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Interaction between calponin and smooth muscle myosin

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Calponin is a thin filament-associated protein in smooth muscle that has been shown to bind actin, tropomyosin and calmodulin, and has been implicated to play a role in regulation of smooth muscle contractility. Using a centrifugation assay we found that calponin interacts with unphosphorylated filamentous smooth muscle myosin. We found that this calponin–myosin interaction is reversed by Ca^{2+}-CaM, and depends on ionic strength. At 50 mM NaCl the binding constant and the stoichiometry of this interaction were estimated to be 2×10^6 M^{-1}, and 1.2–2.4 calponin per myosin, respectively. We suggest that the calponin–myosin interaction could be involved in regulation of smooth muscle contractility by anchoring myosin to actin.

Calponin; Myosin; Smooth muscle

1. INTRODUCTION

Calponin is a 33 kDa thin filament-associated protein that has been suggested to play a role in regulation of smooth muscle contractility [1,2]. It is known to be capable of binding to actin, tropomyosin and CaM [1,3,4], and of inhibiting the actin-activated myosin ATPase activity [2,5]. This inhibitory effect of calponin was found to be reversed upon the addition of Ca^{2+}-CaM [5] or by phosphorylation [2]. Calponin has also been shown to inhibit actin translocation over myosin heads in in vitro motility assays [6,7]. It was also found that calponin enhances the affinity between actin and myosin in in vitro motility assay [7], raising the possibility that calponin interacts directly with myosin as well as with actin. The mechanism whereby calponin inhibits actomyosin ATPase is still under investigation [5,8,9]. Although it is commonly assumed that the inhibitory effect is exerted via actin, it has been suggested that it can also occur via a direct interaction between calponin and myosin [8].

This study was designed to examine whether calponin indeed interacts with smooth muscle myosin directly. We found that calponin co-sediments with unphosphorylated, filamentous myosin via an equilibrium binding process. This binding is reversed by Ca^{2+}-CaM and is weakened at salt concentrations higher than 50 mM NaCl. The binding constant and stoichiometry of this interaction was estimated to be 2×10^6 M^{-1} and 1.2–2.4 calponin per molecule of myosin, respectively. We speculate that calponin may function to anchor myosin to actin in smooth muscle contractility.

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Abbreviations: CaM, calmodulin; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GCaN, gizzard calponin; RαCaN, recombinant α-isoform of calponin; Tn-C, troponin-C; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Materials*

Ovalbumin and other commonly used reagents were from Sigma. Materials from gel electrophoresis were from BioRad.

2.2. *Proteins*

GCaN [1] and myosin [10] were isolated from chicken gizzards. CaM and Tn-C were isolated from bovine brain [11] and rabbit skeletal muscle [12], respectively. Expression, purification and characterization of RαCaN is described elsewhere [13]. Here we briefly mention that RαCaN is a non-fusion protein whose amino acid composition and N-terminal sequence is identical to that derived from the nucleotide sequence, and that like GCaN, RαCaN is fully capable of binding to Ca^{2+}-CaM, Ca^{2+}-Tn-C, tropomyosin, actin, and of inhibiting skeletal muscle acto-subfragment-1 ATPase activity. Protein concentration was determined either by the Lowry method or spectrophotometrically using $A_{1\%,1cm}$ values of 2.0 at 276 nm for CaM [14], 11.3 at 277 nm for calponin [2], 4.5 at 280 nm for myosin [15], and 1.8 at 280 nm for Tn-C [16].

2.3. *Sedimentation assay*

For most of experiments proteins at a final concentration of 6 μM were incubated in 150 μl of 20 mM Tris-HCl, 50 mM NaCl, 2 mM NaN_3, 1 mM DTT, pH 7.5, for 20 min at 4°C, then centrifuged at $100,000 \times g$ for 20 min at 4°C in a Beckman TL 100 ultracentrifuge. Aliquots of the incubation mixtures prior to centrifugation, supernatants and solubilized pellets were then subjected to SDS-PAGE electrophoresis.

2.4. *Quantification of protein content from bands on SDS-PAGE gels*

The amounts of materials in the incubates, supernatants and pellets

were quantified by densitometry using a laboratory-built image analysis system. The images of Coomassie blue-stained gels were captured using a Dage-MTI (Michigan City, IN, USA) model CCD-72 camera, equipped with high resolution optics. The area under each band was then computed using the program IMAGE by Wayne Rosbard distributed by the National Institutes of Health, Research Series Branch. Standard curves for calponin and myosin were constructed to establish the linear concentration range. The 20 kDa myosin light chain was used for the calculation of myosin concentration.

