Calponin reduces shortening velocity in skinned taenia coli smooth muscle fibres

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Abstract Calponin (4.1–5.9 μM, pig stomach) inhibited maximal shortening velocity (V_{max}) by 20–25% with only minor influence on force in skinned smooth muscle from guinea-pig taenia coli activated at different Ca^{2+} levels and with thiophosphorylation. Similar results were obtained with a fragment of the N-terminal 1–228 amino acids engineered using a mouse cDNA construct (5.4 μM). Both the native calponin and the fragment inhibited actin filament sliding in a graded manner in an in vitro motility assay. We conclude that calponin influences the kinetics of the actin–myosin interaction in the organised smooth muscle contractile system and that engineered fragments of calponin can be used to probe its action in muscle fibres. The effects can be due to an introduction of an internal load during filament sliding, possibly by decreasing the detachment rates and increasing the cross-bridge time spent in the attached state.

Key words: Calponin; Motility assay, in vitro; Shortening velocity; Smooth muscle

1. Introduction

Calponin, a 34 kDa protein first isolated from chicken gizzard and bovine aortic smooth muscle [1,2], has been proposed to be a regulatory protein involved in smooth muscle contraction. Calponin binds in vitro to a variety of proteins including actin, tropomyosin, calmodulin and myosin [1,2,3,4]. In smooth muscle cells the protein is found to be associated with the actin filaments [5,6]. In native thin filaments calponin:actin ratios of about 1:10–16 have been reported [7,8]. The interaction with other thin filament proteins is not clarified at present but binding studies have suggested that calponin and caldesmon compete for the same binding site on actin [9], raising the possibility that these proteins are associated with different classes of thin filaments [10]. Recent immunocytochemistry of chicken gizzard smooth muscle [11] has revealed that, whereas caldesmon is co-localised with the contractile proteins, calponin is associated with both the contractile and cytoskeletal proteins.

In vitro calponin inhibits actin-activated myosin Mg-ATPase activity [12,13], reportedly via a predominant effect on the V_{max} of the ATPase with only a minor effect on the K_m [7,14]. The inhibitory action of calponin is not due to an inhibitory effect on myosin phosphorylation [12,13] and is not dependent on the presence of tropomyosin [12], although an interaction between these proteins may occur due to co-operative phenomena [14]. The inhibition of the actomyosin/Mg-ATPase activity can be reversed by Ca^{2+}-calmodulin [13] or calcium-caltropin [15] and by phosphorylation of calponin [12]. The role of these regulatory mechanisms in vivo is, however, unknown and in part also controversial [8,16,17,18,19].

Whereas the in vitro inhibitory effects of calponin on actomyosin/ATPase activity are well documented, information regarding its effects on the organised contractile system is sparse. Itoh et al. [20] recently reported that exogenously added calponin reduced active tension in smooth muscle. In this report we describe the application of native calponin as well as a recently engineered fragment of the molecule [15] to chemically skinned taenia coli muscles activated by calcium or thiophosphorylation. The activity of the protein preparations was also assayed in parallel in actin filament sliding assays, which have formerly shown the inhibitory effects of calponin on filament velocity [21,22]. Our results show that the maximal shortening velocity (V_{max}) is inhibited to a larger extent than force by calponin which might reflect an introduction of an internal load during filament sliding, possibly by slowing cross-bridge detachment reactions.

2. Materials and methods

2.1. Muscle preparation and solutions

Taenia coli preparations were obtained from guinea-pigs (300–400 g). The animals were killed by cervical dislocation and the muscles were dissected and immediately skinned with Triton X-100 as earlier described [23]. The preparations were stored in a glycerol solution at −15°C until the experiments were performed. Thin preparations (5–7 mm long and with diameter 0.1–0.2 mm) were cut out and mounted as described below.

