The Effects of Smooth Muscle Calponin on the Strong and Weak Myosin Binding Sites of F-actin*

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We have investigated the mechanism of inhibition of the actomyosin MgATPase by the smooth muscle protein calponin. We have shown previously the specific interaction of calponin with Glu334 of actin (EL-Mezgueldi, M., Fattoum, A., Derancourt, J., and Kassab, R. (1992) J. Biol. Chem. 267, 15943–15951). This residue is within the sequence 332–334 which has been proposed to be an important part of the strong myosin binding site (Raymond, L., Holden, H. M., Whitaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1993) Science 261, 58–65). Therefore, we suggested that calponin will affect the strong binding actin-myosin interaction.

To test this hypothesis we have investigated the effect of calponin on the strong binding of S-1-MgAMP-PNP (5′-adenylyl imidodiphosphate) and on the weak binding of S-1-MgADP-P, to actin. We found that an inhibitory concentration of calponin decreased the binding of S-1-MgAMP-PNP to actin but had no effect on the binding of S-1-MgADP-P. Similar experiments were obtained with skeletal muscle and smooth muscle S-1. In competition experiments calponin was found to displace S-1-MgAMP-PNP and S-1-MgADP-P from the actin filament. S-1 displaced calponin from actin in the rigor state, in the presence of MgADP, and in the presence of MgAMP-PNP. We conclude that calponin inhibits the actin activated S-1 ATPase by blocking a strong S-1 binding site on actin and does not block the weak binding site.

Three actin-binding proteins, troponin I, caldesmon, and calponin, have been identified as capable of inhibiting the activation of myosin ATPase by muscle thin filaments (1–3). Although the mechanism of inhibition of the actomyosin ATPase by troponin I and caldesmon has been studied extensively (4–9), fewer studies have approached the mechanism of inhibition of calponin (10, 11). Calponin is a 34-kDa smooth muscle protein that binds actin (3), myosin (12), Ca2+-binding proteins (13, 14), and tropomyosin (15). In vitro calponin inhibits the actomyosin ATPase activity upon binding to actin, and this inhibition is reversed by its phosphorylation (16) or by its interaction with Ca2+-binding proteins such as calmodulin or caltrin (13, 14). Consequently, it has been suggested that calponin may have a role in controlling smooth muscle contraction. Although the actin binding and inhibitory activity of troponin I and caldesmon are affected strongly by tropomyosin (4, 8, 17), calponin binding to actin and its inhibitory activity are not affected by tropomyosin (18, 19). This suggests that calponin inhibits the actomyosin ATPase activity at the molecular level by a mechanism different from that of caldesmon and troponin I.

We have investigated previously the calponin-actin interface using proteolysis, peptide synthesis, and recombinant technology (20–22). Those studies led us to build up a detailed picture of the inhibitory domain of calponin. The actin-calponin interface seems to be a multiple contact site involving the calponin sequence extended from Val142 to Tyr182. One contact site involving the calponin amino acids VKYAEK at position 142–147 seems to be directly responsible for ATPase inhibition (21). On the actin monomer, three sequences have been implicated in calponin binding: (i) the sequence 1–226, a large fragment corresponding mainly to actin subdomains 1, 2, and 4 (23); (ii) the sequence 326–355, which spans parts of subdomain 1 and subdomain 3 (20); (iii) the COOH-terminal three amino acids (24). The sequence 326–355 is of particular interest since this region contains the segment 332–334, which has been proposed to be part of the strong binding site of myosin to actin (25). Moreover, calponin has been cross-linked to Glu334 within this segment using the zero-length cross-linker carbodiimide (20). In contrast Lys61 and the NH2 terminus of actin, proposed to be part of the weak binding site of the myosin head (S-1),1 have been excluded from calponin binding (11). From these structural studies (summarized in Fig. 7) it is reasonable to suggest that calponin would inhibit the strong binding actin-myosin interaction and that calponin and the S-1 myosin head would compete for a common binding site on actin.

