Inhibition by calponin of isometric force in demembranated vascular smooth muscle strips: the critical role of serine-175

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α -Calponin is a thin-filament-associated protein which has been implicated in the regulation of smooth muscle contraction. Quantification of the tissue content of rat tail arterial smooth muscle revealed approximately half the amount of α -calponin relative to actin compared with chicken gizzard and other smooth muscles, suggesting that this tissue would be particularly suitable for investigation of the effects of exogenous α -calponin on the contractile properties of permeabilized muscle strips. Rat tail arterial strips demembranated with Triton X-100 retained $\approx 90\%$ of their complement of α -calponin, and exogenous chicken gizzard α -calponin (which conveniently has a slightly lower molecular mass than the rat arterial protein) bound to the permeabilized muscle, presumably through its high affinity for actin. Exogenous α -calponin inhibited force in demembranated muscle strips in a concentration-dependent manner when added at the peak of a submaximal Ca^{2+} -induced contraction, with a half-maximal effect at $\approx 3\ \mu\text{M}$ α -calponin. Pretreatment of demembranated muscle strips with α -calponin inhibited subsequent force development at all concentrations of Ca^{2+} examined over the activation range. The inhibitory effect of α -calponin was shown to be Ca^{2+} -independent, since exogenous α -calponin also inhibited force in the absence of Ca^{2+} in demembranated muscle

strips containing thiophosphorylated myosin. Phosphorylation of α -calponin on Ser-175 by protein kinase C has been suggested to alleviate the inhibitory effect of α -calponin on smooth muscle contraction. To test this hypothesis, the effects on Ca^{2+} -induced and Ca^{2+} -independent contractions of demembranated muscle strips of phosphorylated α -calponin and three site-specific mutants of α -calponin (in which Ser-175 was replaced by Ala, Asp or Thr) were compared with the effects of unphosphorylated tissue-purified and recombinant wild-type α -calponins. The recombinant wild-type protein behaved identically to the unphosphorylated tissue-purified protein, as did the S175T mutant, which is known to bind actin with high affinity and to inhibit the actin-activated myosin MgATPase *in vitro*. On the other hand, phosphorylated α -calponin and the S175A and S175D mutants, which bind weakly to actin and have little effect on the actin-activated myosin MgATPase *in vitro*, failed to cause significant inhibition of force induced by Ca^{2+} or myosin thiophosphorylation. These results support a role for α -calponin in the regulation of smooth muscle contraction and indicate the functional importance of Ser-175 of α -calponin as a regulatory site of phosphorylation.

INTRODUCTION

The α - or h1- isoform of calponin [1,2] is a smooth-muscle-specific, thin-filament-associated protein which has been implicated in the regulation of smooth muscle contraction [3]. The isolated protein binds to actin with high affinity [4,5] and thereby inhibits the actin-activated myosin MgATPase (cross-bridge cycling rate) [6,7]. Consistent with this effect, α -calponin inhibits the movement of fluorescently labelled actin filaments over immobilized myosin [8,9] and, when added to permeabilized smooth muscle strips, calponin inhibits force development and reduces the unloaded shortening velocity [10–12]. Calponin is phosphorylated *in vitro* by two protein serine/threonine kinases, protein kinase C (PKC) and Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) [6,13], and is dephosphorylated by type 2A [14] and 2B protein phosphatases [15]. Phosphorylation occurs predominantly at Ser-175 [13], although Nakamura et al. [16] concluded that Thr-184 was the principal site of phosphorylation by PKC. Phosphorylation markedly lowers the affinity of α -calponin for actin [6,13] and alleviates inhibition of the cross-

bridge cycling rate [6]; these properties are restored following dephosphorylation [14,15]. Several examples have been reported of an increase in α -calponin phosphorylation in intact smooth muscle in response to contractile stimuli [13,17–20], although some investigators have concluded that α -calponin is not phosphorylated *in vivo* [21–23].

