Stoichiometry and stability of caldesmon in native thin filaments from sheep aorta smooth muscle

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Ca^{2+} -regulated native thin filaments were extracted from sheep aorta smooth muscle. The caldesmon content determined by quantitative gel electrophoresis was 0.06 caldesmon molecule/actin monomer (1 caldesmon molecule per 16.3 actin monomers). Dissociation of caldesmon and tropomyosin from the thin filament and the depolymerization of actin was measured by sedimenting diluted thin filaments. Actin critical concentration was $0.05\ \mu\text{M}$ at $I0.1$ and 0.13 at $I0.05$ compared with $0.5\ \mu\text{M}$ for pure F-actin. Tropomyosin was tightly bound, with half-maximal dissociation at less than $0.3\ \mu\text{M}$ thin filaments (actin monomer) under all conditions. Caldesmon dissociation was independent of tropomyosin and not co-operative. The concentration of thin filaments where 50% of the caldesmon was dissociated (CD_{50}) ranged from $0.2\ \mu\text{M}$ (actin monomer) at $I0.03$ to $8\ \mu\text{M}$ at $I0.16$ in a $5\ \text{mM-MgCl}_2$, pH 7.1, buffer. Mg^{2+} , $25\ \text{mM}$ at constant I , increased CD_{50} 4-fold. CD_{50} was 4-fold greater at $10^{-4}\ \text{M-Ca}^{2+}$ than at $10^{-9}\ \text{M-Ca}^{2+}$. Aorta heavy meromyosin (HMM)·ADP· P_i complex ($2.5\ \mu\text{M}$ excess over thin filaments) strongly antagonized caldesmon dissociation, but skeletal-muscle HMM·ADP· P_i did not. The behaviour of caldesmon in native thin filaments was indistinguishable from caldesmon in reconstituted synthetic thin filaments. The variability of Ca^{2+} -sensitivity with conditions observed in thin filament preparations was shown to be related to dissociation of regulatory caldesmon from the thin filament.

INTRODUCTION

Smooth-muscle contraction is controlled largely by cytoplasmic Ca^{2+} , which acts on switches located on the thick myosin filaments and the thin filaments. When native thin filaments are isolated from vascular smooth muscle by the technique developed in our laboratory [1], they retain the ability to activate myosin MgATPase under the control of Ca^{2+} and also to make a very tight and specific binding interaction with smooth-muscle myosin [2].

The major protein components of smooth-muscle thin filaments are actin, tropomyosin, caldesmon and a Ca^{2+} -binding protein [1,3]. Structural studies have indicated that both tropomyosin and caldesmon are thin elongated molecules located in the grooves of the actin double helix [4].

Studies on the mechanism of control have used isolated protein components. Actin and tropomyosin are involved in the contractile and ATP-hydrolysing interaction with myosin, whereas caldesmon acts as an inhibitor of the rate-limiting step of this reaction and is itself under the control of Ca^{2+} via the Ca^{2+} -binding protein [5–7]. These studies have postulated that inhibition of actin-tropomyosin by caldesmon is consequential on caldesmon binding [7].

The stoichiometry of the caldesmon-actin interaction is a matter of debate. Structural studies have produced a model in which there is one caldesmon molecule to 14 actin monomers [4], whereas direct binding studies using pure caldesmon have suggested stoichiometries of 1:7 [8] and 1:20 ([7] adjusted for the true M_r , 87000). Such studies may be in error, owing to a change in caldesmon properties during isolation, the kinetic model chosen to fit binding data and the assay of protein concentration. In crude smooth-muscle extracts a fraction of caldesmon-containing filaments with a 1:17 ratio was found [4], but in earlier work we reported that purified thin filaments contained less caldesmon [1,10]. It is now known that these measurements were in error, owing to the use of an incorrect M_r (120000; the true M_r

is 87000 [9]) and because actin and caldesmon bind protein stain differently in polyacrylamide gels [4]. In the present study I remeasured the stoichiometry of native thin filaments and found it to be in the range 14–18.

