Uncoupling of GPCR and RhoA-induced Ca^{2+}-sensitization of chicken amnion smooth muscle lacking CPI-17

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Abstract **Ca^{2+}-sensitization of smooth muscle occurs through inhibition of myosin light chain phosphatase (MLCP) leading to an increase in the MLCK:MLCP activity ratio. MLCP is inhibited through phosphorylation of its regulatory subunit (MYPT-1) following activation of the RhoA/Rho kinase (ROK) pathway or through phosphorylation of the PP1c inhibitory protein, CPI-17, by PKC δ or ROK. Here, we explore the crosstalk between these two modes of MLCP inhibition in a smooth muscle of a natural CPI-17 knockout, chicken amnion. GTPγS elicited Ca^{2+}-sensitized force which was relaxed by GDI or Y-27632, however, U46619, carbachol and phorbol ester failed to induce Ca^{2+}-sensitized force, but were rescued by recombinant CPI-17, and were sensitive to Y-27632 inhibition. In the presence, but not absence, of CPI-17, U46619 also significantly increased GTP · RhoA. There was no affect on MYPT-1 phosphorylation at T695, however, T850 phosphorylation increased in response to GTPγS stimulation. Together, these data suggest a role for CPI-17 upstream of RhoA activation possibly through activation of another PP1 family member targeted by CPI-17.**

Keywords: Myosin phosphatase; Ca^{2+}-sensitization; Smooth muscle; CPI-17; Rho guanine nucleotide exchange factors

1. Introduction

Ca^{2+}-sensitization of smooth muscle myosin II, a highly relevant physiological process, reflects the ratio of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) activities that determine the level of myosin light chain phosphorylation (MLC_{20}) and force [1–3]. Agonists induce Ca^{2+}-sensitization by inhibition of MLCP through activation of G-protein coupled receptors (GPCRs) which activate guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP on RhoA, its translocation to the cell membrane and activation of Rho kinase (ROK) [4,5]. Evidence, in vitro [6,7] and in vivo [8], suggests that inhibitory phosphorylation of MYPT-1 may occur at several sites. The relevant sites in smooth muscle tissues are unclear. The identified ROK phosphorylation sites in chicken MYPT-1 (133 kDa) are threonine 695 (T695) and serine 849 (S849) corresponding, respectively, to T696 and S852 in the human sequence. Also, it has been demonstrated in vitro that the phosphorylation of T850 by ROK leads to dissociation of the phosphatase from myosin [7].

MLCP activity can be modulated by other mediators such as CPI-17, a 17-kDa peptide [9], where phosphorylation at Thr-38 inhibits PP1c, the catalytic subunit of MYPT-1. Recently, it has been demonstrated that CPI-17 is a substrate for a number of kinases including PKC [10], Zip-like kinase [11], integrin-linked kinase [12], the Rho effector PKN [13] as well as ROK [14]. ROK phosphorylation of CPI-17 in vitro is inhibited by the ROK inhibitor, Y-27632 [15,10], suggesting a possible link between RhoA and CPI-17 in the Ca^{2+}-sensitization pathway. MLCP activity can also be increased through PKG-mediated inhibition of RhoA [16] or possibly through interaction between the leucine zipper motifs of PKGIα and MYPT-1 [17]. In this study, we have taken advantage of a special preparation, chicken amnion (ASM), which does not express CPI-17, to examine the interdependence of the RhoA/ROK and the CPI-17 signaling pathways in the regulation of Ca^{2+}- sensitization. As the signaling between GPCRs and RhoA activation is poorly understood in smooth muscle, we have also identified the expression of two RhoGEFs, LARG and PDZ-RhoGEF in native smooth muscle providing evidence of a means of activation of RhoA in Ca^{2+}-sensitization.

2. Materials and methods

2.1. Materials and chemicals

α-Toxin was purified from *Staphylococcus aureus*. A23187, U-46619 (Calbiochem, La Jolla, CA), Y-27632 (Mitsubishi Pharma Corp., Yokohama, Japan), anti-Rho A (sc179), anti-$G_{\alpha q/11}$ (sc392), anti-$G_{\alpha 12}$ (sc409), and anti-$G_{\alpha 13}$ (sc410) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-chicken ROK (generous gift from Dr. L. Lim, Singapore), anti-MYPT-1 (MMS-458R) antibody (Covance, Princeton, NJ), anti-pS854 (phospho-serine 849 in chicken) MYPT-1 antibody (generous gift from Dr. K. Kaibuchi, Nagoya University, Japan), anti-pT695 MYPT-1 and anti-pT850 MYPT-1 antibodies (Upstate Biotechnology, Lake Placid, NY). Anti-PP1δ and anti-CPI-17 were prepared as described previously [18]. CPI-17 antibody raised against pig CPI-17 has been shown to react with pigeon CPI-17 [19]. Recombinant RhoA · GDI was prepared as

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reported previously [20]. Anti-PDZ-RhoGEF and anti-LARG antibodies were generously provided by Dr. S. Gutkind, NIH. Eggs were purchased from Truslow Farms Inc. (Chestertown, MD) and maintained in a Turn-X incubator (Lyon Electric Co., Chula Vista, CA).