Bárány and Bárány [22] determined that α -calponin is expressed in a variety of porcine smooth muscle tissues at a molar ratio to tropomyosin of $\approx 1:1$, i.e. $\approx 1:6$ or $1:7$ actin monomers. Takahashi et al. [24] concluded similarly that α -calponin is present in chicken gizzard smooth muscle at the same molar concentration as tropomyosin. From a quantitative analysis of α -calponin expression in a variety of chicken tissues, we found that phasic muscles (e.g. gizzard, intestine and ureter) contained higher levels of α -calponin than tonic muscles (e.g. arteries) [25]. Defining the gizzard α -calponin content as 1.0, levels in chicken intestine, ureter, aorta and veins were 1.8, 0.7, 0.4 and 1.5 respectively. In agreement with variable tissue contents of α -calponin, Bárány and Bárány [22] calculated the concentration of α -calponin to range from 107 to 222 μM in various porcine

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; CaM kinase II, Ca^{2+} /calmodulin-dependent protein kinase II; PKC, Ca^{2+} - and phospholipid-dependent protein kinase C.

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smooth muscle tissues. We sought a smooth muscle which expresses a lower molar ratio of α -calponin/actin. Such a tissue would provide a suitable experimental system for investigating the effects of exogenous α -calponin on the contractile properties of permeabilized smooth muscle strips, since there would be less of a contribution from endogenous α -calponin and presumably more capacity for binding of exogenous α -calponin. It should be noted that purified F-actin filaments can bind 1 molecule of α -calponin per 1–3 actin monomers [4,26].

To address the physiological role of α -calponin and, in particular, the importance of Ser-175, we have compared the effects on the contractile properties of demembranated rat tail arterial muscle strips of phosphorylated and unphosphorylated tissue-purified α -calponin, recombinant wild-type α -calponin and three site-specific mutants of α -calponin in which Ser-175 was replaced by Ala, Asp or Thr. The results show that: (i) rat tail arterial smooth muscle expresses approximately half the amount of α -calponin, relative to actin, compared with other smooth muscle tissues, (ii) calponin inhibits Ca^{2+} -induced isometric force development in a concentration-dependent manner, (iii) the inhibitory effect of calponin on force development is independent of Ca^{2+} , and (iv) Ser-175 plays a critical role in the inhibitory effects of calponin.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (> 5000 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). Triton X-100 was purchased from Calbiochem (La Jolla, CA, U.S.A.), and adenosine 5'- $[\gamma\text{-thio}]\text{triphosphate}$ (ATP[S]), phosphocreatine and creatine kinase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L- α -Phosphatidyl-L-serine and 1,2-diolein were purchased from Serdary Research Laboratories (London, Ontario, Canada). Electrophoresis reagents and molecular mass markers were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). General laboratory reagents were of analytical grade or better and were purchased from CanLab (Edmonton, Alberta, Canada).

Protein purification

The following proteins were purified by methods previously described: bovine brain calmodulin [27], rat brain PKC (a mixture of α , β and γ isoenzymes) [28] and chicken gizzard α -calponin [6]. Wild-type and mutant α -calponins, the latter engineered by PCR mutagenesis, were expressed in *Escherichia coli* and purified as previously described [29].

Electrophoresis and immunoblotting

Rat tail arterial smooth muscle strips, before and after demembranation with Triton X-100 and following incubation with chicken gizzard α -calponin ($5\text{ }\mu\text{M}$) at $20\text{ }^{\circ}\text{C}$ for 1.5 h and washing for 1 h in relaxing solution, were quick frozen in 10% trichloroacetic acid in solid CO_2 /acetone. Trichloroacetic acid was washed out with acetone/10 mM dithiothreitol and the sample was lyophilized. SDS gel sample buffer [30] ($25\text{ }\mu\text{l}$) was added to the muscle strips, which were then homogenized with a hand-operated glass homogenizer and boiled for 3 min. An additional $25\text{ }\mu\text{l}$ of SDS gel sample buffer was added and the samples were subjected to SDS/PAGE in 10–20% acrylamide gradient gels with a 5% stacking gel using the discontinuous buffer system of

Laemmli [30]. Gels were stained in 55% (v/v) ethanol/10% (v/v) acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue R-250, and diffusion destained in 20% (v/v) ethanol/5% (v/v) acetic acid. Actin and α -calponin bands were quantified by densitometric scanning using a Pharmacia Image Master Desktop Scanning System. This method of quantification was also applied to compare endogenous chicken gizzard and rat tail arterial smooth muscle α -calponin contents. This approach is valid given the similarity between the chicken and rat proteins, which exhibit 83% sequence identity; the rat protein is just five amino acids longer [1,30a].