To test this hypothesis we have investigated the effect of calponin on the binding of S-1 to actin in the absence or the presence of MgAMP-PNP, MgADP, or MgATP. We found that an inhibitory concentration of calponin decreased the strong binding of S-1-MgAMP-PNP to actin. Calponin binding to actin and its inhibitory activity are not affected by tropomyosin (18, 19). This suggests that calponin inhibits the actomyosin ATPase activity at the molecular level by a mechanism different from that of caldesmon and troponin I.

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We conclude that calponin binding to actin affects the strong binding of myosin to actin but has no effect on the weak binding.

MATERIALS AND METHODS

Muscle Proteins—Chicken gizzard calponin and its chymotryptic fragment CH22, spanning residues 7–182, were produced as described previously (20). Rabbit skeletal muscle F-actin and S-1 were prepared

1 The abbreviations used are: S-1, chymotryptic subfragment-1 of myosin; AMP-PNP, 5′-adenylyl imidodiphosphate; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, 1,4-dithio-threitol.
Inhibitory Mechanism of Calponin

Results

Effect of Ionic Strength on Calponin Inhibitory Activity—

Before investigating the mechanism of the acto-S-1 ATPase inhibition by calponin we explored the effect of ionic strength on the inhibitory activity of calponin. Fig. 1 shows the effect of increasing the concentration of calponin on the activation of the ATPase activity of smooth and skeletal muscle myosin S-1 by F-actin at two different KCl concentrations. At 10 mM KCl, increasing concentrations of calponin led to a progressive loss of acto-S-1 MgATPase activity, and about 70% inhibition of the ATPase activity is obtained at a calponin concentration that is equimolar to actin for both smooth and skeletal muscle S-1. At this ionic strength, tropomyosin had no effect on the inhibitory activity of calponin (data not shown). This is in agreement with the previously described pattern of ATPase inhibition by calponin at low ionic strength (18, 20, 35).

Increasing the KCl concentration led to a reduction of the calponin ATPase inhibitory activity. 40 μM calponin was needed to reach 60% inhibition at 60 mM KCl and gave only 30% inhibition at 110 mM KCl (data not shown). Raising the KCl concentration to 160 mM abolished the calponin ATPase inhibitory activity even at the highest concentration used with both smooth and skeletal muscle S-1 (Fig. 1). When we measured the binding of calponin to actin, we found that increasing the ionic strength from 10 to 160 mM KCl had only a slight effect on calponin binding to actin.

We then tested the ability of calponin to displace strongly and weakly bound S-1 complexes from actin. Fig. 4 shows that increasing concentrations of calponin led to a progressive displacement of acto-S-1 MgAMP-PNP (Fig. 4A) and acto-S-1 MgADP (Fig. 4B) from actin.

At low concentration (<8 μM) calponin was more effective in displacing S-1 MgAMP-PNP (Fig. 4A) than S-1 MgADP (Fig. 4B). At 10 μM calponin, which gave 68% inhibition in a parallel
MgATPase assay, only 30% of both S-1 strong complexes were displaced. A maximum of 60% displacement was observed at the highest concentration of calponin with no indication of a plateau being reached.

The observed competition between calponin and S-1 strong binding complexes for actin binding is compatible with the structural studies, indicating partial overlap between the calponin binding region and the S-1 binding region on the actin sequence (20, 25).

To define whether the shared site on actin corresponds only to the strong myosin binding site or also includes the weak binding site we investigated the relationship between calponin inhibition and the binding of S-1 to actin. Fig. 4 shows that up to 20 μM calponin did not displace the weak binding S-1 complex from actin even though this concentration range gave 80% inhibition of the MgATPase. Thus calponin does not interfere with the weak binding site at concentrations that gave full inhibition of MgATPase activity and caused 50% displacement of S-1 bound to actin. At higher concentrations of calponin there was displacement of S-1 bound to actin even though this concentration range gave 80% inhibition of the MgATPase. We conclude that calponin inhibition does not require displacement of S-1 from actin.

