Interaction of calponin with actin and its functional implications

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Titration of F-actin with calponin causes the formation of two types of complexes. One, at saturation, contains a lower ratio of calponin to actin (0.5:1) and is insoluble at physiological ionic strength. The another is soluble, with a higher ratio of calponin to actin (1:1). Electron microscopy revealed that the former complex consists of paracrystalline bundles of actin filaments, whereas the latter consists of separate filaments. Ca^{2+} -calmodulin causes dissociation of bundles with simultaneous increase in the

number of separate calponin-containing filaments. Further increase in the calmodulin concentration results in full release of calponin from actin filaments. In motility assays, calponin, when added together with ATP to actin filaments complexed with immobilized myosin, evoked a decrease in both the number and velocity of moving actin filaments. Addition of calponin to actin filaments before their binding to myosin resulted in a formation of actin filament bundles which were dissociated by ATP.

INTRODUCTION

Calponin, a constituent of smooth-muscle thin filaments [1], is considered to be a potential modulator of smooth-muscle contraction [2]. It interacts with actin and, *in vitro*, inhibits the ATPase activity of smooth- and skeletal-muscle actomyosin. Both binding and inhibition of the ATPase can be relieved in the presence of Ca^{2+} by either an excess of calmodulin [3,4] or phosphorylation of calponin mediated by Ca^{2+} /calmodulin-dependent protein kinase II and protein kinase C [5] or both [6].

Molecular cloning and sequencing of chicken gizzard calponin cDNA revealed the existence of two isoforms, α and β , composed of 292 (molecular mass 32.3 kDa) and 252 (molecular mass 28.1 kDa) amino acid residues respectively [7]. The two calponin variants share a common N-terminal sequence (up to position 216), but the shorter form differs from the longer one by a deletion of 40 amino acid residues in the remaining C-terminal part of the protein. With the use of proteolytic cleavage of α -calponin with chymotrypsin, it has been shown that the actin-binding domain resides within 38 amino acid residues (145–182) starting from the middle of the molecule and stretching towards the C-terminus, in the vicinity of the calmodulin-binding domain (positions 52–144) [8].

Zero-length cross-linking of calponin and actin revealed that C-terminal region of actin (positions 326–355) is involved in the interaction between the proteins [8].

The isotherms of calponin binding to skeletal-muscle F-actin showed that saturation of the binding occurs at 1 calponin molecule/1–3 actin monomers [4,9,10], whereas the maximum inhibition of actomyosin ATPase is observed at a ratio of calponin to actin of 1:1–4 [4,5,10–12]. Both parameters are unaffected by tropomyosin. The affinity of binding of calponin and actin is very high ($K_d = 4.6 \times 10^{-8}$ M) [9]. This strong binding causes conformational changes in actin protomers that seems to be responsible for the reduction of their interaction with myosin heads [13].

In the present study we have re-investigated the interaction of

calponin with F-actin as well as its functional implications. We have also analysed the structure of the calponin–actin complexes.

MATERIALS AND METHODS

Preparation of proteins

Calponin was isolated from chicken gizzard muscle by the method of Takahashi et al. [14]. Rabbit skeletal-muscle actin, rabbit skeletal-muscle myosin and chicken gizzard tropomyosin were prepared as described previously ([15], [16] and [15] respectively). Calmodulin was prepared as described by Gopalakrishna and Anderson [17]. The purity of all proteins was checked by SDS/PAGE on 5–20% gradient mini-slabs by the method of Laemmli [18]. Protein concentration was determined by measuring u.v. absorbance with the following absorption-coefficient values: calponin, $\epsilon_{277}^{1\%}$ 11.3 [5]; G-actin, $\epsilon_{290}^{1\%}$ 6.3 [19]; myosin, $\epsilon_{278}^{1\%}$ 5.4; [20]; tropomyosin, $\epsilon_{277}^{1\%}$ 1.9 [21]; calmodulin, $\epsilon_{278}^{1\%}$ 2.0 [17].

