Published in final edited form as:

Biochem Biophys Res Commun. 2010 October 8; 401(1): 75–78. doi:10.1016/j.bbrc.2010.09.011.

G Protein-Mediated Ca^{2+} -Sensitization of CPI-17 Phosphorylation in Arterial Smooth Muscle

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Abstract

CPI-17 is a unique phosphoprotein that specifically inhibits myosin light chain phosphatase in smooth muscle and plays an essential role in agonist-induced contraction. To elucidate the *in situ* mechanism for G protein-mediated Ca^{2+} -sensitization of CPI-17 phosphorylation, α -toxin-permeabilized arterial smooth muscle strips were used to monitor both force development and CPI-17 phosphorylation in response to GTP γ S with varying Ca^{2+} -concentrations. CPI-17 phosphorylation increased at unphysiologically high Ca^{2+} levels of pCa ≤ 6 . GTP γ S markedly enhanced the Ca^{2+} sensitivity of CPI-17 steady-state phosphorylation but had no enhancing effect under Ca^{2+} -free conditions, while the potent PKC activator PDBu increased CPI-17 phosphorylation regardless of Ca^{2+} concentration. CPI-17 phosphorylation induced by pCa 4.5 alone was markedly inhibited by the presence of PKC inhibitor but not ROCK inhibitor. In the presence of calyculin A, a potent PP1/PP2A phosphatase inhibitor, CPI-17 phosphorylation increased with time even under Ca^{2+} -free conditions. Furthermore, as Ca^{2+} concentration increased, so did CPI-17 phosphorylation rate. GTP γ S markedly enhanced the rate of phosphorylation of CPI-17 at a given Ca^{2+} . In the absence of calyculin A, either steady-state phosphorylation of CPI-17 under Ca^{2+} -free conditions in the presence of GTP γ S or at pCa 6.7 in the absence of GTP γ S was negligible, suggesting a high intrinsic CPI-17 phosphatase activity. In conclusion, cooperative increases in Ca^{2+} and G protein activation are required for a significant activation of total kinases that phosphorylate CPI-17, which together overcome CPI-17 phosphatase activity and effectively increase the Ca^{2+} sensitivity of CPI-17 phosphorylation and smooth muscle contraction.

Keywords

CPI-17; Protein kinase C; Myosin phosphatase; Calcium sensitivity; Smooth muscle; Artery

1. Introduction

CPI-17, a potent myosin phosphatase (MLCP) inhibitor protein, plays a critical role in regulating smooth muscle contraction [1,2]. We recently demonstrated that CPI-17 is a remarkable Ca^{2+} -dependent messenger that mediates GPCR stimulation to MLCP regulation in intact arterial smooth muscle [3], which acts on MLC phosphorylation in addition to the classical Ca^{2+} /calmodulin-mediated regulation of MLC kinase (MLCK) [4]. α_1 -Agonist increases CPI-17 phosphorylation levels from negligible values at the resting state to about

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0.4 mol/mol protein within seconds. This marked phosphorylation increase is coupled to the rapid onset of both MLC phosphorylation and muscle contraction and is also associated with agonist-induced SR Ca^{2+} release and PKC activation but not Ca^{2+} influx or ROCK activation [3]. Furthermore, the significant increase in $[\text{Ca}^{2+}]_i$ induced by high K^+ depolarization does not increase CPI-17 phosphorylation. Together, these results indicate that at least one second messenger, very likely diacylglycerol (DAG), works with Ca^{2+} to stimulate Ca^{2+} -dependent CPI-17 phosphorylation through Ca^{2+} -dependent PKC. However, little is known about the role of Ca^{2+} sensitivity and G-protein activity in regulating *in situ* CPI-17 phosphorylation in smooth muscle. Here, the mechanism for Ca^{2+} -dependent CPI-17 phosphorylation and its effect of G protein activation is investigated in α -toxin-permeabilized arterial smooth muscle, where the SR Ca^{2+} was depleted with Ca^{2+} ionophore A23187 and the $[\text{Ca}^{2+}]_i$ concentration was clamped with 10 mM EGTA.

