Bundling of actin filaments by aorta caldesmon is not related to its regulatory function

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Received 22 July 1985; revised version received 29 August 1985

Ca^{2+}-sensitive thin filaments from vascular smooth muscle were disassembled into their constituent proteins, actin, tropomyosin and caldesmon. Caldesmon bound to both actin and to actin-tropomyosin and inhibited actin-tropomyosin activation of skeletal muscle myosin MgATPase. It also promoted the aggregation of actin or actin-tropomyosin into parallel aligned bundles. Quantitative electron microscopy measurements showed that with 1.1 μM actin-tropomyosin, 1.6 $\pm$ 0.5% ($n=3$) of the filaments were in bundles. At 0.073 μM, caldesmon inhibited MgATPase activity by 50%, whereas bundling was 3.0 $\pm$ 1.3% ($n=4$) .At 0.37 μM caldesmon, MgATPase inhibition was 83% while 28.1 $\pm$ 6.9% ($n=4$) of filaments were in bundles. Experiments at 4.4 μM in which MgATPase and bundling were measured in the same samples gave similar results. Small bundles of 2–3 filaments showed the most frequent occurrence at 1.1 μM actin. At 4.4 μM actin the most common bundle size was 3–5 filaments, with the occasional occurrence of large bundles consisting of up to 120 filaments. The incidence of bundling was the same in the presence and absence of tropomyosin. Thus caldesmon can induce the formation of actin bundles but this property bears no relationship to its inhibition of MgATPase activity.

Smooth muscle *Caldesmon* *Actin* *Tropomyosin* *Ca^{2+} regulation*

1. INTRODUCTION

Ca^{2+}-sensitive regulation of vascular smooth muscle (vsm) thin filaments has recently been demonstrated using native thin filaments [1], and with a reconstituted system consisting of F-actin, myosin, tropomyosin, calmodulin and a 120 kDa protein [2,4]. The 120 kDa thin filament associated inhibitory protein is now known to be identical with caldesmon [3]. Inhibition of actin-activated myosin Mg^{2+}-ATPase activity by caldesmon is relieved when Ca^{2+} binds to calmodulin added to the system.

Binding studies show that vsm caldesmon binds to both calmodulin and to actin (or actin-tropomyosin), and that binding to calmodulin is calcium-sensitive [1,12]. The stoichiometry of high affinity binding, inhibition of Mg^{2+}-ATPase activity and the native occurrence of caldesmon in vsm thin filaments all suggest that caldesmon interacts with F-actin at a molar ratio of 1:25–30 [4].

A number of proteins are known to cross-link or to bundle F-actin (e.g. fodrin [5], filamin [6], myosin subfragment-1 [13], troponin-I and myelin basic protein [14]) and recent reports show that caldesmon shares this property [7–9] in addition to an ability to accelerate nucleation and to induce polymerization of G-actin [10].

In this study we investigated whether bundling is the mechanic underlying vsm caldesmon inhibition of actomyosin MgATPase activity using electron microscopy to visualize the filaments and filament bundles. Bundle formation was related to simultaneous measurements of vsm caldesmon binding to actin and to inhibition of actomyosin ATPase activity.

2. MATERIALS AND METHODS

Sheep aorta thin filaments, F-actin, tropomyosin,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

caldesmon, and skeletal muscle myosin were freshly prepared and purified by the methods detailed by Marston and Smith [1] and Smith and Marston [4].

2.1. *Electron microscope studies*

Preliminary experiments showed that electron micrographs of long, separate, randomly orientated filaments, spaced sufficiently for counting, were obtained using grids prepared from about 1 μM F-actin solutions.

