

CPI-17-deficient smooth muscle of chicken

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Ca²⁺ sensitivity of arterial contractility is governed by regulating myosin phosphatase activity in response to agonist stimuli. CPI-17, a myosin phosphatase inhibitor phosphoprotein, is phosphorylated concomitantly with agonist-induced contractile Ca²⁺ sensitization in mammalian artery. CPI-17 has not been detected in chicken artery, but is readily detectable in pigeon artery. To evaluate a role of CPI-17, we compared contractility of the arteries of 'CPI-17-deficient' chicken with those of CPI-17-rich rabbit and pigeon, and studied the effect of CPI-17-reconstitution in chicken artery. Other major regulatory/contractile proteins for Ca²⁺ sensitization are expressed in both chicken and rabbit arteries. Agonists, such as an α_1 -agonist and endothelin-1, produced significant contraction in arteries of all species under physiological Ca²⁺-containing conditions. Depletion of Ca²⁺ abolished these contractions in chicken but partially inhibited them in rabbit and pigeon arteries. Unlike CPI-17-rich tissues, chicken arteries exerted little Ca²⁺ sensitization in response to α_1 -agonist or endothelin-1. GTP γ S produced a slight Ca²⁺ sensitizing effect in chicken artery, but this was significantly smaller compared with CPI-17-rich tissues. A PKC activator (PDBu) did not generate but rather reduced a contraction in both intact and α -toxin-permeabilized chicken artery in contrast to a large contraction in CPI-17-rich arteries. Myosin light chain phosphorylation was reduced by PDBu in chicken but elevated in rabbit artery. Addition of recombinant CPI-17 into β -escin-permeabilized chicken artery restored PDBu-induced and enhanced GTP γ S-induced Ca²⁺ sensitization. Thus, CPI-17 is essential for G protein/PKC-mediated Ca²⁺ sensitization in smooth muscle.

(Resubmitted 15 March 2004; accepted after revision 16 April 2004; first published online 16 April 2004)

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The primary regulatory mechanism for smooth muscle contraction is the reversible phosphorylation of the regulatory myosin light chain (MLC₂₀) at Ser¹⁹ by MLC₂₀ kinase (MLCK) and MLC₂₀ phosphatase (MLCP) (Hartshorne, 1987; Kamm & Stull, 1989). MLCK activity is Ca²⁺/calmodulin dependent, while MLCP functions independently of Ca²⁺ and is regulated by G protein-coupled signalling pathways (Somlyo & Somlyo, 2003). G protein activation leads to inhibition of MLCP and thus an increase in both MLC₂₀ phosphorylation and contraction without change in Ca²⁺ (Kitazawa *et al.* 1991*b*; Kubota *et al.* 1992). This mode of regulation is termed Ca²⁺ sensitization and is an essential process for agonist-induced contraction of smooth muscle as well as for cytoskeletal reorganization and movement of non-muscle cells (Somlyo & Somlyo, 2003). At least two signalling pathways have been proposed for the inhibition of MLCP. First, MLCP activity can be inhibited through phosphorylation of the MLCP regulatory sub-

unit, MYPT1, and this mechanism is thought to involve RhoA/Rho-kinase-dependent pathways (Kimura *et al.* 1996; Fukata *et al.* 2001; Somlyo & Somlyo, 2003). Although Thr⁶⁹⁵ of MYPT1 is likely to be a key residue for MLCP inhibition (Hartshorne *et al.* 1998; Feng *et al.* 1999), the exact signalling mechanisms surrounding this target are still controversial in smooth muscle tissues (Kitazawa *et al.* 2003; Niuro *et al.* 2003).

The second mechanism of MLCP inhibition is through phosphorylation of the smooth muscle-specific MLCP inhibitor protein, CPI-17 (PKC potentiated inhibitor protein-17 kDa; Eto *et al.* 1997; Kitazawa *et al.* 2000). When phosphorylated at Thr³⁸ CPI-17 inhibits MLCP activity by 1000-fold (Eto *et al.* 1997; Senba *et al.* 1999). Selective depletion of CPI-17 by skinning of smooth muscle cells eliminates PKC-induced Ca²⁺ sensitization of artery, and the response can be reconstituted by addition of PKC and CPI-17 together but not by PKC alone (Kitazawa *et al.* 1999). Stimulation of arterial smooth muscle with

not only PKC activators but also several agonists and the non-hydrolysable GTP analogue, GTP γ S, induces phosphorylation of CPI-17 at Thr³⁸ paralleling the contractile Ca²⁺ sensitization (Kitazawa *et al.* 2000, 2003; Niiro *et al.* 2003). This phosphorylation is suppressed by both GF-109203X (a PKC inhibitor) and Y-27632 (a Rho-kinase inhibitor). The expression level of CPI-17 among various types of smooth muscles correlates with the degree of PKC-mediated Ca²⁺ sensitization (Woodsome *et al.* 2001). In tonic artery, which expresses high CPI-17 and low MLCP, CPI-17 is thought to be a dominant player in MLCP inhibition and Ca²⁺ sensitization. These observations strongly suggest that CPI-17 mediates PKC-dependent Ca²⁺ sensitization in vascular smooth muscles.

To further elucidate the function of CPI-17, a crucial experiment would be to remove or down-regulate CPI-17 expression and study its effects on smooth muscle contractility and Ca²⁺ sensitization. Here, we report that CPI-17 is not detected in chicken smooth muscle tissues, such as artery, gizzard and small intestine. We investigated how PKC activation and G protein-coupled receptor activation affect contraction and MLC phosphorylation in the 'CPI-17-deficient' artery. Furthermore, we attempted the reconstitution of contractile Ca²⁺ sensitization in chicken artery with recombinant CPI-17. Our results strongly support the hypothesis that CPI-17 plays a key role in receptor-mediated Ca²⁺ sensitization in vascular smooth muscles. A part of these findings has been presented at the annual Biophysical Society Meeting (Li & Kitazawa, 1998).

Methods

Tissue preparation and force measurement

All animal procedures were approved by the Animal Care and Use Committee of Boston Biomedical Research Institute. Adult male New Zealand White rabbits, chickens (Charles River Laboratories, Wilmington, MA, USA) and homing pigeons (Double T Farm, Glenwood, IA, USA) were killed by halothane overdose. Smooth muscle strips (600 μ m in width, 2–2.5 mm in length and natural wall thickness) were dissected from mesenteric arteries and mounted on a force transducer (AM801, SensoNor, Horten, Norway) set-up as previously described (Masuo *et al.* 1994). Force levels were monitored throughout the experiments.

Solutions

The compositions of external solutions for intact smooth muscle strips have been described previously (Woodsome *et al.* 2001). The standard relaxing solution used for

resting states of the α -toxin- and β -escin-permeabilized strips contained the following (Masuo *et al.* 1994): 74.1 mM potassium methanesulphonate, 2 mM Mg²⁺, 4.5 mM MgATP, 1 mM EGTA, 10 mM creatine phosphate, 30 mM piperazine-*N,N'*-bis(2-ethanesulphonic acid), 1 mM dithiothreitol, pH 7.1 adjusted with KOH at 20°C. In the activating solution, 10 mM EGTA was used and a calculated amount of calcium methanesulphonate was added to give the final desired concentration of free Ca²⁺ ions. Ionic strength of 0.2 M was achieved by appropriately using more or less potassium methanesulphonate. For Triton X-100-skinned smooth muscle, the solution was modified to prevent the deterioration of contraction. The standard relaxing solution for the skinned smooth muscle had 0.13 M ionic strength, and contained 1 mM Mg²⁺, 3.5 mM MgATP, 0.5 μ M calmodulin, 0.1% fatty acid-free BSA, 1 mM EGTA, 10 mM creatine phosphate, 30 mM piperazine-*N,N'*-bis(2-ethanesulphonic acid) and 1 mM dithiothreitol, pH 7.1 (adjusted with KOH). The experiments using intact smooth muscle were carried out at 30°C. All experiments for permeabilized smooth muscles were carried out at 20°C.

Cell permeabilization

After measuring the force of contractions induced by high K⁺ (154 mM) and by phenylephrine (30 μ M) in freshly dissected strips, these strips were incubated in the standard relaxing solution for several minutes. For membrane permeabilization with α -toxin, the strips were treated for 30 min at 30°C with 20 μ g ml⁻¹ of purified *Staphylococcus aureus* α -toxin (List, Campbell, CA, USA) at pCa 6.7 buffered with 10 mM EGTA and then treated with 10 μ M of the Ca²⁺ ionophore A23187 for 20 min at 25°C to deplete the SR of Ca²⁺ and maintain the cytoplasmic Ca²⁺ constant (Masuo *et al.* 1994). For membrane permeabilization with β -escin (Sigma), the strips were treated with 60 μ M β -escin in the relaxing solution for 45 min at 5°C and then for 20 min at 30°C together with 10 μ M A23187 (Masuo *et al.* 1994). To make skinned preparations, we used 0.1% Triton X-100 (Sigma) in the relaxing solution for 30 min at 5°C and for 15 min at 30°C (Kitazawa *et al.* 1999). Experiments in intact smooth muscle preparations were carried out at 30°C. For all permeabilized preparations, experiments were carried out at 20°C to minimize the deterioration of contractility.