2.2. Isometric tension measurement

Chicken amnion sheets (approximately 3×3 mm) from 8 to 14 day eggs were dissected and attached to a fixed hook and force transducer (AE 801; AME, Horten, Norway) to record isometric tension, at room temperature, in HEPES-buffered Krebs solution (according to published protocol [5]).

2.3. Ca^{2+}-sensitization and protein phosphorylation

Amnion sheets were permeabilized in relaxing solution (G1) (containing 1 mM EGTA) with *Staphylococcus aureus* α-toxin (0.1 mg/ml) or β-escin (75 μM) for 30 min at 22 °C and subsequent incubation in the presence of A23187 (10 μM) for 20 min. At the appropriate time point, muscle sheets were rapidly frozen in liquid nitrogen, transferred to pre-cooled 10% TCA/acetone, and processed by freeze substitution as reported previously [21] and centrifuged. Samples in Laemmli sample buffer were separated by SDS–PAGE (7.5% polyacrylamide for MYPT-1 and 15% for MLC_{20}) and transferred to PVDF membranes before Western blotting with monoclonal anti-MYPT-1 (1:10 000) or polyclonal anti-phospho-MYPT-1 antibodies (anti-T695, 1:2000; anti-S854, 1:50; and anti-T850, 1:1000).

2.4. RhoA translocation

In order to separate cytosolic and particulate fractions, two amnion sheets from stimulated and control (approximately 3×3 mm) were used per sample. These were prepared for SDS–PAGE analysis according to the method previously described [5].

2.5. mDia and Rhotekin assay for GTP-RhoA

Active RhoA was affinity precipitated using the Rho binding domain (RBD) of mDia or Rhotekin. pGEX-4T-1 mouse mDia or Rhotekin RBD was introduced into *Escherichia coli*, and the GST fusion protein was expressed, conjugated to glutathione beads, and purified. Following α-toxin or β-escin permeabilization and treatment with the Ca^{2+} ionophore A23187 for 10 min. the samples were transferred to submaximal Ca^{2+} (pCa 6.5) and treated with various agents as indicated for 10 min. Tissues were flash frozen in liquid nitrogen and stored at −80 °C, then homogenized in a buffer containing: 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol (DTT), 0.5% Nonidet P-40, and a protease inhibitor mixture, centrifuged and supernatants incubated with 30 μg of the GST-RBD fusion protein conjugated with glutathione beads for 45 min at 4 °C. Beads were pelleted, washed and supernatants saved. DTT and Laemmli sample buffer were added to the samples, followed by boiling and separation of the beads by centrifugation and electrophoresis on an SDS–PAGE gel. RhoA was detected by Western blot analysis using a polyclonal antibody against RhoA (1:1000). Bound RhoA was normalized to the RhoA in the whole extract of a given sample.

2.6. Western blots for ROK, G_α subunits, PKC isoforms, and RhoGEFs

Supernatants from whole tissue homogenate were solubilized in 1× Laemmli sample buffer and clarified ($800 \times g$, 10 min) before SDS–PAGE. Immunoblotting was carried out as previously described [5]. The primary antibody dilutions were 1:1000 for chicken polyclonal anti-ROK antibody; 1:5000 for G_α antibodies; 1:200 or 1:1000 (as indicated) for PDZ-RhoGEF and LARG antibodies.

2.7. Primary cultures of chicken amnion smooth muscle

Sheets of smooth muscle were dissected from chicken eggs and maintained in culture in DMEM/F12 medium + 10% FBS at 37 °C. Explanted primary cultures were treated as specified before terminating the reaction at the appropriate time point following exchange of the culture media for lysis buffer containing: 1% SDS, 1 mM EDTA, 1 mM Na_3VO_4, 0.4 mM AEBSF, and 50 mM Tris–HCl, pH 7.4. Identification of smooth muscle cells was confirmed by immunostaining using an anti-smooth muscle-α-actin antibody (1:20 000).

