphosphorylated by ILK at Thr\(^{38}\) or Thr\(^{37}\) 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\(^{37}\)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\(^{37}\)Ala treated with ILK in the presence of adenosine 5'-(β,γ-4)-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\(^{37}\) of the 20 kDa light chains (LC\(_{\text{phospho}}\)) 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 integr β subunits and was reported to be involved in cytoskeletal regulation via interaction with actin-binding proteins, such as Paxillin and afilin, via its N-terminal ankyrin repeat domain (see [4] for a review). Smooth-muscle myofilament-associated ILK phosphorylates LC\(_{\text{phospho}}\) 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\(_{\text{phospho}}\), 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; PKC, phosphoinositide 3-kinase; PKD, protein kinase C; PKN, protein kinase N; PP1, type 1 protein serine/threonine phosphatase; ROCK, Rho-associated kinase; ZIP, zipper-interacting protein.

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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\(^{37}\). 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**

\([\gamma-\text{P}]\text{ATP} (> 5000 \text{ Ci/mmol})\) 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 PKC\(\delta\) 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\(\)\_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\(^{29}\)Ala [10] and PHI-1-Thr\(^{25}\)Ala [19], were introduced by the QuikChange protocol (Stratagene) according to the manufacturer’s directions. Sequences of all cDNA inserts were verified by dye sequencing in the Bioluminescent 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\(^{39}\), 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\(^{37}\), was produced using the phosphopeptide CGK\(v\)(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\(^{37}\))] 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 \(\mu\)g of chicken lamin A at 3-week intervals: antigen (100 \(\mu\)l) was mixed with 100 \(\mu\)l 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 \(\mu\)g) was injected intraperitoneally, and 5 days after this booster injection, spleen cells were fused with Sp\(_2\) myeloma cells. Fused cells were distributed into 96-well plates and cultured in RPMI 1640 medium containing 20% 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 x SDS gel sample buffer \([50 \text{ mM Tris/HCl (pH 6.8)} / 1\% (\text{v/v) SDS/30\% (v/v) glycerol/0.01\% (v/v) 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 \(\mu\)g/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 \(\mu\)l of fresh kinase assay buffer, and protein phosphorylation was initiated by addition of 60 \(\mu\)M ATP containing 100 \(\mu\)Ci of \([\gamma-\text{P}]\text{ATP}\). After incubation at 20 °C for 3 h, the gel was washed extensively with 5 \(\%\) (v/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 \(\mu\)l each) were dried and hydrolysed in 100 \(\mu\)l of 5.7 M HCl/0.1 \(\%\) phenol \(\text{in vacuo}\) 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 \(\mu\)M) or PHI-1 (1.8 \(\mu\)M) 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 \(\mu\)M microcystin-LR. Reactions were started by addition of \([\gamma-\text{P}]\text{ATP} (\approx 200 \text{ c.p.m./pmol})\) to a final concentration of 0.4 mM. Samples (10 \(\mu\)l) 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 \(\mu\)M) or PHI-1 (1.8 \(\mu\)M) 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-[\(\gamma\)-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 \(\mu\)M) containing MLCP activity was phosphorylated by incubation for 10 min at 30 °C with MLCK (10 \(\mu\)g/ml) and...
calmodulin (10 μg/ml) in 25 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT, 0.1 mM CaCl₂ and 1 mM [γ-32P]ATP (≈ 100 c.p.m./pmol). An equal volume of CPI-17 or PHI-1 thio-phosphorylation 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 32P 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 Ca²⁺-free Hepes–Tyrode’s buffer [H-T buffer; 140.6 mM NaCl/2.7 mM KCl/1.0 mM MgCl₂/10 mM Heps (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 × 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 MgCl₂, 1.8 mM CaCl₂, 10 mM Heps, 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 Ca²⁺-free H-T buffer, and for 5 min in 30 mM Tes, 0.5 mM DTT, 50 mM KCl, 5 mM KEGTA, 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 KEGTA, 5.83 mM MgCl₂, 7.56 mM potassium propionate, 1 mM NaN₃, 3.9 mM Na₂ATP, 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 MgCl₂, 7.53 mM potassium propionate, 1 mM NaN₃, 3.9 mM Na₂ATP, 0.5 mM dithioerythritol, 16.2 mM phosphocreatine and 15 units/ml creatine kinase (pCa 4.4 solution), to elicit a Ca²⁺-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 Ca²⁺ concentration in the Triton X-100-skinned preparation), and washed in pCa 6.2 solution prior to the addition of thio-phosphorylated protein (CPI-17, CPI-17-Thr⁵²⁷ Ala, PHI-1 or PHI-1-Thr⁵²⁷ Ala) 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 [Ca²⁺] 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-Thr⁵²⁸ Ala or PHI-1-Thr⁵²⁷ 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, 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.