Measurement of MLC phosphorylation

Details of MLC phosphorylation measurements using two-dimensional gel electrophoresis have been previously described (Kitazawa *et al.* 1991a).

Expression and purification of recombinant CPI-17

Recombinant hexahistidine-tagged CPI-17 was expressed in *E. coli* and purified to homogeneity as previously described (Eto *et al.* 1997).

Antibodies

We used three different phosphorylation-independent and one phospho[Thr³⁸]-dependent anti-CPI-17 antibodies. Chicken anti-CPI-17 IgY and rabbit anti-CPI-17 IgG were against hexahistidine-tagged porcine CPI-17 and affinity-purified (Senba *et al.* 1999; Kitazawa *et al.* 2000). Rabbit anti-CPI-17 peptide antibody was made against the residues 35–46 (100% identity between human and mouse) and affinity-purified. The rabbit anti-phospho[Thr³⁸]-CPI-17 (anti-*p*[Thr³⁸]) has been previously described (Kitazawa *et al.* 2000). These antibodies cross-react with pig, rabbit, rat, mouse and pigeon CPI-17 (Eto *et al.* 1999; Woodsome *et al.* 2001). The rabbit anti-phospho[Thr⁶⁹⁵]-MYPT1 (anti-*p*[Thr⁶⁹⁵]) has been previously described (Kitazawa *et al.* 2003). Rabbit polyclonal anti-PP1C δ and PHI-1 antibodies were prepared and affinity-purified (Eto *et al.* 1999). Polyclonal anti-*h*-CaD and anti-*h*-CaP antibodies were provided by Dr K. Mabuchi (Boston Biomedical Research Institute). Monoclonal anti-MLC₂₀, and polyclonal anti-actin, PKC α and PKC ϵ were from Sigma. Monoclonal anti-RhoA, and polyclonal anti-G₁₃ α , anti-ERK1 and anti-p38MAPK were from SantaCruz Biotech (Santa Cruz, CA, USA). Monoclonal anti-Rho-kinase antibody was from Transduction Laboratories (Lexington, KY, USA), polyclonal anti-MYPT1 antibody from Babco (Richmond, CA, USA), and polyclonal anti-Raf1 antibody from Calbiochem. The dilutions of antibody used for Western blots were as follows: chicken anti-CPI-17 IgY antibody ($\times 10\,000$), rabbit anti-CPI-17 ($\times 2000$), anti-phospho[Thr³⁸]-CPI-17 ($\times 2000$), anti-peptide(35–46)-CPI-17 ($\times 500$), anti-PP1C δ ($\times 10\,000$), anti-PHI-1 ($\times 2500$), anti-*h*-CaD ($\times 10\,000$), anti-*h*-CaP ($\times 10\,000$), anti-MLC₂₀ ($\times 2000$), anti-actin ($\times 5000$), anti-PKC α ($\times 10\,000$), anti-PKC ϵ ($\times 7500$), anti-RhoA ($\times 500$), and anti-G₁₃ α ($\times 250$), anti-ERK1 ($\times 1000$) and anti-p38MAPK ($\times 1000$), anti-Rho-kinase ($\times 1000$), and anti-MYPT1 ($\times 5000$).

Western blotting

Immuno-blotting experiments were performed as previously described in detail (Woodsome *et al.* 2001).

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissues using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Purified RNA was eluted in 30 μ l RNase-free dH₂O, and the typical yield of RNA from 15 mg of wet smooth muscle tissue was ~ 25 μ g.

The reverse transcription and the subsequent PCR steps were carried out in a single-tube system using OneStep RT-PCR Kit (QIAGEN) according to the manufacturer's instructions. The primers were designed against the murine CPI-17 sequence as follows. For sense: 5'-GTC ACC GTC AAG TAC GAC CG-3'; and for anti-sense: 5'-GGT CCT GGC GGG GGC TCA GGC TG-3' (Integrated DNA Technologies, Inc., IA, USA). Each reaction consisted of 50 μ l total volume, containing 0.5–1 μ g RNA, 0.6 μ M each of sense and anti-sense primers, 8 U RNase inhibitor (Roche, Germany), 400 μ M dNTP mixture, and the reverse transcriptase–Taq polymerase mixture. The reverse transcription reaction was carried out at 50°C for 30 min. The reaction mixture was directly subjected to the PCR step, which was run for 35 cycles of melting at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 45 s, then further extended at 72°C for 10 min; 10 μ l of the PCR product was analysed on a 1.3% agarose gel. DNA fragments were detected by ethidium bromide staining and the sequence was confirmed on both strands (Tufts Core Facility, Boston, MA, USA).

Statistics

Results are expressed as the means \pm s.e.m. of *n* experiments. Statistical significance was evaluated with Student's two-tailed *t* test ($P < 0.05$).

Results

Expressions of CPI-17 and other proteins in chicken smooth muscle tissues

Three polyclonal anti-CPI-17 antibodies were used to detect CPI-17 in the SDS-extracts from arteries of rabbit (as a representative mammalian species known to express CPI-17 in the smooth muscle tissues; Woodsome *et al.* 2001), chicken and pigeon (as a control avian species). All immunoblots using these antibodies consistently showed no detectable expression of CPI-17 in chicken aorta (Fig. 1A) while high expression was found in rabbit and pigeon aortas. Moreover, RT-PCR was carried out from total RNA isolated from the aortas of all three species with mouse brain as positive control. Using a

primer pair designed against regions of high homology among all known sequences of mammalian species (see Methods), a fragment of approximately 300 bp was generated from rabbit and pigeon aortas, but not from chicken aorta (Fig. 1B). In addition, CPI-17 protein was not detectable in chicken mesenteric artery (MA), gizzard (Gz) and small intestine (SI) from adult (Fig. 1C) and embryo (17-day) (not shown). PHI-1 (a member of the CPI-17 family; Eto *et al.* 1999), and the δ -isoform of type 1 protein phosphatase (a catalytic subunit of MLCP; Hartshorne *et al.* 1998) were expressed in chicken as well as rabbit smooth muscle tissues (Fig. 1C). Expressions of other signalling and contractile proteins such as actin, *h*-caldesmon (*h*-CaD), *h*-calponin (*h*-CaP), MLC₂₀,

G₁₃ α , RhoA, Rho-kinase (ROK α), MYPT1, PKC α and ϵ , p38MAPK, ERK1, and Raf-1 were more or less equivalent in rabbit and chicken tissues (Fig. 1C).

Agonist- and GTP γ S-induced contraction and Ca²⁺ sensitization

We used two types of excitatory agonists: the first class (α_1 -the agonist phenylephrine (PE), endothelin-1 (ET) and histamine) is linked to G protein-coupled Ca²⁺ sensitization (Somlyo & Somlyo, 2003), and the second (ATP) is coupled to ligand-gated non-selective cation channels (Abbracchio & Burnstock, 1998), and thus used as a negative control. Most of agonists except histamine

