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Properties of calponin isolated from sheep aorta thin filaments

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Calponin, a 35 kDa actin-binding protein, was shown to be a normal component of 'native' thin filaments prepared from sheep aorta. Actin, tropomyosin, caldesmon and calponin were present in molar ratios 14 : 2 : 1 : 0.9. Calponin was isolated from thin filaments in yield 0.5 mg/100 mg thin filament protein. Calponin inhibited actomyosin ATPase up to 85%, half maximal at 0.2 calponin/actin. Inhibition did not depend on tropomyosin, Ca^{2+} or Ca^{2+}·calmodulin. Caldesmon inhibited actomyosin with a 10-fold greater potency than calponin in the presence of tropomyosin and inhition could be reversed by Ca^{2+}·calmodulin under certain conditions. Calponin had no effect on caldesmon inhibition or the reversal of inhibition.

Smooth muscle; Caldesmon; Calmodulin; Calponin; Thin filament; Actomyosin

1. INTRODUCTION

Smooth muscle cells contain a number of actin binding proteins, which may be broadly classified into those that are associated with the cytoskeletal domain (e.g. α-actinin, filamin) and those which are associated with the contractile actomyosin domain, such as caldesmon [1,2]. Calponin is a recently discovered actin binding protein, present in all smooth muscle cells, which can also bind tropomyosin, Ca^{2+}, and Ca^{2+}·calmodulin [3–5].

In my laboratory we have developed a procedure for the preparation of native thin filaments from any smooth muscle and have made a detailed study of thin filaments from sheep aorta [6,7]. The thin filaments activate myosin MgATPase activity in a Ca^{2+}-sensitive manner and are primarily composed of actin, tropomyosin and caldesmon [8]. It has been noted that some preparations of thin filaments from chicken gizzard contained calponin [9]. Nishida et al. [10] reported that gizzard thin filaments prepared by the Marston and Smith technique [7] do not contain calponin, but calponin-containing filaments were obtained by a modified procedure. If calponin is normally present in thin filament preparations then it might be involved in their Ca^{2+}-dependent regulation of actomyosin MgATPase activity [4,5,9,11].

In this study, I have used urea/SDS gel electrophoresis to identify and quantitate calponin in preparations of sleep aorta thin filaments. Calponin was then isolated from the thin filaments under mild conditions and its regulatory properties were studied. It was found that calponin was present in thin filaments at approximately the same molar proportion as caldesmon, and that it could inhibit actomyosin MgATPase activity; however these properties did not support suggestions that it has a regulatory function [4,5,9,10].

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2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Native thin filaments, tropomyosin and skeletal muscle myosin were prepared from sheep aorta by standard methods [7,12]. F-actin, calponin and caldesmon were isolated from native thin filaments. Thin filaments, dissolved in 0.8 M KCl, 10 mM Tris, pH 8.0, were centrifuged for 1 h at 250 000 × *g*. The pellet contained pure f-actin and the supernatant contained primarily caldesmon, calponin and tropomyosin (Fig. 1B) [13]. Crude calponin was precipitated from the supernatant with 30% saturated ammonium sulphate. The pellet was dissolved and dialysed in 0.3 M KCl, 20 mM Tris, pH 7.5, 1 mM dithiothreitol. Residual actin was removed by centrifugation (15 min in a Beckman Airfuge). Final purification was by FPLC gel filtration on a superose 6 column. The yield of pure calponin (Fig. 1B) was about 0.5 mg per 100 mg of thin filament protein. Caldesmon was isolated from a 30–50% ammonium sulphate fraction of the high speed supernatant using standard procedures [13].

2.2. *Assay of proteins*

Protein concentration was determined by the Lowry method based on molecular masses of 42 kDa for actin, 35 kDa for calponin [5] and 87 kDa for caldesmon [14]. ATPase activity was measured with an actomyosin made up of 0.5 mg/ml aorta actin and 0.125 mg/ml skeletal muscle myosin [15] in 50 or 90 mM KCl, 10 mM sodium azide, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM PIPES·K_2, pH 7.1, at 28 or 37°C. Samples (150 μl) were incubated for 10 min; the reaction was initiated by adding MgATP to 2 mM, and terminated with 0.5 ml 5% trichloroacetic acid. The quantity of P_i released was assayed as previously described [7,15].