At present it is not known whether the mechanisms of caldesmon control studied in isolated proteins and synthetic thin filaments accurately reflect the native thin filament. A number of anomalies have been pointed out [11,12]: for instance, thin filament Ca^{2+} -sensitivity is greatest at low temperatures and ionic strengths, and also the Ca^{2+} -sensitivity is always much greater when activating smooth-muscle myosin than with skeletal myosin [3,10]. In contrast, in synthetic filaments, regulation does not depend on myosin type and is better at higher temperatures and ionic strengths [7,12]. In the synthetic filaments it has been possible to give a quantitative explanation of regulation based on measurements of actin-tropomyosin-caldesmon and calmodulin-caldesmon binding constants [7,12]. Here I have devised a technique which permits one to determine the tropomyosin and caldesmon affinities for actin in the native thin filament. These data show that caldesmon in native thin filaments has similar affinities to isolated caldesmon and that most of the observed characteristics of thin-filament regulation by Ca^{2+} may be quantitatively explained on this basis.

METHODS

Proteins

Thin filaments were extracted from sheep aorta as described by Smith & Marston [1]. Aorta myosin was isolated, thio-phosphorylated and converted into its soluble derivative, heavy meromyosin (HMM), as described previously [2,13,14]. Skeletal-muscle myosin and HMM were prepared by standard methods [15,16]. Protein concentrations were assayed by the Lowry method, with actin and myosin standards which had been calibrated by total nitrogen determination.

Abbreviation used: HMM, heavy meromyosin; CD, concn. for 50% dissociation.

Fig. 1. SDS/PAGE of aorta thin filaments sedimented at different concentrations

Samples (2 ml) containing native thin filaments from sheep aorta at 0.1–10 μM (actin monomer concentration) were centrifuged and the pellet fraction was redissolved to a concentration equivalent to 1.25 mg of thin filaments/ml before sedimentation. The Figure shows a stained SDS/polyacrylamide slab gel of the pellet fractions of thin filaments sedimented in 50 mM-KCl at 25 °C. Initial thin-filament concentration ([TF], μM) is shown on the Figure. Identified protein bands are: A, actin; CD, caldesmon; Tm, tropomyosin.

Dissociation of the thin filament proteins

Thin-filament preparations were diluted in ATPase buffer [5 mM-dipotassium Pipes (pH 7.1)/5 mM-MgCl₂/1 mM-dithiothreitol/10–120 mM-KCl] in the range 10–0.1 μM -actin monomer concentration [based on an M_r for actin of 42000 and a 62% (w/w) actin content of native thin filaments [1]]. Samples (2 ml) were centrifuged at 120000 g for 1 h. Supernatant was removed and the thin-filament pellet was dissolved in SDS/gel buffer [2.5% (w/v) SDS/5% (v/v) mercaptoethanol/10% (v/v) ethanediol/1 mM-EDTA/10 mM-Tris, pH 8.0] and heated to 90 °C for 1 min. The gel sample volume was adjusted to give a constant concentration equivalent to 1.25 mg of thin filaments/ml in the unspun sample. The samples (15 μl) were run on Pharmacia 4–30%-(w/v)-polyacrylamide slab gels in a running buffer containing 0.2% SDS, 40 mM-Tris, 20 mM-sodium acetate, 2 mM-EDTA, adjusted to pH 7.4, for 80 min at 150 V (Fig. 1). The gels were stained with 0.2% PAGE Blue 83 (BDH) in methanol/acetic acid/water (5:1:4, by vol.), and destained in methanol/acetic acid/water (1:1:8, by vol.). Stained gels were scanned by an LKB 2202 Ultrosan densitometer. Each track was scanned in two places, and the mean value of integrated peak area was used.