F-Actin solutions (1.1 μM) were prepared in buffer A (0.1 mM dithiothreitol, 10 mM NaN_3, 10 mM Tris-Mes, 60 mM KCl, 5 mM $MgCl_2$, 6 mM KH_2PO_4/K_2HPO_4 and 0.1 mM $CaCl_2$ at 4°C and pH 7), shaken for 10 s and left to disperse for a minimum of 30 min. All procedures took place at 4°C, as this was found to preserve actin filaments in better structural detail than preparation at room temperature. Tropomyosin was added to some actin solutions at a molar ratio of 1:7 and caldesmon was added to give a range of molar ratios (1:3–1:100). Solutions were allowed to stand for a period of 15 min before preparation of grids for electron microscopy.

Carbon-strengthened celloidin-coated 300-mesh copper grids (Agar Aids) were used. The central area of these grids is characterized by the letter 'A'. One drop of protein solution was applied to the grid, followed by 3 drops of buffer and 3 drops of 1% uranyl acetate (aqueous). Grids were blotted and left to dry at room temperature. Three replicate grids were prepared from each protein solution. Grids were examined using a Philips 301 electron microscope at an accelerating voltage of 60 kV.

The central A portion of the grid was used to locate 7 separate pre-determined grid squares. Electron micrographs of the top left-hand corner of each square (image dimensions 27.4 × 35.7 μm) were taken and printed to a final magnification of ×84 000. The total number of F-actin filaments (mean ± SE = 60 ± 2, for n = 4 protein preparations) and the number of F-actin filaments involved in bundles were recorded for each micrograph. Bundles were defined as the close parallel alignment of 2 or more actin filaments. Micrographs were counted 'blind' and grids were labelled in a random order. Data from replicate grids were pooled and any F-actin interactions expressed as a percentage of the total number of filaments.

The experiment was repeated with 4 actin, tropomyosin and caldesmon preparations.

2.2. *Comparison of caldesmon inhibition of MgATPase activity, actin binding and bundling*

4.4 μM actin saturated with tropomyosin was incubated with 0–3.5 μM caldesmon covalently labelled with iodo[^{14}C]acetamide (40 cpm/pmol) at 4°C in buffer B (as buffer A, but without phosphate). At each caldesmon concentration, samples were taken (in triplicate) and placed on electron microscope grids for quantitative examination of size and fequency of bundles. Further samples were: (i) mixed with 4.4 μM skeletal muscle myosin for the determination of MgATPase activity [4] at 4°C. The reaction was initiated by the addition of MgATP to 1 mM, and terminated by the addition of trichloroacetic acid to 5% after 30 min incubation. (ii) Used in the sedimentation assay [4] for the measurement of binding. In a total volume of 250 μl, 80-μl aliquots were taken before and after centrifugation for 2 h at 50 000 × g. Samples containing no actin were also run, to account for [^{14}C]caldesmon which sediments in the absence of actin. Samples were assayed by liquid scintillation counting. Caldesmon bound to actin was equated to the difference in supernatant cpm before and after sedimentation.

3. RESULTS

3.1. *Caldesmon binding to actin-tropomyosin and inhibition of actin activation of myosin MgATPase*

In this study all measurements were made at 4°C, since this was optimal for the visualization of filaments using electron microscopy. Under these conditions, low concentrations of vsm caldesmon inhibited actin-tropomyosin activation of myosin MgATPase activity (figs 1 and 2) while less inhibition of actin activation was seen. For example, with 2.5 μM actin, inhibition of actin-tropomyosin activation by 0.12 μM caldesmon (molar ratio of 0.048 caldesmon/actin), was 50% while only 10% inhibition of actin activation was found. With 0.9 μM caldesmon (0.36 caldesmon/actin) inhibition was 90% for actin-tropomyosin and 60% for actin.