**Effect of Calponin on the Weak and the Strong Binding Actin-Myosin Complexes**—We measured the effect of calponin on the strong binding of S-1 to actin by cosedimenting actin and [3H]iodoacetamide-labeled S-1 in the presence of 2 mM MgAMP-PNP and in the absence or presence of an inhibitory concentration of calponin. The same method has been used previously to investigate the effect of caldesmon-tropomyosin and troponin-tropomyosin on the strong binding of S-1 to actin (9, 35). Actin-S-1-MgAMP-PNP complex is considered to be in a strong binding actin-actin cross-bridge state since MgAMP-PNP does not relax muscle mechanically, and its attached structure is similar to that of the rigor state (35, 36).

We found that skeletal muscle S-1-MgAMP-PNP bound to actin with an affinity of 2.5 × 10^5 M^-1 at 10 mM KCl (Fig. 5A). This low affinity of actin-S-1-MgAMP-PNP to actin was decreased strongly (Fig. 5A). From the initial slope we estimated the affinity of skeletal muscle S-1-MgAMP-PNP for actin in the absence of calponin to be 10 times higher than in the presence of calponin. This pattern of results was obtained in five separate experiments. In all of the experiments we noted a slight cooperativity in skeletal muscle S-1-MgAMP-PNP binding to actin in the presence of calponin. We also tested the effect of calponin on the binding of smooth muscle S-1-AMP-PNP to actin. The results are presented in Fig. 5B. Smooth muscle S-1-AMP-PNP bound to actin with an affinity of 6.2 × 10^5 M^-1. At a concentration of calponin which gives 85% inhibition in ATPase assay the binding of smooth muscle S-1-AMP-PNP to actin was reduced significantly (the affinity of smooth muscle S-1-AMP-PNP for actin is seven times higher than for actin-calponin).
We also investigated the effect of calponin on the binding of skeletal muscle S-1 to actin at low ionic strength. Skeletal muscle S-1 MgADP bound actin with a significantly higher affinity, estimated to be $>10^6 \text{M}^{-1}\text{s}^{-1}$. Binding was substantially reduced by inhibitory concentrations of calponin (data not shown). These results show directly that calponin decreases the strong binding of both smooth and skeletal muscle S-1 to actin.

Since calponin weakened the binding of S-1-MgADP-PNP to actin, it seemed possible that calponin might also affect the binding of the weak complex S-1-MgADP-PNP to actin. Previously, assay of the reduction in the EDTA-ATPase activity of S-1 in the supernatant when S-1-MgADP-PNP is cosedimented with actin has been used to measure the weak binding of myosin to actin (37). Skeletal muscle S-1-MgADP-PNP binding was measured over a range of actin concentrations from 0 to 50 \muM. Calponin was added at a fixed molar ratio to actin; at the higher actin/calponin concentrations significant aggregation of filaments occurred which led to irregular measurements of S-1-MgADP-PNP bound. Experiments with 2:1 calponin/actin molar ratio, giving around 65\% inhibition of acto-S-1 MgATPase activity, avoided aggregation, and binding could be measured accurately (Fig. 6). Under these conditions the binding of skeletal muscle S-1-MgADP-PNP to actin in the presence of calponin the curve shows a clear decrease in the binding of both smooth and skeletal muscle S-1-MgADP-PNP to actin.

![Fig. 4. Displacement of S-1 from actin by calponin.](image)

![Fig. 5. Effect of calponin on the binding of S-1-MgAMP-PNP to actin.](image)
Inhibitory Mechanism of Calponin

FIG. 6. Effect of calponin on the binding of S-1-MgADP-P_i to actin. The fraction of skeletal muscle S-1 bound to actin in the presence of MgATP is plotted against the concentration of actin. S-1 bound to actin was determined by the EDTA-ATPase activity of the S-1 remaining in the supernatant after sedimentation with actin filaments as described under "Materials and Methods." Measurements were made in the absence (open circles) or presence (closed circles) of calponin at a molar ratio to actin of 1:1. This gave 65% inhibition in MgATPase assay. Conditions were: 10 mM PIPES, pH 7.0, 10 mM KCl, 2.5 mM MgCl_2, and 0.1 mM DTT.

on the binding of S-1-MgADP-P_i to actin (data not shown).