Sedimentation experiments

Sedimentation experiments were performed in a buffer containing 50 mM NaCl, 0.5 mM 2-mercaptoethanol, 20 mM imidazole/HCl, pH 7.0, and 1 mM EGTA or 0.1 mM CaCl_2 . Before the experiments were performed, calponin, tropomyosin and calmodulin were clarified by ultracentrifugation (at 180 000 g for 0.5 h). Samples containing 4 μM F-actin and various concentrations of calponin or calponin and calmodulin (specified in the Figure legends) were incubated for 0.5 h at room temperature with gentle shaking and centrifuged at 15 500 g for 10 min (Eppendorf 5415 C centrifuge). The supernatants were ultracentrifuged at 150 000 g for 1 h (Sorvall RC M100 ultracentrifuge, RP100-AT2 rotor). Samples of supernatants and resuspended pellets were subjected to SDS/PAGE [18]. Gels were stained with

Coomassie Brilliant Blue, and the amount of calponin and actin was quantified in relation to standard amounts of proteins using a Molecular Dynamics computing densitometer.

Motility assay

The motility assay was performed essentially as described by Kron et al. [22] with minor modifications. The surface of a coverslip was coated with 50 % Sigmacote (Sigma) in pentane. To the flow cells the following solutions were added sequentially: (1) myosin (0.5 mg/ml) in buffer A (0.5 M KCl/25 mM imidazole/HCl, pH 7.6); (2) buffer A; (3) BSA in buffer B [25 mM imidazole/HCl (pH 7.6)/25 mM KCl/4 mM MgCl₂/1 mM EGTA]; (4) unlabelled actin (32 µg/ml) in buffer B; (5) 1 mM ATP in buffer C [5 mM KCl/5 mM imidazole/HCl (pH 7.6)/1 mM MgCl₂/0.2 mM EGTA]; (6) 2 mM ATP in buffer B with increased KCl concentration to 150 mM; (7) buffer B; (8) rhodamine-phalloidin (Molecular Probes)-labelled actin (0.64 µg/ml) either alone or with calponin in buffer B; (9) buffer B containing 1 µg/ml glucose oxidase, 0.2 µg/ml catalase, 30 µg/ml glucose and 100 mM dithiothreitol (GOC/DTT) (for reduction of photobleaching); (10) buffer B containing 2 mM ATP and GOC/DTT; and (11) buffer B containing 2 mM ATP, GOC/DTT and various amounts of calponin (indicated in the Figure legends) if calponin is not added at step 8. Introduction of steps (4)–(6) markedly increased the number of moving filaments and allowed us to omit pelleting of myosin with actin in order to remove 'wrong heads' (which bind irreversibly to actin) during preparation of myosin. Filaments were observed in a Jenalumar (Carl Zeiss, Jena, Germany) fluorescence microscope equipped with a silicone intensified tube camera (C2400-08, Hamamatsu). After recording on to videotape, filaments were tracked and analysed using a personal computer equipped with a Scorpion frame grabber card (Univision Technologies) and using software written by Optotech (Warsaw, Poland) enabling automatic detection and tracking of the objects. Only filaments moving with uniform velocity, i.e. these for which any incremental velocity (at a sampling interval of approx. 0.3 s) did not differ more than 20 % of their mean velocity, were considered in the final calculations.

Electron microscopy

For electron-microscopic observations, aliquots of the samples of calponin with F-actin prepared for sedimentation assays (and diluted to a final actin concentration of 0.1 mg/ml), were placed on Formvar-carbon coated grids and negatively stained with aq. 1 % uranyl acetate. They were examined in a JEM 100B electron microscope operating at 80 kV.