When dilution experiments were done in the presence of HMM, the caldesmon and HMM heavy chain were not well separated. In this case the pellets were dissolved in 0.4 M-NaOH in a volume adjusted to give a constant concentration equivalent to 35 μg of thin filaments/ml added, and then neutralized with an equal volume of 0.4 M-HCl/100 mM-Tris, pH 7.4. The quantity of caldesmon was assayed by dot-blot of 20 μl of sample on to nitrocellulose, using a Bio-Rad Bio-Dot apparatus and immunoassay with anti-caldesmon antibody and ¹²⁵I-Protein A as the detection system. This assay was linear up to 1 μg of applied caldesmon.

Caldesmon content of thin filaments

The ratio of caldesmon to actin in thin-filament preparations was determined by electrophoresis. For each preparation eight samples in the range of 0–70 μg of protein were run on a 4–30% polyacrylamide/0.5% SDS slab gel and stained for 24 h in Procion Blue MX-R (Serva) [0.2% (w/v) in methanol/acetic acid/water (1:1:8, by vol.)]. Destained gels were scanned in the LKB Ultrosan densitometer. Each track was scanned in three places. Peak areas for actin, tropomyosin and caldesmon were plotted against μg of thin-filament protein. Plots were linear, and the molar ratio of caldesmon to actin was calculated from the ratio of the slopes.

RESULTS

Caldesmon content of native thin filaments

Preparations of native thin filaments from sheep aorta were run on SDS/polyacrylamide gels and stained with Procion Blue MX-R. Lehman *et al.* [4] have published absolutely calibrated dye-binding ratios for actin and caldesmon (1 μg of actin produces as much integrated dye area as 0.695 μg of caldesmon). By using the dye-binding ratios, the newly determined M_r for gizzard caldesmon of 87000 [9] and an M_r of 42000 for actin [17], the estimated molar ratio of actin to caldesmon was 15.8, 18.5, 14.7 and 16.3 measured for four separate thin-filament preparations (mean: 16.3 actin monomers per caldesmon molecule; 0.06 caldesmon molecule/actin monomer).

Sedimentation of diluted thin filaments

The pellet fraction from 19 μg of aorta native thin filaments was analysed by SDS/polyacrylamide-gel electrophoresis; it was observed that, when thin filaments were sedimented at high concentration, the pellet-fraction protein content was the same as for the initial solution of thin filaments, but, when the thin filaments were sedimented from dilute solutions, the protein content was different, owing to the dissociation of the proteins actin, tropomyosin and caldesmon, which form the thin filament. An example of this is given in Fig. 1.

At very low thin-filament concentrations, and at low ionic strength, the amount of actin that sedimented was reduced (Fig. 1). This was probably due to actin depolymerization, since plots of total added actin versus sedimented actin were linear, with an x -axis intercept corresponding to a critical actin concentration (result not shown) [18]. Critical concentration was estimated to be 0.05 μM at I 0.1 and 0.13 μM at I 0.05. This compares with 0.5 μM for pure aorta actin at I 0.05, assayed by the same procedure.

Dissociation of caldesmon and tropomyosin

At low concentrations of thin filaments, tropomyosin and caldesmon dissociated from polymeric actin (Fig. 1). This could be clearly demonstrated by determining the quantity of tropomyosin and caldesmon sedimenting relative to polymeric actin, which sedimented as illustrated in Fig. 2. Under normal conditions the tropomyosin was tightly associated with F-actin, and the caldesmon was the first component to dissociate during dilution. There was no evidence for co-operativity in the dissociation reactions, and tropomyosin and caldesmon appeared to dissociate independently of one another (Fig. 2).

Caldesmon dissociation was strongly dependent on ionic strength (Fig. 3); the concentration for 50% dissociation (CD_{50}) ranged from 0.2 μM actin at 0.03 to 8 μM at I 0.1 (Fig. 3). Dissociation was also temperature-dependent; CD_{50} increased 3-fold between 25 and 37 °C.