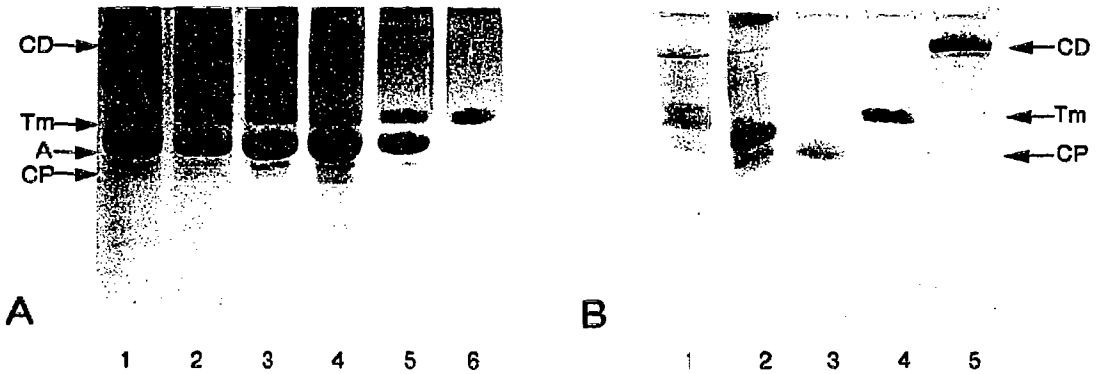

Fig. 1. Urea/SDS gel electrophoresis of aorta thin filaments and calponin. 20 μg samples of protein were loaded onto 8% polyacrylamide/0.1% SDS/8 M urea/10 mM sodium Phosphate, pH 7.0, slab gels and separated by electrophoresis for 3.5 h at 60 V in running buffer (100 mM sodium Phosphate, pH 7.0/0.1% SDS. Staining was in 0.2% PAGE blue 83 (Merck). (A) (1–5) Sheep aorta thin filament preparations, (6) pure tropomyosin. In this gel system tropomyosin migrates anomalously with an apparent mass of 55 kDa. A band migrating at 35 kDa was detected in all thin filament preparations. Identified bands are CD, caldesmon; Tm, tropomyosin; A, actin; CP, 35 kDa protein (calponin). (B) Steps in the purification of calponin from thin filaments. (1) Supernatant following ultracentrifugation of thin filaments in 0.8 M KCl; (2) 0–30% ammonium sulphate fraction from supernatant, major components are actin and calponin; (3) calponin, purified on superose 6 column; (4) tropomyosin; (5) caldesmon.

3. RESULTS

3.1. *Native sheep aorta thin filaments contain calponin*

Calponin could not be distinguished by SDS-gel electrophoresis of sheep aorta thin filaments because of its proximity to the tropomyosin band. However on 8% polyacrylamide/0.1% SDS/8 M urea gels, tropomyosin migrated slower than actin (Fig. 1) and a 35 kDa band was revealed, corresponding to the molecular mass of calponin [5]. This band was observed in all thin filament preparations (Fig. 1A). Twelve thin filament preparations dating from April 1989 to June 1991 were separated on urea/SDS gels and the quantity of 35 kDa protein was determined by densitometric scans. The area of the 35 kDA peak relative to caldesmon was 0.36±0.11. Assuming equal staining per unit mass of protein, this corresponds to 0.9 mol calponin per mol caldesmon (0.056 35 kDa protein per actin).

The 35 kDA protein was isolated from sheep aorta thin filaments (Fig. 1B) and identified as calponin by comparison of its apparent molecular mass, its precipitation by 30% ammonium sulphate and its amino acid composition with the values published by Takahashi [3,5].

3.2. *Calponin inhibition of actomyosin ATPase*

Purified calponin inhibited actomyosin MgATPase up to 85% both in the presence and absence of tropomyosin (Fig. 2). Half maximal inhibition was obtained with 0.2 calponin per actin and maximal inhibition at 0.4 calponin per actin. These figures are similar to recently published results from chicken gizzard caldesmon [9,11,16]. By comparison, caldesmon was 5–10 times more potent at inhibiting actomyosin ATPase activity in the presence of tropomyosin (half maximal inhibition at 0.02 caldesmon per actin (Fig. 2)).

Inhibition by calponin was not dependent on Ca^{2+} concentration (Fig. 3), nor was inhibition significantly reversed by Ca^{2+}·calmodulin (Figs 3,4). In contrast, caldesmon inhibition was reversed by low concentrations of Ca^{2+}·calmodulin when assayed in 90 mM KCl at 37°C (Fig. 4, [15,17].