For immunoblotting, following SDS/PAGE proteins were transferred at 80 V for 3 h to nitrocellulose membranes ($0.45\text{ }\mu\text{m}$) in 25 mM Tris, 192 mM glycine, 20% methanol and 0.1% SDS (pH 8.3). Air-dried membranes were immersed in 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.05% Tween 20 (TTBS) for 2×10 min, and blocked by immersion for 2 h at $20\text{ }^{\circ}\text{C}$ in TTBS containing 5% milk powder and 0.02% NaN_3 . Membranes were then washed (2×10 min) in TTBS prior to incubation overnight at $4\text{ }^{\circ}\text{C}$ in TBS (TTBS minus Tween 20) containing 1% BSA plus rabbit anti-(chicken gizzard α -calponin) prepared as previously described [6]. After three washes (10 min each) in TTBS, membranes were incubated for 2 h at $20\text{ }^{\circ}\text{C}$ with goat anti-(rabbit IgG)–(alkaline phosphatase) conjugate (1:10000 dilution) in TTBS containing 2% milk powder and 0.02% NaN_3 . After three washes (10 min each) with TTBS and one wash (10 min) with TBS, colour development was achieved with 5-bromo-4-chloro-3-indolyl phosphate (0.165 mg/ml)/Nitro Blue Tetrazolium (0.33 mg/ml) in 0.1 M Tris/HCl (pH 9.5), 0.1 M NaCl and 5 mM MgCl_2 .

Phosphorylation of α -calponin by PKC

Chicken gizzard α -calponin ($11.8\text{ }\mu\text{M}$) was incubated at $30\text{ }^{\circ}\text{C}$ for 60 min with PKC ($4\text{ }\mu\text{g/ml}$) in 20 mM Pipes/KOH (pH 7.0), 5 mM MgCl_2 , 0.2 mM CaCl_2 , 370 μM phosphatidylserine, 93 μM 1,2-diolein, 0.03% (w/v) Triton X-100 and 0.2 mM unlabelled ATP or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (≈ 100 c.p.m./pmol). The stoichiometry of phosphorylation of α -calponin was determined, as previously described [31], to be 1.13 mol of P_i /mol of calponin.

Preparation of permeabilized muscle strips and tension measurement

Tail arterial smooth muscle helical strips ($0.5\text{ mm} \times 2\text{--}3\text{ mm}$) were dissected from male Sprague–Dawley rats (250–350 g) into buffered physiological saline solution (see below) at room temperature ($20\text{ }^{\circ}\text{C}$) and the endothelial layer removed mechanically. Muscle strips were chemically demembranated by incubation with 0.5% Triton X-100 for 20 min, stored at $-20\text{ }^{\circ}\text{C}$ in 50% glycerol in relaxing solution (see below) and used within 6 days. Contractile responses of the permeabilized smooth muscle strips were measured at room temperature as described previously [32]. Briefly, muscle strips were maintained horizontally between two hooks and immersed in a pool of solution (0.3 ml) which was rounded by surface tension on a rotation plate of 6 cm diameter. One hook was connected to a force/displacement transducer mounted on a micromanipulator and the other to a second micromanipulator. Solution changes were effected rapidly by rotation of the plate.

Solutions for muscle preparation and tension measurements

Buffered physiological saline solution was composed of 137 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl_2 , 2.2 mM CaCl_2 , 14 mM

glucose and 10 mM Hepes/NaOH (pH 7.4). Relaxing solution (pCa $\approx$ 8.5) contained 94 mM potassium methanesulphonate, 5.1 mM Na₂ATP, 5.3 mM magnesium methanesulphonate, 2 mM EGTA, 10 mM phosphocreatine, 10 units/ml creatine kinase and 20 mM Pipes/KOH (pH 7.0). Free Ca²⁺ concentrations were calculated using the chelation constant of EGTA reported by Martell and Smith [33]. Ionic strengths of solutions were maintained at 0.2 M by adjusting the concentration of potassium methanesulphonate. All tension measurements were carried out in the presence of 1 μ M calmodulin at room temperature (20 °C). Rigor solution contained 150 mM potassium methanesulphonate, 1 mM magnesium methanesulphonate, 2 mM EGTA and 20 mM Pipes/KOH (pH 7.0).

