Correlation between calponin and myosin subfragment 1 binding to F-actin and ATPase inhibition

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Calponin is a thin-filament-associated protein that has been implicated in the regulation of smooth-muscle contractility. It binds to F-actin and inhibits the MgATPase activity of actomyosin. In the present work we have examined the effect of recombinant chicken gizzard α -calponin (R α CaP) on the binding of rabbit skeletal-muscle myosin subfragment 1 (S1) to F-actin and on the inhibition of its actin-activated MgATPase. We have found that binding of one R α CaP molecule to every three to four

actin monomers is sufficient for maximal inhibition of acto-S1 ATPase. At this R α CaP/actin ratio R α CaP does not interfere with S1 binding to F-actin. At higher concentrations, R α CaP displaces S1 from F-actin and a 1:1 R α CaP-actin monomer complex is formed. R α CaP is also able to displace troponin I from its complex with F-actin which may reflect the amino acid sequence similarity between R α CaP and troponin I in their actin-binding regions.

INTRODUCTION

Regulation of smooth-muscle contraction involves phosphorylation of the regulatory light chain of myosin by Ca^{2+} /calmodulin-dependent myosin light chain kinase (for a review see refs. [1–5]). The thin-filament-associated smooth-muscle proteins calponin (CaP), caldesmon and tropomyosin are also thought to be involved in regulation of contraction (for a review see refs. [6–8]), but their specific functions are not well understood.

CaP was first isolated from chicken gizzard smooth muscle by Takahashi et al. [9]. It has been shown that CaP is capable of binding to actin and inhibiting the actin-activated MgATPase activity of actomyosin [9–14]. The stoichiometry of CaP binding to actin appears to be not well defined and values between 1:3 and 1:1 CaP to actin have been reported [9,11,12,15]. The discrepancy between various reports may be related to the formation of two types of CaP-F-actin complexes depending on ionic strength and protein concentration [15,16]. Even though CaP is capable of forming a 1:1 complex with actin, the maximal inhibition of actomyosin ATPase is obtained at significantly lower than equimolar CaP/actin ratios [11,13,17]. Thus it is not clear what the relationship between CaP-actin binding and the inhibition of actomyosin ATPase is. The inhibitory properties of CaP appear to be related to a segment (residues 146–171) that has considerable sequence similarity to the inhibitory region of the skeletal-muscle troponin I (TnI) [18]. Studies using proteolytic fragments and peptide analogues of CaP have indicated that the region comprising residues 145–182 is important for binding to actin [19–21]. These observations suggest that the mechanisms of actomyosin ATPase inhibition by CaP and by TnI may share some common features. However, unlike TnI the binding of CaP to actin and its inhibitory properties are not enhanced by tropomyosin (Tm).

In the present work we have correlated CaP and myosin subfragment 1 (S1) binding to F-actin with the ATPase inhibition. We found that one CaP molecule bound to every three to four actin monomers is sufficient to inhibit acto-S1 ATPase maximally with

little effect on S1 binding to F-actin. At higher concentrations, CaP causes displacement of S1 from F-actin, and a 1:1 CaP-actin complex is formed. CaP also competes with TnI for the binding sites on actin.

MATERIALS AND METHODS

Chemicals

Buffer components were from Research Organics (Cleveland, OH, U.S.A.). Materials for recombinant DNA procedures were from Life Technologies (Gaithersburg, MD, U.S.A.). Materials for gel electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.). Other commonly used reagents were from Sigma (St. Louis, MO, U.S.A.).

Proteins

F-actin [22], S1 [23] and TnI [24] were prepared from rabbit skeletal muscle according to established procedures. Chicken gizzard Tm was prepared as described by Sanders et al. [25]. R α CaP was expressed and purified as described previously [26]. Protein concentration was determined spectrophotometrically using A_{280} (1 %, 1 cm) values of 10.9 for actin, 7.4 for R α CaP, 4.0 for TnI and 7.7 for S1, and A_{277} (1 %, 1 cm) = 2.4 for Tm.

