

## Expression of CPI-17 and myosin phosphatase correlates with $\text{Ca}^{2+}$ sensitivity of protein kinase C-induced contraction in rabbit smooth muscle

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1. Various smooth muscles have unique contractile characteristics, such as the degree of  $\text{Ca}^{2+}$  sensitivity induced by physiological and pharmacological agents. Here we evaluated six different rabbit smooth muscle tissues for protein kinase C (PKC)-induced  $\text{Ca}^{2+}$  sensitization. We also examined the expression levels of myosin light chain phosphatase (MLCP), the MLCP inhibitor phosphoprotein CPI-17, and the thin filament regulator h-calponin.
2. Immunohistochemical and Western blot analyses indicated that CPI-17 was found primarily in smooth muscle, although expression varied among different tissues. Vascular muscles contained more CPI-17 than visceral muscles, with further distinction existing between tonic and phasic subtypes. For example, the tonic femoral artery possessed approximately 8 times the cellular CPI-17 concentration of the phasic vas deferens.
3. In contrast to CPI-17 expression patterns, phasic muscles contained more MLCP myosin-targeting subunit than tonic tissues. Calponin expression was not statistically different.
4. Addition of phorbol ester to  $\alpha$ -toxin-permeabilized smooth muscle caused an increase in contraction and phosphorylation of both CPI-17 and myosin light chain (MLC) at submaximal  $[\text{Ca}^{2+}]_i$ . These responses were several-fold greater in femoral artery as compared to vas deferens.
5. We conclude that the expression ratio of CPI-17 to MLCP correlates with the  $\text{Ca}^{2+}$  sensitivities of contraction induced by a PKC activator. PKC stimulation of arterial smooth muscle with a high CPI-17 and low MLCP expression generated greater force and MLC phosphorylation than stimulation of visceral muscle with a relatively low CPI-17 and high MLCP content. This implicates CPI-17 inhibition of MLCP as an important component in modulating vascular muscle tone.

Smooth muscle is heterogeneous in both structure and function. Based on their electrophysiological and mechanical characteristics, smooth muscles can be broadly classified into tonic and phasic types (Somlyo & Somlyo, 1968). Tonic muscles such as the aorta, pulmonary and femoral arteries, along with the trachea, usually respond to excitatory agonists with a graded depolarization. Prolonged depolarization induced by high  $\text{K}^+$  typically evokes a slowly developing sustained contraction in tonic muscles. In contrast, phasic muscles, which include the portal vein, ileum, bladder and vas deferens, react to excitatory agonists by generating a spike-like action potential. In addition, depolarization with high  $\text{K}^+$  elicits an initial phasic contraction, followed

by a decline to a low steady-state level (Himpens *et al.* 1989). The contractile diversities among different smooth muscles appear to be attributed, at least in part, to variations in cellular protein expression rather than to different time courses in  $[\text{Ca}^{2+}]_i$  (Himpens *et al.* 1988). Myosin heavy chain (see review by Adelstein & Sellers, 1996) and light chain isoforms (see Barany & Barany, 1996) are specifically distributed among smooth muscle tissues and the composition of these isoforms determines myosin motor activity. Moreover, variable expression of other regulatory contractile proteins possibly contributes to the differentiation of smooth muscle function (Khalil, 1992; North *et al.* 1994; Szymanski *et al.* 1998; Dirksen *et al.* 2000).

The primary mechanism that regulates actomyosin ATPase and contraction in all vertebrate smooth muscle is reversible phosphorylation of the 20 kDa myosin light chain (MLC) by MLC kinase (MLCK) and MLC phosphatase (MLCP) (Hartshorne, 1987; Kamm & Stull, 1989). MLCK is a  $\text{Ca}^{2+}$ -calmodulin-dependent kinase that is stimulated by an increase in  $[\text{Ca}^{2+}]_i$ . Although  $\text{Ca}^{2+}$  is the major contractile messenger for all types of smooth muscle, the  $\text{Ca}^{2+}$  sensitivity of MLC phosphorylation and contraction is an equally important factor for the regulation of smooth muscle contraction (see Somlyo & Somlyo, 1994). Tonic muscles (pulmonary and femoral arteries) have a 3-fold greater calcium sensitivity of MLC phosphorylation and contraction as compared to phasic muscles (portal vein and ileum) (Kitazawa *et al.* 1991*a*). The higher  $\text{Ca}^{2+}$  sensitivity of tonic muscle contraction is explained by a 4-fold lower dephosphorylation rate of MLC in tonic muscle, as compared to phasic muscle. This is evident even in phasic tissues with a higher MLCK content, suggesting that *in situ*, the primary determinant of smooth muscle  $\text{Ca}^{2+}$  sensitivity is MLCP rather than MLCK activity (Gong *et al.* 1992).

It is well documented that G protein-coupled receptor activation increases the  $\text{Ca}^{2+}$  sensitivity of MLC phosphorylation and contraction in all types of smooth muscle through the inhibition of MLCP (Kitazawa *et al.* 1991*b*; Kubota *et al.* 1992; see Somlyo & Somlyo, 1994). MLCP is a heterotrimeric enzyme with a 38 kDa type-1 phosphatase catalytic subunit  $\delta$ -isoform (PP1<sub>C</sub>), a 110–130 kDa myosin-targeting subunit (MYPT1) and a 20–21 kDa subunit of unknown function (see Hartshorne *et al.* 1998). Two major pathways have been proposed for the regulation of MLCP. First, RhoA-activated kinase (Rho-kinase) phosphorylates Ser-696 of MYPT1, which inhibits the phosphatase activity (Kimura *et al.* 1996; Feng *et al.* 1999; Kawano *et al.* 1999; see Somlyo & Somlyo, 2000). In smooth muscle cells, this phosphorylation is enhanced by stimulation with the G protein activator GTP $\gamma$ S (Trinkle-Mulcahy *et al.* 1995; Swärd *et al.* 2000; Nagumo *et al.* 2000). In addition to MYPT1 phosphorylation, a second pathway involves an inhibitor protein for MLCP, called CPI-17, which was first isolated from pig aorta smooth muscle (Eto *et al.* 1995) and is only expressed in smooth muscle (Eto *et al.* 1997, 1999). Phosphorylation of CPI-17 at Thr-38 by PKC converts this protein to a potent inhibitor of the catalytic activity of not only PP1<sub>C</sub>, but also the MLCP holoenzyme (Eto *et al.* 1997; Senba *et al.* 1999) and MLCP anchored to myofibrils *in situ* (Li *et al.* 1998; Eto *et al.* 2000). The PKC  $\delta$ -isoform has been isolated as a dominant CPI-17 kinase from pig aorta (Eto *et al.* 2001), and a recombinant PKC  $\alpha$ -isoform can much more rapidly phosphorylate Thr-38 of CPI-17 as compared to calponin, caldesmon, MLC and myosin (Kitazawa *et al.* 1999). Furthermore, selective depletion of CPI-17 by skinning of smooth muscle cells diminishes PKC-induced  $\text{Ca}^{2+}$  sensitization of femoral artery strips and the response can be reconstituted by

addition of PKC and CPI-17 together, but not by PKC alone (Kitazawa *et al.* 1999). These results indicate that CPI-17 is at least a key component in PKC-induced MLCP inhibition and calcium sensitization. It has recently been demonstrated that stimulation of the femoral artery by agonists, GTP $\gamma$ S and PKC activator induces Thr-38 phosphorylation of CPI-17 and contractile  $\text{Ca}^{2+}$  sensitization (Kitazawa *et al.* 2000). A PKC inhibitor (GF109203X) and a Rho-kinase inhibitor (Y27632) partially inhibited the CPI-17 phosphorylation and contraction induced by histamine, suggesting that possibly Rho-kinase and PKC phosphorylate CPI-17 in response to histamine signalling (Kitazawa *et al.* 2000; Koyama *et al.* 2000). In summary, these results imply that the CPI-17–MLCP signalling pathway may be as significant as the Rho-kinase–MLCP pathway in G protein-coupled agonist-induced  $\text{Ca}^{2+}$  sensitization of smooth muscle contraction.