Tropomyosin was strongly associated in the thin filaments. Under all conditions 50% dissociation required dilution of the

Fig. 2. Dissociation of tropomyosin and caldesmon from polymeric actin in native thin filaments

Thin filaments were sedimented in ATPase buffer plus 120 mM-KCl at 25 °C. Estimated degree of association was determined by densitometric scanning of a slab gel of pellet fractions similar to that shown in Fig. 1. Association is defined as area of peak/area of actin peak relative to the value for the original thin-filament preparation. In this experiment caldesmon/actin was 0.12, and tropomyosin/actin was 0.4; 50% of caldesmon was dissociated at 6 μM-actin monomer concentration.

Fig. 3. Effect of ionic strength (I , in mol/litre) and Mg^{2+} on caldesmon dissociation from thin filaments

The concentration of thin filaments at which 50% of the caldesmon was dissociated was determined by scanning SDS/polyacrylamide gels of the pellet fraction of serial dilutions of native thin filaments as shown in Figs. 1 and 2. Conditions: 0–120 mM-KCl, 5 mM-dipotassium Pipes; pH 7.1, 10 mM- $NaNO_3$, 1 mM-dithiothreitol and 25 mM- $MgCl_2$ (□), 5 mM- $MgCl_2$ (■) or no $MgCl_2$ (○) (25 °C).

thin filaments to less than 0.3 μM (actin monomer), and often it appeared that tropomyosin dissociation and actin depolymerization occurred together. There was no evidence of systematic changes in tropomyosin dissociation with temperature or ionic strength in ATPase buffer.

Effect of HMM on thin-filament stability

When vascular-smooth-muscle HMM was present in concentrations 2.5 μM in excess of the actin monomer concentration, the association of caldesmon was greatly enhanced, such that, even at an actin concentration of 0.06 μM, the thin filaments were fully saturated with caldesmon (Fig. 4). This concentration of aorta HMM is sufficient to saturate the 'tight'

Fig. 4. Effect of smooth-muscle HMM on caldesmon dissociation from thin filaments

Thin filaments (0.06–3.5 μM-actin monomers) were sedimented in the presence (■) or absence (▲) of thiophosphorylated aorta HMM (2.5 μM in excess of actin monomer concentration) in ATPase buffer (5 mM- $MgCl_2$) plus 3 mM- $MgATP$ at 25 °C. Pellets were dissolved in 0.4 M- $NaOH$ to a constant 0.5 μM concentration, and the caldesmon quantity was determined by immunoassay. Caldesmon quantity is expressed as a fraction of the amount of caldesmon in the thin filaments before sedimentation. The lines are fitted by linear regressions.

Fig. 5. Effect of Ca^{2+} on the dissociation of tropomyosin and caldesmon from thin filaments

SDS (0.2%)/4–30-(w/v)-PAGE of sheep aorta thin filaments sedimented at 10, 2 and 0.4 μM-actin monomer concentration in the presence of 0.1 mM- $CaCl_2$ (+) or 1 mM-EGTA (pCa 9) (–) in ATPase buffer plus 50 mM-KCl (10.09) at 25 °C. Identified bands are: CD, caldesmon; A, actin; Tm, tropomyosin. The ratio of caldesmon to actin band area determined by densitometry is shown beneath the Figure. Values are means for four scans at different positions on each track; the S.D. was 10–15%.

binding sites on the thin filaments, but only about 10% of the ATPase-activating sites on the thin filaments would be occupied [2]. In contrast, skeletal-muscle HMM at the same concentrations had no effect on caldesmon dissociation.

Effects of varying Ca^{2+} and Mg^{2+}

When measured at a constant ionic strength, Mg^{2+} promoted caldesmon dissociation without any measurable effect on tropo-

myosin dissociation. The effect was relatively small. In direct comparisons, CD_{50} for caldesmon increased 4-fold between 0 and 25 mM-MgCl₂ at constant 10.09.