Solutions contained 30 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES), 2 mM Mg^{2+}, 3.2 mM Mg-ATP, 12 mM phosphocreatine (PCr), 0.5 mg/ml creatine kinase (CK), 4 mM ethylene glycol-bis(β-aminoethyl ether)*N,N,N'N'*-tetraacetic acid (EGTA), 1 mM NaN_3 and 0.5 μM calmodulin. In the relaxation solutions free $[Ca^{2+}]$ was 10^{-9} M (pCa 9.0) and in the contraction solutions $10^{-4.5}$ M (pCa 4.5). The contraction and relaxation solutions were mixed in appropriate amounts to obtain intermediate $[Ca^{2+}]$. The rigor solutions were made without Mg-ATP, PCr and CK. Thiophosphorylation was performed in a rigor solution with pCa 4.5, 1 mM ATP-γ-S and 0.5 μM calmodulin. The ionic strength of all solutions was 150 mM adjusted with KCl and pH was 6.9 adjusted with KOH. Calponin and the CP 1-228 fragment, when added, were introduced from a stock solution in TES as described below. The composition of the solutions were calculated as described in [24].

Pig stomach calponin was purified according to Vancompernolle et al. [25] and lyophilised. Prior to experiments, calponin was dissolved and dialysed for 2 or 12 h at 4°C against 20 mM TES at pH 6.9. This calponin stock solution was used to mix experimental solutions. The dialysis medium served as control solution. The calponin concentration was determined in the experimental solutions according to the Brad-

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ford-method, using bovine serum albumin as a standard, or in the calponin stock solution by measuring the optical density at 276 nm (using an extinction coefficient, $E^{1\%}_{276\ nm}$, of 8.9). A molecular weight for calponin of 33 kDa was used. The N-terminal fragment CP 1-228 was engineered in bacteria using a mouse cDNA construct as described previously [15]. An extinction coefficient, $E^{1\%}_{276nm}$, of 7.7 and a molecular weight of 25 kDa were used. The fragment was dialysed prior to experiments as described above for calponin [15].

2.2. Isotonic quick release experiments

Force–velocity relationships were determined as described by [23] with the preparations mounted in an apparatus with an AE 801 force transducer (SensoNor a.s., Horten, Norway) and an isotonic lever. The muscles were mounted in relaxing solution at a passive load of about 0.1 mN. Force–velocity relations were determined in fibres activated with Ca^{2+} or with irreversible thiophosphorylation of the regulatory myosin light chains. A series of 15–25 releases to different afterloads was performed at the force plateau of contractions. The shortening velocity (V) was determined 100 and 500 ms after the release and then fitted to the Hill force–velocity equation in the form $V = b(1 - P/P_0)/(P/P_0 + a/P_0)$; where a and b = constants, P = afterload and P_0 = isometric force. In fibres activated with Ca^{2+}, the experimental protocol contained an initial 5 min period in relaxation solution followed by activation with a calponin-free solution at pCa 4.5 for 30 min. At the plateau of this control contraction a first series of quick releases was performed. The fibre preparation was relaxed for 10 min and thereafter contracted in either calponin-free or calponin-containing solution at intermediate [Ca^{2+}], pCa 5.75, for 30 min before performing another series of quick releases. Then the muscle was transferred to contraction solution with pCa 4.5 with or without calponin and at the plateau of the contraction a third series of quick releases was performed. In each fibre preparation, both the second and the third contractions were performed in the same kind of solution (either calponin-free or calponin-containing). Force and the maximal shortening velocity (V_{max}) for the second (pCa 5.75) and the third contraction (pCa 4.5) were expressed relative to those during the initial control contraction in calponin-free solution obtained in each preparation.

In experiments on thiophosphorylated preparations the muscles were mounted in relaxation solution as described above and then exposed to a repeated thiophosphorylation protocol as described in [26]. The preparations were treated with the thiophosphorylation solution for 15 min and then transferred to a rigor solution for 5 min before each contraction. The muscles were contracted by transfer to ATP-, PCr-and CK-containing solution with pCa 9.0 (i.e. the relaxation solution above). Four contractions, each preceded by a thiophosphorylation period, were performed on each fibre preparation. The first two contractions were elicited in calponin-free solutions, the third contraction in either calponin-free or calponin-containing solution and the fourth contraction in a calponin-free solution. During the second, third and fourth contractions quick releases were performed after 30 min. The maximal shortening velocities and force were expressed relative to those during the second control contraction. Experiments on the CP 1-228 fragment on thiophosphorylated fibres were performed as described above, except that calponin was replaced by the fragment.

