Functional interrelationship between calponin and caldesmon

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Calponin and caldesmon, constituents of smooth-muscle thin filaments, are considered to be potential modulators of smooth-muscle contraction. Both of them interact with actin and inhibit ATPase activity of smooth- and skeletal-muscle actomyosin. Here we show that calponin and caldesmon could bind simultaneously to F-actin when used in sub-saturating amounts, whereas each one used in excess caused displacement of the other from the complex with F-actin. Calponin was more effective than caldesmon in this competition: when F-actin was saturated with calponin the binding of caldesmon was eliminated almost completely, whereas even at high molar excess of caldesmon one-third of calponin (relative to the saturation level) always remained bound to actin. The inhibitory effects of low concentrations of calponin and caldesmon on skeletal-muscle actomyosin ATPase were additive, whereas the maximum inhibition of the ATPase attained at high concentration of each of them was practically unaffected by the other one. These data suggest that calponin and caldesmon cannot operate on the same thin filaments. Ca^{2+} -calmodulin competed with actin for calponin binding, and at high molar excess dissociated the calponin-actin complex and reversed the calponin-induced inhibition of actomyosin ATPase activity.

INTRODUCTION

Contraction of muscle cells is controlled by the cytoplasmic level of Ca^{2+} . In contrast with striated muscle, where Ca^{2+} -sensitivity is conferred by the troponin-tropomyosin complex associated with thin filaments [1,2], the primary mechanism of smooth-muscle regulation is linked to the thick filament and involves phosphorylation of the 20 kDa myosin light chains by a specific Ca^{2+} /calmodulin-dependent myosin light-chain kinase [3,4]. However, evidence supports the existence of a thin-filament-linked regulatory mechanism [5–7] in smooth muscle, in addition to myosin phosphorylation. The candidates to fulfil this function are two thin-filament proteins discovered in the 1980s: caldesmon [8] and calponin [9]. Both are heat-stable proteins, of 87 kDa and 34 kDa respectively, present in relatively high quantities in smooth muscle and non-muscle cells [8,10–12].

Caldesmon and calponin share some functional properties: (1) they bind to actin, tropomyosin and, in the presence of Ca^{2+} , to calmodulin [8,9,13,14]; (2) they inhibit the actin-activated ATPase activity of myosin [15–18]; (3) the inhibitory effect of both is released by phosphorylation mediated by Ca^{2+} /calmodulin-dependent protein kinase II [18–20] and protein kinase C [18,21,22]. However, whereas caldesmon-induced inhibition of actomyosin ATPase is potentiated by tropomyosin, that by calponin seems to be tropomyosin-independent [18]. So far it is unknown whether calmodulin can release calponin-induced inhibition of actomyosin ATPase in the same way as that induced by caldesmon [8,17,23]. Similarly, the functional relationship between caldesmon- and calponin-mediated inhibition of actomyosin ATPase activity has not been explored. To investigate the functional relationship between caldesmon and calponin, we have studied the effect of caldesmon on the interaction of calponin with actin as well as on the calponin-induced inhibition of actin-activated ATPase of myosin. To avoid the effects of myosin phosphorylation [4] and caldesmon-myosin interaction [24,25] on actomyosin ATPase activity, we have exploited the skeletal-muscle actomyosin system instead of smooth muscle.

The results demonstrate that caldesmon can compete with

calponin for actin binding and that the binding of calponin to actin is abolished by an excess of calmodulin.

A preliminary report of this work has been presented [26].