3.3. *The effect of calponin on caldesmon function*

Calponin had no effect on caldesmon inhibition when added at a ratio equivalent to 4 times that found in native thin filaments (Fig. 3). Higher calponin concentrations inhibited actomyosin ATPase activity in the presence of low caldesmon concentrations as if calponin were acting independently. The possibility that calponin might modulate the reversal of caldesmon inhibition by Ca^{2+}·calmodulin (Fig. 4) was investigated at 25°C in 50 mM KCl and at 37°C in 90 mM KCl. Calponin was added to actomyosin + caldesmon + Ca^{2+}·calmodulin mixtures at ratios up to 0.4 calponin per actin, but no

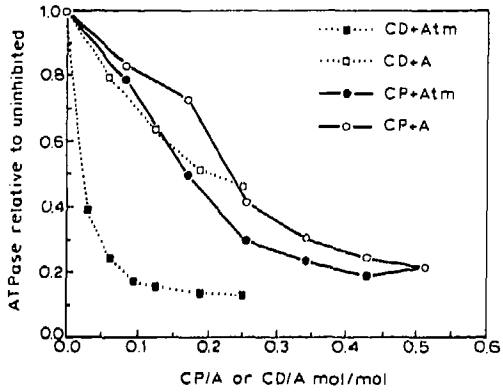

Fig. 2. Inhibition of actomyosin ATPase by calponin and caldesmon. Conditions: 28°C, 50 mM KCl buffer (see section 2), 0.5 mg/ml aorta f-actin, 0.125 mg/ml skeletal muscle myosin, in the presence (closed symbols) or absence (open symbols) of 0.15 mg/ml aorta tropomyosin. Circles (○,●), calponin; squares (□,■), caldesmon.

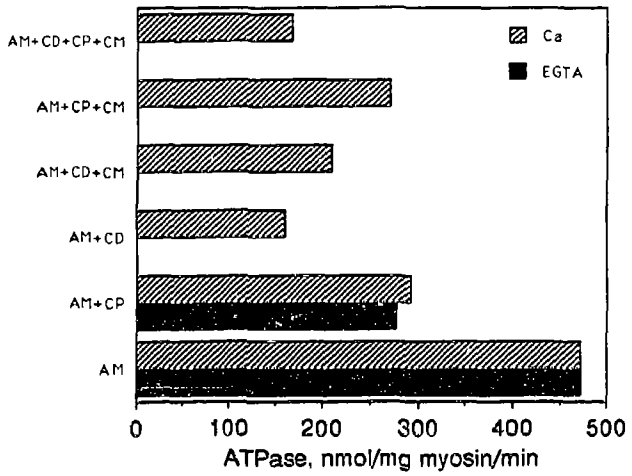

Fig. 3. Effect of Ca^{2+}, Ca^{2+}·calmodulin and caldesmon on calponin. Conditions: 28°C, 50 mM KCl buffer (see section 2) plus 1 mM EGTA (pCa9) or 0.1 mM $CaCl_2$, 0.5 mg/ml aorta actin, 0.125 mg/ml skeletal myosin, 0.15 mg/ml tropomyosin. Calponin was added at 0.2 calponin per actin, caldesmon was added at 0.04 caldesmon per actin and calmodulin at 9 μM. Calponin did not influence the 15% reversal of caldesmon inhibition due to Ca^{2+}·calmodulin.

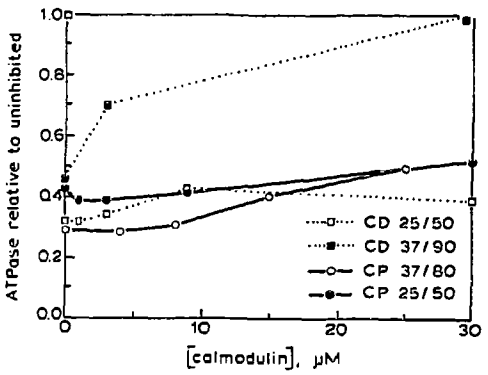

Fig. 4. Reversal of inhibition by Ca^{2+}·calmodulin. Conditions: 0.5 mg/ml aorta actin, 0.125 mg/ml skeletal muscle myosin, 0.12 mg/ml tropomyosin, 0–30 μM calmodulin, 0.1 mM $CaCl_2$. Circles, calponin. (○) 37°C, 90 mM KCl, 0.2 mol calponin/mol actin. (●) 29°C, 50 mM KCl, 0.26 mol calponin/moi actin. Squares, caldesmon. (□) 37°C, 90 mM KCl, 0.19 caldesmon/actin. (■) 29°C, 50 mM KCl, 0.06 caldesmon/ actin.

significant change in actomyosin ATPase activity was observed (Fig. 3).