3. RESULTS AND DISCUSSION

As expected, centrifugation of myosin (6 μM) at 50 mM NaCl produced complete sedimentation of the myosin (Fig. 1, lanes 2–4), since under these conditions myosin is in the readily sedimentable filamentous form. Under the same conditions 11–32% of RαCaN was found in the pellet (Fig. 1; lanes 5–7), indicating RαCaN aggregates to a certain extent under these conditions. When RαCaN was centrifuged together with myosin 67–80% of the RαCaN sedimented (Fig. 1, lanes 8–10). Similar results were obtained for GCaN (Fig. 1, lanes 11–16). These results indicate that both GCaN and RαCaN can co-sediment with the filamentous form of myosin. RαCaN was used in all further experiments.

In order to ascertain whether the observed co-sedimentation of RαCaN and myosin resulted from RαCaN–myosin interaction or from occlusion by myosin aggregates, we compared the sedimentation properties of RαCaN with those of ovalbumin and Tn-C. Both proteins remained in the supernatant regardless of whether they were centrifuged in the presence or absence of myosin (data not shown), indicating that myosin filaments have no tendency to occlude other proteins that are present in solution.

Since Ca^{2+}-CaM is known to reverse the interaction between calponin and actin [5], we examined whether calponin–myosin interaction is likewise reversible upon addition of Ca^{2+}-CaM. Ca^{2+} alone did not inhibit RαCaN-myosin co-sedimentation (Fig. 2, lane 6), but the addition of both Ca^{2+} and CaM produced nearly complete inhibition of RαCaN-myosin co-sedimentation (Fig. 2, lane 7). RαCaN-myosin co-sedimentation was restored when EGTA was added to chelate Ca^{2+} (Fig. 2, lane 8). None of the above conditions affected sedimentation of RαCaN when centrifuged alone (Fig. 2, lanes 1–3).

The strength of calponin–myosin interaction was examined in the presence of increasing NaCl concentration. As expected, complete sedimentation of myosin was observed between 50 and 150 mM NaCl (Fig. 3). Higher salt concentrations inhibit myosin sedimentation, as myosin filaments dissociate into soluble monomers. The presence of RαCaN has no effect on the salt-dependent sedimentation of myosin (data not shown). The amount of RαCaN that co-sediments with myosin decreases rapidly with increasing NaCl concentration: at 150 mM NaCl, only 36% of the added RαCaN co-sediments with myosin compared to 75% at 50 mM NaCl. Within this range of NaCl concentration (50–150 mM) the amounts of sedimented myosin and RαCaN when centrifuged separately remain relatively constant (97–83% for myosin, 23–17% for RαCaN). These results show that the interaction between RαCaN and myosin is salt-dependent; it is relatively strong at 50 mM NaCl, begins to weaken at 100 mM NaCl, and is virtually abolished at 150 mM NaCl.

The affinity and stoichiometry of the RαCaN–myosin interaction were characterized by titrating myosin (0–6 μM) into a constant amount of RαCaN (2 μM) followed by centrifugation. The amounts of RαCaN in the incubates prior to centrifugation, in the pellets and supernatants were then measured by densitometry (see section 2). Fig. 4 shows that the amount of sedimented RαCaN reaches a plateau at high concentrations of myosin, although some amount of RαCaN remains in the supernatant even at the highest myosin concentration that we used. When the data in Fig. 4 were fitted by a non-linear regression program [17] we