RESULTS

Quantitative analysis of the α -calponin content of rat tail arterial smooth muscle as described in the Materials and methods section revealed this tissue to contain only approximately half the amount of α -calponin relative to actin as does chicken gizzard smooth muscle. The α -calponin/actin molar ratio was determined by densitometric scanning of Coomassie Blue-stained SDS/polyacrylamide gels of tissue extracts to be 0.11 ± 0.03 (mean $\pm$ S.D., $n = 10$) for rat tail artery and 0.21 ± 0.04 (mean $\pm$ S.D., $n = 6$) for chicken gizzard.

The protein composition of rat tail arterial smooth muscle strips was compared by SDS/PAGE prior to and following demembranation with Triton X-100, as described in the Materials and methods section. Figure 1 shows the Coomassie Blue-stained gel of a muscle strip before (lane 1) and after (lane 2) Triton X-100 treatment. Several protein bands were lost by the skinning procedure, as expected since all membranes have been destroyed and soluble proteins washed out. The contractile machinery, however, is retained as shown by the preservation of actin and

Figure 1 α -Calponin is retained in rat tail arterial smooth muscle strips demembranated with Triton X-100

The protein composition of rat tail arterial strips was analysed by SDS/PAGE before (lane 1) and after (lane 2) Triton X-100 treatment as described in the Materials and methods section. Purified chicken gizzard α -calponin (5 μ g) is shown in lane 3 for comparison. Lanes 1–3, Coomassie Blue-stained gels; lane 4, immunoblot of sample as in lane 2 (rat tail artery after Triton X-100 treatment) with anti- α -calponin; lane 5, immunoblot of purified chicken gizzard α -calponin with anti- α -calponin. Tm, tropomyosin; CaP_A, arterial α -calponin; CaP_G, gizzard α -calponin. Some breakdown products of gizzard α -calponin are evident in lanes 3 and 5 due to its extreme sensitivity to proteolysis [34].

Figure 2 Exogenous α -calponin binds to demembranated rat tail arterial smooth muscle strips

Coomassie Blue-stained gel of: lane 1, molecular mass markers (numbers on the left indicate sizes in kDa); lane 2, purified chicken gizzard α -calponin (2 μ g); lane 3, Triton X-100-demembranated rat tail arterial strip; lane 4, demembranated strip incubated with chicken gizzard α -calponin and washed as described in the Materials and methods section. Tm, tropomyosin; CaP_A, arterial α -calponin; CaP_G, gizzard α -calponin.

tropomyosin. α -Calponin was identified by immunoblotting using an antibody raised against chicken gizzard α -calponin (Figure 1, lane 4). We have shown previously that this antibody recognizes mammalian and amphibian as well as avian α -calponins [35]. A Coomassie Blue-stained gel of purified chicken gizzard α -calponin and the corresponding immunoblot are shown in lanes 3 and 5 respectively of Figure 1 for comparison. Rat tail arterial α -calponin clearly has a significantly higher molecular mass than the chicken gizzard protein, as expected for mammalian α -calponins: from the deduced amino acid sequences, the molecular masses of rat and chicken α -calponins are 33342 and 32333 Da respectively [1,30a]. Densitometric scanning of lanes 1 and 2 in Figure 1 revealed that $\approx$ 90% of the α -calponin was retained in the muscle strip following Triton X-100 treatment, consistent with its physical association with the thin filaments [35,37].