3. Results and discussion

3.1. Agonist-induced responses of intact amnion smooth muscle

A wide variety of agonists leading to contractile responses in smooth muscle mediated through increases in cytosolic [Ca^{2+}] and/or Ca^{2+}-sensitization were explored in intact amnion (Table 1). The muscarinic agonist, carbachol (10 μM), and 5-hydroxytryptamine (5-HT) (10 μM) elicited fast, phasic contractions in intact chicken amnion, whereas the α-adrenergic agonist phenylephrine (100 μM), the thromboxane analog U-44619, a strong Ca^{2+} sensitizer, and oxytocin (600 nM) were without effect, indicating selectivity for some GPCR agonists in chicken amnion. PDBu (1 μM), a phorbol ester, consistently caused a small relaxation in intact ASM along with the PKC activator arachidonic acid (300 μM).

3.2. Ca^{2+}-sensitization of α-toxin permeabilized amnion smooth muscle

Surprisingly, despite responses of intact amnion to carbachol and 5-HT, these agonists did not induce Ca^{2+}-sensitization in the α-toxin permeabilized amnion consistent with responses in the intact amnion being due to Ca^{2+} release and influx. In contrast, GTPγS (10 μM), a non-hydrolyzable form of GTP, elicited a robust Ca^{2+}-sensitization (Fig. 1A, a) as did AlF_4^- (10 μM $AlCl_3$/3 mM NaF), a non-specific activator of heterotrimeric G-proteins and RhoA (reviewed in [3]) (Table 1; Fig. 1A, c). The amplitude of Ca^{2+}-sensitization by GTPγS was $29.4 \pm 2.4\%$ ($n = 11$) of the maximum tension (pCa 4.5) (Fig. 1A, e). The ROK inhibitor, Y-27632 (10 μM), rapidly

Table 1
Agonist-induced contractile responses in intact versus permeabilized chicken amnion

Agonist	Concentration	Response		
		Intact	Permeabilized	
			(−)CPI-17	(+)CPI-17
Carbachol	10–100 μM	+++	–	+
5-HT	10 μM	++	–	nt
Phenylephrine	100 μM	–	–	nt
U-46619	0.3–1 μM	–	–	++
Oxytocin	600 nM	–	–	nt
PDBu	1 μM	r	–	+
Arachidonic acid	300 μM	r	–	nt
GTPγS	10–300 μM	–	+++	
AlF_4^-	10 μM	–	++	

'+' denotes contraction, '–' denotes no response, 'r' denotes relaxation and 'nt' denotes not tested.