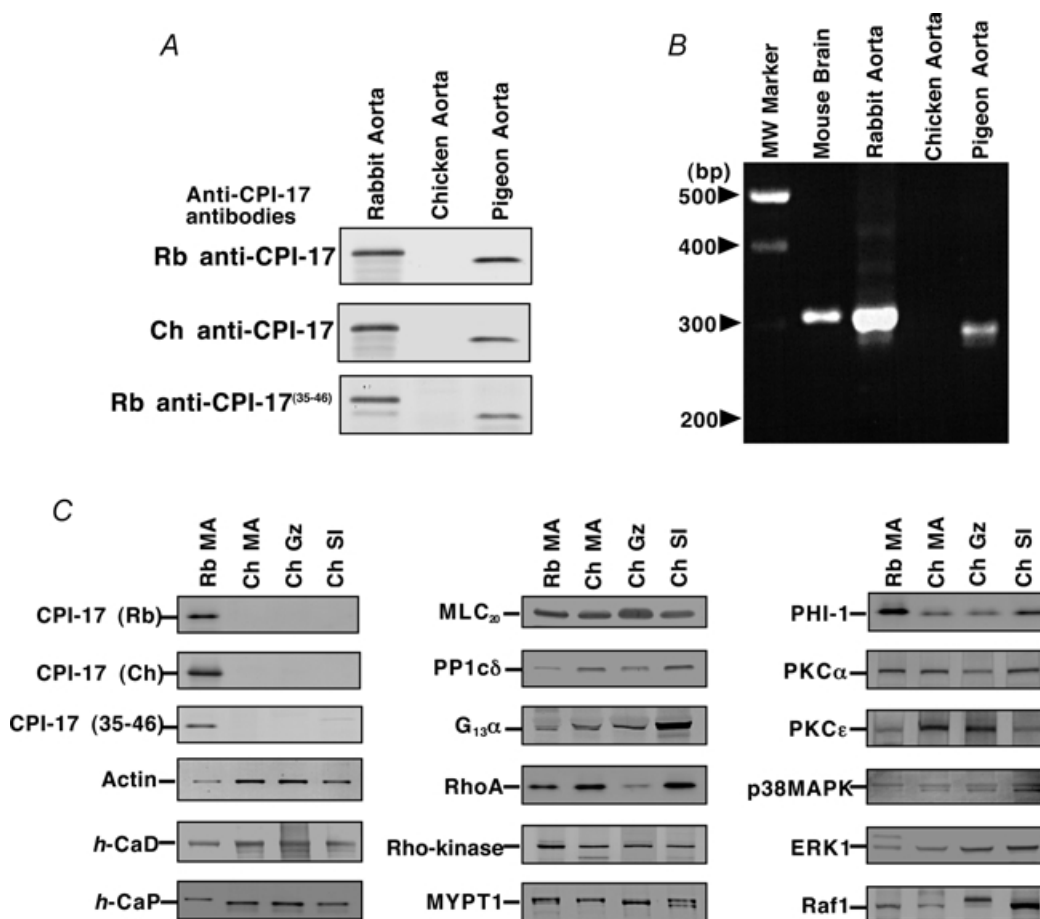


Figure 1. Expression of CPI-17 and other proteins in rabbit, pigeon and chicken smooth muscle tissues
A, CPI-17 protein expression in rabbit, chicken and pigeon aortas. Top, a representative immunoblot probed using rabbit (Rb) anti-CPI-17 IgG antibody; middle, using chicken (Ch) anti-CPI-17 IgY; and bottom, using rabbit anti-CPI-17-peptide⁽³⁵⁻⁴⁶⁾ IgG. Equal amounts of total protein (20 μ g per lane) were added. The experiments were repeated three times using different animals of each species. **B**, CPI-17 mRNA expression. RT-PCR was performed from aortas of rabbit, chicken and pigeon. The mouse brain RNA was used as control. **C**, expression of other contractile and signalling proteins in Rb and Ch mesenteric artery (MA), chicken gizzard (Gz) and small intestine (SI). Per lane, 0.5 μ g of total protein for actin and CaP, 2 μ g for CaD and MLC₂₀, and 20 μ g for all others were applied. The experiments were repeated 4–6 times.

evoked a large contraction to a level similar to that of high K^+ (membrane depolarization) under the normal Ca^{2+} -containing conditions in rabbit, pigeon and chicken mesenteric arteries (Fig. 2). It is known that, in rabbit artery, most of agonists can produce a large contraction even in the absence of Ca^{2+} influx (Himpens *et al.* 1990). Figure 2D and E summarizes the agonist-induced contractions and effect of a dihydropyridine Ca^{2+} -channel blocker, nifedipine, in rabbit and chicken arteries, respectively. Nifedipine ($0.2 \mu M$) markedly diminished the development of high K^+ -induced contraction in both rabbit and chicken arteries. The blocker also markedly reduced PE- and ET-1-induced contractions in chicken artery (Fig. 2E), suggesting a large dependence on Ca^{2+} influx via dihydropyridine-sensitive Ca^{2+} channel. In rabbit artery, in contrast, agonist-induced contractions were only partially inhibited (Fig. 2D). ATP produced significant contraction in the presence of the blocker in both rabbit and chicken arteries. Removal of extracellular Ca^{2+} and addition of 2 mM EGTA under depolarized conditions almost abolished agonist-induced contractions in intact chicken artery (Fig. 3B). On the average, PE and ET-1 in the absence of Ca^{2+} produced only $3 \pm 1\%$ and $9 \pm 2\%$ ($n = 5$), respectively, of control high K^+ -induced contraction. In the CPI-17-rich arteries, however, even after removal of extracellular Ca^{2+} and further treatment with ryanodine to deplete the SR of Ca^{2+} , significant contractions were induced by agonists without Ca^{2+} influx and Ca^{2+} release. In rabbit artery (Fig. 3A), PE and ET-1 evoked $21 \pm 3\%$ and $60 \pm 7\%$ of high K^+ -induced contraction ($n = 5$), respectively, and in pigeon artery (Fig. 3C), $75 \pm 6\%$ ($n = 6$) and $52 \pm 6\%$ ($n = 3$), respectively. These indicate significant contractile Ca^{2+} sensitization in rabbit and pigeon arteries. ATP-induced contraction was almost totally abolished under the Ca^{2+} -free conditions in arteries of all species as predicted (not shown). These results together suggest little or no Ca^{2+} sensitization by agonists in intact chicken artery.

Figure 4 displays representative force traces showing effects of excitatory agonists and G protein activator on the contractile response of α -toxin-permeabilized and A23187-treated rabbit, chicken and pigeon vascular smooth muscles, in which intracellular Ca^{2+} stores were depleted and cytoplasmic Ca^{2+} concentration was maintained with 10 mM EGTA (Kitazawa *et al.* 1991a). In rabbit mesenteric artery at constant pCa 6.7, a single dose of a saturated concentration of PE and ET-1 produced significant contractions from 8 ± 1 to 52 ± 3 and $44 \pm 6\%$ ($n = 3$) of the maximum induced at pCa 4.5, respectively. In the pigeon artery at pCa 6.9, PE and ET-1 similarly potentiated from 19 ± 3 to 54 ± 7 ($n = 6$) and $56 \pm 8\%$

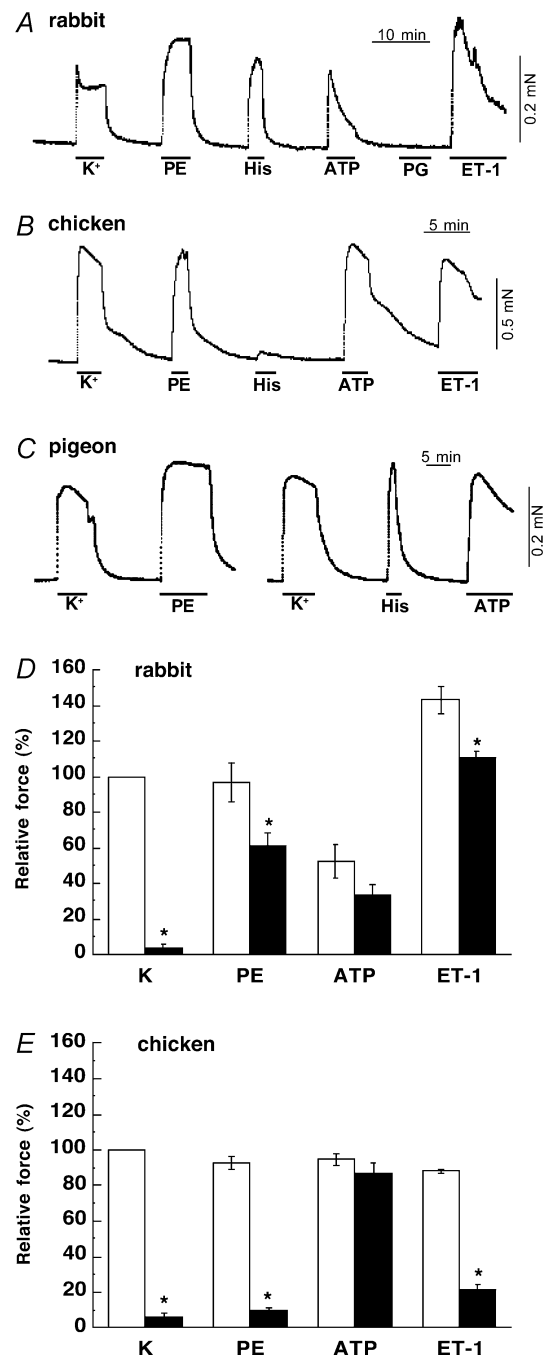


Figure 2. Effect of agonists in intact mesenteric arteries

Contractions induced by high K^+ and agonists in intact rabbit (A), chicken (B) and pigeon arteries (C) under the normal Ca^{2+} (2 mM)-containing conditions ($n = 6$). Before the experiments were started, strips were repeatedly stimulated with a cycle of high K^+ for 3 min and rest for 10 min until the steady state contraction was obtained. PE, 30 μM phenylephrine; His, 30 μM histamine; ATP, 200 μM ; PG, 1 μM prostaglandin F $_{2\alpha}$; ET-1, 1 μM endothelin-1. D and E show a summary of high K^+ - and agonist-induced contractions under the normal conditions (open column) and in the presence of 0.2 μM nifedipine (filled column) in rabbit and chicken MA, respectively ($n = 4$). *Significant difference from respective control contraction in the absence of nifedipine.