Prior to investigation of the effects of exogenous α -calponin on demembranated rat tail arterial strips, we verified that added chicken gizzard α -calponin was indeed incorporated into such muscle strips. Comparison of lanes 3 and 4 in Figure 2 shows that, following incubation of demembranated rat tail artery with purified α -calponin and washout of unbound protein, a significant amount of the exogenous α -calponin was incorporated into the muscle. Given the high affinity of smooth muscle actin for α -calponin, the specificity of this interaction and the maximal binding stoichiometry of 1 calponin:1–3 actin monomers achievable *in vitro* [4,5,24,26,38], we conclude that the bound exogenous α -calponin is probably associated with the thin filaments in the demembranated muscle strips. It is also apparent from Figure 2 that the amount of all proteins in lane 4 is somewhat lower than in lane 3. This is attributable to differences in size of the muscle strips and to some degree of loss of all tissue proteins during the extensive washing following addition of exogenous α -calponin. However, the relative amounts of α -calponin, tropomyosin and actin remain constant, as shown by scanning densitometry.

Figure 3(A) shows the contractile response of a Triton X-100-

Figure 3 α -Calponin inhibits force in permeabilized rat tail arterial smooth muscle strips

(A) Triton X-100-permeabilized muscle strips in relaxing solution (R2G) were induced to contract submaximally by transfer to pCa 6.3 solution and subsequently relaxed slowly at pCa 6.3 or rapidly upon return to relaxing solution. The gradual loss of force at pCa 6.3 was observed whether or not the solution was changed at the peak of the contraction. (B) α -Calponin (5 μ M) was added at the peak of the Ca^{2+} -induced contraction at pCa 6.3, causing a marked reduction in force. (C) Concentration-dependent inhibition of force at pCa 6.3 by α -calponin. Key to symbols: ∇ , control ($n = 9$); $\blacktriangle$, 1 μ M α -calponin ($n = 5$); $\blacksquare$, 3 μ M α -calponin ($n = 4$); $\bullet$, 5 μ M α -calponin ($n = 6$); $\blacktriangledown$, 10 μ M α -calponin ($n = 4$). (D) Plot of force at time 150 min relative to the peak of the pCa-6.3-induced contraction as a function of α -calponin concentration (data taken from C). Data in (C) and (D) are expressed as means $\pm$ S.E.M.

demembranated rat tail arterial smooth muscle strip to a submaximal concentration of Ca^{2+} (pCa 6.3). Following addition of Ca^{2+} , force rose quite rapidly to a peak and slowly declined over a prolonged period of time. Addition of α -calponin at the peak of the contraction induced by Ca^{2+} resulted in a much greater loss of force (Figure 3B). Removal of Ca^{2+} in the absence or presence of α -calponin then resulted in rapid and complete relaxation (Figures 3A and 3B). Figure 3(C) depicts the inhibitory effects of increasing concentrations of α -calponin. The protocol was as in Figure 3(B), and 100% relative force refers to the maximal force induced by increasing the free Ca^{2+} concentration to pCa 6.3; the indicated concentrations of α -calponin were added to the medium bathing the muscle strips at the peak of the Ca^{2+} -induced contraction (time 0). α -Calponin clearly inhibited force in a concentration-dependent manner. This effect is emphasized in Figure 3(D), a plot of force remaining at time 150 min as a function of α -calponin concentration. A half-maximal reduction in force was observed at $\approx 3 \mu\text{M}$ α -calponin.

The effects of tissue-purified α -calponin (phosphorylated and unphosphorylated), recombinant wild-type α -calponin and three site-specific mutants of α -calponin are compared in Figure 4(A). The protocol again was as in Figure 3(B), with the various calponins added at the peak of the Ca^{2+} -induced contraction.

Force was inhibited, as before, by α -calponin purified from chicken gizzard. Similar effects were seen with bacterially expressed wild-type α -calponin and the S175T mutant. On the other hand, the inhibitory effects of phosphorylated chicken gizzard α -calponin and the S175A and S175D mutants were significantly lower. Figure 4(B) shows the force remaining at time 150 min in the absence of α -calponin (control) and in the presence of the indicated α -calponin species. Statistically significant reductions in force were observed only with the tissue-purified and recombinant wild-type α -calponins and the S175T mutant.