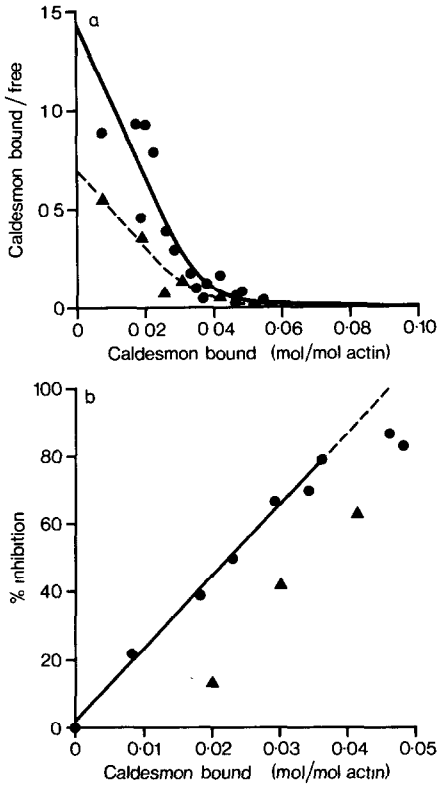


Fig.1. (a) Scatchard plot of binding of vsm iodo[^{14}C]-acetamide labelled caldesmon (0–3.5 μM) to 4.4 μM vsm F-actin (▲) or to actin-tropomyosin (●) at 4°C. Binding was determined by the sedimentation assay. The data was analysed on the basis of 2 classes of independent binding sites. An initial fit was obtained by a visual iterative procedure [11,15]; solid lines on graph. For actin-tropomyosin the weaker class of binding was estimated as K_m = 6 μM, B_m = 0.12 caldesmon/actin and the strong binding was estimated as K_m = 25 nM, B_m = 0.034 caldesmon/actin. The contribution of the weak binding was calculated and subtracted from the total binding and the residual (strong) binding was further analysed by fitting to the equation for a single class of binding sites [16] to yield K_m = 24 ± 4 nM, B_m = 0.035 ± 0.001 (mean ± SE, n = 15 observations). The curve fitted to the actin data has the parameters: strong, K_m = 46 nM, B_m = 0.032; weak, K_m = 6.0 μM, B_m = 0.12 caldesmon/actin. (b) Correlation of caldesmon binding with inhibition of actin activation of myosin MgATPase. Data from figs 1a,2. Actin (▲), actin-tropomyosin (●). The line drawn was calculated after least-squares linear regression analysis of data in the rage 0–80% inhibition. Parameters for actin-tropomyosin are: correlation coefficient, 0.995; intercept 100% inhibition, 0.041 caldesmon/actin; slope, 2111% per molar ratio caldesmon/actin; abscissa intercept, 2%.