($n = 4$), respectively. These potentiated contractions by different agonists are additive (Kitazawa *et al.* 1991) and the mixture of the agonists produced a larger contraction than a single agonist alone, but the amplitude was limited by the maximum force capacity (Fig. 4A and C). The non-selective G protein activator, GTP γ S, markedly enhanced a submaximal contraction from 13 ± 3 to $77 \pm 3\%$ ($n = 5$) in rabbit artery (Fig. 4A) and from 16 ± 3 to $78 \pm 3\%$ ($n = 5$) for pigeon (Fig. 4C), indicating very significant G protein-mediated Ca $^{2+}$ sensitization. In chicken artery, however, PE did not produce any significant contraction at constant Ca $^{2+}$ ($n = 7$; Fig. 4B). ET-1 and GTP γ S did significantly enhance a submaximal contraction at pCa 6.8 from 27 ± 3 to $44 \pm 2\%$ ($n = 3$) and from 32 ± 2 to $58 \pm 2\%$ ($n = 9$), respectively (Fig. 4B). This enhancement

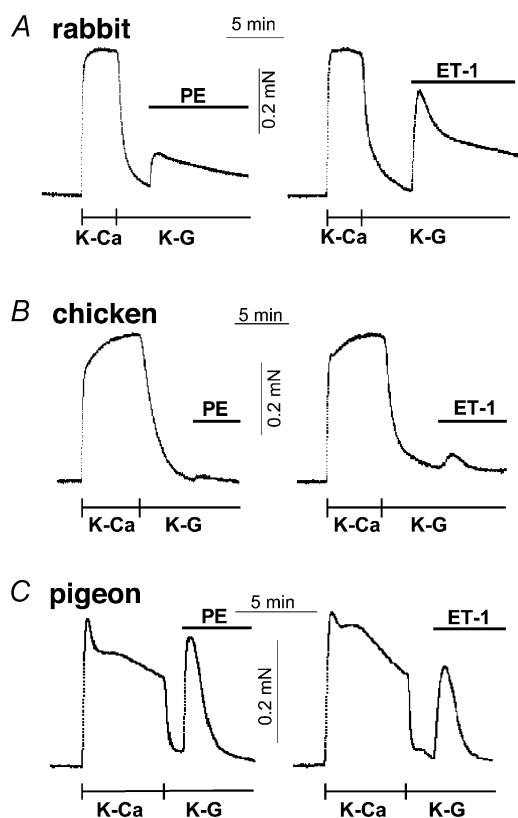


Figure 3. Agonist-induced contractions under the Ca $^{2+}$ -free, depolarized conditions in intact rabbit (A), chicken (B) and pigeon arteries (C)

High K $^{+}$ -induced contraction was abolished with removal of Ca $^{2+}$ and addition of 2 mM EGTA. Then, 30 μ M PE or 1 μ M ET-1 was added. K-Ca, high-K $^{+}$ solution containing 2 mM Ca $^{2+}$. K-G, Ca $^{2+}$ -free, high-K $^{+}$ solution containing 2 mM EGTA. Rabbit and pigeon arteries were pre-treated with 1 μ M ryanodine in the K-G solution containing 30 mM caffeine for 30 min to deplete the SR of Ca $^{2+}$ (Himpens *et al.* 1990). ($n = 3-6$.)

was significantly smaller as compared to those of rabbit and pigeon artery.

The Rho-kinase inhibitor, 10 μ M Y-27632, more effectively inhibited basal and GTP γ S-induced contractions in α -toxin-permeabilized chicken artery (Fig. 4B) than those of rabbit (Uehata *et al.* 1997; Kitazawa *et al.* 2000). In chicken artery, the pCa 6.8-induced contraction was insensitive to 3 μ M GF-109203X (Kitazawa *et al.* 2000), a PKC inhibitor ($n = 3$), but markedly decreased by Y-27632 from 51 ± 4 to $1 \pm 1\%$ of maximum ($n = 6$). The basal Ca $^{2+}$ sensitivity was significantly decreased but maximal contraction at pCa 4.5 was not altered ($n = 3$). In rabbit artery, the basal contraction at pCa 6.5 was significantly reduced from 14 ± 2 to $7 \pm 3\%$ ($n = 3$). In the presence of Y-27632, GTP γ S only minimally increased the reduced contraction to $6 \pm 4\%$ in chicken artery ($n = 6$; Fig. 4B) while still significantly increased to $46 \pm 5\%$ in rabbit artery ($n = 3$). In contrast, a G protein inhibitor, GDP β S (0.3 mM), did not significantly inhibit the basal Ca $^{2+}$ -activated contraction in chicken artery (54 ± 9 versus $53 \pm 7\%$ at pCa 6.7; $n = 3$).

PKC-induced contraction and Ca $^{2+}$ sensitization

In rabbit and pigeon mesenteric arteries, the PKC activator, phorbol 12,13-dibutyrate (PDBu, 1 μ M), generated a contraction at rest to a significant level ($60 \pm 10\%$ of high K $^{+}$ -induced contraction in rabbit and $85 \pm 9\%$ for pigeon; $n = 6$) (Fig. 5A and C). In the chicken mesenteric artery, in contrast, even 10 μ M PDBu evoked no measurable contraction at rest ($n = 22$) and, rather, inhibited high-K $^{+}$ -induced contraction (Fig. 5B). In α -toxin-permeabilized, A23187-treated rabbit and pigeon arteries, PDBu but not an inactive isomer, 4 α -PDBu (Masuo *et al.* 1994), significantly enhanced a contraction (from $4 \pm 1\%$ at pCa 6.7 to $64 \pm 6\%$ of maximum contraction obtained at pCa 4.5 for rabbit and from $12 \pm 3\%$ at pCa 6.9 to $78 \pm 4\%$ for pigeon; $n = 8$) (Fig. 5D and F) and increased the contractile Ca $^{2+}$ sensitivity (Fig. 5G and I). In the permeabilized chicken artery, in contrast, PDBu failed to increase and rather decreased a contraction (from $44 \pm 2\%$ at pCa 6.7 to $11 \pm 2\%$ of maximum; $n = 13$) (Fig. 5E) similar to that in intact preparations and reduced the Ca $^{2+}$ sensitivity (Fig. 5H) whereas 4 α -PDBu had no effect. A synthetic diacylglycerol (DAG), 1,2-*sn*-dihexanoylglycerol (Masuo *et al.* 1994), at 10 μ M also enhanced a submaximal contraction in rabbit (from $11 \pm 4\%$ at pCa 6.7 to $68 \pm 4\%$; $n = 4$) and pigeon arteries (from $18 \pm 1\%$ at pCa 6.9 to $71 \pm 6\%$; $n = 4$) but reduced a submaximal contraction in

chicken artery (from $39 \pm 5\%$ at pCa 6.7 to $6 \pm 1\%$; $n = 3$) similar to the effect of phorbol ester. PDBu significantly increased MLC₂₀ phosphorylation from 23 ± 3 to $49 \pm 4\%$ of total MLC₂₀ ($n = 5$) at pCa 6.7 in rabbit artery, while PDBu significantly decreased MLC₂₀ phosphorylation from 48 ± 2 to $35 \pm 2\%$ ($n = 4$) at pCa 6.7 in chicken artery, paralleling the effect on contraction. Notably, control Ca²⁺ sensitivity of both contraction and

MLC phosphorylation in chicken mesenteric artery and contractile Ca²⁺ sensitivity in pigeon artery were higher than that of rabbit mesenteric artery (Fig. 5). The high Ca²⁺ sensitivity under control conditions and PDBu-induced Ca²⁺ desensitization were also observed in chicken carotid artery (not shown).

PKC inhibitors, GF-109203X ($3 \mu\text{M}$) and PKC pseudosubstrate inhibitor peptide^(19–31) (PKC-IP; $30 \mu\text{M}$)