The experiments depicted in Figure 5 were designed to determine the effects of α -calponin on contraction of permeabilized rat tail arterial smooth muscle strips at submaximal and maximal Ca^{2+} concentrations. Figure 5(A) shows the control experimental protocol: contraction was induced in Triton X-100-permeabilized strips by incremental addition of Ca^{2+} to the bathing medium as shown. Maximal contraction was observed at pCa 5.5. Relaxation occurred upon removal of Ca^{2+} , and the incremental addition of Ca^{2+} was repeated 90 min later. The sensitivity to Ca^{2+} was unchanged but the force developed was somewhat reduced, as expected from the results of Figures 3 and 4. Following treatment with α -calponin in relaxing solution for 90 min, the force generated by the addition of Ca^{2+} was reduced

Figure 4 Effects of tissue-purified α -calponin (phosphorylated and unphosphorylated), recombinant wild-type α -calponin and three site-specific mutants of α -calponin on force in permeabilized rat tail arterial smooth muscle strips

The experimental protocol was as in Figure 3(B). At the peak of the pCa-6.3-induced contraction (time zero), the following additions were made: none (Control; $n = 9$; ▽); tissue-purified (unphosphorylated) α -calponin (CaP; $n = 6$; ●); phosphorylated α -calponin (P-CaP; $n = 4$; △); recombinant wild-type α -calponin (WT; $n = 5$; ○); S175A ($n = 4$; □); S175D ($n = 5$; ■); or S175T ($n = 4$; ▲). Where present, α -calponin was at $5 \mu\text{M}$. (A) Time courses of force reduction under control conditions and in the presence of the indicated α -calponin species. (B) Force remaining at $t = 150$ min in the presence of the indicated α -calponin species, expressed as a percentage of the peak of pCa-6.3-induced force (recorded at $t = 0$). Data are expressed as means $\pm$ S.E.M. Asterisks indicate a significant difference from the control as assessed by Student's t test ($P < 0.05$).

relative to the control at each free Ca^{2+} concentration (Figure 5B). This was confirmed by quantitative analysis of three independent experiments (Figure 5C).

To determine whether or not the relaxant effect of α -calponin is Ca^{2+} -dependent, its effect on permeabilized strips containing thiophosphorylated myosin was examined in the absence of Ca^{2+} . Figure 6(A) summarizes the experimental protocol: maximal contraction was first elicited by addition of Ca^{2+} to pCa 5, followed by relaxation upon return to EGTA-containing relaxing solution. ATP and ATP-regenerating-system components were then removed by washing in rigor solution. Addition of ATP[S] in the presence of Ca^{2+} (pCa 6.3) then allowed myosin thiophosphorylation to occur; very little force was elicited under these conditions since ATP[S], being a non-hydrolysable analogue of ATP, is not a substrate of actomyosin and therefore does not support cross-bridge cycling. A second wash with rigor

Figure 5 α -Calponin reduces the force generated by the addition of Ca^{2+} at each free Ca^{2+} concentration in permeabilized rat tail arterial smooth muscle strips

(A) and (B) Triton X-100-permeabilized muscle strips in relaxing solution were transferred sequentially to solutions of steadily increasing free Ca^{2+} concentrations as indicated. Following maximal contraction at pCa 5, muscle strips were returned to relaxing solution without (A) or with (B) $5 \mu\text{M}$ α -calponin and incubated for a prolonged period of time as shown, prior to repeating the Ca^{2+} titration protocol. (C) Force-pCa relationships following incubation without (○) or with (●) $5 \mu\text{M}$ α -calponin; force is expressed as a percentage of the force elicited at pCa 5 in the initial Ca^{2+} titration. Values represent means $\pm$ S.E.M. of three independent measurements.

solution then served to remove the ATP[S] and Ca^{2+} , following which addition of ATP in the absence of Ca^{2+} elicited an immediate and close-to-maximal contractile response. As in earlier experiments, force slowly decayed with time. Figure 6(B) shows that α -calponin enhanced the loss of force in thiophosphorylated muscle strips in the absence of Ca^{2+} , as it did earlier with phosphorylated muscle strips in the presence of Ca^{2+} (Figure 3A).

Figure 6 α -Calponin-mediated inhibition of force in permeabilized rat tail arterial smooth muscle strips is independent of Ca^{2+}

A control pCa-5-induced contraction was followed by relaxation in relaxing solution (R2G). ATP and the ATP-regenerating system were removed by washing in rigor solution (r). ATP[S] (ATP γ S; 1 mM) was then added in pCa 6.3 solution to thiophosphorylate the myosin light chains and was washed out with rigor solution. A return to relaxing solution elicited a rapid, near-maximal, contraction followed by slow relaxation (A). In (B), α -calponin (5 μ M) was added at the peak of the Ca^{2+} -independent contraction and caused a marked inhibition of force.