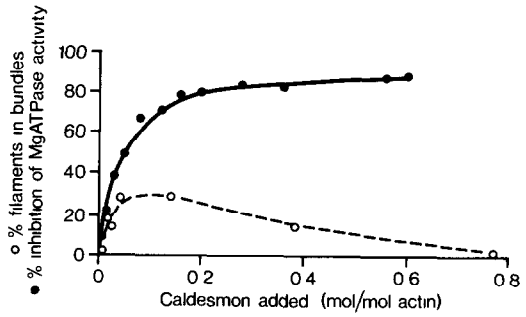
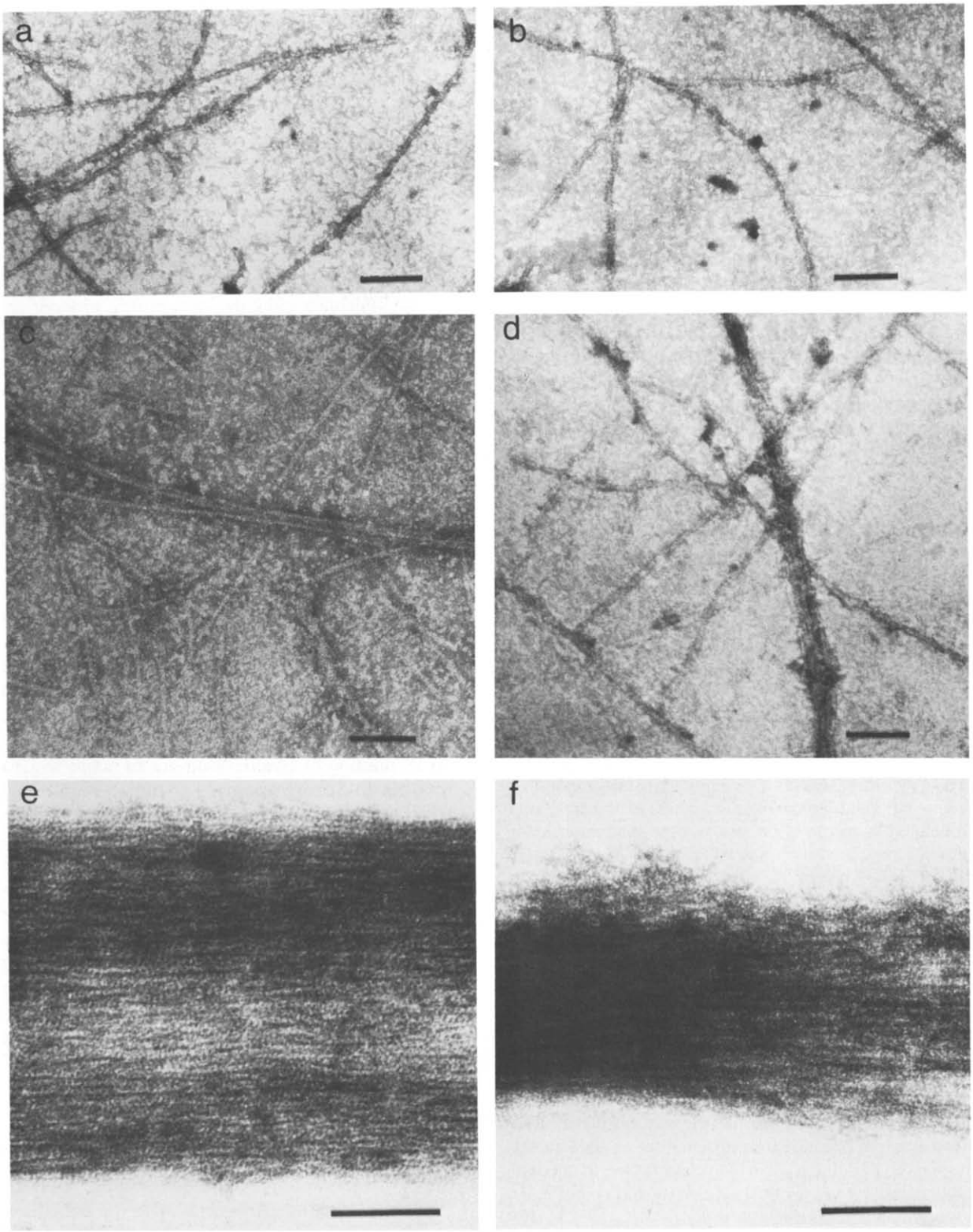


Fig.2. Comparison of bundling and inhibition induced by vsm caldesmon using the same protein preparation. 4.4 μM vsm actin-tropomyosin was incubated with 0–3.5 μM caldesmon at 4°C in buffer B. Samples were taken for measurement of bundle size and frequency (○) and activation of skeletal myosin (4.4 μM) MgATPase activity (●). Results are plotted in terms of percentage inhibition of the MgATPase activation found in the absence of caldesmon (30 nmol/min per mg myosin). Samples were also taken for measurement of actin-caldesmon binding – results shown in fig.1.

Measurement of the binding of caldesmon (iodo-[^{14}C]acetamide-labelled) to actin-tropomyosin showed that binding was not simple. It was analysed in terms of 2 classes of binding site by the procedures which have proved successful with binding measurements at 25°C [4,11,15] (fig.1a). The stronger class of binding sites had an estimated binding constant of 4.1 × 10^7 M^{-1} and a maximum binding of 1 caldesmon per 29 actins (fig.1). Binding to actin-tropomyosin and the inhibitory effect of caldesmon were closely related. Linear regression analysis of the data (fig.1b) gave a correlation coefficient of 0.995 and a slope which extrapolated to 100% inhibition with a total caldesmon bound of 1 per 22 actins. This relationship has also been obtained at 25 and 37°C and confirms that inhibition is due to caldesmon binding to the 'strong' sites [4,11,15]. A few measurements of caldesmon binding to actin gave a similar pattern of results, but binding to the (strong) sites was 2–5-fold weaker and was not as closely related to inhibition (fig.1) as with actin-tropomyosin.