MATERIALS AND METHODS

Preparation of proteins

Caldesmon was isolated from chicken gizzard by method of Bretscher [27]. Calponin was purified from chicken gizzard either by the original method of Takahashi *et al.* [9] or by a modified procedure including sequential steps of chromatographic purification: on DEAE-cellulose, SP-Sephadex C-50, and calmodulin- or troponin C-Sepharose 4B column. The pellet after $(\text{NH}_4)_2\text{SO}_4$ fractionation (to 30% saturation) was dissolved in and dialysed against buffer I (20 mM-imidazole/HCl, pH 7.0, 50 mM-NaCl, 1 mM-EGTA, 1 mM-dithiothreitol, 0.2 mM-phenylmethanesulphonyl fluoride). After filtration, the protein solution was applied on a DEAE-cellulose column (30 cm $\times$ 2.5 cm) equilibrated with buffer I. The flow-through fraction was collected and immediately applied on a SP-Sephadex C-50 column (30 cm $\times$ 2.5 cm) equilibrated with the same buffer. The column was washed with buffer I, and proteins were eluted with a linear NaCl gradient (50–300 mM) in buffer I. Calponin-containing fractions were pooled and, after adding CaCl_2 to 2 mM final concentration were further purified by affinity chromatography on a preparative calmodulin- or troponin C-Sepharose 4B column (15 cm $\times$ 1.5 cm). When a troponin C-Sepharose 4B column was used, the sample was diluted 3-fold with buffer II (40 mM-Tris/HCl, pH 7.5, 0.2 mM- CaCl_2 , 3 mM- MgCl_2 , 1 mM- β -mercaptoethanol, 0.2 mM-phenylmethanesulphonyl fluoride) to decrease NaCl concentration. The column was washed with buffer II containing 50 mM-NaCl (troponin C-Sepharose) or 200 mM-NaCl (calmodulin-Sepharose) and then eluted with the same buffer in which 1 mM-EGTA was used instead of CaCl_2 . Rabbit skeletal-muscle myosin was prepared as described by Perry [28] and further purified by the procedure of Kielley & Bradley [29]. Rabbit skeletal-muscle actin and chicken gizzard tropomyosin were prepared by procedures described

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elsewhere [30]. Calmodulin was prepared from bovine brain by the method of Gopalakrishna & Anderson [31].

The purity of all these proteins was checked by SDS/PAGE on 12.5%-acrylamide slab gels (Laemmli [32]).

Protein determination

Protein concentrations were determined by measuring u.v. absorbance, by using the following absorption coefficients and molecular masses: caldesmon, $A_{280}^{1\%} = 3.8$, 87 kDa [33]; calponin, $A_{277}^{1\%} = 11.3$, 34 kDa [18]; G-actin, $A_{290}^{1\%} = 6.3$, 42 kDa [34]; myosin, $A_{278}^{1\%} = 5.4$, 470 kDa [35]; chicken gizzard tropomyosin, $A_{278}^{1\%} = 2.2$, 72 kDa; calmodulin, $A_{278}^{1\%} = 2.0$, 16.7 kDa [37].

Sedimentation experiments

Sedimentation experiments were performed at 25 °C in a buffer containing 10 mM-Tris/HCl, pH 7.4, 0.1 M-KCl, 1 mM- β -mercaptoethanol, 2 mM-MgCl₂ and 0.1 mM-CaCl₂ or -EGTA. Before the experiment, calponin and tropomyosin were spun down at 150 000 *g* for 30 min in a Beckman LP42Ti rotor at 25 °C to sediment possible aggregates. Samples were mixed in a total volume of 100 μ l. F-actin was added last, and mixtures were incubated for 30 min at 25 °C. Samples were ultracentrifuged at 150 000 *g* for 30 min in a LP42Ti rotor at 25 °C. Then 75 μ l of supernatant was withdrawn for SDS/PAGE. To the pellet 75 μ l of water was added. Both pellets and supernatants were supplemented with 25 μ l of electrophoresis sample buffer, consisting of 0.5 M-Tris/HCl, pH 6.8, 8% (v/v) SDS, 40% (v/v) glycerol, 3 M- β -mercaptoethanol and 0.004% Bromophenol Blue, and boiled for 3 min. Care was taken to dissolve pellets completely. SDS/PAGE was performed in 0.75 mm-thick 12.5%-polyacrylamide slab minigels (Laemmli [32]). Samples were applied in a volume of 10 μ l. After a 1 h run at 175 V, gels were fixed in 30% (v/v) propan-2-ol/10% (v/v) acetic acid for 10 min, stained with 0.1% Coomassie Brilliant Blue R-250 in 30% propan-2-ol/10% acetic acid for 20 min, and destained in 10% propan-2-ol/5% acetic acid until the background staining was removed. Gels were quantified by densitometry using a LKB Ultrascan laser densitometer equipped with a Hewlett-Packard integrator. Two or three independent scans were taken from each track, and data were averaged. In separate experiments the linear range of densitometer readings was established to extend from 0.1 to 5.5 μ M-actin per band. Thus our highest protein loadings were within the linear range of the densitometer. Samples from a given titration experiment and samples for direct comparison were run in the same gel, or at least in the same run, and gels were processed simultaneously. Titration experiments were repeated two or three times with independent protein preparations. The fraction of caldesmon, calponin and tropomyosin sedimenting in the absence of F-actin never exceeded 5% of the total protein added, and was not considered in the calculations. The reproducibility of results was not less than 80%. Each point in Figs. 1(a)–1(c) and 4(a) represents the mean $\pm$ S.D. of 4–9 determinations.