2.3. In vitro motility assays

Avian smooth muscle actin was purified according to [27]. Rabbit skeletal muscle myosin was prepared by a procedure modified from [28], and HMM made from fresh myosin according to [29]. HMM was aliquoted, frozen in liquid nitrogen and stored at −70°. HMM prepared in this way exhibited activity after thawing comparable to fresh HMM.

The in vitro motility assay was performed at 36°C essentially according to [30] with the following variations: after actin had bound to the immobilised HMM on the glass surface, calponin or the CP 1–228 fragment in assay buffer (containing 4 mM $MgCl_2$, 1 mM EGTA, 80 mM KCl and 25 mM imidazole with pH 7.4) was perfused into the chamber and allowed to bind to actin. Perfusion was then continued with the same mixture supplemented with 1 mM Mg-ATP and 0.6% methyl cellulose, 30 s was allowed for equilibration within the chamber, then three separate fields were recorded for 20 s each using a Sony Umatic VO-5800PS video recorder. Actin filament velocity analysis was performed on a Macintosh Quadra 650 computer using a macro program for tracking (written by Kurt Anderson) which runs under NIH Image (written by Wayne Rasband at the US National Institutes of Health, and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd. Springfield, VA 22161, part number PB93-504868). All of the filaments were tracked in a given twenty second series. For all points more than 20 filaments were tracked, except for the slowest velocities, where 10 filaments were tracked. The optimal ratio of HMM, actin and methyl cellulose for the maximum velocity and minimal filament shredding was determined in control experiments and held constant throughout.

2.4. Statistics

Results are given as mean ± S.E.M. with the number of observations given within parenthesis. Statistical comparisons were made according to the Student's t-test for unpaired data and with the Bonferroni method when more than two means were compared.

3. Results

Both intact calponin and the engineered fragment composed of amino acids 1–228 produced a dose-dependent inhibition of the velocity of movement of smooth muscle actin filaments over skeletal muscle HMM. Velocity histograms showed that calponin caused a dose-dependent shift in the velocity distribution towards lower velocities rather than introducing a population of non-moving filaments. This suggests that the effects occurred gradually, rather than in an all-or-none fashion. In Fig. 1 the mean velocity is plotted against concentration of calponin and CP 1-228. A complete arrest of movement was observed at about 0.5 μM for calponin and 1.5 μM for the CP 1-228 fragment.

Fig. 2 shows the maximal shortening velocity (V_{max}) at 100 ms plotted against the isometric force at different activation levels. Force and velocity data are expressed relative to those during the initial control contraction in each fibre (the cross in Fig. 2 indicates this point with V_{max} and force = 1). Open symbols show data obtained in the absence of calponin. Intermediate activation by pCa 5.75 (open circle) was associated with lower force and V_{max} compared to pCa 4.5 (open square). The