3.2. *Bundling of actin filaments by caldesmon*

Electron micrographs prepared from 1.1 μM actin or actin-tropomyosin showed long, separate, randomly orientated filaments with the occasional parallel alignment of filaments into bundles (fig.3a,


a
b
c
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b). This accounted for 3.5 ± 0.9% (mean ± SE, $n=4$) and 1.6 ± 0.5% ($n=3$) of the total number of filaments counted, respectively. In 4 separate preparations, caldesmon induced an increase in the incidence of bundles (figs 3c, d and 4). Fig.4 shows where this increase reached statistical significance (Student's paired t-test) compared with controls. On the basis of the direct relationship between binding and inhibition (fig.1b), it may be calculated that about 50% inhibition of MgATPase is achieved at 0.073 μM caldesmon (molar ratio of 1:15 caldesmon/actin, 1.1 μM actin), where caldesmon-induced bundling (3.0 ± 1.3%, $n=4$) was not significantly different from basal levels. With 0.37 μM caldesmon (1:3 molar ratio, the maximum tested) 90% inhibition of MgATPase activity was achieved, yet only 28.1 ± 6.9% ($n=4$) of actin-tropomyosin filaments were in bundles (increase statistically significant, $p<0.05$, compared with actin-tropomyosin controls). Furthermore, in the absence of tropomyosin there was no significant difference in bundling at any of the caldesmon concentrations tested (fig.4), yet caldesmon is a more potent inhibitor in the presence of tropomyosin (fig.1b). The increased potency for actin-tropomyosin inhibition over actin is more pronounced at 25°C [4,15].

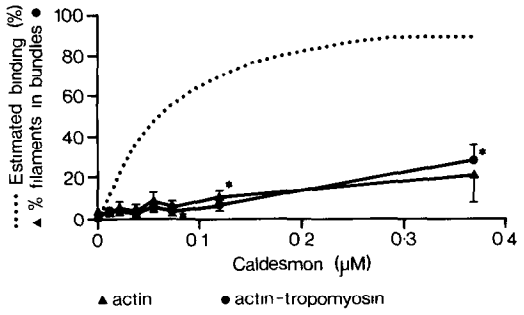

Fig.4. Bundling of 1.1 μM F-actin (▲) or 1.1 μM F-actin-tropomyosin (●) by vsm caldesmon. (···) The binding predicted for vsm caldesmon to high-affinity binding sites on actin-tropomyosin (see fig.1). Mean ± SE values are shown. The increase in percentage bundling was compared with actin or actin-tropomyosin controls using the paired Student's t-test ($n=4$). An asterisk marks a probability level of $p<0.005$.

In our analysis the distribution of bundle size (0.37 μM caldesmon, 1.1 μM actin or actin-tropomyosin) was as follows (mean values given); 2 filaments, 22 (actin) and 53% (actin-tropomyosin); 3 filaments, 37 and 14.5%; 4 filaments, 21 and 13%; 5 filaments, 13.5 and 3%; 6 or more filaments, 6 and 16%. The largest bundles observed were of 14 filaments (0.5%, actin) and 24 filaments (2.5%, actin-tropomyosin). Thus, the most common number of actin filaments involved in a bundle was 2 (actin-tropomyosin) or 3 (actin) filaments. At lower caldesmon/actin ratios a similar pattern of bundle size distribution was seen, with the frequency of occurrence of larger bundles (6 or more filaments) decreasing as the total percentage of filaments bundled became reduced. Electron microscopy of native thin filaments showed them to be long and randomly orientated, with occasional parallel alignment. The degree of bundling observed was independent of calcium concentration.