Changes in Ca²⁺ from 10⁻⁹ to 10⁻⁴ M had a small effect on caldesmon dissociation (Fig. 5). Ca²⁺ promoted dissociation of caldesmon with a mean 4-fold increase in CD_{50} (four experiments) but, at 10 μ M-thin filaments, caldesmon was fully associated at any Ca²⁺ concentration (Fig. 5).

DISCUSSION

Caldesmon content of native thin filaments

Two methods have been used to prepare native thin filaments from smooth muscles. The method of Marston & Smith [1] has been shown to give Ca²⁺-regulated thin filaments with similar tropomyosin and caldesmon contents from a wide variety of smooth muscles [10], whereas the method of Marston & Lehman [10] has largely been used for gizzard smooth muscle and produces filaments with a lower caldesmon content [4] and low to zero Ca²⁺-sensitivity [19]. The general objective of our research has been to obtain thin filaments in a state as close as possible to native, and we have therefore used 'Marston & Smith' [1] thin filaments for our studies over the last 6 years. The caldesmon content of this type of preparation from sheep aorta has been reassessed on the basis of the correct M_r and dye-binding ratios on Procion Blue-stained SDS/polyacrylamide gels, and it was found to be one caldesmon molecule per 16.3 actin molecules (0.06 caldesmon molecule/actin molecule). In previous studies we have found that the caldesmon content of native thin filaments from chicken gizzard, rabbit stomach, trachea and uterus, and sheep aorta smooth muscle, is the same [10,29]; therefore the ratio of 16–17 actin molecules per caldesmon molecule is common to native thin filaments in all the smooth-muscle types investigated.

Studies on the structure of smooth-muscle thin filaments have established that caldesmon is an elongated molecule which is stretched out along the actin double helix in register with the tropomyosin and that the most probable model has a stoichiometry of 1 caldesmon molecule per 14 actin molecules (2 tropomyosin molecules) [4]. It has also been reported that, when caldesmon-containing thin filaments are immunoprecipitated from whole-muscle homogenates without any purification, the caldesmon content was 1 molecule per 17 actin molecules [4]. The most likely stoichiometry for caldesmon-containing thin filaments *in vivo* is thus 1:14. My isolated thin-filament preparations contain a similar amount of caldesmon, and therefore the isolation procedure probably selects for caldesmon-containing thin filaments. This does not exclude the possibility that many of the thin filaments in intact muscles contain filamin rather than caldesmon, and thus the whole-tissue caldesmon content is often less than 1:16 [30,31].

Stability of the thin filament

The major components of the smooth-muscle thin filament all dissociate at low concentrations. In the buffers used here, the actin double helix depolymerizes with a critical concentration somewhat lower than that of pure F-actin, which indicates a contribution of tropomyosin to actin polymer stability. Tropomyosin was found to be stably associated with the actin polymer; usually significant dissociation was not observed until most of the caldesmon was dissociated and actin concentration was less than 0.3 μ M, equivalent to a tropomyosin molecule concentration of 0.043 μ M (Fig. 2). This would appear to be a significantly tighter binding than that observed in synthetic mixtures of smooth-muscle tropomyosin and skeletal-muscle actin reported by Sanders & Smillie [20] (50% saturation of

0.5 μ M-tropomyosin) or with skeletal-muscle tropomyosin ([21,22]; 50% dissociation at 0.1–0.3 μ M). At present it is not possible to decide whether this is a result of using proteins from a different muscle type or a change in tropomyosin–actin binding properties when the proteins are purified and reassembled into filaments.

Caldesmon was less tightly associated with the thin filaments than tropomyosin, and its dissociation appeared to be independent. The effects of ionic strength (Fig. 3) and temperature in weakening the association parallel their effects on the affinity of pure caldesmon for actin–tropomyosin [7]. Two groups have estimated the affinity of the caldesmon actin–tropomyosin interaction with broadly similar results [7,8]. In Table 1 I have compared measurements made with aorta proteins under the same conditions as used in the present work with approximate determinations of the dissociation constant of caldesmon from native thin filaments. The dissociation constants are quite similar over a range of conditions. On this basis it would be reasonable to hypothesize that caldesmon reconstituted with tropomyosin and actin behaves the same as the native thin filament. This would further indicate that results obtained with the purified proteins should be applicable to the native thin filament.