The converse experiment is shown in Fig. 4C. At a constant actin and S-1-MgADP-P_i concentration, addition of calponin inhibited actin activation but did not change the amount of S-1-MgADP-P_i bound.

We conclude from these experiments that calponin inhibits the actin-activated S-1-MgATPase without affecting the weak binding of S-1-MgADP-P_i to actin.

DISCUSSION

Regulation of cross-bridge cycling in smooth and striated muscles involves regulatory proteins that act either on the thick or the thin filaments. Despite the diversity of regulatory proteins it is generally observed that they control cross-bridge cycling by a common mechanism that involves regulating the transition from the weak (actin-myosin-ADP-P_i) to the strong (actin-myosin-ADP) complex (38). This may be a direct effect, as in the regulation by myosin light chain phosphorylation (37), or an indirect mechanism in which the state of acto-tropomyosin is controlled and the block of the strong site is due to tropomyosin (1). Troponin and caldesmon are examples of this kind of regulation (4, 9). Calponin is a component of smooth muscles associated with actin filaments which is an inhibitor of acto-myosin cross-bridge cycling in vitro and which has therefore been proposed as a potential muscle regulatory protein (3). Our goal in carrying out this work was to determine the mechanism by which calponin inhibits the acto-S-1 ATPase at the molecular level.

From published work it is evident that calponin inhibition of actin filament activity involves a mechanism that is different from striated muscle troponin or smooth muscle caldesmon, since inhibition is not mediated by tropomyosin (18, 19). Nevertheless, calponin could act upon the weak to strong transition directly. To determine how calponin inhibits the actin-activated myosin MgATPase we used binding methods that have been used to study the effect of troponin-I-tropomyosin (4) and caldesmon-tropomyosin (9) on the weak and strong binding of S-1 to actin.

We found that calponin inhibition was maximal at low ionic strength and was diminished rapidly as ionic strength was increased, irrespective of whether measurements were made using skeletal or smooth muscle myosin subfragments. This made it necessary to do our studies under low ionic strength conditions. The observation that regulation of acto-S-1 by calponin is the same with the smooth and skeletal muscle S-1, despite the 50-fold difference in their V_max and the strikingly different ionic strength dependences of the acto-S-1 interactions (39), indicates that calponin acts only upon the properties of the actin filament. Therefore we are justified in using smooth and skeletal muscle S-1 interchangeably; it should be noted that the majority of previously published experiments on calponin have used skeletal muscle S-1 and assumed that results would be valid for smooth muscle S-1.

A direct measurement of the affinity of the strong binding complex S-1-AMP-PNP for actin showed that inhibitory concentrations of calponin strongly reduced the binding affinity, showing that calponin did indeed inhibit the weak to strong transition. The binding experiment was backed up by competition experiments showing that calponin and strong binding myosin complexes such as S-1-AMP-PNP compete for a common binding site on actin. In contrast, neither competition nor binding experiments showed any evidence for an effect of calponin on the weak binding of S-1-MgADP-P_i to actin. Together, these results suggest that calponin inhibits the acto-S-1 ATPase by blocking an actin-S-1 contact site exclusively involved in the strong binding of S-1 to actin and crucial for cross-bridge cycling.

This conclusion is consistent with previously published works. Horiuchi and Chacko (10) reported from kinetic studies of smooth muscle acto-S-1 MgATPase that calponin inhibition corresponds mainly to a decrease of the velocity (V_max) with only a slight effect on the affinity of S-1 for actin (K_m). This suggests that calponin has only a slight effect on the binding of the myosin head to actin; instead it affects primarily the rate-limiting step (presumably corresponding to the weak to strong transition). Furthermore, calponin inhibited maximal shortening velocity with only a minor effect on force in skinned Taenia coli smooth muscle fibers (40). In the in vitro motility assay, calponin decreased the fraction of motile actin filaments (34, 41), which was also observed with troponin and caldesmon, both known to inhibit by blocking the strong binding state (42, 43).