ATPase activity

The actin-activated ATPase activity of S1 was measured at 25 °C for 20 min in a reaction mixture (375 μ l) containing 3 μ M F-actin, 2 μ M S1 (also 0.5 μ M Tm where indicated), 50 mM NaCl, 5 mM MgCl_2 , 1 mM dithiothreitol, 1 mM NaN_3 and 20 mM Hepes, pH 7.5. The reaction was initiated by adding ATP to a final concentration of 2 mM, and terminated by adding SDS and EDTA to final concentrations of 3.3 % and 30 mM respectively. Liberated P_i was determined by the method of Fiske and SubbaRow [27].

Abbreviations used: CaP, calponin; R α CaP, recombinant α -isoform of chicken gizzard CaP; TnI, rabbit skeletal-muscle troponin I; S1, chymotryptic subfragment 1 of rabbit skeletal-muscle myosin; Tm, chicken gizzard tropomyosin; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate.

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Binding assay

The binding of R α CaP to F-actin was studied by adding R α CaP (0–15 μ M final concentration) to a solution containing 4 μ M F-actin (also 0.5 μ M Tm when present), 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM NaN₃ and 20 mM Hepes, pH 7.5. In some experiments involving S1, 2 mM ADP or 2 mM adenosine 5'-[β,γ -imido]triphosphate (p[NH]ppA) was also included. The competition experiments were performed under similar conditions except that the complexes of F-actin (4 μ M) with R α CaP, S1 or TnI (4 μ M) were formed before the addition of the competing protein (0–15 μ M). In all experiments the samples were incubated for 20 min at 25 °C, then centrifuged at 176000 *g* for 20 min at 25 °C in a Beckman TL100 ultracentrifuge. The pellets were solubilized with 1 % SDS and subjected to SDS/PAGE [28] followed by staining with Coomassie Blue R-250. Proteins on gels were quantified by video image densitometry using a Sony XC-77 CCD video camera interfaced with a MacIntosh computer. Gel images were analysed with the program Image from the National Institutes of Health, Research Series Branch. For calibration, various amounts of R α CaP, S1 and TnI were mixed with a constant amount of actin (5.3 μ g loaded on gel), which was used as an internal standard. Protein amount for each band was expressed as a ratio to actin of the peak area in the absorbance graphs obtained for 1–2 mm-wide strips of the gel. The relative protein concentration was plotted as a function of the relative peak area and fitted with linear (for TnI) or second-order polynomial (for S1 and R α CaP) equations. The fitted equations were used for calculation of the amount of protein in unknown samples. The amounts of R α CaP and TnI bound to actin were calculated by subtracting the amounts of these proteins that sedimented alone (in this case actin was added to the pellets) from the amounts that cosedimented with actin. For determination of apparent binding constants the binding curves were analysed as described by Morris and Lehrer [29] using non-linear least-squares fitting with the program Kaleida-Graph (Abelbeck Software).

RESULTS

We have used a high-speed sedimentation assay to measure the binding of R α CaP to F-actin in the presence and absence of Tm. The data are consistent with a simple binding equilibrium with no co-operativity, with or without Tm present (Figure 1). The apparent binding constants obtained from the fit are 7×10^5 M⁻¹ and 1×10^6 M⁻¹ for binding in the absence and presence of Tm respectively. In both cases the binding reaches saturation at a CaP/actin ratio of approx. 1:1. Thus Tm has no effect on R α CaP binding, consistent with the observations reported by others [8,15].

In Figure 2 we have compared the inhibitory effect of R α CaP on the acto-S1 ATPase activity in the presence and absence of Tm. Although the initial ATPase activity is much higher in the presence than in the absence of Tm the maximal level of inhibition and the concentration of R α CaP needed for maximal inhibition are similar. It is clear from Figure 2 that most of the inhibition occurs at very low levels of actin saturation with R α CaP. Analysis of the dependence of ATPase inhibition by R α CaP on the amount of R α CaP bound to actin indicates that one R α CaP molecule is capable of inhibiting up to three to four actin monomers (Figure 2 inset). Binding of R α CaP in excess of 0.3 R α CaP/actin does not cause any further change to the ATPase activity.