CPI-17 expression is almost exclusively confined to smooth muscle tissues, and we believe that it is an endogenous mediator for the PKC signalling pathway that induces contractile  $\text{Ca}^{2+}$  sensitization. Yet between different smooth muscle tissues (aorta *vs.* urinary bladder), differential expression of CPI-17 is suggested by Northern and Western blot analyses (Eto *et al.* 1997, 1999). In preliminary experiments at submaximal  $[\text{Ca}^{2+}]_i$  (Woodsome & Kitazawa, 2000), we observed that in the phasic vas deferens and urinary bladder, phorbol ester-induced contractions were several times smaller than those caused by GTP $\gamma$ S. In contrast, the tonic femoral artery displayed equal responses to both phorbol 12,13-dibutyrate (PDBu) and GTP $\gamma$ S (Masuo *et al.* 1994). Therefore we hypothesized that the variation in PDBu-induced  $\text{Ca}^{2+}$  sensitization may possibly be due to differential expression of CPI-17 and the phospho-CPI-17 target MLCP. In this study, we used immunoblotting techniques to measure the tissue-specific expression and concentration of CPI-17, MLCP and h-calponin in six different rabbit smooth muscle tissues, including vascular tonic or phasic and visceral tonic or phasic subphenotypes. These results were then compared to the contractile response and MLC phosphorylation induced by PKC or G protein activation at a given level of  $\text{Ca}^{2+}$  in the various smooth muscles. The preliminary results were presented at the February 2000 Biophysical Society Meeting in New Orleans (Woodsome & Kitazawa, 2000).

## METHODS

### Tissue preparation

The Animal Care and Use Committee of Georgetown University approved all procedures. White male albino rabbits (2–3 kg) were killed with an overdose of halothane, and the desired tissues were dissected and cleaned of non-smooth muscle media. The isolated smooth muscle was then cut into strips 750  $\mu\text{m}$  wide and 3 mm long with natural wall thickness for the femoral artery (FA), aorta (AO) and portal vein (PV). Tissue strips were 250  $\mu\text{m}$  in diameter and 2.5–3 mm long for the urinary bladder (UB), vas deferens (VD) and

trachea (TR). Vascular endothelium was removed by gentle rubbing with a razor blade (Kitazawa *et al.* 1991*a*). Silk monofilaments were used to attach the strips to two tungsten needle tips, one of which was mounted to a force transducer (AM801, SensoNor, Horten, Norway). The strips were then immersed in a bubble plate (Horiuti, 1988) to allow fairly rapid solution exchange (< 1 s) and freezing for phosphorylation measurements.

### Solutions

Normal external solution comprised (mM): 150 NaCl, 4 KCl, 2 calcium methanesulphonate (Ca-Ms), 2 magnesium ethanesulphonate, 5.6 glucose and 5 Hepes buffer. Depolarizing external solution had potassium methanesulphonate (K-Ms) substituted equally for NaCl with other chemicals in the same concentrations. Both solutions were neutralized by Tris buffer to a pH of 7.4. Standard intracellular relaxing solution used for the resting state was composed of (mM): 74.1 K-Ms, 2 Mg<sup>2+</sup>, 4.5 Mg-ATP, 1 EGTA, 30 Pipes buffer and 1 DTT (Kitazawa *et al.* 1991*a*). The activating solution was 10 mM EGTA, and an appropriate amount of Ca-Ms was added to adjust final [Ca<sup>2+</sup>] (Horiuti, 1988). All solutions had a pH of 7.1 and ionic strength was kept at 0.2 M.

### Proteins

Pig aorta CPI-17 and His 6-tagged CPI-17 were prepared as described previously (Eto *et al.* 1995, 1997). Recombinant untagged CPI-17 was prepared from lysates of *E. coli* BL21 (DE3) including pig CPI-17 cDNA at *Nde*I/*Eco*R I sites of the pET30 vector (Novagen).

### Antibodies

Anti-CPI-17 IgG and IgY antibodies were raised, respectively, in rabbit sera and chicken eggs, using His 6-tagged porcine CPI-17 as an antigen (Senba *et al.* 1999; Kitazawa *et al.* 2000). Both antibodies were further purified using Affigel-10 resin that was conjugated with untagged CPI-17. Monoclonal MYPT1 antibodies were from BAbCO. Monoclonal antibodies against  $\alpha$ -actin and h-calponin were from Sigma. Secondary antibodies against chicken were from Promega. Anti-mouse secondary antibodies were from Sigma, and anti-rabbit secondary antibodies from Chemicon.

### Measurement of force development

The force of contraction was first recorded with high [K<sup>+</sup>]<sub>o</sub> (154 mM), in the absence and presence of 100  $\mu$ M phenylephrine, and the strips were then immersed in the relaxing solution for several minutes. The strips were then treated for 30 min at 30°C with 20  $\mu$ g ml<sup>-1</sup> of *Staphylococcus aureus*  $\alpha$ -toxin (List, Campbell, CA, USA) at pCa 6.3, buffered with 10 mM EGTA, in order to permeabilize the membrane whilst retaining cytosolic proteins (Masuo *et al.* 1994). The Ca<sup>2+</sup> ionophore A23187 (10  $\mu$ M; Calbiochem) was then applied to the strips for 20 min at 20°C in order to remove Ca<sup>2+</sup> from the sarcoplasmic reticulum. The force of contraction was then recorded at pCa 6.3 for the PV, TR, UB and VD, and at pCa 6.7 for the FA and AO, for 10 min, and then with either 1  $\mu$ M PDBu (Gibco BRL) or 30  $\mu$ M GTP $\gamma$ S (Boehringer Mannheim).

### Measurement of MLC phosphorylation

Muscle strips were prepared as described above, and subjected to the same experimental conditions. Permeabilized strips were initially immersed in the appropriate pCa solution and then subjected to PDBu or GTP $\gamma$ S. The tissues were then rapidly frozen in liquid chlorodifluoromethane, cooled by liquid N<sub>2</sub>. The strips were then placed on top of frozen acetone containing 10% trichloroacetic acid (TCA) overnight at -80°C. Then the strips and the TCA-acetone solution were gradually warmed to -20°C, then 4°C, and finally to room temperature. TCA was removed by washing with acetone. After air drying the strips, the tissue was homogenized in a buffer containing 0.1% SDS, 20 mM DTT, 10% glycerol and 0.1 mg ml<sup>-1</sup> bovine serum albumin (BSA). The samples were then subjected to

2-dimensional gel electrophoresis as described previously (Kitazawa *et al.* 1991*a*). The isoelectric focusing tube gel was run overnight until a constant current was reached. The tube gels were then run on SDS-PAGE gels, and the proteins transferred to nitrocellulose membrane using a wet transfer technique. The membranes were then washed in phosphate-buffered saline solution (containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.5 mM KH<sub>2</sub>PO<sub>4</sub>) overnight at room temperature. The membranes were rinsed with de-ionized H<sub>2</sub>O, and then stained with colloidal gold (BioRad). The blots were subsequently scanned and analysed (software from Signal Analytics Co., Vienna, VA, USA). The percentage phosphorylation (PP) was evaluated using the following equation:

$$\text{Percentage phosphorylation} = 100(P1 + P2)(U + P1 + P2)^{-1},$$

where U is unphosphorylated, P1 is monophosphorylated, and P2 is diphosphorylated MLC.