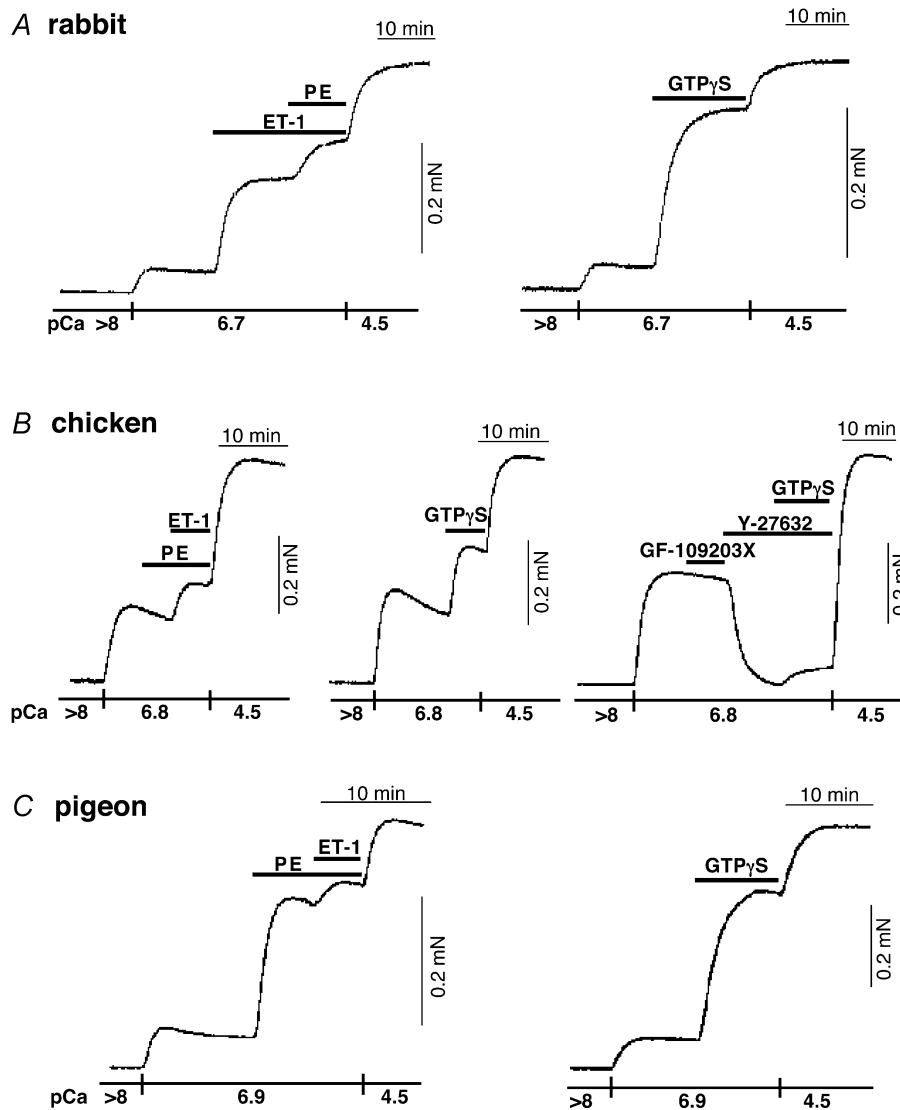


Figure 4. Effects of agonists and GTP γ S on contractile force at clamped Ca²⁺ in α -toxin-permeabilized and A23187-treated mesenteric arteries

A, left, a representative trace of cumulative application of ET-1 ($1 \mu\text{M}$) and PE ($30 \mu\text{M}$) on submaximal contraction at pCa 6.7 in α -toxin-permeabilized rabbit arteries ($n = 3$). Right, GTP γ S ($30 \mu\text{M}$)-induced contraction at pCa 6.7 ($n = 5$). B, left, PE did not increase a submaximal contraction at pCa 6.8 in α -toxin-permeabilized chicken artery ($n = 7$). ET-1 produced a significant contraction at pCa 6.8 ($n = 5$). Middle, GTP γ S-induced contraction at pCa 6.8 ($n = 9$). Right, effect of $3 \mu\text{M}$ GF-109203X and $10 \mu\text{M}$ Y-27632 on pCa 6.8- and $30 \mu\text{M}$ GTP γ S-induced contraction in chicken artery ($n = 6$). C, left, PE- and ET-1-induced contraction at pCa 6.9 in α -toxin-permeabilized pigeon arteries ($n = 3$). Right, GTP γ S-induced contraction at pCa 6.9 ($n = 5$).

both, but not a conventional PKC isoform-specific inhibitor Go6976 ($10 \mu\text{M}$), significantly inhibited PDBu-induced relaxation in α -toxin- and β -escin-permeabilized chicken mesenteric artery, respectively (Fig. 6A and B). PDBu significantly increased phosphorylation levels of several proteins in intact chicken artery monitored by immunoblotting with anti-phosphothreonine antibody (Fig. 6C). Using phospho[Thr³⁸]CPI-17-specific antibody, the immunoblots showing Thr³⁸ phosphorylation of CPI-17 by PDBu was clearly identified in rabbit artery, but not found in chicken artery (Fig. 6D).

Reconstitution of Ca²⁺ sensitization in chicken artery

In Triton X-100-skinned smooth muscle in which endogenous CPI-17, if any, was depleted (Kitazawa *et al.* 1999), addition of $5 \mu\text{M}$ pre-thiophosphorylated recombinant CPI-17 (*tp*CPI-17) consistently enhanced a basal contraction to near maximum levels at pCa 6.7 in Triton X-100-skinned artery smooth muscles from all three species (for rabbit, from 4 ± 1 to $74 \pm 4\%$ of maximum at pCa 4.5; for pigeon, from 24 ± 2 to $96 \pm 2\%$; for chicken, from 7 ± 2 to $77 \pm 4\%$; $n = 3$). This result suggests that

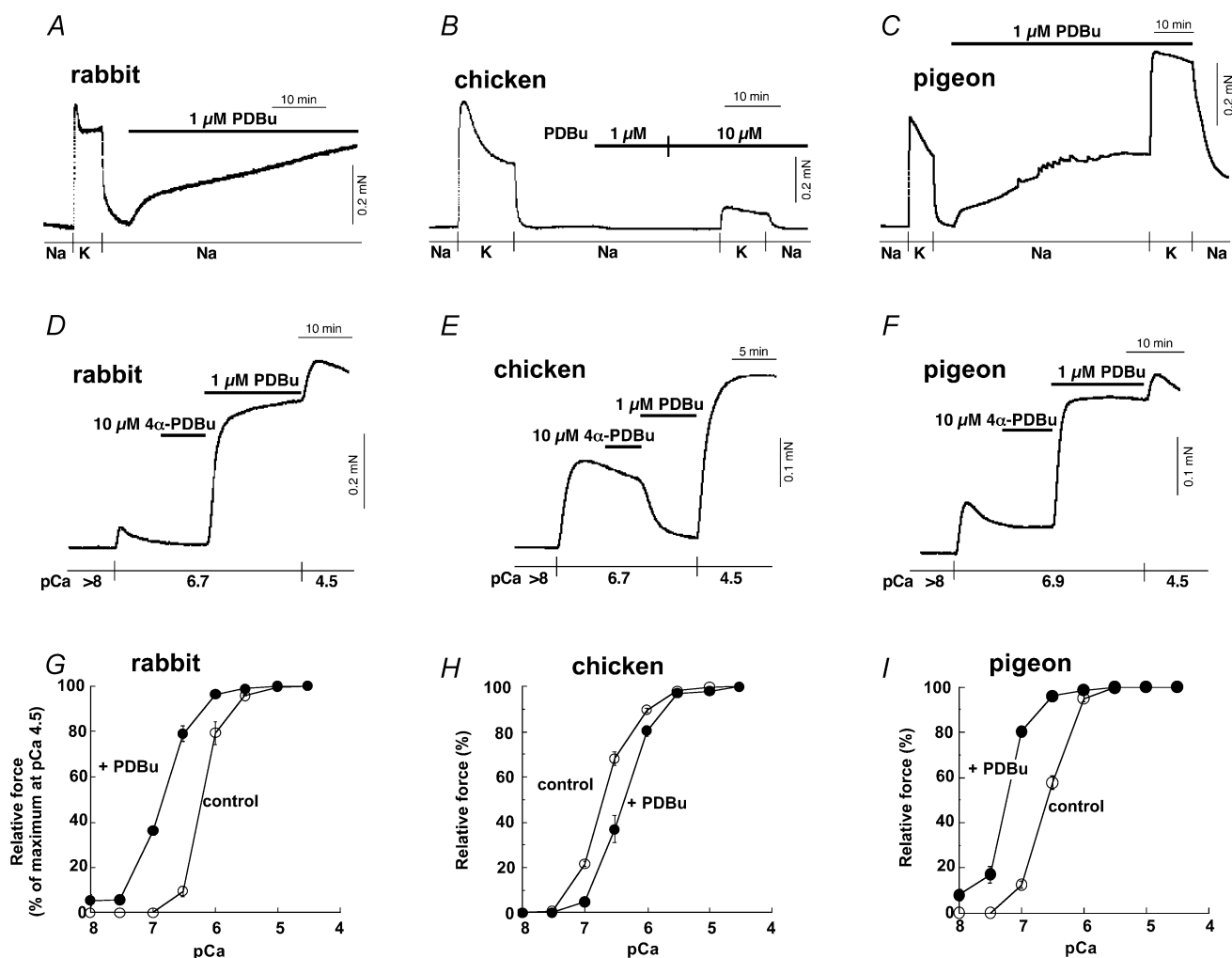


Figure 5. Effect of phorbol ester (PDBu) on contractile force in mesenteric arteries

Time course of contractile effect of $1 \mu\text{M}$ PDBu following high K⁺-induced contraction in intact rabbit (A), chicken (B) and pigeon MA (C). Na, normal extracellular solution; K, high (154 mM)-K⁺ solution. Middle panel, effect of $1 \mu\text{M}$ PDBu on Ca²⁺-induced contraction in α -toxin-permeabilized rabbit (D), chicken (E) and pigeon MA (F). Height of pCa 4.5-induced contraction represents a maximal force in each arterial strip. Bottom panel ($n = 4$), control contractile Ca²⁺ sensitivity (○) and effect of $1 \mu\text{M}$ PDBu (●) in rabbit (G), chicken (H) and pigeon MA (I).

the activated CPI-17 if expressed is able to inhibit the *in situ* MLCP and induce a significant Ca^{2+} sensitization in chicken artery similar to rabbit and pigeon arteries.