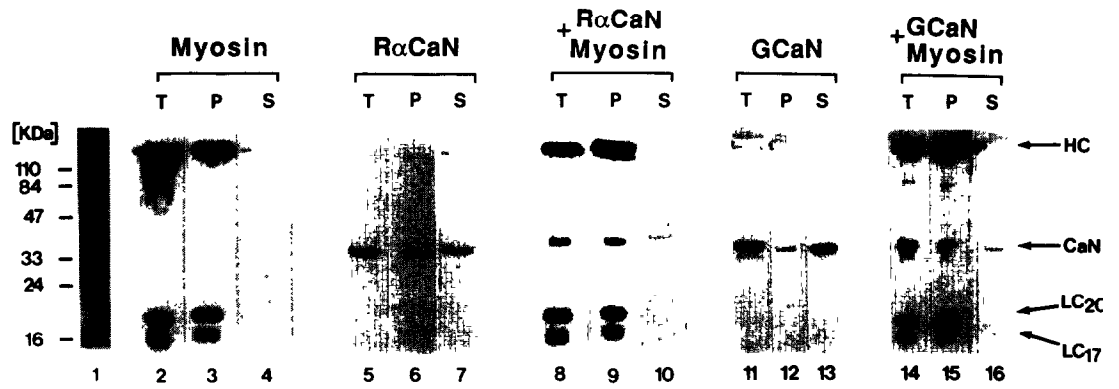

Fig. 1. Sedimentation of calponin in the presence and absence of smooth muscle myosin. Coomassie blue-stained 12% SDS-PAGE of: incubates before centrifugation (T), pellets (P) and supernatants (S). (Lane 1) Molecular weight standards; (lanes 2–4) myosin alone; (lanes 5–7) RαCaN alone; (lanes 8–9) RαCaN plus myosin; (lanes 11–13) GCaN alone; (lanes 14–16) GCaN plus myosin. Concentrations of all proteins are 6 μM. HC, LC_{20} and LC_{17} indicate myosin heavy chain, 20 kDa myosin light chain and 17 kDa myosin light chain, respectively.

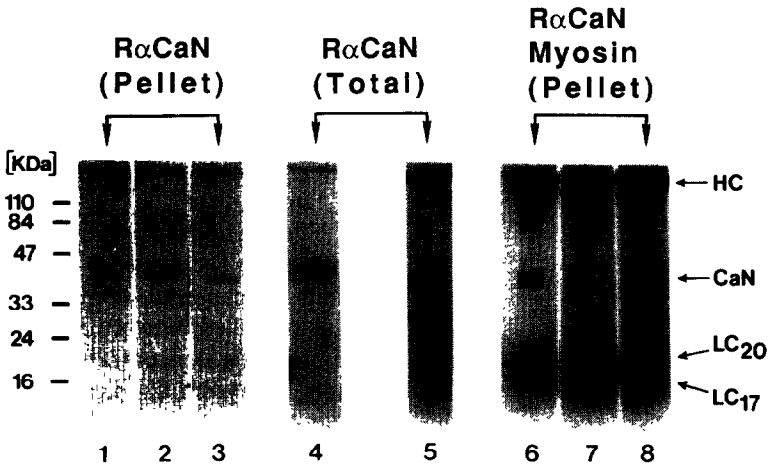

Fig. 2. Effect of Ca^{2+}-CaM on RαCaN-myosin co-sedimentation. (Lanes 4 and 5) RαCaN before centrifugation; (lanes 1–3) RαCaN in pellets when sedimented alone; (lanes 6–8) RαCaN and myosin in pellets when centrifuged together. Incubations were performed with 2 μM RαCaN in the presence or absence of 2 μM myosin in 100 μM Ca^{2+} (lanes 1 and 6); 100 μM Ca^{2+} and 20 μM CaM (lanes 2 and 7), and 100 μM Ca^{2+}, 20 μM CaM and 1 mM EGTA (lanes 3 and 8). Assay conditions and symbols are identical to those in Fig. 1.

obtained a binding constant of 2×10^6 M^{-1}, and a concentration of myosin at saturation of 0.82 μM.

The actual stoichiometry of the RαCaN–myosin interaction could not be determined unequivocally because it is not clear how much of the total RαCaN pool is capable of binding myosin. If we assume that the entire 2 μM pool of RαCaN can bind myosin, then the RαCaN:myosin stoichiometry would be 2.4. However, as shown in Fig. 4, ~17% of RαCaN does not sediment even at saturating myosin concentrations. Assuming that all the remaining RαCaN (1.7 μM) is capable of binding to myosin, the RαCaN:myosin stoichiometry would be 2.0. The origin of this RαCaN fraction that does not bind myosin is not clear; it is likely to be RαCaN that was denatured during the purification procedure. It is possible that the fraction of RαCaN that sediments in the absence of myosin (~34%) is also incapable of binding myosin. Assuming that the remaining fraction of RαCaN (1.3 μM) is the only pool that can bind myosin, the RαCaN:myosin stoichiometry would be 1.2. Although the stoichiometry of the RαCaN–myosin interaction cannot be determined precisely in these experiments, it appears to fall within a reasonable range of either one RαCaN per molecule of myosin (480 kDa) or one RαCaN per mole of myosin heavy chain.