### Measurement of protein expression

Three of the selected tissues (FA, PV and VD) possessed smooth muscle layers that could be microscopically dissected from the extracellular non-smooth muscle media. However, as a result of their anatomy, tissue from the aorta (AO), trachea (TR) and urinary bladder (UB) prohibit the removal of the non-smooth muscle component. Thus, the tissues from AO, TR and UB contain significant amounts of non-smooth muscle cells and the measured values for concentrations of h-calponin, CPI-17 and MYPT1, all three of which are smooth muscle specific, are underestimated when total protein content is matched. Our experiments on the first three tissues (FA, PV and VD) indicated an approximate equality concerning smooth muscle-specific h-calponin expression (see Results). Therefore we assumed that h-calponin was equally expressed in smooth muscle tissues of different types and thus we compared CPI-17 and MYPT1 expression using the h-calponin-matched AO, FA, PV, TR, UB and VD samples in an attempt to equalize the smooth muscle cell content in the Western blots. The tissues were cleaned of connective tissue and immersed intact in 10% TCA-acetone overnight. After TCA treatment, the tissues were washed in acetone and allowed to dry. Homogenization buffer (10 mM Tris, 1% SDS, pH 7.4) was then added to the tissues in a volume equal to 5 times the wet weight, and the mixture was ground in a glass hand homogenizer. The resultant homogenates were then centrifuged and the supernatants collected. Protein concentration was measured using a BCA protein assay (Pierce). Laemmli sample buffer (LSB; with final concentrations 62.5 mM Tris, 1% SDS, 15% glycerol, 30 mM DTT and 0.005% Bromophenol Blue) was then added to dilute and equalize the protein concentrations. For h-calponin and  $\alpha$ -smooth muscle actin measurement, the tissue samples were further diluted with LSB. CPI-17, h-calponin and  $\alpha$ -smooth muscle actin were run on 15%, and MYPT1 on 8% polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) using a wet transfer method. For CPI-17, the membranes were blocked in Tris-buffered saline (TBS) solution containing 0.05% Tween-20, 5% non-fat milk and 1% BSA for 3 h at room temperature. For MYPT1, h-calponin and  $\alpha$ -smooth muscle actin, the membranes were blocked overnight at room temperature in TBS-Tween-20 with 5% non-fat milk. After primary antibody treatment, the membranes were incubated with alkaline phosphatase-conjugated secondary antibodies, and the bands were developed with alkaline phosphatase substrate (Sigma). The bands were analysed as described above.

### Calibration of staining for CPI-17 and MYPT1

Quantitative immunoassays for comparing the protein content in various smooth muscles were established. For CPI-17 (Fig. 1*A*), we applied 0–45 ng of recombinant purified protein, which produced a range of staining densities that encompassed the protein band staining from actual tissue samples. For MYPT1 (Fig. 1*B*), due to the

lack of stability of the recombinant protein, we instead used 0–45  $\mu\text{g}$  of total VD protein, which rendered a range spanning the staining densities of the tissue samples. For all Western blotting, we applied between 25 and 37.5  $\mu\text{g}$  of total protein, which fell on the more linear portion of the curve. However, as some of the samples stained on the lower end of the curve, we calibrated our results to reflect the non-linear staining of the MYPT1 antibody. MYPT1 in muscles consisted of two bands, but often the separation was not distinct. MYPT1 has been shown previously to consist of two isoforms (Shimizu *et al.* 1994). When these were clearly evident as shown here, the staining densities of the bands were added together. These assays allowed comparison of the relative expression of CPI-17 and MYPT1 in multiple tissue samples.

#### Estimation of CPI-17 content

Samples with known tissue wet weight and total protein concentration were subjected to Western blotting and their bands were compared to that of samples of purified CPI-17. For each tissue of each animal, at least three blots were carried out to determine the correlating amount of CPI-17. The amount of cellular CPI-17 in the various tissues was then estimated by multiplying by the dilution factor of the original wet sample and then dividing by the molecular weight (16 700) of CPI-17.

#### Immunohistochemistry

Immunohistochemical analysis was essentially performed as described previously (Everett *et al.* 2000). Briefly, individual tissues from male rats were fixed in 4% paraformaldehyde solution for 20 min at room temperature then paraffin embedded for sectioning. Separate tissue sections were incubated with the CPI-17 affinity-purified rabbit polyclonal antibody. Control slides were incubated with non-specific mouse IgG (Vector Laboratories, Burlingame, CA, USA) using the same concentration as the primary antibody. Sections were blocked with horse serum, followed by incubation with the primary antibody. Primary antibody binding was visualized using the ABC method with a red alkaline phosphatase substrate as a fluorescent substrate (Vector Laboratories), according to the manufacturer's protocol. The sections were then mounted between a

glass slide and a coverslip with Vectorshield mounting medium (Vector Laboratories). Images were acquired with a Nikon Microphoto-FXA/SA fluorescence microscope attached to a Hamamatsu CCD camera (Eto *et al.* 2000).

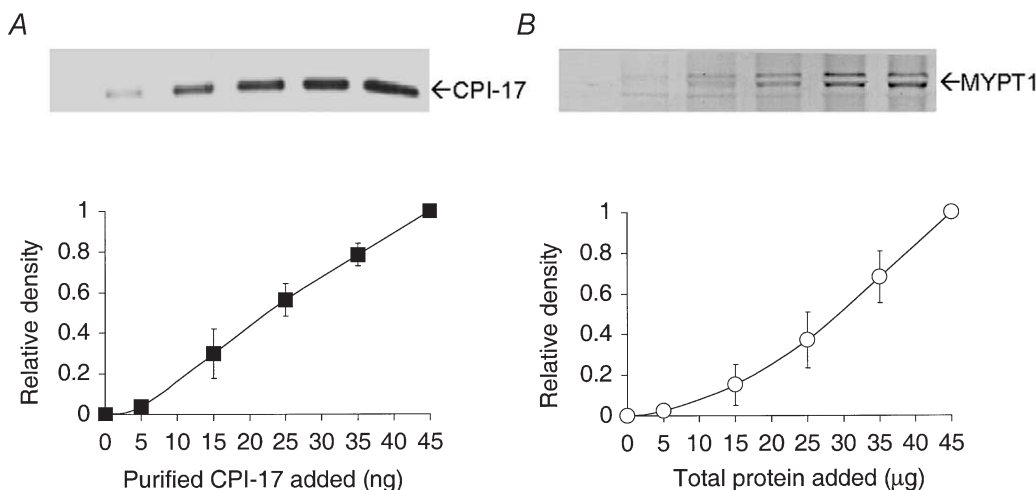
#### Statistics

Results shown are the means  $\pm$  S.E.M. of  $n$  experiments. The statistical significance was evaluated with Student's two-tailed  $t$  test with  $P < 0.05$  considered significant.