2. Materials and Methods

2.1. Tissue preparation, force measurement, and cell permeabilization

All animal procedures were approved by the Animal Care and Use Committee of the Boston Biomedical Research Institute. Strips of rabbit femoral artery smooth muscle were prepared and mounted for force measurements and quick-freezing using liquid nitrogen-cooled propane, as described previously in detail [3,5]. Briefly, adventitia-free and de-endothelialized smooth muscle strips (70 μm thick, 0.75 mm wide, and 3 mm long) were dissected from rabbit femoral arteries and mounted on a force transducer assembly. Force levels were monitored throughout the experiments. The compositions of external and intracellular solutions were described previously and Ca^{2+} concentrations in the intracellular solutions were clamped with 10 mM EGTA at pH 7.1 [5,6]. For cell membrane permeabilization, strips were treated for 30 min at 30 °C with 20 $\mu\text{g}/\text{ml}$ purified *Staphylococcus aureus* α -toxin (List, Campbell, CA) at pCa 6.7 and further treated with 10 μM Ca^{2+} -ionophore A23187 for 20 min at 25 °C to deplete the sarcoplasmic reticulum of Ca^{2+} and maintain constant cytoplasmic Ca^{2+} as described previously [6,7]. The pCa is defined as $-\log(\text{molar concentration of free } \text{Ca}^{2+})$. Thereafter, the temperature was maintained at 20°C.

2.2. Immunoblotting

Permeabilized femoral artery strips were rapidly frozen and treated as previously described [1,5]. The strips were dried and homogenized in electrophoresis sample buffer and equal amounts of the same tissue extracts were loaded onto two 15% (w/v) polyacrylamide gels, and the separated proteins transferred to the same nitrocellulose membranes. The membranes were blocked in Tris-buffered saline solution containing 0.05% Tween 20 and 5% nonfat milk and incubated with a primary antibody followed by an alkaline phosphatase-conjugated secondary antibody. The immunoblots were developed with an alkaline phosphatase substrate solution to visualize immunoreactive proteins. The alkaline phosphatase product bands were digitized with a color scanner and analyzed with image processing software (Signal Analytics Co., Vienna, VA). Western blotting experiments were always carried out in duplicate. We compared the ratio of phosphorylated CPI-17 at Thr38 to the total amount of CPI-17 in the paired set of Western blots.

2.3. Statistical analysis

Where applicable, results are expressed as the mean $\pm$ SEM. Significance was evaluated using one-way ANOVA or Student's t-test. A level of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Ca^{2+} sensitivity of CPI-17 phosphorylation

To investigate the Ca^{2+} sensitivity of CPI-17 phosphorylation, we used α -toxin-permeabilized smooth muscle to control free $[\text{Ca}^{2+}]_i$. In contrast to other cell permeabilization methods, endogenous small proteins, including CPI-17, are retained in α -toxin-permeabilized preparations at levels similar to intact tissues while the cytoplasmic concentration of small molecules such as ATP and EGTA can be controlled [8]. The free Ca^{2+} concentration was buffered with 10 mM EGTA and intracellular Ca^{2+} stores were depleted with A23187 [6,7]. When Ca^{2+} was increased from pCa <8 (no added Ca^{2+} in 10 mM EGTA-containing solution) to pCa 6.7, minimal force was detected (Fig. 1A). Upon increasing to pCa 6, force developed to a level near the maximum level induced by pCa 4.5. The G protein activator GTP γ S (30 μ M) and PKC activator PDBu (3 μ M) markedly enhanced contraction at pCa 6.7 while the enhancing effect of both activators was minimal at pCa >8 and pCa 4.5, suggesting that those activators primarily increase the Ca^{2+} sensitivity of smooth muscle contraction.