RESULTS

Co-sedimentation of calponin with F-actin

Titration of actin (4 µM) by calponin induced the appearance of faint precipitate. Therefore we have applied two-step centrifugation, first at low speed (which does not pellet F-actin itself), followed by the second at high speed. Low-speed centrifugation pellet obtained at saturating amounts of calponin contained 45 % of actin (Figure 1). F-actin remaining in the supernatant sedimented at high-speed. Analysis of both pellets by SDS/PAGE revealed that they contain calponin and actin, although at different molar ratios. In the low-speed pellets a molar ratio of calponin to actin at saturation was about 0.5:1. In the high-speed pellets maximum binding was observed at equimolar ratio of calponin to actin monomer (Figure 1). The increase in ionic

Figure 1 Co-sedimentation of calponin with actin

▽, Percentage of actin sedimenting with calponin during low-speed centrifugation; ○ and ●, molar ratio of calponin to actin in complexes sedimented by low-speed (○) and high-speed (●) centrifugation. Sedimentation experiments were performed at 25 °C in a mixture containing 4 µM actin, various concentrations of calponin, 20 mM imidazole/HCl, pH 7.0, 50 mM NaCl and 1 mM EGTA as described in the Materials and methods section.

strength (from 50 to 100 mM NaCl) or the presence of tropomyosin (at the molar ratio to actin of 1:6) did not significantly affect neither the amount of actin sedimenting at low-speed centrifugation nor the calponin to actin ratio in both pellets (results not shown).

Electron microscopy of negatively stained samples withdrawn before the first low-speed centrifugation of calponin/actin mixtures revealed, besides separate actin filaments, highly ordered bundles of filaments, whereas the supernatants after the low-speed centrifugation contained only separate filaments, indistinguishable from F-actin (Figure 2). These results indicate that calponin is able to form two structurally distinct complexes with F-actin: actin bundles with a low calponin content and actin filaments with a high calponin content.

Titration of calponin–F-actin complexes with calmodulin

Titration of calponin–actin complexes obtained by mixing 4 µM F-actin and 2 µM calponin with Ca²⁺-calmodulin resulted in dissociation of the bundles, as indicated by decrease in the amounts of calponin and actin sedimenting on low-speed centrifugation (Figure 3a). Saturation of this process took place at a molar ratio of calmodulin to calponin of about 3:1. In parallel, calmodulin caused release of calponin from actin filaments, although this process was saturated at higher calmodulin/calponin ratios (about 8:1) (Figure 3b). Thus, initially, Ca²⁺-calmodulin caused dissociation of the bundles, producing actin filaments with additional calponin-binding sites. These sites were saturated by free calponin, giving rise to the pool of calponin-containing actin filaments sedimenting on high-speed centrifugation. A further increase in the calmodulin concentration caused complete release of calponin from actin filaments. A transient increase in the content of calponin in a small number of actin bundles was probably due to their partial dissociation and binding of additional calponin molecules to non-cross-linked parts of the filaments (Figure 3b).

Figure 2 Electron microscopy of calponin-actin complexes

Panels **a-c**, calponin/actin mixture of equimolar ratio; panel **d**, supernatant obtained by low-speed sedimentation of samples used in panels **a-c**. The actin concentration was 0.1 mg/ml. The scale bar represents 100 nm.

Figure 3 Effect of Ca^{2+} -calmodulin on calponin-actin complexes

(a) Low-speed centrifugation of calponin-actin complexes. Δ , Content of calponin in the pellet; ∇ , content of actin in the pellet; $\circ$, molar ratio of calponin to actin in the pellet. (b) High-speed centrifugation of calponin-actin complexes. $\blacktriangle$, Content of calponin in the pellet; $\blacktriangledown$, content of actin in the pellet; $\bullet$, molar ratio of calponin to actin in the pellet; $\blacklozenge$, free calponin present in supernatant. Sedimentation experiments were performed at 25 °C in a mixture containing 4 μM actin, 2 μM calponin, various concentrations of calmodulin, 20 mM imidazole/HCl, pH 7.0, 50 mM NaCl and 0.1 mM CaCl_2 , as described in the Materials and methods section.