Actomyosin Mg²⁺-ATPase assay

ATPase activity of rabbit skeletal-muscle actomyosin (reconstituted from 40 μ g of F-actin/ml and 160 μ g of myosin/ml) was assayed at 30 °C in a medium containing 50 mM-KCl, 2 mM-MgCl₂, 2 mM-ATP, 2 mM-EGTA or 0.1 mM-CaCl₂, and 10 mM-imidazole/HCl (pH 7.0). Chicken gizzard tropomyosin, when present, was added at a 1:7 molar ratio to actin monomer. The concentrations of calponin, caldesmon and calmodulin are given in the Figure legends. The amount of P_i liberated was measured by the method of Fiske & SubbaRow [36]. Experiments were performed in triplicate with independent protein preparations.

RESULTS

Binding of calponin and caldesmon to F-actin

The interaction of calponin with skeletal-muscle F-actin was assessed by high-speed co-sedimentation in the absence and in the presence of Ca²⁺ and other smooth-muscle thin-filament proteins such as tropomyosin and caldesmon. Quantitative analysis of the content of proteins in pellets revealed that approx. 1 molecule of calponin bound per actin monomer at saturation. No difference in the binding of these two proteins was detected in the presence of either Ca²⁺ or tropomyosin (Fig. 1a). However, taking into account the effect of tropomyosin on the caldesmon–actin interaction [39,40], further experiments were performed in the presence of tropomyosin.

Caldesmon up to a molar ratio of 1:14 to actin monomer did not interfere with the binding of calponin to actin. However, when used at higher molar ratio to actin (i.e. 1:7) caldesmon caused inhibition of calponin binding (Fig. 1a). Titration of actin saturated with calponin (i.e. at 1:1 molar ratio) by caldesmon showed that even at high molar excess of the latter protein about one-third of calponin still remained bound to actin (Fig. 1d). Consequently, in the presence of calponin less caldesmon was required to saturate actin (Fig. 1c). Conversely, titration of actin saturated with caldesmon (i.e. at a molar ratio of 1 caldesmon per 7 actin monomers) by calponin caused gradual elimination of binding of caldesmon to actin. At a molar ratio of calponin to actin of 1:1, only about 5% of caldesmon was bound (Fig. 1b).

Effect of calponin and caldesmon on actomyosin ATPase activity

Fig. 2 shows the effects of caldesmon on calponin-induced inhibition and calponin on caldesmon-induced inhibition of skeletal-muscle actomyosin ATPase activity. Calponin produced a concentration-dependent inhibition of actomyosin ATPase independent of the presence of Ca²⁺. By linear extrapolation of the initial part of the titration curve it was calculated that about 2 calponin molecules per actin monomer were required to obtain maximum (80%) inhibition of actomyosin ATPase (Fig. 2a). The minimum value of the ATPase activity was the same regardless of the presence of tropomyosin (results not shown).