Next we examined the effect of S1 on R α CaP binding to F-actin in the presence and absence of Tm. Figure 3 shows that S1 readily displaces R α CaP from the R α CaP-actin complex. Tm

Figure 1 Binding of R α CaP to actin in the presence (▲) and absence (△) of Tm

Various concentrations of R α CaP were added to rabbit skeletal F-actin (4 μ M) and the complex was sedimented by ultracentrifugation. The amount of R α CaP bound to actin was measured by densitometric scanning of the gels obtained from pellets. The data are fitted with a simple binding isotherm as described by Morris and Lehrer [29].

appears to have little effect on the displacement of R α CaP, although the amounts of both S1 and R α CaP that sediment with F-actin in the presence of Tm are slightly, but systematically, higher than those sedimenting with actin in the absence of Tm. These results suggest that the nucleotide-free S1 competes with R α CaP for the binding site on F-actin.

In the above experiments the S1-F-actin interaction corresponds to that in the rigor state (no nucleotide). To evaluate to what extent the effect of CaP depends on the strength of myosin-actin interaction, we studied the competition between

Figure 2 Inhibition of rabbit skeletal-muscle acto-S1 ATPase activity by R α CaP in the presence (▲) and absence (△) of Tm

Assays were performed in the presence of 3 μ M actin and 2 μ M S1, and 0.5 μ M Tm when present. The inset shows the dependence of ATPase inhibition by R α CaP on the amount of R α CaP bound to actin. The percentage inhibition is calculated from $100 \times (V_{\max} - V)/(V_{\max} - V_{\min})$, where V is the ATPase rate at each R α CaP concentration and $V_{\max}$ and $V_{\min}$ are the ATPase rates at zero and infinite concentrations of R α CaP respectively. The amount of R α CaP bound to F-actin at each concentration was calculated using the binding parameters obtained from the best fit of the data in Figure 1.

Figure 3 Effect of myosin S1 on R α CaP binding to F-actin in the presence (▲, ■) and absence (△, □) of Tm

Various amounts of S1 in the absence of nucleotide were added to F-actin (4 μ M) preincubated with R α CaP (4 μ M). Abscissa shows molar ratios of added S1 to actin. Left and right ordinates show molar ratios to actin of bound R α CaP (△, ▲) and S1 (□, ■) respectively.

Figure 4 Effect of R α CaP on S1 binding to F-actin

Total [F-actin] and [S1], 4 μ M each. The abscissa shows the ratio of R α CaP to actin after each addition. Left and right ordinates are the ratios of S1 (solid symbols) and R α CaP (open symbols) respectively to actin. Experiments were performed in the absence of nucleotide (□, ■) and in the presence of 2 mM ADP (△, ▲) or 2 mM p[NH]ppA (○, ●).

R α CaP and S1 for F-actin in the presence of 2 mM ADP or 2 mM p[NH]ppA, a non-hydrolysable analogue of ATP. Although both S1-ADP and S1-p[NH]ppA are believed to form the so-called strong binding cross-bridges with F-actin, the binding in the presence of p[NH]ppA is 10–30-fold weaker than that in the presence of ADP [30]. As shown in Figure 4, with increasing concentrations of R α CaP the amount of S1 sedimenting with actin decreases only slightly for the nucleotide-free S1; the decrease is more pronounced for S1-ADP, and even more pronounced for S1-p[NH]ppA. In all cases there is a lag phase, and the amount of S1 sedimenting with actin begins to decrease only after the level of actin saturation with R α CaP exceeds 0.2. A comparison of the data in Figure 4 with those in Figure 1 indicates that the binding of R α CaP to F-actin is essentially not inhibited by S1-p[NH]ppA. For example, at 4 μ M R α CaP (a 1:1 molar ratio of R α CaP to actin) there are 0.42 and 0.39 mol of R α CaP bound per mol of actin in the

Figure 5 Competition between R α CaP and TnI for binding to actin

The solutions contained 4 μ M F-actin and 4 μ M TnI. The abscissa shows the molar ratio of added R α CaP to actin. Left and right ordinates are molar ratios of bound TnI (●) and R α CaP (▲) respectively to actin.

absence and presence of S1-p[NH]ppA respectively. On the other hand the R α CaP-F-actin binding is inhibited by S1-ADP and to a greater extent by nucleotide-free S1. These results indicate that a displacement of S1 by R α CaP from F-actin occurs regardless of whether or not a nucleotide is bound to S1. The extent of displacement of S1 by R α CaP appears to be correlated with the affinity of the S1-nucleotide complex for actin. The displacement occurs, however, at higher levels of actin saturation with R α CaP than those needed for the inhibition of acto-S1 ATPase activity.