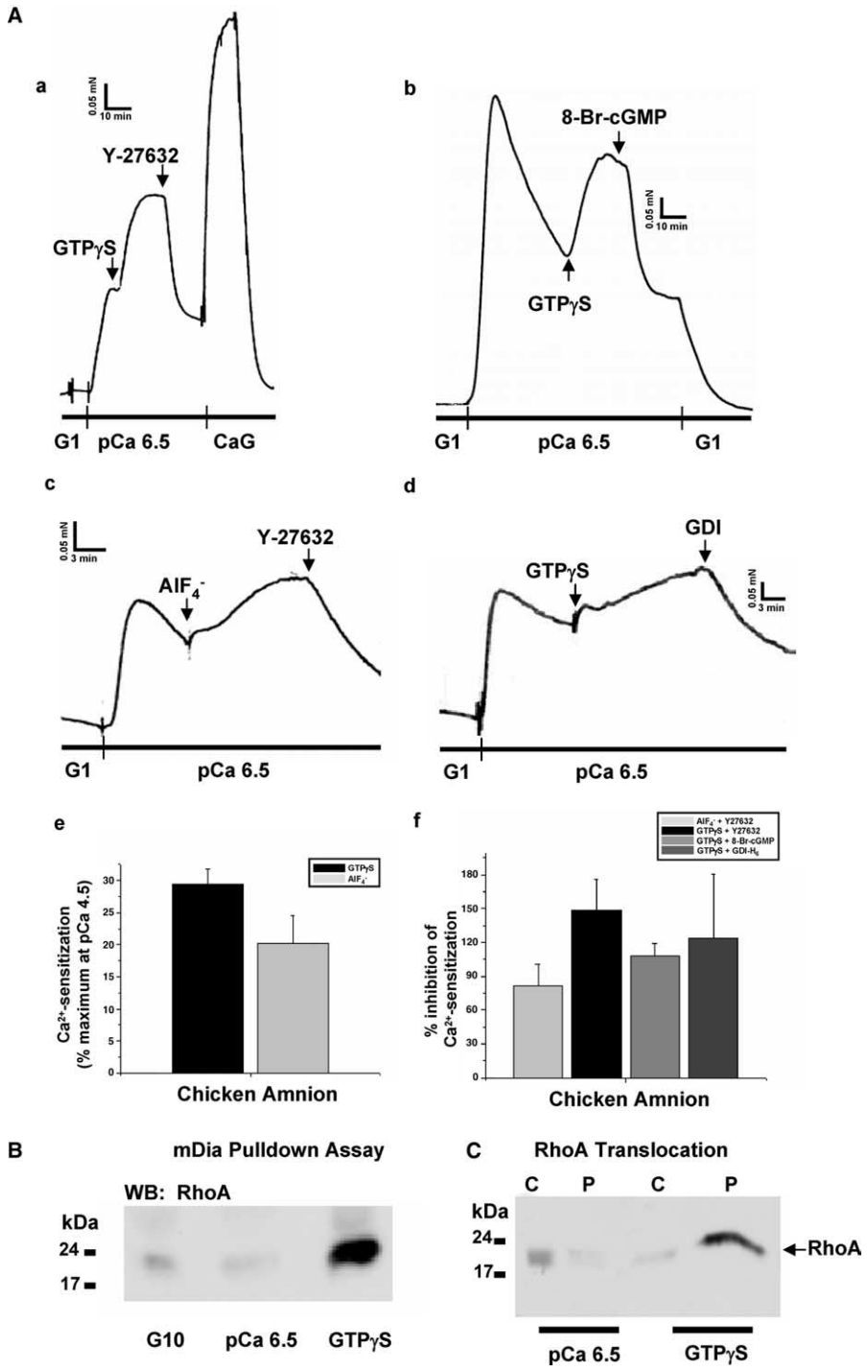

Fig. 1. Representative tension traces illustrating Ca^{2+}-sensitization in α-toxin permeabilized chicken amnion smooth muscle strips. (A) Ca^{2+}-sensitization can be induced by GTPγS (a) and AlF_4^- (c) and inhibited by ROK inhibitor, Y-27632 (10 μM) (a, c) or 8-bromo-cGMP (5 μM) (b). GTPγS-induced sensitization can also be reversed by GDI (d). Data summary (e and f). GTPγS induced increase in RhoA-GTP assayed using the Rho binding domain of mDia (B) and RhoA translocation from the cytosol, C, to the particulate, P, fraction (C).

and reversibly relaxed the GTPγS-induced sensitization (Fig. 1A, f). AlF_4^- caused a significant increase in Ca^{2+}-sensitization ($20.2 \pm 4.3\%$ of maximum at pCa 4.5; $n = 10$) (Fig. 1A, e) and was inhibited by Y-27632 ($81.5 \pm 19\%$ of AlF_4^--induced sensitization) (Fig. 1A, f). Thus, it can be concluded from these data that the signaling steps downstream of activated RhoA are intact in the amnion, but it is not clear whether coupling between heterotrimeric G protein activation and RhoA activation leading to Ca^{2+} sensitization is functional; as AlF_4^- may also activate RhoA directly [22] and GTPγS activates heterotrimeric and monomeric GTPases. GTPγS-induced contraction of ASM was attenuated by addition of 8-br-cGMP, a non-hydrolyzable analog of cGMP (Fig. 1A, b and f). We have shown previously that 8-bromo-cGMP can induce

Ca^{2+}-desensitization by stimulation of cyclic GMP-dependent kinase thus increasing MLCP activity, decreasing RLC_{20} phosphorylation and counteracting the inhibition of GTPγS stimulated phosphatase activity [23]. Thus, the mechanisms responsible for inhibition and activation of MLCP appear normal in ASM.

To further verify that GTPγS-induced Ca^{2+} sensitization in ASM, as in other smooth muscles, was via a pathway that involves RhoA and ROK, we investigated whether addition of the guanine exchange dissociation inhibitor, recombinant Rho · GDI, could reduce GTPγS-induced contraction in ASM. As expected from data in rabbit smooth muscles [24], Rho · GDI completely reversed the GTPγS-induced sensitization (Fig. 1A, d and f). GTPγS caused an increase in activated RhoA (GTP · RhoA) detected in a pulldown assay using the Rho-binding domain of mDia, a RhoA effector [25], as a GST fusion protein (Fig. 1B). We further determined whether activation of RhoA is also accompanied, as in other cells, by its translocation to the plasma membrane. GTPγS caused Ca^{2+}-independent translocation of RhoA from the cytosol to the particulate fraction in permeabilized ASM (Fig. 1C). These results indicate that the downstream signaling pathways of activated RhoA and ROK are functional in ASM. Thus, the deficiency of agonist-induced sensitization is most likely due to lack of upstream coupling, with the "uncoupling" occurring upstream of RhoA activation.

