Agonists Trigger G Protein-mediated Activation of the CPI-17 Inhibitor Phosphoprotein of Myosin Light Chain Phosphatase to Enhance Vascular Smooth Muscle Contractility*

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Myosin light chain phosphatase (MLCP) plays a pivotal role in smooth muscle contraction by regulating Ca\textsuperscript{2+} sensitivity of myosin light chain phosphorylation. A smooth muscle phosphoprotein called CPI-17 specifically and potently inhibits MLCP in vitro and in situ and is activated when phosphorylated at Thr-38, which increases its inhibitory potency 1000-fold. We produced a phosphospecific antibody for this site in CPI-17 and used it to study in situ phosphorylation of endogenous CPI-17 in arterial smooth muscle in response to agonist stimulation. In the intact femoral artery, CPI-17 phosphorylation was negligible at the resting state and was not increased during contraction induced by K\textsuperscript{+} depolarization. The Ca\textsuperscript{2+}-sensitizing agonists histamine and phenylephrine induced nearly equivalent contractions, but histamine generated significantly higher levels of CPI-17 phosphorylation. In α-toxin-permeabilized strips at pCa 6.7, contractile force and CPI-17 phosphorylation were proportional in response to histamine, guanosine 5′-O-(γ-thiotriphosphate), and histamine plus guanyl-5′-y1 thiophosphate, implying that histamine increased CPI-17 phosphorylation through activation of G proteins. Inhibitors of Rho-kinase (Y27632) and protein kinase C (PKC; GF109203X) reduced contraction and CPI-17 phosphorylation in parallel, suggesting that CPI-17 functions downstream of Rho kinases and PKC. The results show that agonists such as histamine signal through phosphorylation of CPI-17 to produce Ca\textsuperscript{2+} sensitization of smooth muscle contraction.

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Reversible phosphorylation of myosin light chain (MLC)\textsuperscript{1} at Ser-19 by Ca\textsuperscript{2+}/calmodulin-regulated MLC kinase (MLCK) and MLC phosphatase (MLCP) is the principal mechanism that regulates actomyosin ATPase and contraction of smooth muscle (1, 2). Although a rise in intracellular Ca\textsuperscript{2+} triggers the phosphorylation of MLC during smooth muscle contraction, the Ca\textsuperscript{2+} sensitivity of MLC phosphorylation and contraction is also dynamically modulated (3, 4). Excitatory agonists initially increase intracellular Ca\textsuperscript{2+} levels leading to an activation of MLCK. This action is followed immediately by a decrease in MLCP activity, which increases the Ca\textsuperscript{2+} sensitivity (4–7), resulting in a cooperatively evoked smooth muscle contraction. The agonist-induced Ca\textsuperscript{2+} sensitization is mimicked by G protein activation (using GTP\textsubscript{yS} and blocked by G protein inhibition (using GDP\textsubscript{βS}) (8, 9). Multiple second messengers/signaling pathways have been proposed for the Ca\textsuperscript{2+}-sensitizing mechanism. These include the Rho A/Rho-kinase (10, 11), protein kinase C (PKC)/CPI-17 (12), and arachidonic acid pathways (13). However, the precise mechanism for regulating MLCP is not yet fully elucidated.

The holoenzyme of MLCP is composed of three subunits (see Ref. 14): a 38-kDa catalytic subunit (PP1c), a large 110–130-kDa regulatory subunit (M130), and a small 20-kDa subunit. The M130 is the key subunit involved in binding and activation of PP1c and in targeting myosin. RhoA, a Ras-related monomeric small GTPase, is thought to play a major role in the agonist-induced Ca\textsuperscript{2+} sensitization of smooth muscle contraction (14). Active but not inactive RhoA produces a Ca\textsuperscript{2+} sensitizing effect (15, 16). Inactivation of the endogenous Rho proteins by ADP-ribosylation or glucosylation inhibits the agonist- and/or GTP\textsubscript{yS}-induced Ca\textsuperscript{2+} sensitization (7, 14). Several proteins have been identified as RhoA targets, including Rho-kinase (17–19) and the M130 (10, 14). Rho-kinase phosphorylates the M130 subunit, and this leads to an inhibition of the catalytic activity of the MLCP holoenzyme (10). Y27632 is a new compound that selectively inhibits Rho-kinase and reduces agonist-induced Ca\textsuperscript{2+} sensitization of contraction in smooth muscle (11). However, despite the several reports of Rho-kinase activity toward M130 in cultured cells (20, 21), to date phosphorylation of M130 during agonist-induced Ca\textsuperscript{2+} sensitization in smooth muscle tissues has not been shown.