In view of the similarities between R α CaP and TnI it was of interest to examine whether the two proteins would compete for F-actin. We found that addition of increasing amounts of R α CaP to the TnI-F-actin complex causes a decrease in the amount of TnI that sediments with F-actin (Figure 5). Conversely, when increasing concentrations of TnI were added to the R α CaP-F-actin complex, the amount of R α CaP bound decreased as the amount of TnI bound increased (results not shown). Unlike the S1 displacement by R α CaP, the TnI displacement did not exhibit a lag phase. The displacement of TnI from actin by R α CaP occurred more readily than that of R α CaP by TnI, suggesting that the affinity of R α CaP for actin is higher than that of TnI. Assuming simple binding isotherms, the data in Figure 5 can be used to calculate the ratio (R) of the apparent association constants for TnI (K_a^{TnI}) and R α CaP (K_a^{CaP}) binding to F-actin:

$$R = K_a^{\text{TnI}} / K_a^{\text{CaP}} = \text{TnI}_b(\text{CaP}_t - \text{CaP}_b) / \text{CaP}_b(\text{TnI}_t - \text{TnI}_b),$$

where the subscripts t and b indicate concentration of total and bound protein respectively. We have obtained $R = 0.29$, which, in combination with the data in Figure 1, gives an estimated association constant for TnI binding to actin in the absence of Tm, $K_a^{\text{TnI}} = 2 \times 10^5 \text{ M}^{-1}$.

DISCUSSION

The data presented in this work indicate two different mechanisms of interaction between R α CaP and F-actin, each having a different effect on myosin S1-F-actin binding and on the inhibition of acto-S1 ATPase activity. One R α CaP bound to every three to four actin monomers in F-actin filaments is sufficient to inhibit maximally acto-S1 ATPase. At these saturation levels there is no competition between S1 and R α CaP. At higher con-

centrations, up to one R α CaP molecule can bind to each actin monomer causing a displacement of S1 from actin. Under neither of these conditions is there a significant effect of Tm.

Our calculations showing that at low saturation one CaP molecule is capable of inhibiting three or four actin monomers is consistent with the results obtained previously by Winder and Walsh [13], Marston [17] and Horiuchi and Chacko [11]. Such a property is not surprising in view of the elongated shape (approx. length 16.2 nm) and flexibility of CaP [31]. It is not clear, however, whether CaP actually makes contact with all the actin molecules that it is capable of inhibiting. A longitudinal interaction of CaP with F-actin would require at least two interaction sites. The actin-binding site in CaP has been attributed to a rather short stretch comprising residues 145–182 [19]. An even shorter synthetic peptide (residues 145–163) was found to bind actin and to inhibit ATPase [20]. In a recent report, deletion of a hexapeptide adjacent to the postulated actin-binding site (residues 142–147) was found to have little effect on actin binding of CaP, but it abolished its ATPase-inhibitory properties [32], consistent with our results and the previously postulated effect of CaP on $V_{\max}$ of actomyosin ATPase [10,33,34]. In another report, phosphorylation of CaP at Ser-175 was found to cause both a loss of the inhibitory properties of CaP and a significant decrease in affinity for actin [35]. A loss of inhibitory properties was also observed on replacement of Ser-175 with Asp [36] or with Cys [37]. It is clear that the two essential properties of CaP, i.e. actin binding and the ATPase inhibition, can be uncoupled under some conditions, but under others both properties can be affected simultaneously. It appears that both types of behaviour can be reconciled in view of our present results showing that, depending on its concentration, CaP may either displace S1 from F-actin or inhibit the ATPase activity without any effect on S1 binding. In view of our results it becomes important to distinguish which type of inhibition mechanism is being tested. This is especially important whenever weakly binding synthetic peptides or deletion mutants are used.