## RESULTS

### Smooth muscle-specific localization of CPI-17

To examine the specific expression of CPI-17, various tissues were harvested from rats for immunohistochemical analysis. Figure 2 shows immunolocalization of CPI-17 in aorta (A), heart (B) and lung (C) as compared to their phase-contrast images. In confirmation of previous Northern analysis of pig RNA (Eto *et al.* 1997) and Western blot analysis of rat protein (Eto *et al.* 1999), CPI-17 was expressed in smooth muscle cells of the aortic media but neither in endothelial nor in adventitial cells (Fig. 2A). It should be noted that CPI-17 also appears to be highly expressed in small blood vessels of the peri-adventitial fat (as indicated by arrows in Fig. 2A). Immunostaining of the heart (Fig. 2B) detected CPI-17 with specific localization within the cross-section of the medial wall of a blood vessel, while no CPI-17 was seen in the surrounding cardiac myocytes. In the lung (Fig. 2C), CPI-17 was highly expressed in the smooth muscle layer of a blood vessel and also in smooth muscle along the walls of the alveolar ducts. In Western blotting analysis, small but significant CPI-17 bands were seen for the lung and brain, but not in the heart, liver, kidney, testes, spleen,



**Figure 1.** Relationship between amount of samples and density of Western blots for CPI-17 and MYPT1

A shows a representative Western blot using polyclonal anti-CPI-17 antibodies against purified recombinant CPI-17; B shows a blot using monoclonal anti-MYPT1 antibodies against a control sample extracted from VD ( $n = 3$  for both). The Western blots were prepared with 0–45 ng of purified protein for CPI-17 and 0–45  $\mu\text{g}$  of total VD protein for MYPT1. Values for no protein were indiscernible from background values.

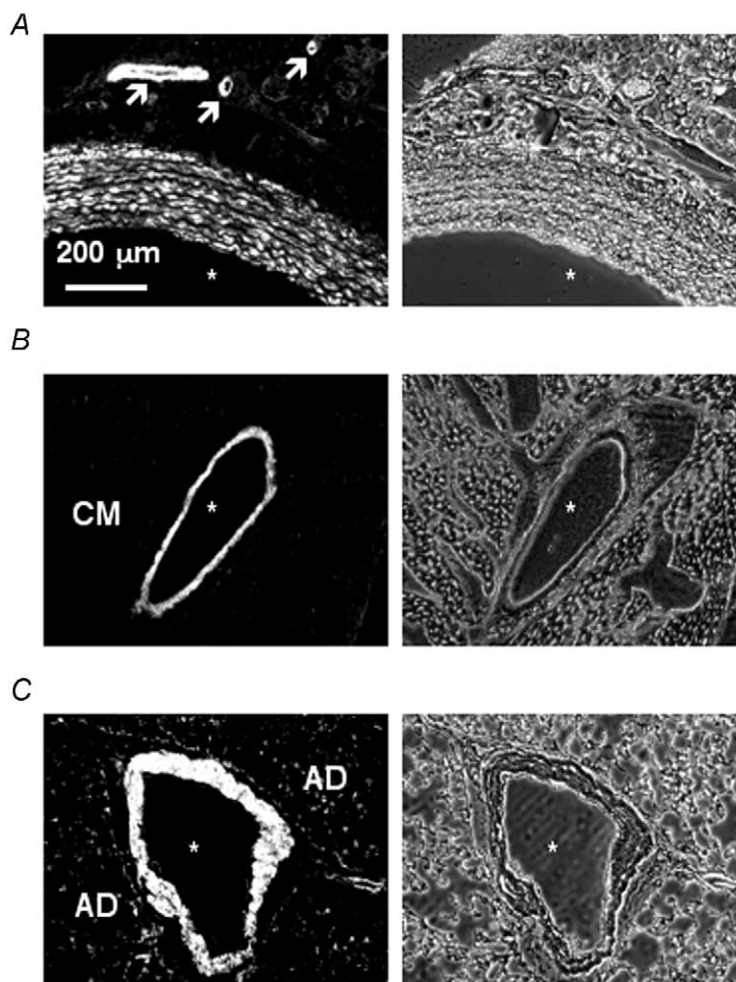
thymus, or skeletal muscle when total protein was matched (Eto *et al.* 1999). In the lung, the observation of small amounts of CPI-17 is probably due to the large amount of smooth muscle within the organ (Fig. 2C). This is supported by the fact that the lung also had higher levels of h-calponin and  $\alpha$ -smooth muscle actin expression (not shown). However, the brain did not express similar levels of these two smooth muscle-specific proteins (not shown). Western blotting patterns were reproducible using either rabbit or chicken anti-CPI-17 in both the rat and rabbit (not shown).

#### Expression of CPI-17, MYPT1, h-calponin and $\alpha$ -actin in smooth muscle tissues

Using quantitative Western analysis, the expression levels of several proteins known to be important for PKC-induced calcium sensitization were first examined when total protein concentration was matched in tissue extracts. Figure 3A illustrates the representative Western blots of  $\alpha$ -smooth muscle actin, h-calponin, CPI-17, and MYPT1 in extracts from smooth muscle layers of three selected tissues: tonic FA, phasic PV and phasic VD. In further agreement with earlier studies (Fatigati & Murphy, 1984; Szymanski *et al.* 1998), we found that the expression of  $\alpha$ -smooth muscle actin was slightly but significantly greater in tonic as compared to phasic

smooth muscles. However, calponin expression did not differ significantly ( $P > 0.05$ ) among the three different smooth muscles (Fig. 3A and B). In contrast, CPI-17 expression varied greatly among the tissues (Fig. 3A). The CPI-17 content was significantly higher in the tonic FA as compared to the phasic PV, and markedly greater than the phasic visceral VD. When the densities of the FA blots were normalized to 1, the relative value for the PV was  $0.70 \pm 0.07$  and that for the VD was  $0.16 \pm 0.08$  ( $n = 4$ ). For the evaluation of MLCP content, we determined that the relative expression of MYPT1 among smooth muscle tissues was an appropriate measure of MLCP content as MYPT1 interaction with  $PP1_C$  is critical to MLCP function (Hartshorne *et al.* 1998). In contrast to CPI-17 expression, MYPT1 content was significantly higher in the phasic PV and VD as compared to the tonic FA, with values of  $3.77 \pm 0.47$  for the PV and  $2.65 \pm 0.35$  for the VD ( $n = 4$ ).

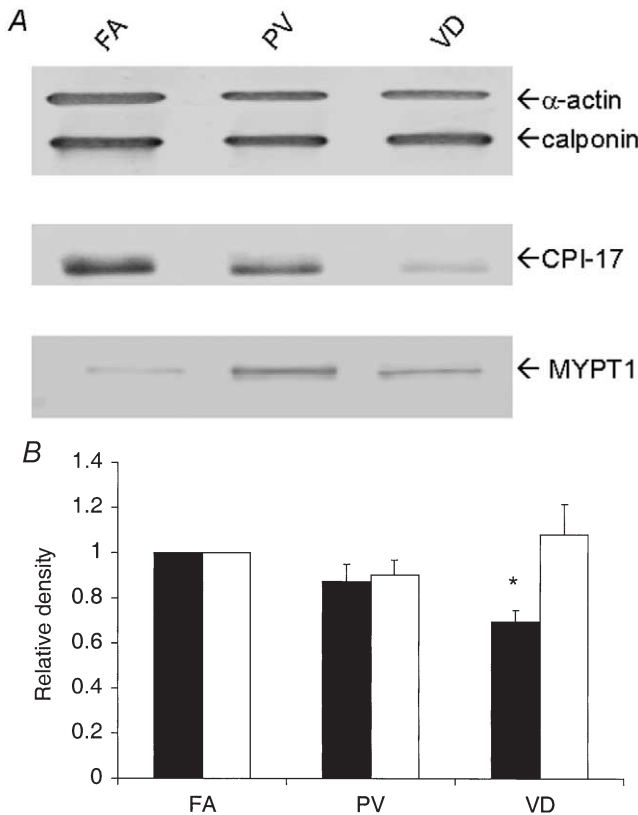
As h-calponin appeared to be equally expressed in smooth muscle regardless of phenotype (Fig. 3B), we applied this observation to experiments for the tonic AO, phasic UB and tonic TR smooth muscle. These were tissues that did not allow for complete removal of the non-smooth muscle component (see Methods). These three new tissues, in addition to those previously discussed (FA, PV and VD),