Under control conditions, CPI-17 phosphorylation was negligible from pCa >8 to 6.7, and significantly increased upon further increases in Ca^{2+} concentration to pCa 6 and 4.5 (Fig. 1B). Similar to force enhancement, GTP γ S at 30 μ M, which is known to produce a maximal effect on MLC phosphorylation and contraction [9], strikingly increased CPI-17 phosphorylation at Ca^{2+} concentrations ranging from pCa 6.7 to 4.5 but not at pCa >8, suggesting an increase in Ca^{2+} sensitivity. In contrast, PDBu increased CPI-17 phosphorylation to a maximal level even at pCa >8, suggesting that the activity of an *in situ* Ca^{2+} -independent CPI-17 kinase, such as a novel PKC isoform is increased.

3.2. Effect of inhibitors on Ca^{2+} -induced CPI-17 phosphorylation

CPI-17 phosphorylation at pCa 4.5 was strongly reduced by PKC inhibitor GF-109203X (3 μ M) and partially by G protein inhibitor GDP β S (1 mM), while ROCK inhibitor Y-27632 (10 μ M) did not significantly affect phosphorylation levels (Fig. 2A) suggesting that high Ca^{2+} activates PKC partially through a G protein-dependent pathway. Elimination of CPI-17 phosphorylation by GF-109203X at pCa 4.5 only slightly affected the maximum contraction (Fig. 2B), suggesting that MCLK was near maximally activated at pCa 4.5 [9].

3.3. Rate of CPI-17 phosphorylation when CPI-17 phosphatase is inactivated

CPI-17 phosphatase is a PP1 type phosphatase [10]. To examine the mechanism of the CPI-17 phosphorylation reaction *in situ*, CPI-17 dephosphorylation was blocked by treatment with the potent PP1/PP2A inhibitor, calyculin A [11]. Permeabilized strips were first incubated in MgATP-free, CP (creatine phosphatase)-free, and Ca^{2+} -free solution to dephosphorylate CPI-17 and block kinase activity. Thirty minutes after ATP removal, 1 μ M calyculin A was added to inhibit CPI-17 phosphatase activity. Thirty min after calyculin A addition under rigor conditions, 4.5 mM MgATP, 10 mM CP-, and 1 μ M calyculin A-containing solution at the indicated Ca^{2+} concentration was exchanged for the rigor solution to initiate the phosphorylation reaction. CPI-17 was time-dependently phosphorylated even at pCa >8 (Fig. 3). Increases in Ca^{2+} to pCa 7 and 6 increased the rate of CPI-17 phosphorylation, suggesting that *in situ* CPI-17 kinase(s) is active even at near resting Ca^{2+} . GTP γ S in the presence of calyculin A significantly enhanced CPI-17 phosphorylation even at pCa >8 (Fig. 3) whereas increases in CPI-17 steady-state phosphorylation upon addition of GTP γ S were minimal under identical conditions except in the absence of phosphatase inhibitor (Fig. 1B). GTP γ S addition resulted in a large increase in the rate of CPI-17 phosphorylation at pCa 7, corresponding to a large enhancement of steady-state phosphorylation at the same pCa in the absence of inhibitor (Fig. 1B). PDBu at 3 μ M

increased the rate of phosphorylation regardless of Ca^{2+} concentration in the absence of calyculin A (not shown).