Cumulative data are shown in Figure 7(A), which also demonstrates that bacterially expressed wild-type α -calponin and the S175T mutant had quantitatively similar effects as the tissue-purified protein, whereas phosphorylated α -calponin and the S175A and S175D mutants were without effect. These conclusions are emphasized by comparison of the force remaining at time 150 min in the absence of α -calponin and in the presence of the various species of α -calponin (Figure 7B).

DISCUSSION

Many properties of α -calponin suggest that it plays a physiological role in thin-filament-mediated regulation of smooth muscle contraction: (i) it is associated with actin filaments *in situ* [17,37,39]; (ii) it binds to purified actin or actin/tropomyosin with high affinity [4,5,16]; (iii) through its interaction with actin it inhibits the actin-activated myosin MgATPase [6,7]; (iv) it inhibits the movement of actin filaments over immobilized phosphorylated or thiophosphorylated smooth muscle myosin [8,9,20]; (v) when applied to permeabilized smooth muscle strips, it attenuates force and reduces shortening velocity [10–12]; and (vi) a synthetic peptide corresponding to Phe-173–Arg-185 of α -calponin, which binds to actin but does not inhibit the actin-activated myosin MgATPase, blocks the inhibitory effect of α -

calponin in permeabilized smooth muscle strips and enhances the contraction elicited at submaximal Ca^{2+} concentrations, presumably by displacing α -calponin from the thin filaments [40]. Furthermore, a similar peptide (corresponding to Leu-166–Gly-194) elicited a concentration-dependent contraction of single isolated ferret aortic smooth muscle cells permeabilized with saponin, presumably by alleviating the inhibitory effect of endogenous α -calponin [41].

Phosphorylation of calponin lowers its affinity for actin [6,13] and alleviates its inhibition of actin-activated myosin MgATPase activity [6], actin filament movement [20], force and unloaded shortening velocity [10,12]. We have shown that Ser-175 is the principal site of phosphorylation by PKC or CaM kinase II [6,13], but Nakamura et al. [16] claimed that PKC preferentially phosphorylated Thr-184. To assess further the role of α -calponin in the regulation of smooth muscle contraction, and particularly the importance of Ser-175, we have compared the effects of the application of exogenous unphosphorylated α -calponin, phosphorylated α -calponin and three site-specific mutants of α -calponin, in which Ser-175 was replaced by Ala, Asp or Thr, on force development in Triton X-100-demembranated rat tail arterial smooth muscle strips. Rat tail artery was selected as the tissue of choice since it has a low endogenous α -calponin content. When demembranated rat tail arterial strips were incubated with chicken gizzard α -calponin, the exogenous α -calponin bound to the muscle strips, presumably localizing to the actin filaments, without displacing endogenous α -calponin. Helical strips of permeabilized rat tail artery contracted rapidly in response to Ca^{2+} and then very slowly relaxed in the continued presence of Ca^{2+} . Application of unphosphorylated α -calponin (tissue-purified or recombinant) to the bathing medium at the peak of the Ca^{2+} -induced contraction inhibited isometric force in a concentration-dependent manner, with a half-maximal effect at $\approx 3 \mu\text{M}$ α -calponin. Consistent with the known effects of phosphorylation, α -calponin phosphorylated by PKC caused a slight, but not significant, inhibition of force compared with the control. Similarly, the α -calponin mutants S175A and S175D had no significant effect on force, consistent with their low affinities for actin and their inability to inhibit the actin-activated myosin MgATPase [29]. On the other hand, the α -calponin mutant S175T which, like native α -calponin, binds to actin with high affinity and inhibits the actin-activated myosin MgATPase, inhibited force to the same extent as native α -calponin.