Titration of actomyosin ATPase activity with calponin performed in the presence of caldesmon (at constant ratio of caldesmon to actin at which half-maximum inhibition is obtained) revealed that at low caldesmon concentrations (up to a molar ratio of 1 caldesmon per 7 actin monomers) the inhibition of the ATPase caused by these two proteins was additive (Fig. 2a). At higher concentrations of caldesmon, however, calponin was practically unable to potentiate the inhibition caused by caldesmon. Similar results were obtained when actomyosin ATPase activity was titrated with caldesmon in the presence of intermediate and high levels of calponin (Fig. 2b). In the first case caldesmon potentiated calponin-mediated inhibition of the ATPase; in the second the inhibition remained at the level seen with calponin alone.

Effect of ATP on calponin–actin complex

Titration of the calponin–actin complex with ATP (Fig. 3) showed that, in agreement with previously published data [39,40], ATP releases calponin from its complex with actin. In the presence of 2 mM-ATP (the concentration used in ATPase assays) around 40% of calponin is released from its complex with actin.

Effect of calmodulin on calponin-induced inhibition of actomyosin ATPase activity

The Ca²⁺–calmodulin complex inhibited the interaction of calponin and actin (Fig. 4a) in a manner similar to that observed

Fig. 1. Competitive binding of calponin and caldesmon to skeletal-muscle actin

(a) Binding of calponin to actin in the absence (○) and presence (□) of tropomyosin (1 molecule per 7 actin monomers) and in the presence of tropomyosin and caldesmon used at two molar ratios to actin, i.e. 1:14 (△) and 1:5 (▲) respectively. (b) Reversal of the binding of caldesmon to actin by calponin. The amounts of calponin and caldesmon bound to actin shown in (a) (▲) and in (b) were estimated from the same gel. (c) The binding of caldesmon to actin in the presence of calponin used at a molar ratio to actin of 1:1. (d) Reversal of the binding of calponin to actin by caldesmon. The amounts of calponin and caldesmon bound to actin shown in (c) and (d) were estimated from the same gel. The binding was assayed at 25 °C in a mixture containing 8.5 μM-actin, 10 mM-Tris/HCl, pH 7.4, 0.1 M-KCl, 2 mM-β-mercaptoethanol, 2 mM-MgCl₂ and 0.1 mM-CaCl₂ or -EGTA as described in the Materials and methods section. Abbreviations: CaD, caldesmon; CaP, calponin; A, F-actin.

Fig. 2. Effects of calponin on caldesmon-induced inhibition (a) and of caldesmon on calponin-induced inhibition (b) of ATPase activity of skeletal-muscle actomyosin

The ATPase activity was measured in the presence of caldesmon used at molar ratios to actin of 0.07:1 (△) and 0.20:1 (▲) (in a) and in the presence of calponin at molar ratios to actin of 1:1 (■) and 5:1 (□) (in b), respectively, in a mixture containing skeletal-muscle actomyosin (reconstituted from 40 μg of F-actin/ml and 160 μg of myosin/ml), tropomyosin (at a molar ratio to actin of 1:7), 50 mM-KCl, 2 mM-MgCl₂, 2 mM-ATP, 2 mM-EGTA and 10 mM-imidazole/HCl (pH 7.0). Control ATPase (●) was assayed in the presence of calponin or caldesmon alone. Abbreviations are as in Fig. 1.

with caldesmon [41,42]. Complete release of calponin from its actin complex took place at a molar ratio of calmodulin to calponin of about 10:1. Consequently, the inhibitory effect of calponin on actomyosin ATPase activity was reversed by calmodulin in the presence of Ca²⁺ (Fig. 4b). Full release of the ATPase inhibition took place at a molar ratio of calmodulin to calponin corresponding to that required for full dissociation of the calponin–actin complex.