Effect of calponin on the *in vitro* motility of F-actin over myosin-coated surface

The effect of calponin on the *in vitro* motility of actin filaments was studied by applying different sequences of addition of the proteins. In the first case, calponin was added after binding of rhodamine-phalloidin-labelled F-actin to myosin-coated surface and initiation of the movement of actin filaments by ATP; in the second case a mixture of calponin with labelled actin was added to myosin and then ATP was infused. Titration of actin filaments complexed with immobilized myosin by calponin in the presence of ATP resulted in a gradual decrease of both the number of moving actin filaments and their average velocity (Figure 4). The concentration of calponin required for full inhibition of the movement was about 0.4 μM .

Addition of calponin to F-actin before its conjugation with immobilized myosin led to the formation of actin bundles with characteristic loops. They bound to myosin in the absence of ATP. Infusion of 2 mM ATP caused slow disaggregation of the bundles and the appearance of short separate filaments that did not move.

Figure 4 Effect of calponin on the *in vitro* motility of F-actin over immobilized myosin

Calponin added to preformed F-actin-myosin complex together with ATP. $\bullet$, Relative number of the moving filaments (among 100 filaments selected at random); $\circ$, relative average velocity of the moving filaments (of 30 filaments selected at random); ----, 'motility index' estimated as the number of moving filaments multiplied by their average velocity. Motility assays were performed at 25 °C in a solution containing 25 mM imidazole/HCl, pH 7.6, 25 mM NaCl, 1 mM EGTA, 4 mM MgCl_2 and 2 mM ATP. The detailed protocol used in the experiments is given in the Materials and methods section.

DISCUSSION

Reversible changes in interaction of calponin with actin filaments is an essential condition for its functioning in actin-linked regulatory system of actin-myosin interaction. In the present paper we have demonstrated that the binding of calponin to F-actin *in vitro*, like the binding of troponin [23], is accompanied by precipitation of the complex in the form of paracrystalline bundles of actin filaments. They are formed at relatively low proportion of calponin to actin monomer (0.5 molecule/actin monomer) and are in equilibrium with actin filaments containing higher amount of calponin (1 molecule/actin monomer). The existence of the two types of complexes between calponin and actin may explain the discrepancies in the stoichiometry of these proteins, from 1:3 [9] to close to 1:1 [4,10], reported by different laboratories. Taking into account the percentage of actin involved in formation of bundles (low-speed pellet) and in filaments (high-speed pellet) and a ratio of actin to calponin in both, the resultant proportion at saturation, under conditions used in the present work, was 0.7–0.8 molecule of calponin/actin monomer.

Calponin-induced formation of actin bundles was independent of the presence of tropomyosin, strongly dependent on ATP and, in the presence of Ca^{2+} , on calmodulin. Calmodulin exerted dual effect on the calponin-actin complexes. At a molar ratio to calponin of 3:1 it dissociated bundles, and at much higher excess it caused complete release of calponin from filaments. This could reflect the two different-affinity calmodulin-calponin binding sites reported by Wills et al. [24], or the existence in the calponin molecule of two, different-affinity, binding sites for actin.

Two ways of calponin-induced bundling of actin filaments can be considered. The first may involve oligomerization of calponin. Since at higher concentrations calponin has a tendency to precipitate (J. Kolakowski, unpublished work), one cannot exclude the possibility that our preparations still contain small aggregates that are responsible for actin bundle formation. The second way, which is more probable, can be attributed to the existence of two actin binding sites on the calponin molecule.