Calponin has been shown to inhibit the ATPase activity of the carbodiimide cross-linked skeletal acto-S-1 complex at low ionic strength (11). In this complex S-1 is attached covalently to the NH_2 terminus of actin and is permanently bound to actin, so it is impossible to change the initial weak binding of S-1 to actin. Hence any inhibition observed must be due to inhibition of other steps in the ATPase cycle. We have observed that smooth muscle S-1 cross-linked to actin is also inhibited by calponin at low ionic strength and that this inhibition is abolished at higher ionic strength (data not shown) as it was for the reversible complex (Fig. 1). Caldesmon-tropomyosin and troponin-tropomyosin can also inhibit the carbodiimide cross-linked acto-S-1; once again this experiment emphasizes that calponin inhibits the same step in cross-bridge cycling as caldesmon and troponin.

Previously we have shown the specific interaction of calponin with actin Glu334 (20). In their model of the actin-myosin interaction Raymont and co-workers (25, 44, 45) predicted the myosin segment 332–334 as an important part of the actin-myosin interface and proposed this contact site to be part of the strong binding site in the actin-myosin complex (see Fig. 7). Our finding that calponin decreases exclusively the strong binding of S-1 to actin confirms the participation of this actin-S-1 contact site exclusively in the strong binding state. It is noteworthy that calponin is the only actin-binding protein that...
has been cross-linked to the putative strong binding site on actin. This seems to be fortuitous as only calponin has the right amino acid at the interface with actin to permit this cross-linking.

Our findings are also compatible with previous evidence indicating that the calponin binding region on actin does not overlap functionally with the weak binding sites of S-1 (see Fig. 7). Cleavage of actin at Met47-Gly48 yields actin split into 9- and 36-kDa fragments and reduces the affinity of actin for myosin (46) but has no effect on the binding of calponin to actin (20). This supports the lack of influence of the NH2-terminal actin segment on the interaction of actin with calponin. In addition, modification of the actin NH2 terminus by ethylenediamine decreased the binding of S-1-ADP·P to actin (47) but had no effect on the binding of calponin to actin (11).

These results confirm and complete our view of the calponin-actin interface (21). There are clearly two types of interactions; the inhibitory interaction is strongly salt-dependent and is competitive with myosin strong binding, whereas the other interaction is salt-independent. Our previous work (21) suggests that the calponin sequence VKYAEEK at position 142–147 is an essential part of this interaction, but the sequence 148–182 also binds to actin and could be responsible for calponin binding at higher salt concentrations. Although the actin sequence around Glu334 probably binds the calponin inhibitory sequence, it is not clear where on actin the noninhibitory sequence of calponin binds; it cannot be in a position that blocks the weak binding site.

Although calponin inhibition seems to control the same step in the actomyosin pathway as troponin and caldesmon, it involves a different mechanism. Full inhibition of the acto-S-1 ATPase activity is achieved at a molar ratio to actin of 1:1 for troponin and 1:14 for caldesmon in the presence of tropomyosin (8), whereas the same inhibition is obtained at an equimolar ratio of calponin to actin (Fig. 2). Tropomin and caldesmon bind to subdomain 1 on actin and block the weak binding site of myosin on the actin monomer involved in their attachment. However, their effect on the strong binding site of the 14 monomers for caldesmon and the 7 monomers for troponin is mediated and propagated by tropomyosin (6, 8, 9). Calponin acts directly upon the strong binding of S-1 at a ratio to actin of 1:1 and appears to be unique in that it specifically blocks only the strong site on actin. This property could make calponin a useful reagent to probe the cross-bridge cycle and its regulation.

It is not clear whether calponin plays a significant role in regulating smooth muscle actomyosin in vivo since its location, quantity, and phosphorylation pattern do not seem to be compatible with a physiological role (48–51). In the present study we also show that at physiological ionic strength calponin has no inhibitory activity, and this is observed with both smooth and skeletal muscle myosin. It is possible that in smooth muscle the concentration of proteins and the presence of other regulatory proteins may modulate calponin’s effect on the actomyosin ATPase. Nevertheless, our study of the mechanism of ATPase inhibition by calponin confirms the general principle of regulation in actomyosin systems, namely that the regulatory molecules control the weak to the strong transition in the cross-bridge cycle (38).

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