α -Calponin also inhibited force development when applied to demembranated rat tail arterial strips prior to Ca^{2+} . In agreement with the findings of Itoh et al. [10] with rabbit mesenteric artery, α -calponin inhibited force at every Ca^{2+} concentration examined over the activation range (pCa 7–5). Furthermore, the inhibitory effect of α -calponin was shown to be Ca^{2+} -independent, since application of α -calponin, in the absence of Ca^{2+} , to permeabilized muscle strips containing thiophosphorylated myosin inhibited force to an extent comparable with that observed at pCa 6.3. Again, tissue-purified and recombinant α -calponins and S175T were equally effective, whereas phosphorylated α -calponin, S175A and S175D were completely ineffective.

α -Calponin has been shown to bind calmodulin in a Ca^{2+} -dependent manner, suggesting that the inhibition of isometric force observed may be due to α -calponin binding to calmodulin, thereby reducing the amount of calmodulin available for activation of myosin light chain kinase and resulting in a reduction in myosin phosphorylation and force. This is unlikely for the following reasons. (i) Actin competes with Ca^{2+} /calmodulin for binding to α -calponin and, while the affinity of α -calponin for Ca^{2+} /calmodulin ($K_d = 52 \text{ nM}$) [42] is similar to that for actin ($K_d = 43 \text{ nM}$) [4], the concentration of actin (1.6 mM) [43] is

Figure 7 Effects of tissue-purified α -calponin (phosphorylated and unphosphorylated), recombinant wild-type α -calponin and three site-specific mutants of α -calponin on force in permeabilized, thiophosphorylated, rat tail arterial smooth muscle strips

The experimental protocol was as in Figure 6. At the peak of the Ca^{2+} -independent contraction (time zero), the following additions were made: none (control; $n = 12$; ∇); tissue-purified (unphosphorylated) α -calponin (CaP; $n = 5$; $\bullet$); phosphorylated α -calponin (P-CaP; $n = 5$; $\triangle$); recombinant wild-type α -calponin (WT; $n = 5$; $\circ$); S175A ($n = 4$; $\square$); S175D ($n = 5$; $\blacksquare$); or S175T ($n = 5$; $\blacktriangle$). Where present, α -calponin was at $5 \mu\text{M}$. (A) Time courses of relaxation under control conditions and in the presence of the indicated α -calponin species. (B) Force remaining at $t = 150 \text{ min}$ in the presence of the indicated α -calponin species, expressed as a percentage of the peak of the Ca^{2+} -independent contraction (recorded at $t = 0$). Data are expressed as means $\pm$ S.E.M. Asterisks indicate a significant difference from the control as assessed by Student's t test ($P < 0.05$).

much higher than that of calmodulin ($20\text{--}50 \mu\text{M}$) [43]. (ii) In an *in vitro* system composed of actin ($6 \mu\text{M}$), myosin, tropomyosin, myosin light chain kinase and calmodulin, α -calponin ($2 \mu\text{M}$) inhibited the actin-activated myosin MgATPase to the same extent in the presence of 0.6 or $30 \mu\text{M}$ calmodulin without affecting myosin phosphorylation [42]. (iii) Exogenous calmodulin ($1 \mu\text{M}$) was added to all demembrated muscle strips. (iv) α -Calponin also inhibited Ca^{2+} -independent isometric force in permeabilized muscle strips containing thiophosphorylated myosin.

Quantitatively, the effects of α -calponin on isometric force in demembrated rat tail arterial muscle strips seen here are greater than those observed with saponin- or β -escin-permeabilized rabbit mesenteric arterial smooth muscle [10], or Triton X-100- [11] or Triton X-100 plus glycerol-permeabilized guinea pig taenia coli [12]. This is most likely due to the low

endogenous level of α -calponin in the rat tail artery. The availability of more binding sites for α -calponin would then account for the larger degree of inhibition observed upon addition of exogenous α -calponin. The low α -calponin content of the rat tail artery compared with other smooth muscles may cause faster contractile responses in this tissue than in, for example, the aorta. It will be of interest in the future to compare the contractile properties, e.g. maximal shortening velocity and peak force development, of rat tail artery and rat aorta to test this hypothesis.

In conclusion: (i) α -calponin inhibits force in vascular smooth muscle in a Ca^{2+} -independent manner through its interaction with actin, (ii) phosphorylation alleviates this inhibitory effect, and (iii) Ser-175 plays a critical role in α -calponin function.

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