3.3. *Simultaneous measurement of bundling, binding and inhibition*

In experiments using 4.4 μM actin or actin-tropomyosin, inhibition, binding and bundling could be measured using the same protein mixtures (figs 2 and 3e,f). At lower caldesmon concentrations (0–0.1 mol/mol actin), inhibition of MgATPase activity and the percentage of filaments involved in bundles both increased. However maximum bundling, involving less than 30% of actin filaments, was measured over the range 0.05–0.15 caldesmon/actin (mol/mol). At higher caldesmon concentrations there was a decrease in bundling (fig.2), while inhibition of MgATPase activity was maintained. In our analysis, the most frequent bundle size measured was of 3–5 filaments, depending upon the vsm caldesmon concentration used. Large bundles of up to 120 filaments, as described by Bretscher [7], were occasionally observed.

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Fig.3. F-Actin bundles induced by vsm caldesmon. Panels a, actin (1.1 μM); b, actin-tropomyosin (1.1 μM); c, actin (1.1 μM) plus caldesmon (0.37 μM); d, actin-tropomyosin (1.1 μM) plus caldesmon (0.37 μM), e, actin (4.4 μM) plus caldesmon (1.36 μM); f, actin-tropomyosin (4.4 μM) plus caldesmon (1.36 μM). No bundles are present either in a or b; c and d show the smaller bundles commonly seen at 1.1 μM actin in the presence of caldesmon, while e and f show the less frequently occurring large bundles (4.4 μM actin). Marker bars represent 0.1 μM.

4. DISCUSSION

Caldesmon is a component of thin filaments isolated from all smooth muscles and is proposed to regulate the Ca^{2+}-dependent thin filament contractile interaction with myosin [2–4,11,12,15]. The key regulatory properties of caldesmon, upon which this proposal is based, are the inhibition of the myosin-actin-tropomyosin interaction and control of this inhibition by Ca^{2+} and calmodulin [4,8,12,15].

How does caldesmon inhibit actin-tropomyosin? One proposal is that it aggregates actin filaments into bundles and that this prevents interaction with myosin [7,8]. In this report we tested this possibility.

We found that at 4°C caldesmon bound tightly to actin-tropomyosin and that this 'strong' binding correlated with inhibition (figs 1,2). However under the same conditions we observed only a small increase in bundling due to caldesmon. In 2 series of experiments, at 1.1 and 4.4 μM actin-tropomyosin, where inhibition was up to 90%, the fraction of actin filaments in bundles never exceeded 30% (figs 2,4). With pure actin filaments, caldesmon caused less inhibition but the same amount of bundling.

Native thin filaments contain 1 caldesmon per 25 actins [1], sufficient on the basis of this work for inhibition but not for significant bundling. In practice we found that thin filament preparations were not bundles in Ca^{2+} or Ca^{2+}-free (EGTA) solutions, even though their interaction with myosin was Ca^{2+}-regulated.

We therefore conclude that it is unlikely that bundling of filaments by caldesmon is involved in its regulatory function.

Although bundles were small and infrequent under the conditions of our binding and Mg-ATPase measurements (fig.4), we did observe larger and more frequent bundling at higher actin concentrations and caldesmon/actin ratios (fig.4), corresponding to those described by Bretscher [7] and by Dabrowska et al. [6–8]. We suggest that such bundling may be related to occupation of the 'weak' binding sites that are not involved in regulation. In agreement with the latter authors and in contrast to Sobue et al. [9], bundling was observed with fresh protein preparation.

It seems that many actin binding proteins can induce bundling, including proteins such as fodrin [5], filamin [6], myosin subfragment-1 [13], troponin-I and myelin basic protein [14]. Although bundling properties might provide some interesting insight into the nature of actin-caldesmon interactions, the real inhibitory mechanism must lie within the smooth muscle thin filament.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council and the British Heart Foundation. We are grateful to A.M. Slade for technical advice and help with the elctron microscopy.

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