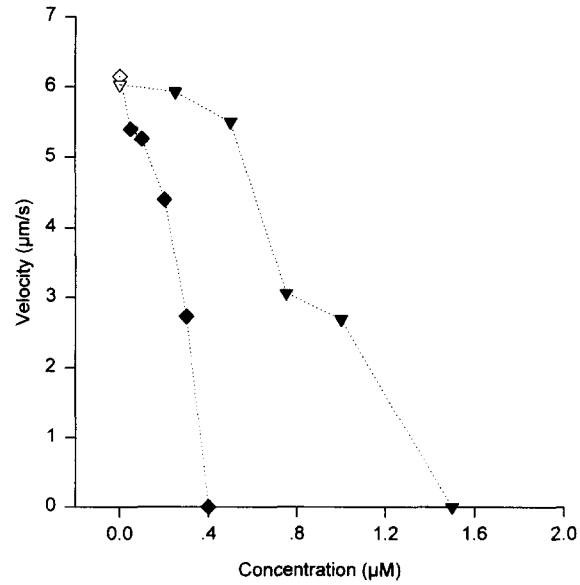

Fig. 1. Inhibitory effect of increasing concentrations (μM) of native calponin (filled diamonds) and the N-terminal CP 1-228 fragment (filled triangles) on the mean velocity of smooth muscle actin filaments over skeletal HMM. Control velocities obtained in the absence of calponin and the fragment are shown with open symbols.

force–velocity relation obtained during the contraction in pCa 4.5 (open square) was similar to that obtained during the initial control contraction (cross) showing that no irreversible alterations in mechanical behaviour occurred during the experiment. Calponin, (4.1 ± 0.3 μM, $n = 5$) significantly decreased V_{max} in both maximally and submaximally activated fibres (filled circle: pCa 5.75, $P < 0.01$ and filled square: pCa 4.5, $P < 0.001$) without affecting the isometric force. Table 1 shows the average force and maximal shortening velocity (V_{max}) in absolute units (muscle lengths per s) as well as the parameters b and a/P_0 of the Hill force–velocity equation. In accordance with earlier studies [23], we chose to measure velocity at 100 ms after release. The V_{max} at 500 ms after release are also given in Table 1. Since velocity at this point in time was decreased by calponin at pCa 4.5, the influence of calponin on the shortening velocity was not restricted to the initial shortening phase after the release. The decrease in V_{max} was associated with a small increase in b and an increase in a/P_0 which shows that the force–velocity relation had become less concave in the presence of calponin.

Force–velocity data obtained in thiophosphorylated muscles are inserted in Fig. 2 (diamonds and triangle). In these maximally activated preparations calponin (5.9 ± 0.8 μM, $n = 5$, filled diamond) caused a significant ($P < 0.01$, Bonferroni method) reduction in V_{max} compared to the thiophosphorylated muscle in the absence of calponin (open diamond). The decrease was of the same magnitude as in the calcium activated muscles. In addition, a small (about 10%) but significant decrease of force ($P < 0.01$, Bonferroni method) was observed compared to the thiophosphorylated muscle in the absence of calponin (open diamond). Velocity in muscle lengths per second at 500 ms was lower although not significantly (Table 1). When expressed relative to the control velocity at 500 ms obtained in each fibre the V_{max} at 500 ms, was significantly reduced in the presence of calponin (0.66 ± 0.05) compared to the controls (0.92 ± 0.08, $P < 0.05$).

Force–velocity data obtained in the presence of the CP 1-228 fragment (5.4 ± 1.2 μM, $n = 5$, filled triangle in Fig. 2) revealed a significant reduction in V_{max} of the same magnitude ($P < 0.05$, Bonferroni method) as that observed for the native calponin and a small reduction in active force. Force velocity data in absolute units are shown in Table 1.

4. Discussion

Calponin caused a pronounced inhibition of the maximal shortening velocity of the skinned smooth muscle fibres with a minor influence on force. Since the effect on velocity was observed in both calcium activated and thiophosphorylated fibres, we consider it to be due to a direct effect of calponin on the actin–myosin interaction, rather than to an influence on myosin phosphorylation, which is consistent with previous biochemical studies [12]. In smooth muscle fibres, variations in $[Ca^{2+}]$ and myosin light chain phosphorylation influence both force and V_{max} ([24,31], Fig. 2 and Table 1). The modulation of the force–velocity relation by calponin appears to be different from that caused by variations in $[Ca^{2+}]$ and phosphorylation since calponin causes a reduction in V_{max} with only a minor influence on force. Also, the influence on the shape of the force velocity relation appears to be different; a reduction in V_{max} by lowering $[Ca^{2+}]$ is associated with a reduction in the parameter b whereas calponin addition causes a small increase in b and an increase in a/P_0 reflecting a less concave relationship. A change in the a/P_0 parameter can be considered to reflect a change in the ratio between the number of cross-bridges exerting positive and the number of cross-bridges exerting negative force [32]. Since V_{max} is considered to be rate limited by cross-bridge detachment reactions [32] the inhibition of V_{max} by calponin could be due to a slowing of product release steps leading to an increase in the time the cross-bridges spend in attached states, introducing an internal load by increasing the number of cross-bridges exerting negative force during shortening. An influence on product release steps is consistent with results of biochemical studies showing that calponin influences catalytical reactions rather than the actin–myosin binding [7,14]. However, since calponin also seems to be associated with the cytoskeleton in the smooth muscle cells [11] a structural change in this cellular domain introduced by calponin influencing filament sliding might also be involved.