**Figure 2.** Smooth muscle-specific expression of CPI-17

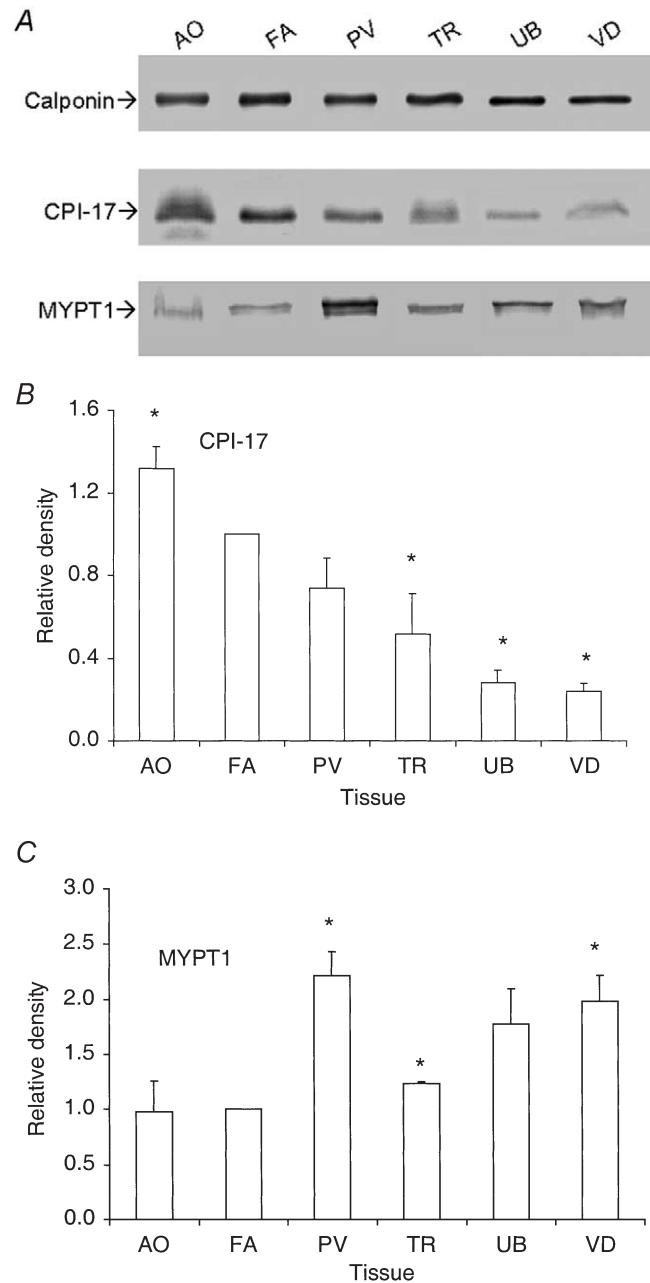
Tissue sections of adult rat aorta (A), heart (B) and lung (C) are shown with both the immunostained image (left) and the corresponding phase contrast image (right). Asterisks mark blood vessel lumens. Arrows in A indicate small blood vessels that stain positively for CPI-17. CM stands for cardiac muscle (B), which does not contain CPI-17. AD stands for alveolar ducts (C), which show some positive staining. Scale bar represents 200  $\mu\text{m}$ .

were subjected to Western immunoblotting as shown in Fig. 4. Calponin content was matched to within  $\pm 10\%$  across the six different tissues (shown in the upper panel of Fig. 4A). Although the total protein concentration was not equal, the representative h-calponin-matched Western blots of CPI-17 and MYPT1 are shown in the middle and lower panel of Fig. 4A. Figure 4B(CPI-17) and C(MYPT1) gives a graphical representation of the data. The AO contained significantly more CPI-17 than the FA, while the TR, UB and VD expressed significantly less CPI-17 ( $n = 3$ ). When the staining density of the FA blot was normalized to 1, the relative values for the AO, PV, TR, UB and VD were, respectively,  $1.32 \pm 0.11$ ,  $0.74 \pm 0.15$ ,  $0.52 \pm 0.20$ ,  $0.28 \pm 0.06$  and  $0.24 \pm 0.04$ . For MYPT1 expression, the FA was again normalized to 1 ( $n = 3$ ). The phasic muscles PV, UB and VD were found to contain more MYPT1 ( $2.22 \pm 0.21$ ,  $1.77 \pm 0.33$  and



**Figure 3. Relative expression of  $\alpha$ -smooth muscle actin, h-calponin, CPI-17 and MYPT1**

Contractile proteins from three rabbit smooth muscle tissues (FA, femoral artery; PV, portal vein; and VD, vas deferens) were probed. For  $\alpha$ -actin and h-calponin,  $1.25 \mu\text{g}$  of total protein was subjected to SDS-PAGE and Western blotting (upper lane in A). For anti-CPI-17 (middle lane) and anti-MYPT1 (lower lane),  $25 \mu\text{g}$  of total protein was used. In B, filled bars are staining intensity for  $\alpha$ -actin and open bars are for h-calponin. The densities for the FA were set to 1 and the relative densities for the PV and VD are plotted ( $n = 4$ ). \* Significant difference from the value for FA.



**Figure 4. Relative expression of CPI-17 and MYPT-1 in six smooth muscle tissues**

The Western blots of h-calponin, CPI-17 and MYPT1 for six different smooth muscle tissues are shown in A. To better compare CPI-17 and MYPT1 expression among different tissues, the samples were first h-calponin matched using the h-calponin density values. The volumes of the tissue sample for subsequent CPI-17 and MYPT1 blots ( $n = 3$ ) were adjusted appropriately to give equal amounts of h-calponin in each tissue lane as shown in A. B(CPI-17) and C(MYPT1) are graphical representations of the h-calponin-matched blots. \* Significant difference from the FA, which was normalized to 1. AO, aorta; TR, trachea; and UB, urinary bladder.

$1.98 \pm 0.23$ , respectively), whereas the tonic AO, TR and FA expressed significantly less (respectively,  $0.98 \pm 0.28$ ,  $1.23 \pm 0.02$  and 1). It is worth mentioning that the phasic PV expressed a relatively high level of CPI-17 with significantly elevated MYPT1 expression. The tonic visceral TR contained less CPI-17 than the tonic arteries, and less MYPT1 than the phasic visceral UB and VD. These results show that the amounts of MLCP and its specific inhibitor CPI-17 vary independently in different smooth muscle tissues.

### Concentration of CPI-17 in smooth muscle cells

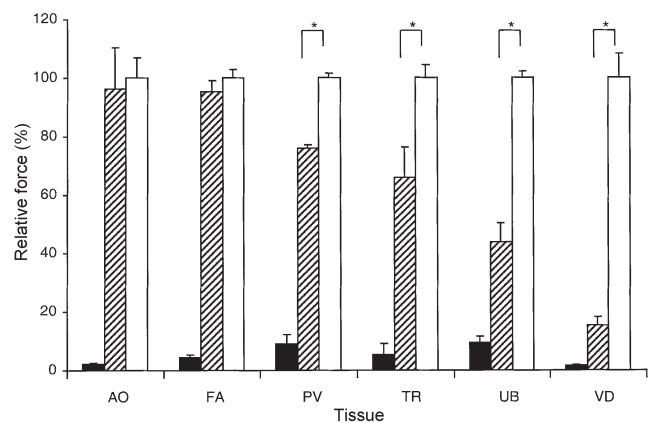
To estimate the intracellular concentration of CPI-17 in different smooth muscles, samples from the FA, PV and VD were probed along with purified CPI-17 protein. As before, these three tissues were carefully cleaned of non-muscle tissue and the samples were then matched for total protein. A volume equal to  $25 \mu\text{g}$  of total protein was applied for the FA and PV and  $50 \mu\text{g}$  for the VD. The greater volume for the VD was necessary to ensure that the blot fell on the linear portion of the curve of the densities for the purified protein (see Fig. 1). The amount of smooth muscle cell volume as compared to the total smooth muscle layer was allowed for in the dilution factor. We assumed muscle cell volume to be 39% for the FA (Gong *et al.* 1992) and 69% for the PV (Todd *et al.* 1983). The VD was also assumed to be 69% as this was the volume previously found for the ileum (Gong *et al.* 1992). Our results found CPI-17 concentrations in the FA, PV and VD samples to be  $6.7 \pm 1.1$ ,  $4.4 \pm 0.7$  and  $0.8 \pm 0.1 \mu\text{M}$ , respectively.