DISCUSSION

Caldesmon and calponin are components of thin filaments of avian and mammalian smooth muscle [8,9,12,14,43]. The molar ratios of caldesmon and calponin to actin monomer in intact thin filaments from chicken gizzard have been reported to be 1:10 and 1:12 respectively [44]. *In vitro*, both calponin and caldesmon inhibit actomyosin ATPase activity, and the inhibition can be

Fig. 3. Effect of ATP on binding of calponin to actin

The binding was determined under conditions given in the legend to Fig. 1. The concentrations of ATP were equimolar to MgCl_2 .

Fig. 4. Effect of calmodulin on the binding of calponin to actin (a) and on the inhibition of skeletal-muscle actomyosin ATPase activity (b) by calponin

The binding and the ATPase were assayed in the presence of 0.1 mM- CaCl_2 (●) or 1 mM-EGTA (○) as described in the legend to Figs 1 and 2 respectively. Abbreviation: CaM, calmodulin; other abbreviations as in Fig. 1.

reversed either by Ca^{2+} -calmodulin complex or by phosphorylation catalysed by Ca^{2+} /calmodulin-dependent protein kinase [15–20]. Thus they seem to be good candidates for the role of mediators of the control of smooth-muscle thin filaments by Ca^{2+} . To understand the mechanism of this control, we have studied interaction of calponin with actin in the absence and presence of caldesmon, tropomyosin and calmodulin.

The binding isotherms of caldesmon to smooth- and skeletal-muscle actin showed that saturation of the binding occurs at about 1 caldesmon molecule per 7 actin monomers [23,45–47].

The binding of calponin to skeletal-muscle actin was saturated at a stoichiometry of 1 calponin molecule per 1 actin monomer (Fig. 1a), and preliminary results (R. Makuch, unpublished work) indicated the same stoichiometry for smooth-muscle actin. The affinity constants for the binding of calponin and caldesmon are in a similar range (10^6 – 10^7 M^{-1}) [41,42,48].

Our results indicate that both calponin and caldesmon can bind simultaneously to F-actin at sub-saturating concentrations. Bringing either protein to saturation causes the displacement of the other one from F-actin. Calponin, however, seems to be more effective in displacing caldesmon. The possible explanation for these observations may be based on the differences in stoichiometry of caldesmon and calponin binding to F-actin. Even at saturation, caldesmon is restricted to bind no closer than to every seventh actin monomer, possibly owing to steric hindrance imposed by adjacent caldesmon molecules. Hence the smaller calponin, which is capable of stoichiometric binding to actin, may occupy available binding sites. On the contrary, when calponin is brought to saturation, the binding sites on every actin monomer are likely to be engaged, and this could result in substantial competitive displacement of caldesmon.

Binding of calponin to F-actin, like that of caldesmon, was accompanied by inhibition of smooth-muscle actin- or thin-filament-activated ATPase activity of myosin [18,44,49]. The maximum inhibition (about 80%) of rabbit skeletal-muscle actomyosin ATPase by calponin (Fig. 2a) occurred at a higher ratio of calponin to actin than that corresponding to the maximum binding of these two proteins (i.e. 1 mol per actin monomer). The lack of strict correlation between the binding of calponin to actin and calponin-induced inhibition of the ATPase activity might result from the effect of ATP on calponin-actin interaction, as reported previously [39,40]. Titration of calponin-actin complex with ATP confirmed this suggestion (Fig. 3). Moreover, some disagreement (probably not significant) between the binding and ATPase-inhibition experiments might be due to slightly different conditions used during determination of these two parameters.

The maximum inhibition of the ATPase of skeletal-muscle actomyosin took place at much higher concentration of calponin (2 calponins per actin monomer) than that observed for smooth muscle actomyosin (1 calponin per 3–4 actin monomers) [18]. This is consistent with the different affinity of calponin for skeletal- and smooth-muscle actin noticed by Sutherland *et al.* [48].