The small decrease in force observed in the thiophosphorylated fibres could be due to the somewhat higher concentration of calponin in those experiments (5.9 μM in comparison with 4.1 μM). One possibility is that calponin reduces cross-bridge cycling leading to a longer occupancy in

Table 1
Effects of calponin and the CP 1-228 fragment on force–velocity characteristics at different levels of activation with calcium or after thiophosphorylation Isometric force (P_0), relative to that during an initial control contraction, V_{max} in muscle lengths (ML) per s measured at 100 ms and 500 ms after release and the parameters b and a/P_0 at 100 ms after release are shown. Significant differences between the calponin/fragment- and the respective calponin/fragment-free groups are indicated (Student's t-test for the Ca^{2+} activated and the Bonferroni method for thiophosphorylated group). Concentrations of calponin and CP 1-228 are given in section 3.

	P_o	V_{max}, 100 ms (ML/s)	b, 100 ms (ML/s)	a/Po, 100 ms	V_{max}, 500 ms (ML/s)
pCa 5.75 (n = 5)	0.68 ± 0.07	0.144 ± 0.014	0.013 ± 0.005	0.094 ± 0.043	0.043 ± 0.007
pCa 5.75 + calponin (n = 5)	0.75 ± 0.06	0.122 ± 0.018	0.026 ± 0.003 $p < 0.05$	0.224 ± 0.018 $p < 0.05$	0.049 ± 0.008
pCa 4.5 (n = 5)	1.00 ± 0.06	0.172 ± 0.008	0.019 ± 0.006	0.116 ± 0.041	0.052 ± 0.005
pCa 4.5 + calponin (n = 5)	1.07 ± 0.08	0.135 ± 0.013 $p < 0.05$	0.030 ± 0.010	0.214 ± 0.060	0.039 ± 0.005 $p < 0.05$
Thiophos. (n = 6)	0.96 ± 0.01	0.152 ± 0.014	0.014 ± 0.002	0.097 ± 0.019	0.063 ± 0.003
Thiophos. + calponin (n = 6)	0.86 ± 0.02 $p < 0.01$	0.115 ± 0.012	0.016 ± 0.003	0.150 ± 0.045	0.055 ± 0.008
Thiophos. + CP 1-228 (n = 5)	0.90 ± 0.03	0.113 ± 0.009 $p < 0.05$	0.031 ± 0.005 $p < 0.05$	0.280 ± 0.047 $p < 0.01$	0.041 ± 0.003 $p < 0.05$