### Tissue-specific PDBu-induced contractions and correlation with CPI-17 expression

To compare the calcium sensitization caused by activation of the PKC–CPI-17 pathway alone as compared to stimulation of all types of G protein, which may activate multiple calcium-sensitizing pathways including RhoA–Rho-kinase–MYPT1 as well as PKC–CPI-17 pathways, we used the PKC activator PDBu and the G protein agonist GTP $\gamma$ S in  $\alpha$ -toxin-permeabilized smooth muscle. The use of an  $\alpha$ -toxin preparation, in contrast to other permeabilization methods such as  $\beta$ -escin, saponin and Triton X-100, had the advantage of retaining endogenous CPI-17 and other small regulatory proteins, whilst allowing control of the intracellular composition and concentration of low molecular weight solutes (Kitazawa *et al.* 1999). In the permeabilized tissue strips,  $[\text{Ca}^{2+}]$  was buffered with 10 mM EGTA and intracellular  $\text{Ca}^{2+}$  stores were eliminated with A23187. The higher  $\text{Ca}^{2+}$  sensitivity of the FA and AO mandated using a lower  $[\text{Ca}^{2+}]$  to produce a submaximal level of control contraction near the threshold, similar to the control contractions of the other smooth muscle strips at higher  $[\text{Ca}^{2+}]$  (Kitazawa *et al.* 1991*a*). Use of the same pCa for all tissues would not allow for a clear indication of the  $\text{Ca}^{2+}$ -sensitizing effects of these agonists. Figure 5 depicts the responses of the various smooth muscle tissues to the addition of PDBu

and GTP $\gamma$ S ( $n = 3–11$ ). As GTP $\gamma$ S markedly potentiated a small force of contraction at a submaximal  $[\text{Ca}^{2+}]$  in all  $\alpha$ -toxin-permeabilized tissues, the control and PDBu-induced contractions were normalized to the force induced by GTP $\gamma$ S for each tissue type. The AO and FA had the most potent responses to PDBu ( $96 \pm 14$  and  $95 \pm 4\%$  of the contraction induced by GTP $\gamma$ S, respectively), and these contractions did not significantly differ from those caused by GTP $\gamma$ S. However, in the PV, TR, UB and VD, the contraction induced by PDBu was significantly less than that caused by GTP $\gamma$ S ( $76 \pm 2$ ,  $66 \pm 10$ ,  $44 \pm 7$  and  $15 \pm 3\%$ , respectively). In summary, we found the vascular tonic muscles showed the greatest response to PDBu and the visceral phasic muscles displayed the weakest reactions to PDBu as compared to GTP $\gamma$ S.

Figure 6 illustrates the relationship between the CPI-17/MYPT1 expression ratios and PDBu-potentiated contractions for the various tissues. The values used for contraction  $(\text{PDBu-induced contraction} - \text{control}) / (\text{GTP}\gamma\text{S-induced contraction} - \text{control})$  were normalized to the FA, the



**Figure 5. Calcium sensitization of contractile response of various rabbit smooth muscle tissues**

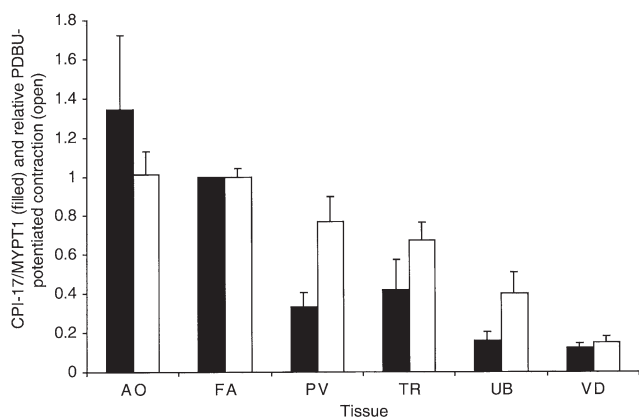
Contractions for each tissue ( $n = 3–11$ ) were normalized to each tissue's contractile force in response to GTP $\gamma$ S treatment (set as 100%). Control contractions were measured at pCa 6.7 for the FA and AO and pCa 6.3 for the PV, TR, UB and VD. The temperature for each condition was 20 °C. Either  $1 \mu\text{M}$  PDBu or  $30 \mu\text{M}$  GTP $\gamma$ S was added. \* Significant difference between the PDBu- and GTP $\gamma$ S-induced contractions in each smooth muscle tissue. Filled bars are  $\text{Ca}^{2+}$  alone, hatched are  $\text{Ca}^{2+}$  + PDBu and open bars are  $\text{Ca}^{2+}$  + GTP $\gamma$ S. The results show different PDBu-induced contractions in different smooth muscles. The GTP $\gamma$ S-induced contractions were  $55 \pm 3.8\%$  for AO,  $64 \pm 1.8\%$  for FA,  $64 \pm 1.0\%$  for PV,  $69 \pm 2.9\%$  for TR,  $82 \pm 1.7\%$  for UB and  $70 \pm 2.0\%$  for VD when compared to contractions induced by pCa 4.5 + GTP $\gamma$ S. Note that in phasic tissues, due to high phosphatase activity, the contractions at pCa 4.5 + GTP $\gamma$ S are still less than full maximal contraction (Kitazawa *et al.* 1991*a*).

result being the relative PDBu-potentiated contractions for each tissue. The PDBu-potentiated contractions tended to increase as the CPI-17/MYPT1 ratio increased.

### Phosphorylation of MLC and CPI-17 by PDBu and GTP $\gamma$ S

To further examine the PKC–CPI-17 signalling pathway leading to inhibition of MLCP, we measured the phosphorylation of MLC and CPI-17 at constant submaximal  $[Ca^{2+}]$  (pCa 6.7 for the FA, 6.3 for the PV and VD) in  $\alpha$ -toxin-permeabilized smooth muscle. MLC phosphorylation levels were significantly increased by GTP $\gamma$ S in the three different tissues (Fig. 7A). As was found in the experiments with the contractile force (Fig. 5), MLC phosphorylation in response to the PKC activator PDBu varied among these smooth muscles ( $n = 3–6$ ). In the FA, PDBu nearly doubled the MLC phosphorylation, from  $35 \pm 1\%$  to a level of  $59 \pm 4\%$  of total MLC, which was not significantly different from that induced by GTP $\gamma$ S ( $60 \pm 3\%$ ). In the PV, PDBu elevated the level of MLC phosphorylation significantly from  $20 \pm 3\%$  to a value of  $34 \pm 4\%$ , which was somewhat less than the GTP $\gamma$ S-induced phosphorylation ( $47 \pm 6\%$ ). PDBu did not significantly increase phosphorylation of MLC in the VD ( $17 \pm 2\%$  as compared to  $20 \pm 4\%$ ). In addition, the PDBu-induced MLC phosphorylation in the VD was significantly less than that induced by GTP $\gamma$ S ( $31 \pm 2\%$ ).