In summary, our results show that calponin can bind to unphosphorylated, filamentous form of smooth muscle myosin. This interaction is reversible by Ca^{2+}-CaM. The strength of the interaction depends on NaCl concentration, being relatively strong at 50 mM NaCl and

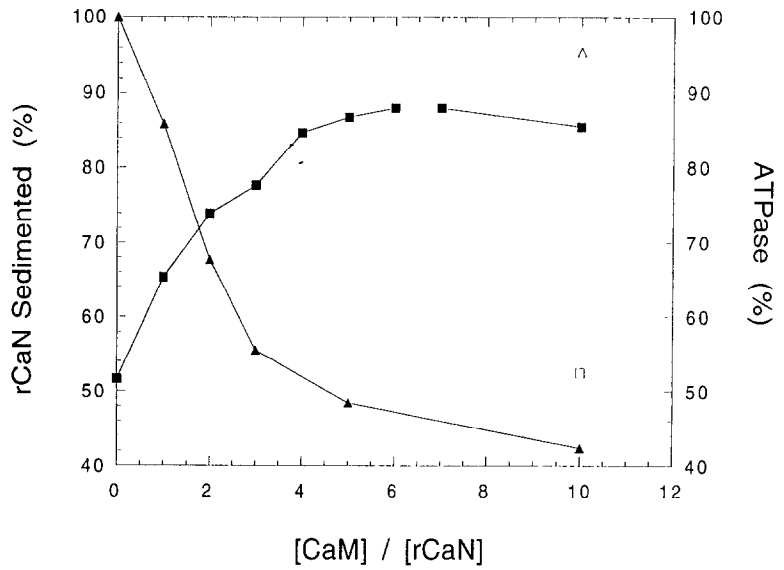

Fig. 3. Salt-dependent sedimentation of myosine alone (open circles), RαCaN alone (closed diamonds) and RαCaN in the presence of myosin (closed squares). The abscissa is the amount of RαCaN in pellet divided by the total amount. Each value represents the mean of 2–4 separate experiments.

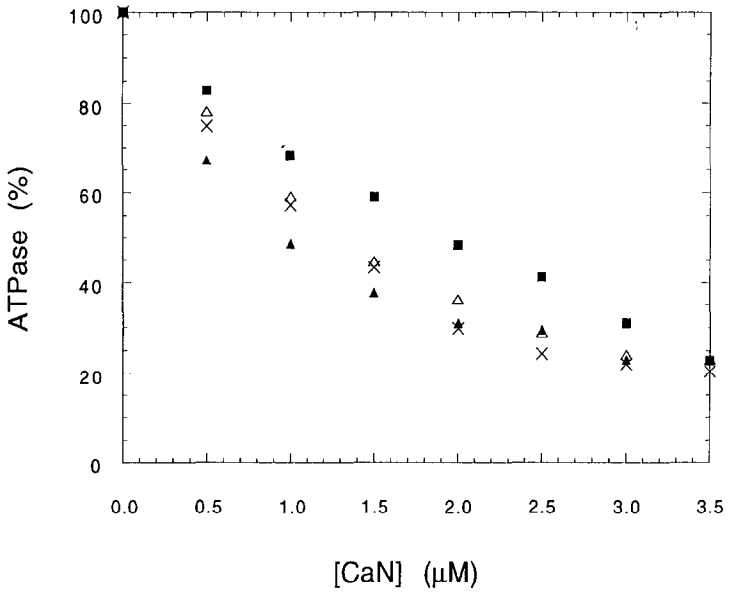

Fig. 4. Sedimentation of RαCaN (2 μM) with the increasing concentrations of myosin (0–6 μM). The abscissa is the difference between total amount of RαCaN in the incubation mixture and the amount that remained in the supernatant, divided by the total amount. Each value represents the average of two independent experiments. The line represents the best fit obtained by non-linear regression [17].

virtually undetectable at 150 mM NaCl. The binding constant between RαCaN and myosin is approximately 2×10^6 M^{-1} mM NaCl. The stoichiometry of this interaction is in the range of 1.2–2.4 RαCaN per myosin.

The physiological significance of this interaction is not clear at this point. It suggests, however, that CaN can bind to both myosin and actin, so that it may play a role in regulation of smooth muscle contractility by anchoring myosin to actin. This is the first report describing a direct interaction between calponin and myosin. Further studies directed towards clarifying the physiological significance of this interaction are in progress.

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