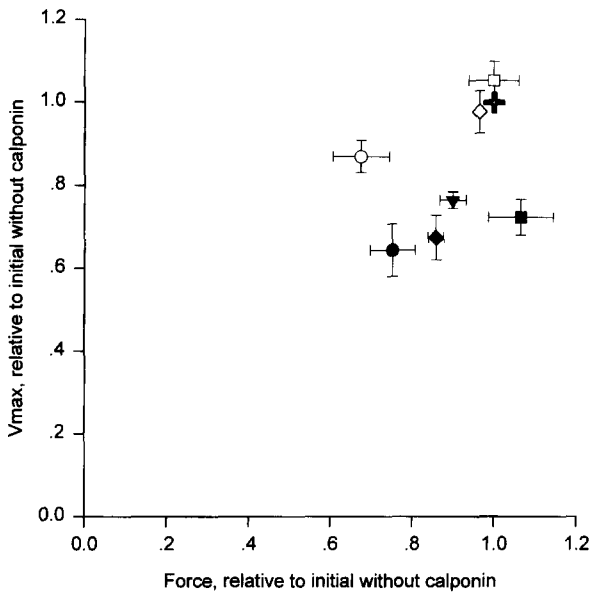

Fig. 2. Relation between maximal shortening velocity (V_{max}) and isometric force at different levels of activation in the absence (open symbols) and in the presence of calponin (filled circle, square and diamond) or the CP 1-228 fragment (filled triangle). The preparations were either activated at pCa 5.75 (circles), at pCa 4.5 (squares) or irreversibly thiophosphorylated (diamonds, triangle). All force and V_{max} values of calcium activated and thiophosphorylated preparations are normalised relative to those obtained during the respective initial control contractions (cross, relative force and V_{max} = 1). n = 5–10.

attached states as discussed above. An increased number of attached heads, in the presence of calponin, might cooperatively turn on the actin filament as suggested by Horiuchi and Chacko [14]. If the thin filament system is not cooperatively turned on in the calcium activated muscles to the same extent as in the thiophosphorylated fibres, a slowing of the cross-bridge cycle might have different influence on the force in these two types of activation.

In a previous report on the effects of calponin on saponin and β-escin skinned smooth muscle a substantial depression of force in the presence of calponin (about 25% inhibition by 5.6 μM) was found [20] which is larger than the influence on force observed in the present study. We have at present no detailed information regarding the concentration dependence of the mechanical effects of calponin or the fragment. Although V_{max} was affected to a much larger extent than force for the protein concentrations we could obtain in the present study it is possible that higher protein concentrations might also influence force generation. In our study an average inhibition of force of 10% was observed at 5.9 μM calponin in thiophosphorylated muscles and no effect was noted at 4.1 μM calponin after Ca^{2+} activation (Table 1). The difference between studies could reflect differences in the skinning methods or smooth muscle fibre preparations used (rabbit blood vessels vs. guinea pig intestinal muscle, possibly containing different composition of thin filaments) or the source of calponin (avian from chicken gizzard vs. mammalian from pig stomach).

In conclusion, our results regarding the effects of calponin on the mechanics of smooth muscle fibre preparations are in general agreement with data obtained from in vitro motility assay studies. Both Shirinsky et al. [21] and Haeberle [22] describe an inhibition of filament movement by calponin in the micromolar range which is similar to that obtained in the present study. The N-terminal fragment from the 1–228 mouse sequence was active at slightly higher concentrations, showing that the effect of the native protein can be mimicked by this truncated form containing proposed binding regions for actin, calcium binding proteins and tropomyosin (cf. [33]). The study of Shirinsky et al. [21] suggested that the inhibition of motility by calponin occurred in an all-or-none fashion. Our motility data suggest, however, a graded action of calponin and the fragment, although with a steep concentration dependence. The force and V_{max} of the smooth muscle fibre preparation reflects the behaviour of a large population of contractile filaments and a switch-like inhibition, as opposed to a graded inhibition, cannot be distinguished. The inhibition of V_{max} and the small effect on force are consistent with the inhibition of the dissociation of the high-affinity actomyosin complex proposed by Haeberle [22]. Since our data are based on experiments where calponin is introduced to a contractile apparatus already containing endogenous calponin the exact nature of its regulatory role in the intact tissue awaits further studies. Our data show, however, that calponin can act as a modulator of cross-bridge turnover kinetics in smooth muscle fibre preparations and that engineered calponin fragments can be used for probing its action in in vitro motility assays and in smooth muscle fibres.

In a recent preliminary report [34], independent of our study, an inhibition of unloaded shortening velocity by calponin is described which is similar to some of the results of the present study.

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