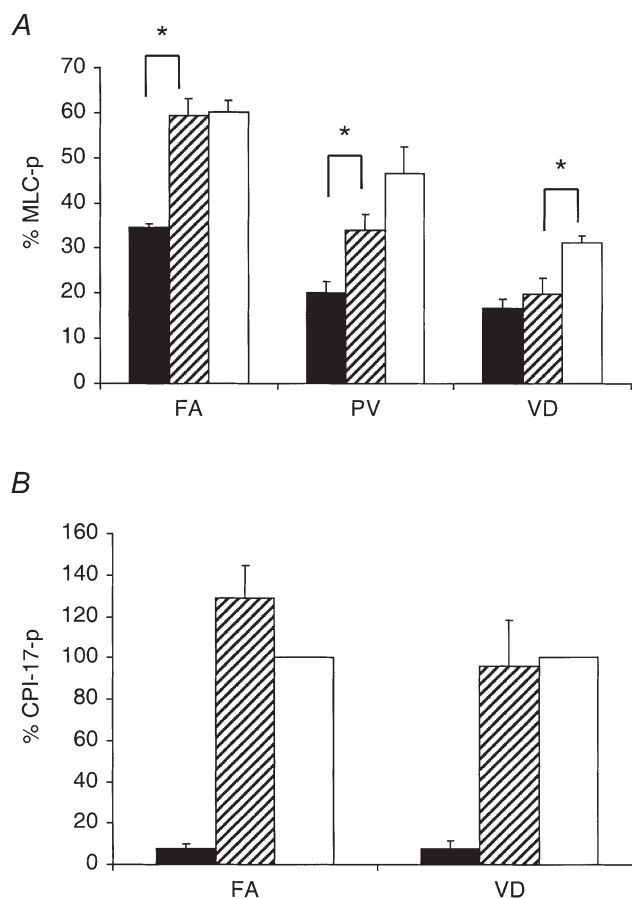
Using an antibody that specifically recognized only CPI-17 phosphorylated at Thr-38 (Kitazawa *et al.* 2000), we found that PDBu and GTP $\gamma$ S both greatly increased levels of phosphorylated CPI-17 in the FA to, respectively, 18- and 14-fold the control phosphorylation



**Figure 6. Correlation between relative expression ratio of CPI-17/MYPT1 and PDBu-potentiated contraction**

PDBu-potentiated contractions (open bars) were plotted along with relative expression of CPI-17 to MYPT1 (filled bars) obtained from data in Fig. 4. PDBu-potentiated contractions were normalized with the FA = 1. Units assigned to the ratio are arbitrary and do not indicate a molar ratio.

level of CPI-17 at pCa 6.7 alone (Fig. 7B;  $n = 3$ ). In the phasic VD, PDBu and GTP $\gamma$ S both increased phosphorylation of CPI-17 at pCa 6.3 by a factor of 13 (Fig. 7B,  $n = 3$ ). For these experiments the blot densities for the GTP $\gamma$ S-treated samples were normalized to 100%. The increase in FA CPI-17 phosphorylation closely paralleled both MLC phosphorylation (Fig. 7A) and force development (Fig. 5). However, in the VD, despite a large increase in relative CPI-17 phosphorylation in response to both PDBu and GTP $\gamma$ S (Fig. 7B), which mirrored the effects seen in the FA, contraction and MLC phosphorylation were significantly increased only with GTP $\gamma$ S but not with PDBu (Fig. 7A).



**Figure 7. Phosphorylation levels of MLC and CPI-17 induced by Ca<sup>2+</sup>, Ca<sup>2+</sup> + PDBu and Ca<sup>2+</sup> + GTP $\gamma$ S**

A illustrates MLC phosphorylation expressed as a percentage of total MLC (% MLC-p;  $n = 3–6$ ) with conditions identical to those of the contraction experiments in Fig. 5. Filled bars are Ca<sup>2+</sup> alone control, hatched bars are control + 1  $\mu$ M PDBu and open bars are control + 30  $\mu$ M GTP $\gamma$ S. \* Significant difference between various treatments. B shows phosphorylation of CPI-17 (CPI-17-p) at Thr-38 in the FA and VD, at control pCa 6.7 (FA) or pCa 6.3 (VD) alone, control + PDBu and control + GTP $\gamma$ S. Values were normalized with GTP $\gamma$ S-induced phosphorylation (100%).



## DISCUSSION

This study clearly demonstrates singular expression of CPI-17, a smooth muscle MLCP inhibitor protein. CPI-17 is specifically localized in smooth muscle, yet expression is highly heterogeneous among individual smooth muscle tissues. CPI-17 is generally expressed more in vascular than visceral tissues regardless of contractile characteristics. Within vascular or visceral tissues, the tonic muscle phenotype appears to contain more CPI-17 than phasic muscles, e.g. the tonic AO and FA contain more CPI-17 than the phasic PV, and the tonic TR more CPI-17 than the phasic UB and VD. The relative order of CPI-17 expression found in the different tissues was identical using either calponin-matched or total protein-matched samples. It is also noteworthy that high expression of CPI-17 was observed, using immunohistochemical analyses, in small blood vessels supplying several organs including the aorta (Fig. 2A), brain, heart, lung and small intestine (data not shown). Therefore, expression of CPI-17 is strictly controlled in smooth muscle. CPI-17 is also expressed in cultured aortic smooth muscle cells, but the degree of expression is markedly decreased upon cell passages (M. Eto, unpublished data), suggesting a dependence on contractile phenotype. It is not detected in cultured fibroblasts including REF52, NIH3T3 and COS7 cells (Eto *et al.* 1999, 2000). CPI-17 expression contrasts with that of another MLCP inhibitor, Rho-kinase, which is ubiquitously expressed *in vivo* and also in various cultured cells (see Somlyo & Somlyo, 2000).

One major downstream effect of the PKC pathway is inhibition of MLCP with a resultant increase in the calcium sensitivity of MLC phosphorylation and contraction (Itoh *et al.* 1993; Masuo *et al.* 1994; Gailly *et al.* 1997). The amount of MLCP might therefore be one of the modifying factors of PKC activator-induced  $\text{Ca}^{2+}$  sensitization of contraction. This study indicates differential expression of MYPT1 (for MLCP) in various smooth muscle tissues. Relative expression correlates to the mode of contraction and relaxation with higher MLCP content in phasic compared to tonic smooth muscle (Kitazawa *et al.* 1991a; Gong *et al.* 1992; this study). The rate of MLC dephosphorylation was about 4 times slower in the  $\alpha$ -toxin-permeabilized FA as compared to the PV (Gong *et al.* 1992), consistent with several times less MLCP content in the former as compared to the latter. However, the correlation between MLCP content and PKC-induced contraction is very low, which suggests little association between PKC-induced  $\text{Ca}^{2+}$  sensitization and MLCP content alone. Since MLCP activity can be regulated by CPI-17, the balance between CPI-17 and MLCP expression may be related to the various levels of PKC-induced  $\text{Ca}^{2+}$  sensitization in different tissues. We estimated the cellular concentration of CPI-17 to be from 0.8  $\mu\text{M}$  in the phasic visceral VD up to 6–7  $\mu\text{M}$  in the tonic arterial FA. Attempts to estimate the concentration of MLCP were frustrated by the instability of recombinant

