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Ca^{2+}–calmodulin binding to caldesmon and the caldesmon–actin–tropomyosin complex

Its role in Ca^{2+} regulation of the activity of synthetic smooth-muscle thin filaments

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We measured the concentration of calmodulin required to reverse inhibition by caldesmon of actin-activated myosin MgATPase activity, in a model smooth-muscle thin-filament system, reconstituted *in vitro* from purified vascular smooth-muscle actin, tropomyosin and caldesmon. At 37 °C in buffer containing 120 mM-KCl, 4 μM-Ca^{2+}–calmodulin produced a half-maximal reversal of caldesmon inhibition, but more than 300 μM-Ca^{2+}–calmodulin was necessary at 25 °C in buffer containing 60 mM-KCl. The binding affinity (K) of caldesmon for Ca^{2+}–calmodulin was measured by a fluorescence-polarization method: $K = 2.7 \times 10^6$ M^{-1} at 25 °C (60 mM-KCl); $K = 1.4 \times 10^6$ M^{-1} at 37 °C in 70 mM-KCl-containing buffer; $K = 0.35 \times 10^6$ M^{-1} at 37 °C in 120 mM-KCl- containing buffer (pH 7.0). At 37 °C/120 mM-KCl, but not at 25 °C/60 mM-KCl, Ca^{2+}–calmodulin bound to caldesmon bound to actin–tropomyosin ($K = 2.9 \times 10^6$ M^{-1}). Ca^{2+} regulation in this system does not depend on a simple competition between Ca^{2+}–calmodulin and actin for binding to caldesmon. Under conditions (37 °C/120 mM-KCl) where physiologically realistic concentrations of calmodulin can Ca^{2+}-regulate synthetic thin filaments, Ca^{2+}–calmodulin reverses caldesmon inhibition of actomyosin ATPase by forming a non-inhibited complex of Ca^{2+}–calmodulin–caldesmon–(actin–tropomyosin).

INTRODUCTION

The activity of the contractile apparatus in smooth muscle is regulated by the intracellular concentration of free Ca^{2+} ion; Ca^{2+} exerts its control via mechanisms based on both the thick (myosin) filaments [1] and the thin (actin-based) filaments [2]. The activation of the MgATPase activity of myosin by thin filaments prepared in the intact, 'native', state from smooth muscle depends on Ca^{2+} concentration in the physiological range [2]. Caldesmon [3] is vital to this mechanism [4,5]. Native smooth-muscle thin filaments contain actin, tropomyosin and caldesmon in molar proportions of 28:4:1 [6,7]. Purified caldesmon inhibits the activation of myosin MgATPase by actin–tropomyosin: this inhibition correlates with the binding of caldesmon to high-affinity sites on actin–tropomyosin, with a stoichiometry of 1 caldesmon molecule per 28 actin molecules [8]. Synthetic thin filaments reconstituted from pure actin, tropomyosin and caldesmon are never Ca^{2+}-sensitive, but they can be Ca^{2+}-regulated by exogenous calmodulin [4,9] because Ca^{2+}–calmodulin binds to caldesmon, reversing caldesmon's inhibitory effect on the actomyosin ATPase [4,8]. We have found that in the reconstituted thin filaments caldesmon can be Ca^{2+}-regulated at 37 °C by little more than stoichiometric amounts of Ca^{2+}–calmodulin, yet Ca^{2+} regulation at 25 °C requires calmodulin concentrations more than 25-fold higher [8].

This striking dependence on experimental conditions presumably reflects underlying changes in the interactions of the proteins involved. The release of caldesmon inhibition may be accompanied by a dissociation of caldesmon from the actin–tropomyosin filaments, because of a competition between Ca^{2+}–calmodulin and F-actin for binding to caldesmon [9–11], but Ca^{2+}–calmodulin does not fully dissociate caldesmon from actin–tropomyosin under all conditions [4,11]; indeed, under conditions where Ca^{2+}–calmodulin is most effective in releasing inhibition by caldesmon, its effect in releasing caldesmon from actin–tropomyosin is least [8].

The interactions between caldesmon and calmodulin thus require clarification, in particular regarding the reasons for the marked effect of experimental conditions. The reconstituted system using calmodulin should be a useful model for the mechanism of Ca^{2+} regulation in the native filaments [8]. An alternative regulatory mechanism based on caldesmon phosphorylation has been proposed by Walsh and co-workers [11,12]. However, Ca^{2+} regulation in the 'native' thin filaments, which are our source of regulatory proteins, does not involve the phosphorylation of caldesmon [13]. We have measured caldesmon–calmodulin binding, using a fluorescence-polarization technique, and calmodulin binding to reconstituted thin filaments (actin–tropomyosin, plus caldesmon) by a sedimentation method. We are now able to account for the way in which experimental conditions influence the mechanism of Ca^{2+} regulation in this system reconstituted *in vitro*.