CPI-17 is a novel phosphoprotein selectively expressed in smooth muscle tissues that specifically inhibits the trimeric MLCP (22–24). PKC phosphorylates CPI-17, enhancing its inhibitory potency 1000-fold, providing an independent pathway for inhibiting MLCP in vitro and in situ without phosphorylation of the M130 subunit (22–25). The action of phospho-CPI-17 increases the Ca\textsuperscript{2+} sensitivity of both MLC phosphorylation and contraction (12, 25). Therefore, CPI-17 could participate in Ca\textsuperscript{2+} sensitization of contraction if it was phosphorylated in response to agonist stimulation. The phosphorylation of CPI-17 at the single site Thr-38 is sufficient to induce inhibition of MLCP and a concurrent increase in the Ca\textsuperscript{2+} sensitivity of...
MLC phosphorylation and contraction in smooth muscle (12). Thus, phosphorylation at Thr-38 is a useful indicator of CPI-17 activity in vivo. We generated an antibody that specifically recognizes CPI-17 phosphorylated at Thr-38 (p[T38]CPI-17) and used it to analyze the phosphorylation of CPI-17 in intact and α-toxin permeabilized smooth muscle. We measured both phosphorylation of CPI-17 and tension induced by different Ca\(^{2+}\)-sensitizing agonists and found significant differences in CPI-17 phosphorylation between agonists that utilize G protein-coupled pathways to enhance contraction.

**MATERIALS AND METHODS**

**Tissue Preparation, Force Measurement, and Cell Permeabilization**—Smooth muscle strips (70 μm thick, 750 μm wide, and 3 mm long) were dissected from rabbit femoral arteries and mounted on a force transducer set up as described previously (26). Force levels were monitored throughout the experiments. The compositions of external solutions were described previously (8). For cell permeabilization, strips were treated for 30 min at 30 °C with 20 μg/ml purified *Staphylococcus aureus* α-toxin (List, Campbell, CA) at pCa 6.7 buffered with 10 mM EGTA and further treated with 10 μM Ca\(^{2+}\)-ionophore A23187 for 20 min at 25 °C to depolarize the sarcoplasmic reticulum of Ca\(^{2+}\) and maintain constant cytoplasmic Ca\(^{2+}\) as described previously (26). Details of the solutions used for the permeabilized strips have been described previously (26).

**Proteins**—Recombinant hexahistidine-tagged CPI-17 protein was expressed in *Escherichia coli* and purified to homogeneity as described previously (23). Phosphorylation of the CPI-17 was carried out using porcine brain PKC as detailed previously (25).

**Antibody Preparations**—Different polyclonal antibodies against CPI-17 were prepared. First, polyclonal IgY antibody (anti-CPI) was raised in chicken eggs against the full-length recombinant porcine CPI-17 and purified using column chromatography on CPI-17 conjugated to Affi-Gel-10. Second, polyclonal rabbit IgG antibody (anti-p[T38]CPI-17) was generated against the phosphopeptide ARV(pT)VKYDRREL (Qual-Controlled Biochemicals Inc.), which corresponds to the sequence of amino acids 35–46 of porcine CPI-17. Antibodies were absorbed from the antiserum onto unphosphorylated peptide conjugated to resin, prior to affinity purification of phosphospecific antibodies, using phosphopeptide resin. The specific antibody was further purified using phosphorylated CPI-17 by the modified method of Talian et al. (27).