4. DISCUSSION

4.1. *Can calponin regulate smooth muscle native thin filaments?*

Ca^{2+} has been shown to control the activation of smooth or skeletal myosin MgATPase by native thin filaments isolated from smooth muscles such as sheep aorta [2,6,7,17,19]. These thin filaments contain tropomyosin and caldesmon and in this paper it is shown that they also contain calponin approximately equimolar with caldesmon (Fig. 1A). This result is in accord with earlier observations on gizzard thin filaments [9]. However Takahashi did not find any calponin in thin filament preparations unless the ATP concentration was greatly reduced. The reason for this discrepancy is not clear [10] although Marston and Smith [7] did show that thin filament composition was critically dependent on the solvent composition and protein concentration during sedimentation. Unlike caldesmon, which is present in ratio of one per 14–16 actins [8], the calponin content of thin filament preparations seems to be rather variable: the average value was one per 16 actins, but the standard deviation was nearly 30% (n=12) (Fig. 1A). The maximum reported figure is 1 per 10 actins [10]. The sheep aorta calponin described in this paper is as potent an inhibitor of aorta actin + skeletal muscle myosin MgATPase (Fig. 2) as any of the gizzard calponin preparations previously described [4,9,16,18] which were assayed with smooth muscle myosin. Thus the myosin type does not seem to significantly influence calponin inhibition of actin. Even at a ratio of 1 calponin per ten actins no more than 20% inhibition of actomyosin ATPase has been observed; this suggests that calponin is unlikely to modulate native thin filaments in vitro. Moreover calponin inhibition shows no sign whatsoever of being under the control of Ca^{2+} or Ca^{2+}·calmodulin (Figs. 3,4). Phosphorylation has been reported to module calponin, but only in the direction of making it even less inhibitory [9].

In contrast, caldesmon is an effective inhibitor of both smooth and skeletal muscle actomyosin ATPase at the ratios found in native thin filaments (Fig. 2, [17]) and its inhitition may be regulated by Ca^{2+} and calmodulin in synthetic filament systems (Fig. 4 [15,17]). In addition there is direct evidence that caldesmon is necessary for the Ca^{2+}-dependent regulation of smooth muscle thin filament activation of myosin MgATPase activity [19].

4.2. *Does calponin modulate caldesmon-mediated regulation?*

It has been noted that the regulatory properties of synthetic filaments reconstituted with actin, tropomyosin, caldesmon and calmodulin (see Fig. 4) are not the same at those of native thin filaments. For instance, activation of myosin MgATPase by native thin filaments is Ca^{2+}-dependent at 25°C, whilst activation by synthetic thin filaments is not (Fig. 4 [2,15,20–22]). Since calponin is equimolar with caldesmon in native thin filament preparations it is possible that calponin, whilst having no direct regulatory effect, may function by modulating caldesmon inhibition and its interaction with Ca^{2+}·calmodulin. In fact, calponin, even at ratios in excess of those in thin filaments, had no significant effect on caldesmon inhibition, nor did it alter the reversal of caldesmon inhibition by Ca^{2+}·calmodulin (Fig. 3). There is therefore no evidence that calponin is the modulator protein which was proposed to be a component of native thin filaments [21,23].

4.3. *Cellular location of calponin*

Since calponin has a high affinity for actin and will also bind to tropomyosin [5,9] it is not surprising that thin filaments isolated from smooth muscle homogenates contain bound calponin, but this does not necessarily mean that calponin is a component of thin filaments. Calponin is an extremely basic protein and this property alone may be sufficient to account for actin binding and inhibiting properties [11]. There is, as yet, no direct evidence that calponin co-localises with caldesmon in the actin filaments of the contractile domain of smooth muscles. Indeed, in vitro it appears that calponin and caldesmon are competitive for actin binding [18] whilst Lehman has recently reviewed evidence that calponin and caldesmon are segregated into separate populations of thin filaments within the cell [24]. In recent studies using antibodies to calponin in cultured smooth muscle cells, calponin was seen to stain stress fibres, primarily in the central portion of the cell, but its location was not precisely defined [25,26].

A number of papers have made the suggestion that calponin might regulate actomyosin ATPase on the basis of some of its in vitro properties [4,5,9,10]. However if one takes into account the quantity present in native thin filament preparations, its weak unregulated inhibitory function, its lack of interaction with caldesmon, and the doubt as to whether calponin is located in the portion of thin filaments which interact with myosin, then it is apparent that the evidence to support a regulatory function is lacking.

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