It is interesting that, in spite of different functional properties of R α CaP–F-actin complexes at low and high saturation levels, the binding data (Figure 1) could be fitted with a single binding constant, suggesting a single class of binding sites. Recently Lu et al. [15] analysed CaP–F-actin binding using the McGhee and von Hippel model originally developed for ligand binding to a one-dimensional lattice and found a 1:2 CaP/actin binding with a slight positive co-operativity at 150 mM ionic strength, but a 1:1 binding at lower ionic strengths. Thus using a simple binding isotherm in fitting our data appears justified and it is unlikely to introduce any significant error. The apparent association constant for R α CaP binding to F-actin calculated from our data ($7 \times 10^5 \text{ M}^{-1}$) is not significantly different from that reported by Lu et al. [15] ($1.9 \times 10^6 \text{ M}^{-1}$). A complication in the analysis of the binding data is related to the ability of CaP to cause actin bundling [15,16,38]. According to Lu et al. [15], bundling occurs at CaP/actin ratios in excess of 0.3. It is perhaps significant that this is the range in which we observe mutual displacement between CaP and S1. It is plausible that bundling facilitates the displacement of S1 by CaP *via* steric restriction of S1-binding sites on actin. Another possibility is that, at high saturation levels, a different type of complex is formed in which CaP directly interferes with S1 binding.

Another interesting finding of this work is that the binding of R α CaP and TnI to F-actin is mutually exclusive. It is well established that proteolytic fragments or synthetic peptides corresponding to the so-called 'inhibitory region' of TnI (residues 96–114) are capable of binding to actin and inhibiting actomyosin ATPase activity [39–41]. It was pointed out by Takahashi and

Nadal-Ginard [18] that the region in CaP comprising residues 146–171 exhibits considerable similarity to the inhibitory region of TnI, although strict homology could not be established. Our finding that R α CaP and TnI displace each other from the complex with actin is consistent with the idea that they compete for the same site and thus share some structural similarity. However, an indirect mechanism involving a conformational change in actin cannot be excluded, especially in view of the report by Noda et al. [42], who showed that CaP affects the fluorescence of a probe attached to Cys-374 of actin and interpreted this effect as indicating a fluorescence change in actin induced by CaP. It appears that the functional similarity between CaP and TnI occurs only at high levels of actin saturation, i.e. when CaP exerts a competitive inhibition on acto–S1 ATPase. Unlike TnI, the inhibitory properties of which are greatly enhanced by Tm, those of CaP are not. On the other hand CaP alone is significantly more potent than TnI in that it can inhibit three or four actins, as discussed above.

The observation that myosin heads and R α CaP displace each other from F-actin raises the possibility of a common site on actin for these proteins. A binding site in actin of a nucleotide-free S1 was established, by zero-length cross-linking studies, to be the N-terminal residues 1–12 [43]. The N-terminal segment of actin was also indicated in the weak binding of myosin S1 [44]. Likewise a binding site in actin for TnI was shown to be the same N-terminal region of actin [45,46]. The fact that R α CaP displaces S1 and TnI from actin may suggest that CaP also binds to the N-terminal region of actin. However, modification of the N-terminal acidic residues in actin had no effect on the binding of CaP to actin [34], indicating minimal interaction between CaP and the N-terminal segment of actin. Reports by Noda et al. [42] that binding of CaP affects the fluorescence of pyrene attached at the penultimate C-terminal cysteine of actin, and by Mezgueldi et al. [19] that CaP can be zero-length cross-linked to a fragment corresponding to residues 326–355 point to the C-terminal segment of actin as the CaP-binding site. In view of the spatial proximity of the N- and C-termini of actin, it is likely that the two proteins displace each other *via* steric restriction even though their binding sites are different. For S1, recent molecular modelling studies of the rigor S1–F-actin structure [47] indicate that, aside from the N-terminal region, nucleotide-free S1 makes extensive contact with the lower portion of subdomain 1 in actin (following the nomenclature defined in Kabsch et al. [48]). This region comprises residues 341–354 and 144–148, and is in close proximity to both the C- and N-termini of actin. Thus both CaP and TnI may easily make contact with this portion of the actin molecule and with S1.

In summary, we have found that R α CaP inhibits acto–S1 ATPase without any effect on S1–actin interaction at low levels of R α CaP/actin saturation (< 0.3) and displaces S1 from F-actin at high saturation (0.3–1.0). The inhibition induced by R α CaP does not involve Tm, a property distinguishing the regulatory properties of CaP from those of TnI. R α CaP is also able to displace TnI from its complex with F-actin.

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