Additive inhibitory effects of calponin and caldesmon on actomyosin ATPase activity at sub-saturating levels of both proteins agree well with the sedimentation data. The lack of the effect of each of them on the maximum inhibition induced by the other is due to roughly the same maximum level of inhibition produced by each of them, although in fact during titration one is exchanged by another.

Ca^{2+} , despite its binding to calponin (cf. [14]), affected neither binding of calponin to actin nor calponin-induced inhibition of skeletal-muscle (results not shown) and smooth-muscle [18] actomyosin ATPase.

Both caldesmon and calponin interact with tropomyosin [13,49–52]. However, whereas for caldesmon this interaction influences the binding of caldesmon to actin and the ATPase-inhibitory activity [13,17,41,49,50], for calponin it seems to have no effect on these two functional properties of calponin. Winder & Walsh [18] also did not observe any effect of tropomyosin on calponin-induced inhibition of smooth-muscle actomyosin ATPase. Therefore it can be suggested that calponin interaction with actin, but not with tropomyosin, confers the ATPase-inhibitory property. Moreover, our sedimentation data show that treatment of the calponin-actin-tropomyosin complex with

Ca^{2+} -calmodulin causes release of calponin to the supernatant (Fig. 4a). It indicates that tropomyosin does not anchor calponin to actin filaments under conditions when the calponin-actin complex dissociates. Further work is required to elucidate whether calponin binds to tropomyosin on the thin filaments.

The actin-binding and actomyosin-ATPase inhibition properties of calponin and caldesmon are both reversed by an excess of calmodulin in the presence, but not in the absence, of Ca^{2+} (Fig. 4) [17,41,42]. This indicates that Ca^{2+} -calmodulin competes with F-actin for calponin. Abe *et al.* [53] also demonstrated reversal of calponin-induced inhibition of smooth-muscle actomyosin ATPase by calmodulin, although, compared with our data, less Ca^{2+} -calmodulin was required to overcome the inhibition. This is presumably related to much lower inhibition of actomyosin ATPase by calponin (25%) observed by those authors [53]. The large excess of calmodulin required to neutralize the inhibitory effect of calponin makes it doubtful whether calponin is regulated through direct binding of Ca^{2+} -calmodulin. Winder & Walsh [18] observed dissociation *in vitro* of calponin from its complex with actin upon Ca^{2+} -calmodulin-dependent phosphorylation. Both ways lead to the same effect, but the latter would require much less calmodulin. However, after the completion of the present work Barany *et al.* [54] reported that phosphorylation of calponin does not occur during the contraction-relaxation cycle of arterial smooth muscle *in vivo*.

Our demonstration of the direct competition between caldesmon and calponin for the binding to actin raises the questions of whether both of them can function on the same filament, or whether they are distributed between two classes of actin filaments. Both caldesmon and calponin are found in smooth-muscle extracts and isolated thin filaments in lower proportions to actin than that required for saturation of actin [44,55]. On the other hand, immunolocalization studies revealed that caldesmon in smooth muscle is associated only with those thin filaments that constitute the so-called actomyosin domain, but is excluded from thin filaments belonging to the cytoskeletal domain (that also contain filamin) [56]. These two classes of smooth-muscle thin filaments can be separated on the basis of their interaction with antibodies specific either to caldesmon or to filamin [57]. Recently Lehman [58] also succeeded in separating calponin-rich and caldesmon-rich thin filaments by immunoprecipitation. Relatively little is known about the localization of calponin in cell and its co-localization with other proteins. Gimona *et al.* [59] showed that calponin is located in stress fibres of cultured smooth-muscle cells, although only in the central part of the actin cytoskeleton but not at the ends stained by anti-vinculin antibody, whereas caldesmon is present in the entire length of stress fibres [11]. All these data suggest that *in vivo* caldesmon and calponin are present in distinct classes of thin filaments. The mutual exclusion of caldesmon and calponin from thin filaments reported in the present paper is strongly compatible with this idea.

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