In β -escin-permeabilized chicken mesenteric artery strips, in which G protein-coupled signal transduction and PKC signalling were still retained (Kobayashi *et al.* 1989), $10 \mu\text{M}$ *tp*CPI-17 markedly increased a pCa 6.7-activated contraction from $17 \pm 4\%$ to $66 \pm 5\%$ of maximum contraction at pCa 4.5 with a half-time ($t_{1/2}$) of 11 ± 1 min ($n = 3$; Fig. 7B compared to control Fig. 7A), comparable to those in β -escin-permeabilized rabbit femoral artery (Li *et al.* 1998) and also in Triton X-100-skinned chicken mesenteric artery. Unphosphorylated CPI-17

(*uCPI-17*) slowly ($t_{1/2}$ of 17 ± 1 min) increased the basal contraction to a significantly lower level (from 19 ± 3 to $45 \pm 5\%$ of maximum; $n = 4$; Fig. 7C) than that of *tp*CPI-17 (Fig. 7B). PKC-IP ($30 \mu\text{M}$) inhibited the *uCPI-17*- (Fig. 7D) but not *tp*CPI-17-induced contraction (not shown), suggesting that spontaneous activity of endogenous PKC is responsible for the main fraction of *uCPI-17*-induced contraction. In the presence of *uCPI-17*, $1 \mu\text{M}$ PDBu significantly enhanced the contraction from 43 ± 5 to $66 \pm 2\%$ ($n = 3$; Fig. 6C). PDBu alone, however, reduced the basal pCa 6.7-induced contraction (Fig. 7E) similar to that in α -toxin-permeabilized artery (Fig. 5E). The *uCPI-17*, but not a

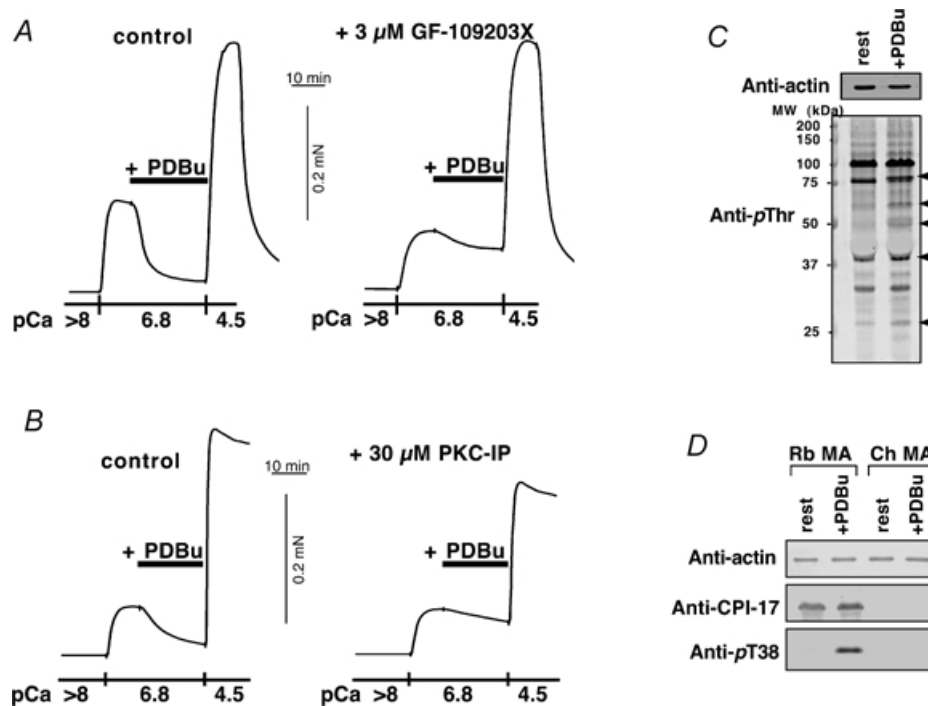


Figure 6. Effect of PKC inhibitors on PDBu-induced relaxation and immunoblots using anti-phosphoprotein antibodies in chicken mesenteric artery

A, effect of $3 \mu\text{M}$ GF-109203X on $1 \mu\text{M}$ PDBu-induced relaxation in α -toxin-permeabilized chicken mesenteric artery ($n = 3$). Left trace, control PDBu-induced relaxation. Right trace, $3 \mu\text{M}$ GF-109203X was added 10 min before and present throughout the experiment. B, effect of $30 \mu\text{M}$ PKC pseudosubstrate inhibitor peptide^(19–31) (PKC-IP; the peptide of 2000 Da cannot penetrate cells through α -toxin pores but can through β -escin pores; Kobayashi *et al.* 1989) on $1 \mu\text{M}$ PDBu-induced relaxation in β -escin-permeabilized chicken artery ($n = 3$). Left, control. Right, $30 \mu\text{M}$ PKC-IP was added 10 min before increase in Ca^{2+} and was present throughout the experiment. C, protein phosphorylation by PDBu in chicken artery ($n = 3$). Intact chicken artery strips were exposed to either the normal external solution (rest) or $1 \mu\text{M}$ PDBu-containing solution for 10 min and quickly frozen. The SDS-extracts were used to monitor phosphorylation levels by immunoblotting using anti-phosphothreonine (anti-pThr) antibody. For immunoblotting for actin, the extracts were diluted 30 times. Arrowheads show more than twice increase in blot density. D, phosphorylation of CPI-17 at Thr³⁸ by $1 \mu\text{M}$ PDBu in intact rabbit (Rb) and chicken (Ch) mesenteric artery (MA) ($n = 3$). Top panel shows immunoblots using anti-actin, middle panel using anti-CPI-17, and lowest panel using anti-pThr³⁸ CPI-17 antibodies. SDS-extract containing $20 \mu\text{g}$ of total protein was applied in each lane for anti-CPI-17 and anti-pT38 immunoblottings. For actin, the extracts were diluted 40 times. Phosphorylation level of CPI-17 at Thr³⁸ was dramatically increased in the PDBu-treated rabbit MA (13, 16). Either polyclonal anti-CPI-17 or anti-pThr³⁸ antibody did not show any bands in chicken MA extracts in the presence and absence of PDBu.

mutant [T38A] CPI-17 (Kitazawa *et al.* 1999), enhanced the PDBu-reduced contraction ($7 \pm 0\%$) to higher level ($71 \pm 4\%$) with a shorter $t_{1/2}$ of 13 ± 0 min ($n = 3$; Fig. 7E) than those in the absence of PDBu (Fig. 7C before addition of PDBu). The *u*CPI-17 in the presence of PDBu significantly reduced the speed of relaxation ($t_{1/2}$ was changed from control 2.4 ± 0.1 min to 5.5 ± 0.2 min; $n = 3$) from pCa 4.5-induced contraction when MLCK activity was blocked by removal of Ca^{2+} and addition of 10 mM EGTA and 0.2 mM ML-9, a MLCK kinase inhibitor (Masuo *et al.* 1994), suggesting inhibition of MLCP by

CPI-17. Addition of unphosphorylated recombinant PHI-1 ($10 \mu\text{M}$) caused very slow development ($t_{1/2} > 40$ min) of contraction at pCa 6.7 to a low level from 13 ± 5 to $25 \pm 5\%$ of maximum at 80 min (Fig. 7F). Unlike in the presence of unphosphorylated CPI-17, PDBu did not potentiate the contraction in the presence of unphosphorylated PHI-1 (25 versus $24 \pm 5\%$; $n = 3$).