3.3. Expression of known players in the Ca^{2+} sensitization pathway

Western blot analysis of $G_{\alpha q/11}$ revealed no qualitative differences in its expression relative to total protein between homogenates of ASM and rabbit portal vein, which is known to express $G_{\alpha q/11}$ (Fig. 2A). $G_{\alpha 12}$ and $G_{\alpha 13}$ were also detectable in ASM preparations, with $G_{\alpha 13}$ showing higher expression (Fig. 2A). Expression of ROK was also verified by Western blot analysis in ASM, using a polyclonal antibody that was specific to the chicken sequence for ROKα, but that also cross-reacted with rabbit ileum (Fig. 2B). CPI-17 was not detectable in ASM but was present in the positive control, rabbit ileum, consistent with the lack of detection of CPI-17 mRNA in the chicken using RT-PCR [19]. It was also shown that the CPI-17 antibody reacts well with CPI-17 in another avian species, the pigeon, but shows no detectable levels of expression in chicken gizzard smooth muscle [19]. Analysis of the PKC isoforms showed expression of a conventional (Ca^{2+}-dependent) isoform of PKC, PKC β1, and two non-conventional (Ca^{2+}-independent) isoforms, PKC ζ and PKC δ (Fig. 2C). A possible mechanism that could account for the lack of response in ASM to agonist stimulation could be the lack of a functional GEF for RhoA [3]. Two known RhoGEFs, PDZ-RhoGEF and LARG, were detected in amnion lysates, as well as in a smooth muscle tissue screen, by immunoblotting (Fig. 2D). Thus, the presence of PDZ-RhoGEF and LARG plus the mDia assay demonstrating nucleotide exchange suggests that the GEF necessary for functional RhoA is present in amnion.

3.4. Regulation of myosin light chain phosphatase (MLCP) in chicken amnion

To elucidate the mechanism by which GTPγS-induced Ca^{2+}-sensitization occurred in ASM; we focused on the inhibition of myosin light chain phosphatase (MLCP). GTPγS caused a significant increase in MLC_{20} phosphorylation at constant [Ca^{2+}] in chicken amnion (data not shown) as in other cells including smooth muscle [2,3,5] or non-muscle myosin II [2], consistent with inhibition of MLCP activity leading to Ca^{2+}-sensitization. A regulatory subunit, MYPT-1, has been shown to contain a number of phosphorylation sites for ROK including T695, S849 and T850 (chicken sequence) [6,7,26] and (at least in vitro) by a variety of other kinases, yet the phosphorylation relevant sites are unclear. In tension-monitored amnion preparations, MYPT-1 phosphorylation at T695, S849 and T850 was determined. Surprisingly, there was no detectable