Current estimates of the stoichiometry of caldesmon binding to actin–tropomyosin differ: Smith *et al.* [7] reported 18.9 ± 5.3 (means $\pm$ S.D., $n = 28$) actin monomers per caldesmon molecule bound at the high-affinity (inhibitory) sites, on the basis of the true M_r of caldesmon, a finding which is not significantly different from our observation in native thin filaments (14–18 actin monomers per caldesmon molecule). Velaz *et al.* [8] reported 1 actin monomer per 7–10 actin molecules with gizzard proteins. Both these estimates involved fitting the experimental data to complex equations; further investigation will be needed to determine if the discrepancies represent any real difference between native and synthetic systems.

Caldesmon–myosin interaction

Caldesmon dissociation from the native aorta thin filament is greatly diminished in the presence of low concentrations (2.5 μ M) of aorta HMM·ADP·P_i complex, but is not affected by skeletal HMM (Fig. 4). I have shown previously that, under these conditions, a tight binding interaction ($K_d < 1 \mu$ M) occurs between native thin filaments and aorta HMM (but not skeletal-muscle HMM) which is distinct from the binding at the ATPase site (K_d 30–50 μ M) [2]. It has been reported that, in synthetic

Table 1. Comparison of caldesmon dissociation from native thin filaments and from synthetic actin–tropomyosin–caldesmon

K_d values for the actin–tropomyosin–caldesmon (ATC) complex (units μ M-caldesmon) are mean values taken from the work of Smith *et al.* [7], which was done under the same conditions as the present work. K_d of caldesmon dissociating from native thin filaments (NTF) is defined as the concentration of free caldesmon when caldesmon is 50% dissociated from the thin filaments. Since there are 17 actin monomers per caldesmon molecule, K_d is equal to $0.5 \times CD_{50}/17$. CD_{50} values are obtained from the experiments described in Figs 1, 2 and 3.

Temperature (°C)	[KCl] (mM)	K_d	
		ATC	NTF
37	70	111 nM	66 nM
25	60	70 nM	60 nM
25	120	480 nM	530 nM

Table 2. Comparison of native-thin-filament activation of myosin Mg-ATPase under different conditions

Synthetic actomyosins containing thin filaments and myosin (4:1, w/w) were made as described in [1]. ATPase activity was measured in ATPase buffer plus 60 mM-KCl, 2 mM-MgATP, myosin (0.5 mg/ml), thin filaments (2 mg/ml) and 0.1 mM-CaCl₂ or 1 mM-EGTA as described in [1,10]

Myosin	Temperature (°C)	Activation of myosin Mg-ATPase activity (nmol of P _i /min per mg)		Ca ²⁺ -sensitivity
		pCa 4	pCa 9	
Smooth muscle (aorta)	37	96	6	16 ×
Skeletal muscle (rabbit)	37	1295	811	1.6 ×
Skeletal muscle	22	131	30	4.3 ×
Skeletal muscle	12	26	7	3.8 ×

filaments, tight binding is also observed, but only in the presence of caldesmon [23,24]; furthermore, a direct binding interaction between caldesmon and smooth-muscle myosin has been demonstrated [25,26]. Hemric & Chalovich [24] demonstrated a coupled equilibrium between actin binding caldesmon and smooth HMM. The observation in native thin filaments confirms that there is a similar coupling between caldesmon and smooth-muscle HMM binding to the native thin filament (i.e. caldesmon promotes tight HMM binding, and HMM promotes tight caldesmon binding) and therefore also confirms that the tight binding interaction is mediated by caldesmon. A consequence of this interaction is that the smooth-muscle-thin-filament-myosin·ADP·P_i complex is exceptionally stable, and we have recently been able to demonstrate this stability by obtaining electron-microscopic images of the complex in solutions containing as little as 0.2 μM thin filaments (S. Marston, W. Lehman, L. Frado & R. Craig, unpublished work).