Figure 8 summarizes the effects of inhibitors on CPI-17 plus $\text{GTP}\gamma\text{S}$ -mediated Ca^{2+} sensitization of β -escin-permeabilized chicken artery at pCa 6.7. PKC-IP had no effect on the basal contraction even in the

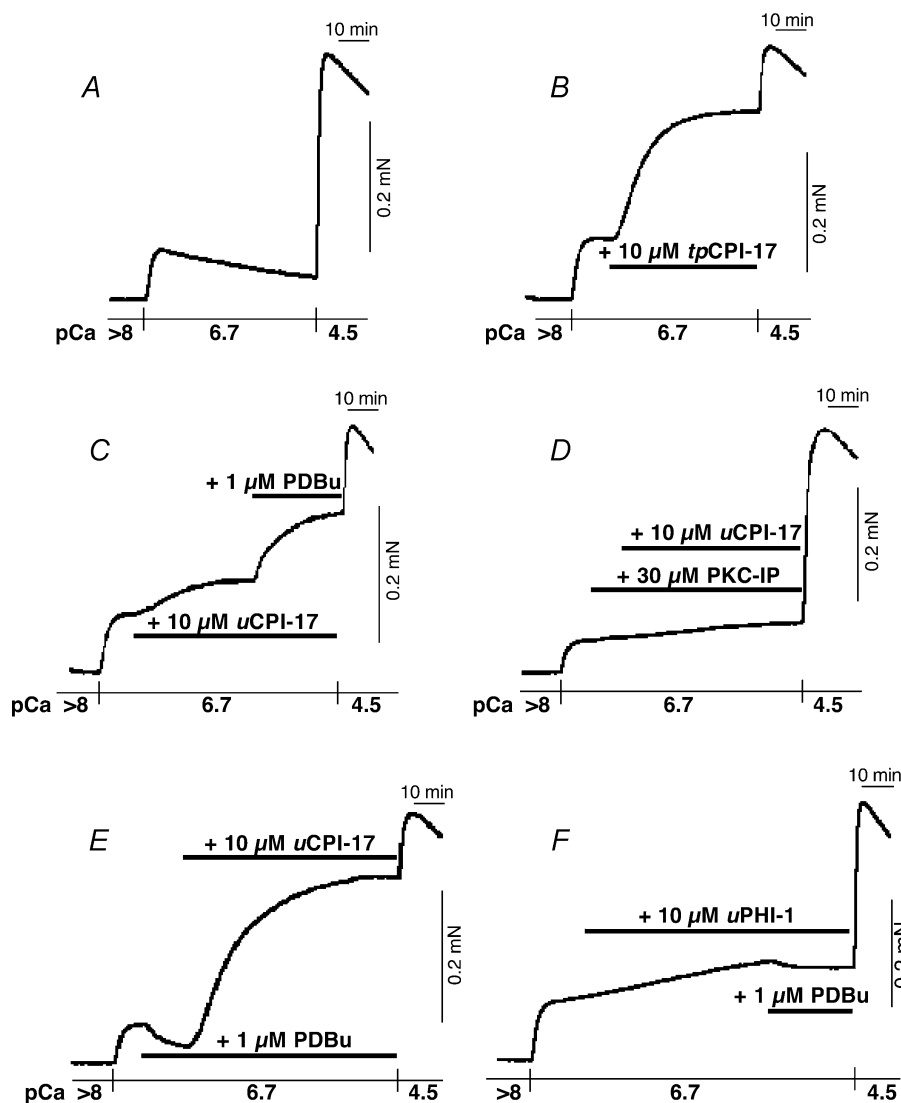


Figure 7. Reconstitution of PKC-induced contractile Ca^{2+} sensitization in β -escin-permeabilized chicken mesenteric artery

A, control force trace at pCa 6.7. Height of contraction at pCa 4.5 shows a maximum force. B, effect of $10 \mu\text{M}$ pre-thiophosphorylated recombinant CPI-17 (*tp*CPI-17) on submaximal contraction at pCa 6.7. C, effect of $10 \mu\text{M}$ unphosphorylated recombinant CPI-17 (*u*CPI-17) and $1 \mu\text{M}$ PDBu. D, effect of *u*CPI-17 in the presence of $30 \mu\text{M}$ PKC-IP. E, effect of *u*CPI-17 in the presence of $1 \mu\text{M}$ PDBu. F, effect of $10 \mu\text{M}$ *u*PHI-1 and PDBu. Each force trace is a representative of three to four similar experiments.

presence of $\text{GTP}\gamma\text{S}$ ($n = 3$) while either $10 \mu\text{M}$ Y-27632 or $3 \mu\text{M}$ H-1152 (a more selective Rho-kinase inhibitor; Sasaki *et al.* 2002) strongly diminished the basal pCa 6.7-activated contraction even in the presence of $\text{GTP}\gamma\text{S}$ to $1 \pm 0\%$ ($n = 4$) and $0 \pm 0\%$ ($n = 3$) similar to those in α -toxin-permeabilized preparations (Fig. 4B). $\text{GTP}\gamma\text{S}$ ($30 \mu\text{M}$) alone significantly but only partially potentiated the basal contraction at pCa 6.7 from 16 ± 1 to $27 \pm 4\%$ of maximum ($n = 3$), similar to that observed in α -toxin-permeabilized arterial smooth muscle (Fig. 4B). In the presence of $\text{GTP}\gamma\text{S}$, $u\text{CPI-17}$ rapidly ($t_{1/2}$ of 10 ± 1 min) enhanced the contraction to $64 \pm 3\%$ (Fig. 8) not significantly different from those of $tp\text{CPI-17}$ (Fig. 7B). The amplitude and speed of potentiation were significantly ($P < 0.05$ and 0.01 , respectively) higher than those of $u\text{CPI-17}$ alone (Fig. 7C). Addition of PKC-IP partially eliminated the contractility induced by $u\text{CPI-17}$ in the presence of $\text{GTP}\gamma\text{S}$ to $41 \pm 2\%$ ($n = 3$). Y-27632 also partially reduced contraction induced by $u\text{CPI-17}$ plus $\text{GTP}\gamma\text{S}$ at pCa 6.7– $36 \pm 5\%$. Addition of Y-27632 and PKC-IP together suppressed the contractility induced by $u\text{CPI-17}$ plus $\text{GTP}\gamma\text{S}$ to a level not significantly different from the basal level ($P = 0.89$; $n = 5$).

Discussion

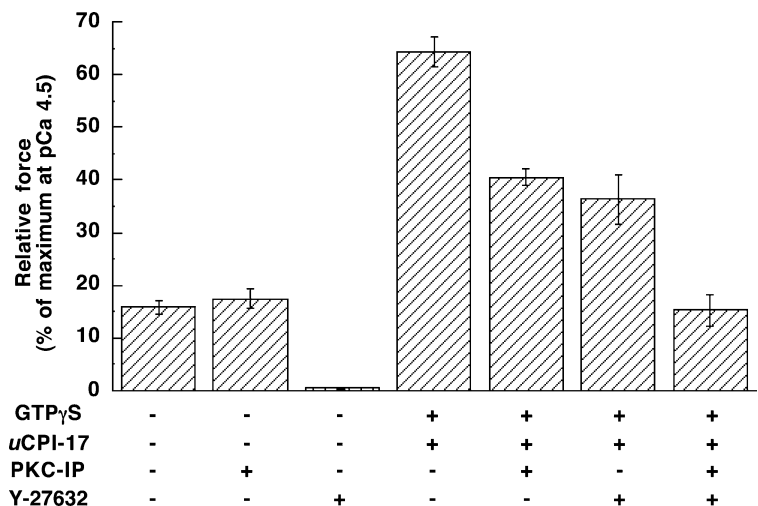
CPI-17 was undetectable in chicken smooth muscles (aorta, mesenteric artery, gizzard and small intestine) by immunoblotting or RT-PCR, whereas it was expressed in mammalian smooth muscle tissues, such as rabbit femoral artery (at $7 \mu\text{M}$) and rabbit vas deferens (at $0.8 \mu\text{M}$) (Woodsome *et al.* 2001). The antigen of three antibodies and the primers used for RT-PCR in this study are derived from known mammalian CPI-17 sequences. The possibility therefore remains that CPI-17 was not

detected in chicken smooth muscle tissues due to substantial sequence differences from known mammalian CPI-17 sequences at both the nucleotide and amino acid levels. Yet, the fact that CPI-17 was easily detected in another avian species (pigeon) together with the results of the functional studies strongly supports the idea that CPI-17 is deficient in chicken smooth muscle tissues examined. Interestingly, MYPT1 of MLCP is known to undergo phenotypic switch around the time of hatching from leucine zipper positive to negative isoforms in chicken gizzard smooth muscle (Khatri *et al.* 2001), and this coincided with the development of cGMP-resistant phenotype in the tissue. However, we did not observe changes in CPI-17 immuno-detectability between embryonic and adult chicken smooth muscle tissues. Therefore, the 'deficiency' of CPI-17 in chicken smooth muscle may not be developmentally regulated, and instead this might be a result of extensive inbreeding of cloned strains for agricultural purposes. Further genomic study is needed to clarify the nature of the mutation in chicken.