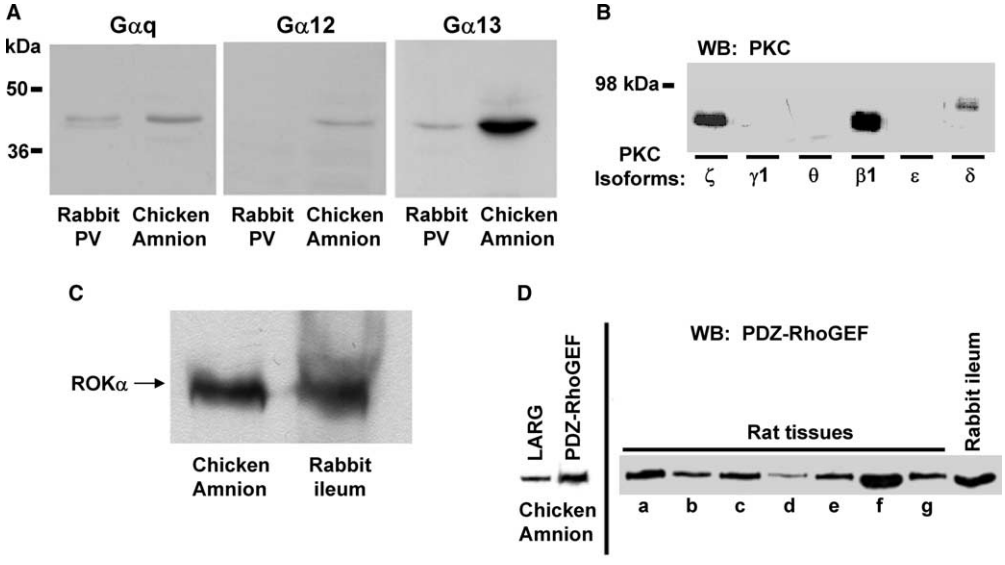

Fig. 2. Expression of proteins known to participate in Ca^{2+}-sensitization in smooth muscle. (A) Western blot for heterotrimeric G protein expression of 40 μg total protein per lane. (B) ROKα is expressed in chicken amnion to similar levels as in rabbit ileum. (C) PKC isoforms expressed in chicken amnion represent both Ca^{2+}-dependent (β_1) and Ca^{2+}-independent (ζ and δ) isoforms. (D) Left, Western blot showing the expression of LARG and PDZ-RhoGEF in chicken amnion; right, expression of PDZ-RhoGEF in various smooth muscle beds (a: portal vein; b: cerebral artery; c: thoracic aorta; d: abdominal aorta; e: bladder; f: ileum; g: femoral artery; and h: rabbit ileum).

change in the level of phosphorylation at T695 or S849 in the presence of GTPγS ± Y-27632, compared to pCa 6.5 alone (Fig. 3A), despite large differences in their relative amplitudes of tension (Fig. 1A). Moreover, there was a significant increase in T850 phosphorylation following stimulation with GTPγS (43.8 ± 19% of control; $n = 4$), which was inhibited by Y-27632 by 52.9 ± 11.6% ($n = 4$) of the GTPγS-induced increase in phosphorylation (Fig. 3A) and by 8-bromo-cGMP (10 μM (Fig. 3B) with a corresponding decrease by 108 ± 11.4% in the GTPγS-induced sensitization (Fig. 1A, b). CPI-17, the inhibitor of the catalytic subunit of the phosphatase, is not expressed in any chicken tissues, including gizzard and amnion (Fig. 3B). Therefore, it appears that phosphorylation of T850 in smooth muscle tissue is the relevant site for inhibition of MLCP, yet there are caveats to this conclusion. Recently, it has been shown that phosphorylation of T696 (human sequence) is inhibited by S695 phosphorylation via PKA/PKG [27]. Furthermore, it is not known whether the phospho-T696 antibody recognizes phospho-T696 in the presence of phospho-S695.Curiously, if chicken amnion smooth muscle cells are enzymatically isolated or explanted and placed into culture, there was a marked difference between intact and passaged cell cultures in Y-27632 mediated reduction of MYPT-1 phosphorylation at both T695 and T850. Y-27632 reduced T695 phosphorylation to 25.3 ± 1.3% of control values in cultured amnion cells, but was without effect in amnion tissue (93.2 ± 23.7% of control). T850 phosphorylation was reduced to 23.4 ± 4.1% ($n = 7$) of control in cultured cells) (Fig. 3D; $P < 0.002$). These data indicate that not only is ROK involved in the phosphorylation of MYPT-1 at both T695 and T850 in cultured cells, but also that culturing at early passages causes a fundamental change as compared to intact tissue where T850 is the significant ROK substrate and which could account for differences in relevant phosphorylation sites reported in the literature.

3.5. Recombinant CPI-17 restores agonist-induced sensitization through generation of GTP · RhoA in chicken amnion

Permeabilized ASM showed no contraction when challenged with a variety of G-protein coupled agonists or PDBu (Table 1). However, activation of $G_{12/13}$ family of GPCRs with the thromboxane A_2 analog, U46619, was rescued if recombinant CPI-17 (10 μM), in the physiological range found in other smooth muscle [28], was added to tissue strips of ASM (20.0 ± 4.4% of pCa 6.5 + CPI-17; $n = 12$) (Fig. 4A and B) that was sensitive to inhibition by 10 μM Y-27632 (106 ± 18.8% of the Ca^{2+} sensitization) (Fig. 4C). Recombinant CPI-17 alone induced a small component of Ca^{2+}-sensitized force as reported previously [29]. It is not clear whether the recombinant CPI-17 becomes phosphorylated by a basal kinase activity. The CPI-17-induced rescue of U46619-induced Ca^{2+}-sensitized force was accompanied by a 10-fold increase in GTP · RhoA (Fig. 4D) when compared with levels of GTP · RhoA in the presence of either U46619, CPI-17 alone or pCa 6.5, strongly suggesting that CPI-17 has some previously unrecognized activity upstream of RhoA leading to its activation. CPI-17-rescued U46619-sensitized force may reflect inhibition of MYPT-1 by the classical CPI-17 pathway (i.e., through phospho-CPI-17 binding to PP1C),