Although it is commonly assumed that the actin-binding site is located in a sequence between amino acid residues 145 and 182 of calponin [8], another region of the molecule situated between residues 48 and 134 which shows sequence similarity to the F-actin-binding region of α -actinin, *Dictyostelium* gelation factor, dystrophin and calpactin I [25], can be considered as a potential binding site of calponin responsible for bundling of F-actin. This site, like α -actinin [26], should interact with the N-terminal amino acid residues of actin. Location of calmodulin within the putative actin bundling site [8] is compatible with the dissociating effect of calmodulin on actin filament bundles demonstrated in the present study.

In conclusion, our results support the existence of two actin binding sites on the calponin molecule, one responsible for bundling and another for inhibition of actomyosin ATPase. The inhibitory actin-binding region of calponin [7] shows sequence similarity to the inhibitory region of troponin I which interact with actin and troponin C [27]. However, actin interfaces which are involved in the interaction with calponin and troponin I are distinct; whereas troponin I, like α -actinin, interacts with negatively charged N-terminal amino acid residues [28], calponin seems to interact with the C-terminal region of actin [8].

The actin-bundling activity of calponin seems not to be exclusively responsible for the observed *in vitro* calponin-induced inhibition of actomyosin ATPase activity, since calponin is able to inhibit the ATPase of cross-linked actin-myosin head [29], i.e. under conditions when bundling cannot evoke steric hindrance of the access of myosin heads to actin. Our results (Figure 4) and those of others [30,31] showing that calponin is able to inhibit movement of actin filaments over myosin-coated surface, when bundling of actin filaments does not occur, are additional evidence for bundling-independent influence of calponin on actin-myosin interaction. Similarly to Shirinsky et al. [30], we have observed (Figure 4) a decrease in both the number of moving filaments as well as in their average velocity with increasing calponin concentrations, and the decrease in the number of moving filaments preceded that of moving-filament velocity. However, under the conditions used by Shirinsky et al. [30], actin filaments behaved in more 'all or none' fashion. This difference is probably mainly attributed to the higher myosin concentration used in our motility assay, since Haeberle [31] observed a strong dependence of the average filament velocity in the presence of calponin on the myosin concentration used for surface coating (density of myosin heads).

Interaction of calponin [8] and caldesmon [32-34] with C-terminal end of actin explains the previously observed competition between these two proteins for the binding to actin filaments [4]. The lower potency of caldesmon in displacing of calponin than the converse could be due to the bundling ability of calponin (at subsaturating ratio to F-actin) and thus its displacement only from non-cross-linked actin filaments.

Recent immunocytochemical studies revealed the existence, in chicken gizzard smooth muscle, of two classes of actin filaments: cytoskeletal and contractile. The former class is composed of the cytoplasmic (β) and the latter of the muscle (γ) isoform of actin respectively [35]. While caldesmon is associated exclusively with contractile filaments, calponin is more concentrated in the domain containing cytoskeletal filaments [36,37]. The bundling of actin filaments by calponin reported in the present paper might be related to the dynamics of cytoskeleton in living cells. Moreover, calponin is also present together with α -actinin in the cytoplasmic dense bodies as well as in the adhesion plaques at the cell surface [37], where it presumably serves a role in the structural arrangement of actin filaments or/and their anchorage.

It has been demonstrated by Lehman [38] that, in the absence

of Ca^{2+} , but not in its presence, thin filaments isolated from chicken gizzard smooth muscle aggregate into a network. This observation led Lehman to conclude that thin filaments bundling on a decrease in the Ca^{2+} concentration may be responsible for sustained tension observed *in vivo* during isotonic contraction of smooth muscle. The ability of calponin to form bundles of F-actin that dissociate in the presence of Ca^{2+} and calmodulin (or another Ca^{2+} -binding protein), as well as recent demonstrations, in *in vitro* motility assays, that calponin not only decreases the rate of cross-bridge cycling (Figure 4) [30,31], but also increases maximum force production by smooth-muscle myosin [31], strongly suggest that calponin may have a dual, cytoskeleton- and contractile machinery-linked, role in maintenance of force in smooth muscle.

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