Discussion

In intact arterial smooth muscle, steady-state CPI-17 phosphorylation is negligible at rest. Upon stimulation with α_1 -agonist, CPI-17 phosphorylation levels rapidly increase in response to SR Ca^{2+} -release, leading to MLCP inhibition and a robust increase in MLC phosphorylation [3] mediated by Ca^{2+} /calmodulin-dependent MLCK [4]. This study, using permeabilized arterial smooth muscle, presents three critical findings: 1) Elimination of CPI-17 phosphatase activity revealed a hidden CPI-17 kinase activity that persists even under Ca^{2+} -free conditions. This Ca^{2+} -independent kinase activity was enhanced by G protein activation. However, the Ca^{2+} -independent basal and GTP γ S-enhanced kinase activities together appear to be insufficient to overcome intrinsic CPI-17 phosphatase activity, and, thus, the steady-state phosphorylation level in the presence of GTP γ S under Ca^{2+} -free conditions in the absence of calyculin A was still negligible (Fig. 1B); 2) Raising Ca^{2+} concentrations to unphysiologically high levels (pCa <6) markedly increased the rate of phosphorylation in the presence of calyculin A and moderately but significantly enhanced steady-state phosphorylation in the absence of calyculin A, which was strongly inhibited by the PKC inhibitor (Fig. 2B). Physiological $[\text{Ca}^{2+}]_i$ (from pCa 7 to pCa >6) appreciably stimulated CPI-17 kinase activity compared to that measured for Ca^{2+} -free conditions (Fig. 3) although both steady-state CPI-17 phosphorylation levels at pCa 6.7 in permeabilized muscle without GTP γ S [1; this study] and during high K^+ -induced contraction in intact muscle [3] was minimal, suggesting that the basal and Ca^{2+} -dependent kinase activity within the physiological Ca^{2+} concentration range without G protein activation still could not overcome the intrinsic phosphatase activity. These results suggest that the *in situ* CPI-17 phosphatase activity is higher than the kinase activity at pCa 6.7 and lower than that at pCa 6 in permeabilized arterial smooth muscle. Free Ca^{2+} concentration at pCa 6.7 in the presence of PDBu can evoke a large contraction that is sensitive not only to Ca^{2+} -independent but also Ca^{2+} -dependent PKC inhibitors in arterial smooth muscle [12], supporting the idea that physiological Ca^{2+} levels can at least partly stimulate Ca^{2+} -dependent PKC; 3) GTP γ S markedly increased the rate of CPI-17 phosphorylation by several fold at both pCa >8 (Ca^{2+} -free conditions) and 7 (Fig. 3). Incremental GTP γ S-induced increases in phosphorylation after 2 min in the presence of pCa 7 were considerably greater than the sum of phosphorylation levels at pCa 7 alone and of GTP γ S-induced changes for Ca^{2+} -free conditions at the same time point, suggesting that G protein activation enhances not only basal but also Ca^{2+} -dependent CPI-17 kinase activities. The steady-state phosphorylation in the presence of GTP γ S under Ca^{2+} -free conditions, however, was negligible while that at pCa 6.7 was maximal, suggesting that the GTP γ S-enhanced rate of phosphorylation is still lower under Ca^{2+} -free conditions but much higher at pCa 6.7 than that of intrinsic phosphatase activity. In intact arterial smooth muscle, when agonist-induced Ca^{2+} increases are blocked with ryanodine and nicardipine, CPI-17 phosphorylation only slowly increases to low levels [3], consistent with the present results. This study cannot exclude the possibility that G protein activation inhibits CPI-17 phosphatase activity, resulting in an increase in apparent kinase activity. Without considering the possible regulation of CPI-17 phosphatase activity, however, the present data can be explained by a combination of the GTP γ S-induced increase in Ca^{2+} -dependent and -independent CPI-17 kinases and the constitutively active phosphatase as discussed above. If the phosphatase activity were reduced by G protein activation without changing the kinase activity, the rate of CPI-17 phosphorylation would not be changed with GTP γ S in the presence of calyculin A, but this is not the case (Fig. 3). Phosphorylation of CPI-17 Thr38 is known to occur in response to agonists following activation of PKC and ROCK in arterial [1,3,12] and integrin-linked kinase (ILK) in intestinal smooth muscles [13]. PDBu, but not GTP γ S,

enhanced the steady-state phosphorylation of CPI-17 under Ca^{2+} -free conditions, suggesting that the total activation of Ca^{2+} -independent CPI-17 kinases (nPKC, ROCK, and possibly ILK) by a maximal activation of G protein cannot overcome the intrinsic phosphatase activity and is considerably lower than PDBu-induced activation of nPKC in arterial smooth muscle. If the Ca^{2+} -independent CPI-17 kinase were totally inactivated, the Ca^{2+} sensitivity of CPI-17 phosphorylation would be shifted to much higher than physiological Ca^{2+} concentrations, suggesting a physiological role for Ca^{2+} -independent activity. Other Ca^{2+} -independent kinases, such as zipper-interacting kinase and p21-activated kinase, can also phosphorylate isolated CPI-17 at Thr38 [14,15]. Further studies are needed to identify which kinase is responsible for the increased *in situ* CPI-17 phosphorylation under different conditions.