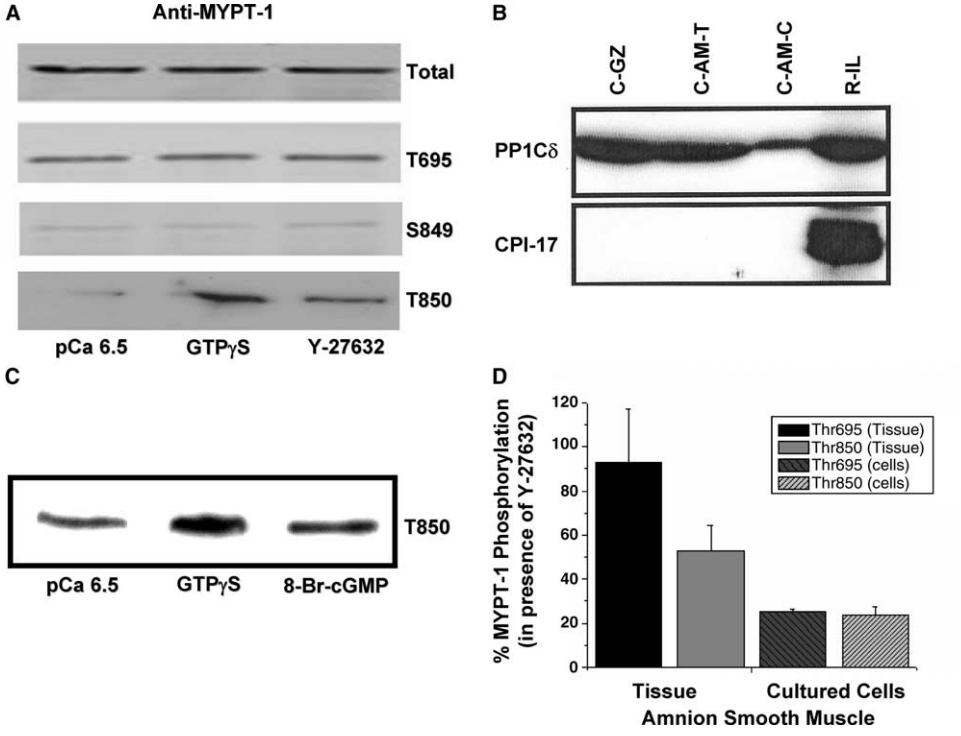

Fig. 3. MLCP regulation in chicken amnion. (A) MYPT-1 phosphorylation at putative ROK sites following GTPγS-induced Ca^{2+}-sensitization and subsequent treatment with Y-27632 (10 μM) in α-toxin permeabilized amnion. MYPT-1 phosphorylation is unchanged at two sites (T695 and S849), but increased at T850 with subsequent inhibition by Y-27632 and 8-Bromo-cGMP (B). Total MYPT-1 was the same in each lane. (C) Western blot analysis showing no detectable level of CPI-17 expression in chicken gizzard (C-GZ), chicken amnion tissue (C-AM-T) or cultured amnion cells (C-AM-C) as compared to expression levels in rabbit ileum (R-IL). 20 μg of total protein were loaded per lane. (D) Summary data of MYPT-1 phosphorylation in tissue versus cultured cells.