**Immunoblotting**—Nontreated intact or permeabilized femoral artery strips were rapidly frozen and treated as previously described (27).

**RESULTS AND DISCUSSION**

**Phosphospecific Antibodies against Thr-38 in CPI-17**—The specificity of phosphorylation-dependent antibodies for p[T38]CPI-17 is demonstrated in Fig. 1. Western blotting with affinity-purified chicken anti-CPI-17 antibodies (Fig. 1A) showed a linear response to the amount of recombinant CPI-17 protein (Fig. 1C). There was no difference in reactivity between the unphosphorylated and phosphorylated forms of CPI-17. This antibody was used to determine the total CPI-17 recovered from tissues. In contrast, affinity-purified rabbit anti-phosphopeptide antibodies directed against the phosphorylation site at Thr-38 (anti-p[T38]CPI-17) did not react with unphosphorylated recombinant CPI-17 (Fig. 1, B, upper panel, and D) but did give a linear response with phospho-CPI-17 (Fig. 1, B, lower panel, and D), with about the same relative sensitivity as the chicken anti-CPI-17 antibodies. CPI-17 is phosphorylated by PKC at two sites, Thr-38 and Ser-12 (22). However, the anti-p[T38]CPI-17 antibody did not recognize a CPI-17 mutant where Thr at position 38 was replaced with Ala, even after PKC phosphorylation of Ser-12 (not shown). The rabbit antibody was both phosphospecific and site-specific for p[T38]CPI-17 and was used in Western blotting to gauge the level of phosphorylation of Thr-38 in CPI-17 recovered from tissues.

**Contraction of Intact Femoral Artery and Phosphorylation of CPI-17 in Response to Depolarization and Ca\(^{2+}\)-sensitizing Agonists**—We used this phosphospecific antibody to determine the in situ phosphorylation of Thr-38 in CPI-17 during stimulation of intact smooth muscle. Rabbit femoral artery strips were treated with 30 mM and 145 mM K\(^+\) and with 30 mM K\(^+\) plus phenylephrine or histamine. Histamine and the α₁-agonist phenylephrine were tested in the presence of 30 mM K\(^+\) to avoid oscillatory and/or transient contractions induced by the agonists alone and to maintain consistent levels of contraction. Fig. 2 compares relative force development and relative CPI-17 phosphorylation under the conditions tested. Histamine plus 30 mM K\(^+\) produced the largest contraction and the highest level of Thr-38 phosphorylation, and therefore the results were normalized to this response. The histamine-induced contraction and CPI-17 phosphorylation were reversed to the resting levels 15 min after the agonist was removed, showing that CPI-17 was rapidly dephosphorylated in intact tissue (data not shown). In Ca\(^{2+}\)-free conditions contractile activity was completely depressed, and the phosphorylation of CPI-17 was negligible (4 ± 2%). Depolarization with high K\(^+\) produced submaximal but sustained contraction, without significant phosphorylation of Thr-38 in CPI-17 (Fig. 2). In comparison, both histamine and phenylephrine produced approximately double the level of contraction elicited by depolarization with 154 mM K\(^+\), and both significantly elevated the levels of CPI-17 phosphorylation. There was no significant difference in the...
Contractile force evoked by histamine versus phenylephrine. However, the phosphorylation of CPI-17 in response to histamine (set as 100%) was higher than that in response to phenylephrine (52 ± 17%). These results suggest the possibility that these agonists engage different signaling mechanisms to enhance contraction of smooth muscle.