MYPT1. As reported previously, the concentration of MLCP in smooth muscle has been estimated, based on specific activity, to be 'at least' sub-micromolar in myofibrils from phasic chicken gizzard muscle (Alessi *et al.* 1992), suggesting a similar order of magnitude in cellular concentration as compared to CPI-17. In the FA, the majority of CPI-17 was phosphorylated by PDBu (Kitazawa *et al.* 2000), and the rate of MLC dephosphorylation was inhibited to half of control by a saturated concentration of PDBu (Masuo *et al.* 1994). Thus we propose that the functional concentration of MLCP is roughly as much as functional CPI-17 in tonic arterial smooth muscle. This results in a large calcium sensitization of MLC phosphorylation and contraction by a PKC activator. Therefore the concentration of CPI-17 is sufficient to act as a physiological inhibitor for MLCP in tonic arterial smooth muscle. Yet in the VD, relative CPI-17 expression is several times less and MYPT1 expression several times more than in the tonic arteries, suggesting that the CPI-17 content is significantly less than the MLCP content. Furthermore, despite a similar relative increase in the amount of CPI-17 phosphorylation in the FA and VD, only the FA had a concurrent increase in MLC phosphorylation and contraction by PDBu. This implies that CPI-17 content limits the effects of PKC activation, and that other G protein pathways are responsible for the GTP $\gamma$ S-induced calcium sensitization in phasic visceral tissue. Application of 5  $\mu\text{M}$  thiophosphorylated exogenous CPI-17 to Triton X-100-demembrated TR, VD and UB visceral smooth muscle also caused a large calcium sensitization (data not shown), as seen in demembrated FA smooth muscle (Li *et al.* 1998; Kitazawa *et al.* 1999). This suggests that endogenous CPI-17 in visceral smooth muscle has the same  $\text{Ca}^{2+}$ -sensitizing effect as in the FA, and that both the cellular concentration of CPI-17 and the ratio between CPI-17 and MLCP are important factors in the regulation of  $\text{Ca}^{2+}$  sensitivity. Therefore tonic arterial smooth muscle with high CPI-17 and low MLCP content responds to PKC stimulation with a large increase in  $\text{Ca}^{2+}$  sensitivity. In contrast, phasic smooth muscle with low CPI-17 and high MLCP content has a small to insignificant response to a PKC activator at constant  $[\text{Ca}^{2+}]$ . A tissue such as the phasic PV with relatively high CPI-17 and high MLCP content, or the tonic TR with relatively low CPI-17 and low MLCP content should have a moderate increase in calcium sensitivity due to PKC stimulation. We thus propose that the unique order of the CPI-17/MLCP ratio correlates to the degree of  $\text{Ca}^{2+}$  sensitization of contraction and MLC phosphorylation induced by the PKC activator phorbol ester.

In addition to expression levels of CPI-17 and MLCP, protein kinase action on CPI-17 is another factor in the regulation of MLCP under stimulation by  $\text{Ca}^{2+}$ -sensitizing agonists. Phorbol ester, GTP $\gamma$ S and excitatory agonists, but not high  $\text{K}^+$  alone, significantly increase the level of

phospho-CPI-17 found in  $\alpha$ -toxin-permeabilized FA (Kitazawa *et al.* 2000; this study). Histamine-induced contraction and phosphorylation of CPI-17 is blocked by the G-protein inhibitor GDP $\beta$ S, and is partially inhibited by either the Rho-kinase inhibitor Y27632 or the PKC inhibitor GF109203X. These results suggest the existence of complex pathways for calcium sensitization. PKC- $\delta$  has been isolated as a dominant kinase for CPI-17 from pig AO, and this kinase is also partially inhibited by Y27632 (Eto *et al.* 2001). Thus, CPI-17 could be phosphorylated by PKC- $\delta$  in histamine-stimulated arterial smooth muscles, even though this response is sensitive to different kinase inhibitors. Recently it has been reported that CPI-17 can be phosphorylated at Thr-38 by Rho-kinase and another RhoA-activated protein kinase (PKN) *in vitro* (Koyama *et al.* 2000; Hamaguchi *et al.* 2000). In spite of a low specific activity compared to PKC, phosphorylation of CPI-17 by RhoA-associated kinases might be physiological. We propose that CPI-17 is a point of mutual convergence, where various Ca<sup>2+</sup>-sensitizing pathways meet for inhibition of MLCP in a particular smooth muscle type.

In tonic arterial smooth muscle, the G protein activator GTP $\gamma$ S increases phosphorylation of *in situ* CPI-17 to a level similar to that invoked by PDBu (this study), inhibits MLCP activity by about 50%, which is also similar to PDBu action (Kitazawa *et al.* 1991*b*; Masuo *et al.* 1994), and increases the calcium sensitivity of MLC phosphorylation and contraction to a level close to that induced by PDBu (Masuo *et al.* 1994; this study). These results imply that in vascular smooth muscle with a high CPI-17/MLCP expression ratio, the G protein-mediated inhibition of MLCP is mainly through the CPI-17–MLCP signalling pathway. Contractile Ca<sup>2+</sup> sensitivity in response to a PKC activator appears higher in the arterial smooth muscle of spontaneously hypertensive rats (Silver *et al.* 1992; Sasajima *et al.* 1997) and in coronary artery vasospasm in a swine model (Kadokami *et al.* 1996). These findings suggest that CPI-17 may be involved in vascular tone disorders, although RhoA/Rho-kinase signalling is another strong candidate for these smooth muscle abnormalities (Kandabashi *et al.* 2000; Sato *et al.* 2000).

In contrast, the expression of CPI-17 in phasic visceral smooth muscle is significantly less as compared to MYPT1, and the PDBu-induced MLC phosphorylation and contraction are much lower than those caused by GTP $\gamma$ S. This indicates that another mechanism(s) for the G protein-mediated inhibition of MLCP exists in phasic visceral smooth muscle. MYPT1 phosphorylation is another crucial factor involved in the regulation of MLCP. Several G protein-coupled receptor agonists and the direct G protein-activator GTP $\gamma$ S significantly increase MLC phosphorylation through inhibition of MLCP in phasic as well as tonic smooth muscle, and even in cultured smooth muscle and fibroblast cells (see review

by Somlyo & Somlyo, 2000). It has recently been shown that MYPT1 is phosphorylated in response to GTP $\gamma$ S in permeabilized portal vein (Trinkle-Mulcahy *et al.* 1995), ileum (Swärd *et al.* 2000), cultured aortic smooth muscle cells (Nagumo *et al.* 2000) and in response to lysophosphatidic acid in Swiss 3T3 cells (Feng *et al.* 1999). MYPT1 phosphorylation is associated with inhibition of the catalytic activity of MLCP *in vitro* (Hartshorne *et al.* 1998), and this phosphorylation is inhibited *in situ* by Y27632 and HA1077, another Rho-kinase inhibitor (Feng *et al.* 1999; Swärd *et al.* 2000; Nagumo *et al.* 2000). These results support the hypothesis that activation of the RhoA/Rho-kinase pathway leads to phosphorylation of MYPT1 and is the predominant route in phasic muscle for inhibition of MLCP and the concomitant increase in MLC phosphorylation and contraction.

In conclusion, we describe the signalling pathway for calcium sensitization as consisting of several branches and possible parallel pathways. All tissues respond to GTP $\gamma$ S, which would activate heterotrimeric and monomeric G proteins. When using a specific activator (PDBu) of the PKC portion of G protein-induced calcium sensitization, different smooth muscles showed variable responses. The sensitization of contraction in each smooth muscle is individually modulated, not only by variation in agonist receptors, but also by tissue-specific expression of regulatory/contractile proteins and possibly disease state. Yet this is logical in that smooth muscle is highly specialized in a multitude of ways, and the G-protein pathway for calcium sensitization is another example of the intricacies involved in smooth muscle function.

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