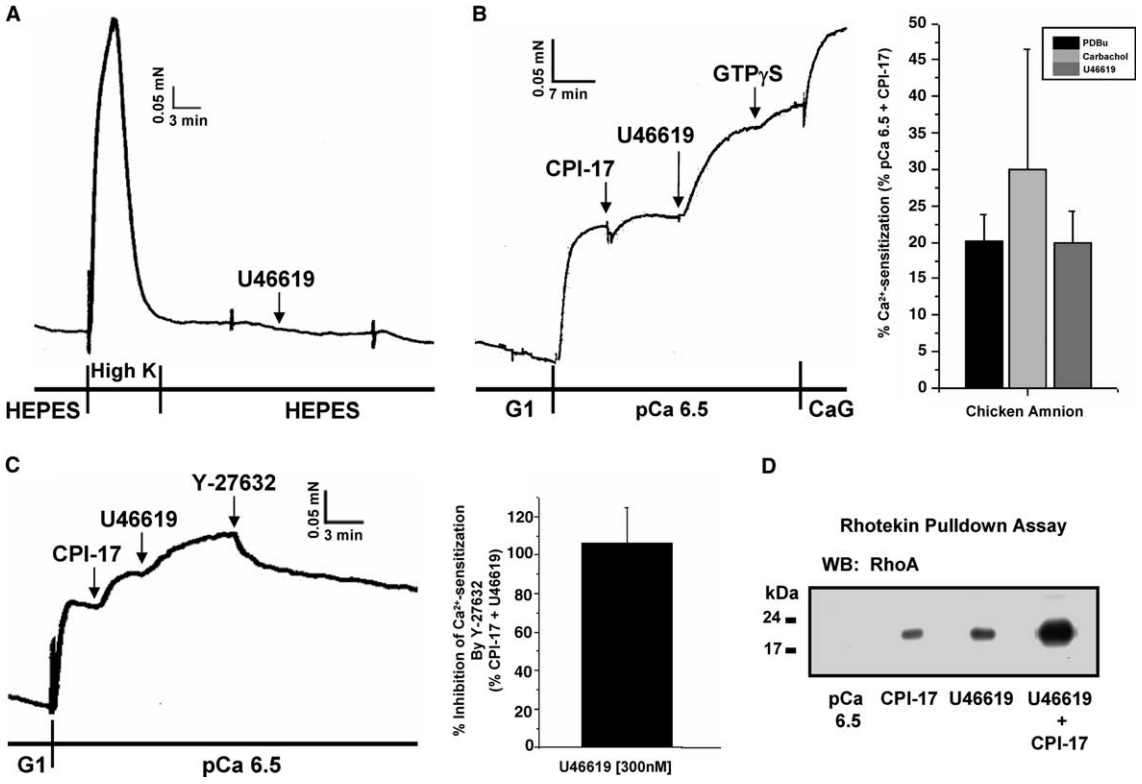

Fig. 4. CPI-17 restores agonist-induced contraction in β-escin permeabilized chicken amnion. (A) U46619, a thromboxane A_2 analog, causes no contraction in intact amnion smooth muscle. (B) Upon addition of CPI-17 (10 μM), U46619 now caused an increase in Ca^{2+}-sensitization comparable to that of GTPγS and AlF_4^- (Fig. 1). (C) U46619-induced Ca^{2+}-sensitization following treatment with CPI-17 could be inhibited by the ROK inhibitor, Y-27632, bar graph (right). (D) U46619 in the presence of CPI-17 causes an increase in GTP · RhoA compared to U46619 or CPI-17 alone.

this would not result in an increase in GTP · RhoA but could contribute to the magnitude of the rescued sensitized force. U46619 induces large contractions with little change in $[Ca^{2+}]$ in mammalian smooth muscle and is thus a potent Ca^{2+} sensitizer, largely through activation of the RhoA pathway [30]. Ca^{2+}-sensitization with the muscarinic receptor agonist carbachol was also rescued by addition of CPI-17 with an amplitude of $29.9 \pm 16.7\%$ of pCa 6.5 + CPI-17 contraction; $n = 5$ (Fig. 4B, bar graph). Stimulation with PDBu (1 μM) also now caused an increase in contraction and sensitized the tissue to Ca^{2+} ($20.2 \pm 3.7\%$ of pCa 6.5 + CPI-17 contraction; $n = 6$) (Fig. 4B, bar graph), as described previously [29]. Separate upstream and convergent downstream pathways of G-protein- and phorbol ester-mediated Ca^{2+}-sensitization have been demonstrated in mammalian smooth muscle where downregulation of PKCs ablated phorbol ester-induced Ca^{2+}-sensitization, while retaining normal agonist and GTPγS responses and vice versa [31,32]. Evidence for separate upstream pathways also arises from our previous demonstration that RhoA · GDI, which complexes with RhoA, relaxes agonist and GTPγS-induced Ca^{2+}-sensitization, but does not relax force induced by stimulation of PKCs with phorbol ester [24]. Moreover, GTPγS, which activates the RhoA/ROK pathway, resulted in a significant Ca^{2+}-sensitization response in the CPI-17 deficient ASM again pointing to a role for CPI-17 lying upstream of GTP · RhoA. Therefore, CPI-17 is not critical for GTPγS-induced Ca^{2+}-sensitization of ASM. However, the ability of CPI-17 to rescue the carbachol and U46619-induced Ca^{2+}-sensitization as well as the significant increase in GTP · RhoA suggests that CPI-17 activity is necessary for agonist-induced signaling. These data strongly support the hypothesis that CPI-17 can rescue the signaling events upstream of GTP exchange on GDP · RhoA, possibly through the actions of another PP1 family member targeted by CPI-17 and acting, for example, on RhoGEFs, RhoGTPase activating proteins (RhoGAPs) or by regulating the phosphorylation state of GPCRs. This new role for CPI-17 remains to be explored and its targets identified.

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