CPI-17 Phosphorylation in Permeabilized Smooth Muscle—To investigate the signaling mechanisms for CPI-17 phosphorylation, we used α-toxin-permeabilized smooth muscle to control the composition and concentration of low molecular weight soluble components. In contrast to other permeabilization methods (e.g. β-escin and Triton X-100) the endogenous CPI-17 is retained in the α-toxin preparation (12). In muscle permeabilized with α-toxin, the 

Ca$^{2+}$ concentration was buffered with 10 mM EGTA, and intracellular Ca$^{2+}$ stores were removed with A23187. As expected, CPI-17 phosphorylation at pCa > 8 in permeabilized muscle was similar to that in quiescent intact muscle. Increasing the Ca$^{2+}$ concentration to pCa 6.7 produced a detectable contraction that was less than 5% of maximum force, and under these conditions there was a correspondingly low level of CPI-17 phosphorylation (Fig. 3, A and B). Increasing the concentration of Ca$^{2+}$ to pCa 6 significantly increased contraction to over half-maximum but only increased CPI-17 phosphorylation a minimal amount (Fig. 3). We concluded that physiological [Ca$^{2+}$]$^g$ alone was not sufficient to produce phosphorylation of CPI-17.

Involvement of G Proteins in Receptor-mediated Phosphorylation of CPI-17—Histamine increased contraction of permeabilized smooth muscle more than 10-fold at pCa 6.7, and in parallel, histamine increased CPI-17 phosphorylation nearly 10-fold (Fig. 3). The nonhydrolyzable GTP analog GTP[S], which activates G proteins, produced larger contractions and higher levels of CPI-17 phosphorylation than those elicited by histamine (Fig. 3). On the other hand, the compound GDP[S] acts as a G protein inhibitor, and when added to permeabilized smooth muscle it reduced histamine stimulation of contraction and CPI-17 phosphorylation by 80% (Fig. 4). Activation by GTP[S] and inhibition by GDP[S] together strongly implicate G proteins in the histamine-induced increase in CPI-17 phosphorylation.

Protein Kinases Involved in CPI-17 Phosphorylation—A PKC inhibitor GF109203X inhibited histamine-induced contraction
by 50% and reduced CPI-17 phosphorylation by 70% (Fig. 4). A Rho-kinase inhibitor, Y27632, partially blocked histamine-induced contraction and CPI-17 phosphorylation, whereas contraction induced by phenylephrine was more effectively inhibited (from 53 ± 9% to 18 ± 9% of maximum at pCa 4.5; not shown), consistent with the engagement of different signaling pathways for these agonists. Activation of various PKC isoforms with phorbol ester (PDBu) at pCa 6.7 gave high levels of CPI-17 phosphorylation (125 ± 21% versus GTPγS). This phosphorylation was only slightly reduced at pCa > 8, supporting the hypothesis that Ca2+-independent PKC isoforms phosphorylate CPI-17. To maximize CPI-17 phosphorylation we added calyculin A, a potent protein phosphatase-1 and -2A inhibitor, to block dephosphorylation of CPI-17. Calyculin A increased, but not significantly, the PDBu-induced phosphorylation of CPI-17 at pCa 6.7. CPI-17 phosphorylation with GTPγS plus PDBu at high Ca2+ (pCa 4.5) was only slightly higher (by 26 ± 20%) than with PDBu at pCa 6.7. Therefore, G proteins and PKC apparently function in the same or parallel pathways leading to CPI-17 phosphorylation.

This is the first demonstration of reversible phosphorylation of CPI-17 in intact arterial smooth muscle. The experiments revealed that CPI-17 phosphorylation was activated by stimulation of G protein-coupled Ca2+-sensitizing agonists but not by an increase in intracellular Ca2+. Overall, the results support a model for a physiological pathway for CPI-17 activation involving receptors, G proteins, and Ca2+-independent PKC isoforms and/or Rho-activated kinases. CPI-17 may provide a point of convergence for PKC and RhoA signaling pathways. Expression of CPI-17 differs among smooth muscle tissues (23, 28), and CPI-17 phosphorylation responds to different agonists. Therefore the involvement of CPI-17 in agonist-induced Ca2+ sensitization may depend upon tissue type and agonist, and we predict that the CPI-17 signaling pathway to inhibit MLCP will prove to be dominant in a few selective circumstances.

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