Research highlights

- Phosphorylation of smooth muscle-specific CPI-17 increases at only high Ca^{2+} .
- G protein activation increases Ca^{2+} sensitivity of CPI-17 phosphorylation and smooth muscle contraction, but has no enhancing effect under Ca^{2+} -free conditions.
- When phosphatase is blocked, CPI-17 phosphorylation increases with time even at low Ca^{2+} .
- Because of high intrinsic CPI-17 phosphatase activity, cooperative increases in Ca^{2+} and G protein activation are required for a significant activation of CPI-17 kinases and smooth muscle contraction.

Acknowledgments

This study was partly supported by National Institutes of Health grants R01HL51824 and HL70881. I thank Dr. Masumi Eto for his continuous support and invaluable phospho-specific CPI-17 antibody.

Abbreviations

CPI-17	protein kinase C-potentiated phosphatase inhibitor protein 17 kDa
pCa	$-\log(\text{concentration of free } \text{Ca}^{2+} \text{ in molar})$
PKC	protein kinase C
PDBu	phorbol 12,13-dibutyrate
ROCK	Rho-associated kinase
PP1	protein phosphatase type 1
PP2A	protein phosphatase type 2A
MLC	myosin light chain
MLCP	myosin light chain phosphatase
MLCK	myosin light chain kinase
SR	sarcoplasmic reticulum
DAG	diacylglycerol

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Fig. 1.

Effect of 30 μM GTP γ S and 3 μM PDBu on the Ca^{2+} sensitivity of force development (A) and CPI-17 phosphorylation (B) in α -toxin-permeabilized rabbit femoral artery smooth muscle. A: Force levels are expressed as a percentage of contraction produced at pCa 4.5 under control conditions ($n=6$). pCa = $-\log(\text{molar concentration of free } \text{Ca}^{2+})$. pCa >8 is the free Ca^{2+} concentration when no calcium is added to relaxation solution containing 10 mM EGTA at pH 7.1. (B) Paired set of representative western blots for CPI-17 (upper panel) and pThr38 CPI-17 (lower panel) and a summary of phosphorylated levels of CPI-17 measured 7.5 min after addition of Ca^{2+} in the presence and absence of GTP γ S or PDBu expressed as a percentage of total phosphorylation (phosphorylated CPI-17/total CPI-17) at pCa 6.7 in the presence of GTP γ S for 7.5 min ($n=3-6$). ND, not determined.

Fig. 2.

Effect of G protein and protein kinase inhibitors on CPI-17 phosphorylation (A) and maximum contraction (B) at pCa 4.5. Permeabilized arterial smooth muscle strips were pretreated in the presence and absence of 1 mM GDPβS, 3 μM GF-109203X, and 10 μM Y-27632 for 10 min in relaxing solution with 1mM EGTA before Ca²⁺ was increased to pCa 4.5 buffered with 10 mM EGTA for 7.5 min. Panel A shows a paired set of representative western blots for CPI-17 (upper panel) and pThr38 CPI-17 (lower panel) and a summary of CPI-17 phosphorylation levels expressed as the percentage of phosphorylation level at pCa 4.5 for 7.5 min under control conditions (n=4). In Panel B, force levels are expressed as a percentage of maximum contraction produced at pCa 4.5 under control conditions (n=4). * represents statistical significance ($p<0.05$).

Fig. 3.

Effect of Ca^{2+} and $30 \mu\text{M GTP}\gamma\text{S}$ on the CPI-17 phosphorylation rate in the presence of $1 \mu\text{M}$ calyculin A. MgATP (4.5 mM) and CP (10 mM) were added to initiate the phosphorylation reaction after a 30-min incubation in rigor solution containing calyculin A with (filled symbols) or without (open symbols) GTP γ S at the indicated Ca^{2+} concentration ($n=3$ at pCa 7). Ca^{2+} levels were clamped with 10 mM EGTA .