As reported previously [11,14], PKC and ROCK2 phosphorylated predominantly Thr⁵²⁸ of CPI-17 (Figure 3A). ILK-phosphorylated, ³²P-labelled CPI-17 gave the same phosphopeptide mapping pattern as PKC- or ROCK2-phosphorylated [³²P]CPI-17, indicating predominant phosphorylation at Thr⁵²⁸ of CPI-17 by ILK. Phosphorylation at Thr⁵²⁸ of CPI-17 was confirmed by immunoblotting using anti-[P-CPI-17(Thr⁵²⁸)] 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-Thr38Ala (■), wild-type PHI-1 (▼) and PHI-1-Thr57Ala (▲) 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-Thr38Ala + ILK; lanes 7–9, wild-type CPI-17 + ILK; lanes 10–12, PHI-1-Thr57Ala + ILK; lanes 13–15, wild-type PHI-1 + ILK.

identified as Ser12 of CPI-17 [6], suggesting that Ser12 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 [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 Thr57 mutation (Figures 2 and 3B). Only phosphothreonine was detected in ILK-phosphorylated PHI-1 by phospho amino acid analysis (Figure 3B). Thr57 phosphorylation was detected by immunoblotting using anti-[P-PHI-1(Thr57)] antibody (B, lowest two panels).
Phosphorylation of phosphatase inhibitors by integrin-linked kinase

Figure 5 Confirmation of the phosphorylation of PHI-1 at Thr57
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-Thr57Ala + ILK; 2, untreated wild-type PHI-1; 3, wild-type PHI-1 + ILK; 4, wild-type PHI-1-Thr57Ala + ILK; 5, PHI-1-Thr57Ala + ILK.

Figure 6 CPI-17 thiophosphorylated at Thr38 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-Thr38Ala + ILK (○). Values represent the means ± S.E.M. (n = 4, each in duplicate).

Figure 7 PHI-1 thiophosphorylated at Thr57 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-Thr57Ala + ILK (●). Values represent the means ± S.E.M. (n = 4, each in duplicate).

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 Ca2+-independent manner to elicit a Ca2+-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 Thr2002, 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-Thr38Ala (B), PHI-1 (C) or PHI-1-Thr57Ala (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\(^{37}\) 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 PPIs, and their activities are regulated by phosphorylation. However, these inhibitors act only on the free catalytic subunit, not the phosphatase holoenzymes. Since all the PPI catalytic subunits (PPIc) 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. Delcomenne et al. [28] showed that ILK is stimulated and regulates protein kinase B in response to phophatidylinositol 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 angiostatin II-induced activation of protein kinase B in smooth-muscle cells [30]. Thus it is quite possible that angiostatin II induces activation of ILK via PI3K stimulation. Therefore, in addition to eliciting an increase in intracellular [Ca\(^{2+}\)], angiostatin 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 PPI, 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 PPI 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 PPI [32]. We postulate, therefore, that PHI-1 phosphorylated by ILK inhibits PPI activity in focal adhesions to mediate integrin signalling.

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