Preparation and Ca²⁺-sensitivity of native thin filaments

It is recognized that the method of preparation of thin filaments from smooth muscles is a compromise between purity and activity [1]. Thin filaments isolated by the Marston & Smith [1] technique have a tropomyosin and caldesmon content which is close to that in intact muscle (see above), but they also contain myosin impurities (see Figs. 1 and 5). This is now shown to be a consequence of the coupling of the interactions between myosin, actin-tropomyosin and caldesmon (Fig. 4). Conditions which dissociate myosin from the filament, namely high KCl and MgCl₂ concentrations, also tend to dissociate caldesmon (Fig. 3). Since caldesmon dissociation is not very Ca²⁺-dependent (Fig. 5), thin filaments may be extracted in either EGTA or Ca²⁺ solutions [28]. Repeated washings of thin filaments as used in [10], and especially in high Mg²⁺ solution [19,27], will result in specific removal of caldesmon from the thin filaments and should therefore be avoided.

The Ca²⁺-sensitivity of smooth-muscle thin filaments activating myosin Mg-ATPase has been found to be rather variable (Table 2). The greatest Ca²⁺-sensitivity is always observed with smooth-muscle myosin or HMM [1,10,29]. This is now shown to be due to the ability of smooth-muscle myosin to prevent caldesmon dissociating from the thin filament (Fig. 4). The activation of skeletal-muscle myosin by native thin filaments in Ca²⁺ was equal to activation by actin-tropomyosin, whereas the relative ac-

tivation in EGTA was elevated compared with that in experiments using smooth-muscle myosin (Table 1) [3]. The pattern of results suggests a deficiency of inhibitory caldesmon. Ca²⁺-sensitivity is further reduced at elevated temperatures and KCl concentrations, whereas it is increased at higher total thin-filament concentration (Table 1 [1]). It is now clear that the loss of Ca²⁺-sensitivity correlates with conditions favouring caldesmon dissociation from the filament (Fig. 3).

Although the work reported here shows that caldesmon, tropomyosin and actin behave the same in native smooth-muscle thin filaments and in synthetic thin filaments, it also reconfirms the proposal [11,12] that the calcium-sensitizing factor of native thin filaments is different from calmodulin, which has been commonly used in synthetic thin filaments [5-7,12]. In the synthetic system actin-tropomyosin-caldesmon-calmodulin, Ca²⁺-dependent reversal of caldesmon inhibition may be observed at near-stoichiometric calmodulin concentrations at elevated temperatures and ionic strengths, but requires a considerable excess caldesmon concentration to ensure adequate caldesmon binding to actin. On the other hand, very high calmodulin concentrations (in excess of 100 μM) are required at 25 °C and 10.09 [5-7,12]. This contrasts with the observations with native thin filaments (Table 2). The low Ca²⁺-sensitivity of thin filaments at higher temperature and ionic strength may be ascribed to caldesmon dissociation (above); however, at 25 °C and moderate ionic strength, the mechanisms of synthetic and native filament regulation are clearly different. In the synthetic thin filament at 25 °C Ca²⁺-calmodulin competes with actin for caldesmon binding [5,7,12], but this does not happen in native thin filaments, since caldesmon association is not highly dependent on Ca²⁺ concentrations (Fig. 5): caldesmon remains fully associated with the native thin filament at the concentrations used for ATPase assay, in both the presence and absence of Ca²⁺. This point is further emphasized by the observation that, in the presence of smooth-muscle myosin, Ca²⁺-dependent regulation of ATPase activation is particularly high (Table 1), and caldesmon association with the thin filament is strong (Fig 4).

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