The absence of CPI-17 in chicken artery presented a useful model system for studying the role of CPI-17 in Ca^{2+} sensitization. In chicken and rabbit/pigeon arteries, agonists, such as PE, ET-1 and ATP, can produce a contraction to a level similar to that induced by high K^+ . Histamine, known to strongly promote phosphorylation of CPI-17 in rabbit femoral artery (Kitazawa *et al.* 2000), failed to induce contraction in chicken mesenteric artery, possibly due to lack of CPI-17, histamine receptor and/or the specific G-proteins coupled to the receptor. In rabbit and pigeon arteries, in the absence of extracellular Ca^{2+} in the ryanodine-treated intact muscles or at clamped Ca^{2+} concentration in the α -toxin-permeabilized, A23187-treated muscles, agonists still produced large contractions, showing significant Ca^{2+} sensitization

Figure 8. Effects of PKC-IP and Y-27632 on $\text{GTP}\gamma\text{S}$ -induced Ca^{2+} sensitization in the presence of $u\text{CPI-17}$ in β -escin-permeabilized chicken mesenteric artery

Strips were pre-activated in pCa 6.7 solutions for 10 min and then $30 \mu\text{M}$ PKC-IP and/or $10 \mu\text{M}$ Y-27632 were applied. After a new steady state level of contraction was achieved in the presence of the inhibitors, $30 \mu\text{M}$ $\text{GTP}\gamma\text{S}$ and $10 \mu\text{M}$ $u\text{CPI-17}$ were added. $n = 3$ –4.



(Himpens *et al.* 1990; Kitazawa *et al.* 1991a). In chicken artery, in contrast, PE and ET-1 evoked only minor contraction under the same conditions. These results suggest that agonist- as well as high K^+ -induced contractions in chicken artery are predominantly regulated by classical Ca^{2+} -dependent mechanism such as MLC_{20} phosphorylation by Ca^{2+} /calmodulin-activated MLCK (Hartshorne, 1987; Kamm & Stull, 1989). The absence of extracellular Ca^{2+} without ryanodine treatment or the presence of Ca^{2+} channel blocker also strongly reduced agonist-induced contractions in chicken artery, further suggesting a minor role for the SR Ca^{2+} release during agonist-induced contraction. The direct G protein activator, $GTP\gamma S$, produced significantly less Ca^{2+} sensitization in chicken than in rabbit and pigeon arteries. The G protein activation with supplement of $uCPI-17$ in chicken artery was able to make a larger and faster Ca^{2+} sensitization similar to those of $CPI-17$ -rich arteries of rabbit and pigeon. These results together suggest that $CPI-17$ is at least partially required for strong G-protein coupled receptor-mediated Ca^{2+} sensitization of smooth muscle.

PKC activation did not evoke significant contraction in either intact or permeabilized chicken artery, while the same PKC activators can elicit a large contraction in rabbit and pigeon arteries. A reasonable interpretation of our data is that the absence of $CPI-17$ is accountable for the lack of PKC-mediated Ca^{2+} sensitization in chicken smooth muscles. Several lines of evidence suggest that PKC itself is functional in chicken artery. First, active but not inactive form of PDBu and also DAG analogue affected (reduced) the Ca^{2+} -activated contraction in chicken artery. Second, both Ca^{2+} -dependent and Ca^{2+} -independent isoforms of PKC and signalling proteins implicated in the PKC pathway (such as MAPKs, Raf-1, $h-CaP$, and $h-CaD$; Morgan & Gangopadhyay, 2001) were expressed in chicken smooth muscles as well as in rabbit artery. Third, PDBu significantly increased phosphorylation levels of several proteins in chicken artery detected by immunoblotting. Finally, PKC inhibitors reduced PDBu-induced relaxation in chicken artery. Therefore, that PKC activation was not capable of developing a contraction in chicken artery strongly suggests that this is due to the lack of $CPI-17$. Yet, chicken artery retains the contractile ability to respond to PKC activation, provided that $CPI-17$ is made available. This is demonstrated by the large Ca^{2+} sensitization induced by PKC activator and recombinant $uCPI-17$ together in chicken artery. It is consistent with our previous finding in rabbit smooth muscles (Woodsome *et al.* 2001), that the expression level of $CPI-17$ is a key factor in determining the contractile profile of PKC-mediated

Ca^{2+} sensitization in various smooth muscle tissues. There are some reports, however, demonstrating that phorbol ester caused a relaxation of high K^+ - and/or agonist-induced contractions in some types of intact mammalian smooth muscle tissues that could express $CPI-17$ (Mitsui & Karaki, 1993; Tajima *et al.* 1997; Chakder *et al.* 2001). In those cases, the major relaxant action was correlated with a reduction in intracellular Ca^{2+} by phorbol ester but not Ca^{2+} desensitization similar to that observed in the $CPI-17$ -deficient chicken artery (this study).

Interestingly, the closely related phosphatase inhibitor protein, $PHI-1$ (Eto *et al.* 1999), is expressed in chicken, suggesting that this protein is unable to couple PKC signalling to contraction despite their similarities. In fact, unlike $CPI-17$, $10 \mu M$ recombinant $PHI-1$ incorporated into chicken artery did not elicit PDBu-induced Ca^{2+} sensitization. This can be also supported by biochemical evidence that phosphorylation of $PHI-1$ by PKC was much slower (about one-fifth; M. Eto, unpublished result) and IC_{50} of phosphorylated $PHI-1$ for MLCP was about one order of magnitude higher than those of $CPI-17$ under similar conditions (Eto *et al.* 1999). These results suggest that $PHI-1$ is not responsible for PKC-mediated MLCP inhibition. The thin filament-associated proteins, $h-CaD$ and $h-CaP$, are also expressed in chicken as well as rabbit smooth muscles. Even under Ca^{2+} -clamped conditions, however, PKC activation caused a relaxation associated with a decrease in MLC_{20} phosphorylation in chicken artery, while in rabbit artery PDBu-induced contraction was developed together with an increase in MLC_{20} phosphorylation (Itoh *et al.* 1993; Masuo *et al.* 1994). Supplement of $uCPI-17$ into chicken artery switched PKC activator-induced relaxation to contraction. This is consistent with the fact that Ca^{2+} sensitization in Triton X-100-skinned rabbit artery (in which endogenous $CPI-17$ and PKC are depleted while $h-CaD$ and $h-CaP$ still remain as in intact preparations) is reconstituted by addition of PKC together with $uCPI-17$ but not PKC alone (Kitazawa *et al.* 1999). Since $CPI-17$ and PKC activator together markedly reduced the relaxation rate even when MLCK was blocked, it strongly supports the model that PKC-induced contraction is mediated through the $CPI-17/MLCP$ pathway in smooth muscles.

PKC is also believed to at least partly mediate G protein-coupled receptor signalling in Ca^{2+} sensitization (Yoshida *et al.* 1994; Brozovich, 1995; Parsons *et al.* 1996; Buus *et al.* 1998; Kitazawa *et al.* 2000). How does $GTP\gamma S$ trigger the Ca^{2+} sensitization in the $CPI-17$ -deficient artery in which PKC activation does not lead to contraction? The Rho-kinase inhibitor, Y-27632 or H-1152, effectively inhibited $GTP\gamma S$ -induced

contractions in chicken artery similar to rabbit smooth muscles (Uehata *et al.* 1997; Sasaki *et al.* 2002), suggesting that the Ca^{2+} sensitizing pathway downstream of G protein requires Rho-kinase activation. However, consistent with the results from rabbit smooth muscles (Kitazawa *et al.* 2003; Niiro *et al.* 2003), phosphorylation of MYPT1 Thr⁶⁹⁵ (an inhibitory site in chicken sequence; Feng *et al.* 1999) was unchanged during GTP γ S-induced contraction in chicken artery (not shown). Signalling pathways involved in Ca^{2+} sensitization mediated by RhoA/Rho-kinase in CPI-17-deficient chicken artery (this study) and CPI-17-minimal rabbit phasic visceral smooth muscles (Woodsome *et al.* 2001; Kitazawa *et al.* 2003) are unclear and remain to be determined. Interestingly Y-27632 and H-1152 also strongly suppressed Ca^{2+} -dependent contractility of chicken artery similar to an appreciable degree of inhibition of the tonic component of high K^{+} -induced contraction (Mita *et al.* 2002; Urban *et al.* 2003). This is consistent with the reports that RhoA and Rho-kinase in rabbit arteries are activated in response to elevation of cytosolic Ca^{2+} concentration (Urban *et al.* 2003; Sakurada *et al.* 2003). Y-27632 might be more sensitive in Ca^{2+} -induced contraction of arterial smooth muscle, as compared to Ca^{2+} sensitization. The CPI-17-deficient chicken smooth muscle will be a useful model for studying not only the role of CPI-17 but also other potential mechanism(s) regulating smooth muscle contractility.

In conclusion, the absence of CPI-17 strongly correlates with the lack of PKC-dependent and at least in part with the minimal agonist-induced Ca^{2+} sensitization of contraction in chicken arterial smooth muscle. Thus, CPI-17 is one of the key players in the G protein-coupled receptor-mediated Ca^{2+} sensitization in vascular smooth muscles.

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Acknowledgements

We thank Dr Katsuhide Mabuchi for kind gift of anti-CaP and anti-CaD antibodies, and also Kazuyo Kitazawa and Mallappa Anitha for technical assistance. We thank Dr Lin Li for two-dimensional gel analysis of myosin light chain phosphorylation in the early stage of the present project. This work was supported by National Institute of Health grants R01HL51824 and HL70881 to T.K and a Scientist Development Grant from the AHA National Center to M.E.