EXPERIMENTAL

Preparation of proteins

Caldesmon and F-actin were prepared from sheep aorta thin filaments [4]. Aorta smooth-muscle tropomyosin was prepared by a modification [6] of the

Abbreviations used: 1,5-IAEDANS, 5({[(iodoacetyl)amino]ethyl}amino)-1-naphthalenesulphonic acid; CMAEDANS, 1,5-AEDANS-labelled calmodulin.

method of Bailey [14]. Rabbit skeletal-muscle myosin was prepared by the method of Perry [15]. Heavy meromyosin was prepared by chymotryptic digestion of thiophosphorylated aorta smooth-muscle myosin [16]. Bovine brain calmodulin was prepared as in ref. [17]. For fluorescence studies, calmodulin was labelled with 1,5-IAEDANS (Sigma) by a procedure based on that of Olwin & Storm [18]. For sedimentation binding studies, calmodulin was radioactively labelled with [^{3}H]*N*-hydroxysuccinimidyl propionate (Amersham), giving [^{3}H]propionyl-calmodulin.

Binding 1,5-AEDANS-labelled calmodulin to caldesmon

Fluorescence anisotropy titrations were carried out with a Perkin–Elmer LS-5 spectrofluorimeter with polarizer accessory. Proteins for binding studies were dissolved in 'ATPase buffer' (KCl 60, 70 or 120 mM; $MgCl_2$, 5 mM; NaN_3, 1 mM; K_2Pipes, 10 mM; dithiothreitol, 1 mM; pH 7.0); 0.1 mM-$CaCl_2$ or 1.0 mM-EGTA was added as required. 1,5-AEDANS-labelled calmodulin (CMAEDANS) was present in one cuvette at 0.1–1.0 μM; a paired cuvette contained buffer only. Equal additions of caldesmon were made to both cuvettes; the maximum dilution was 20%. Fluorescence was excited at 340 nm (slit width 5 nm) and emission was monitored at 480 nm (slit width 10 nm). Calculations of anisotropy and correction for the 'parallel diffraction anomaly' of the instrument were done as in ref. [18]. Background anisotropy due to caldesmon, and the apparent increment in anisotropy arising from the enhancement in emission intensity after binding, were also allowed for [18,19]. The fraction of CMAEDANS bound was calculated as in ref. [19], and the limiting maximum value of probe fluorescence was estimated by using a non-linear least-squares fit to a hyperbola. Binding constants were obtained by fitting values of the fraction of CMAEDANS bound versus free caldesmon, assuming a single non-interacting class of binding sites (ENZFIT computer program; Elsevier Biosoft, Cambridge, U.K.).

Reversal of caldesmon inhibition by calmodulin

Calmodulin was tested for its ability to reverse caldesmon inhibition of actin–tropomyosin-activated myosin MgATPase activity. With skeletal-muscle myosin, assay tubes contained 1–1.5 μM-myosin, 21–25 μM aorta actin and 3.5–4.2 μM aorta tropomyosin (tropomyosin/actin molar ratio 0.16) and 1.5–2.5 μM aorta caldesmon (sufficient for maximal ATPase inhibition; caldesmon was tested at a range of concentrations in preliminary experiments). Calmodulin was included at up to 3 mg/ml (180 μM). Smooth-muscle heavy meromyosin (5 μM) was incubated with 50 μM-actin, 18 μM-tropomyosin, 5 μM-caldesmon and up to 60 μM-calmodulin. Reaction was initiated by adding MgATP to 2 mM and terminated by the addition of 0.5 ml of 5% trichloroacetic acid, and the amount of P_i released was determined as in ref. [8].

Binding of ^{3}H-labelled calmodulin to reconstituted thin filaments

Calmodulin (0.5 μM) labelled with *N*-hydroxysuccinimidyl propionate (500000 c.p.m. per sample) was incubated with 160–200 μM-actin and tropomyosin (sufficient to saturate actin; tropomyosin/actin molar ratio 0.25) in 150 μl of ATPase buffer containing either 120 mM-KCl at 37 °C or 60 mM-KCl at 25 °C. Samples contained either 0.1 mM-$CaCl_2$ or 1 mM-EGTA. Caldesmon was added to concentrations of 0–8 μM. Control tubes contained no actin. After equilibration (30 min) the tubes were centrifuged at 200000 *g* for 30 min at the incubation temperature. The supernatants were then assayed for radioactivity by liquid-scintillation counting. The fraction of calmodulin bound to caldesmon–actin–tropomyosin was calculated as: [(c.p.m. in 'zero-caldesmon' sample) − (c.p.m. in presence of caldesmon)]/(c.p.m. in 'zero-caldesmon' sample). The dissociation constant was determined by using the ENZFIT non-linear regression program, fitting to the equation for a single set of binding sites.

RESULTS

Binding of CMAEDANS to caldesmon

In the presence of 0.1 mM-$CaCl_2$, the initial anisotropy of the 1,5-AEDANS fluorophore covalently attached to calmodulin was 0.043 ± 0.005 (S.D.; $n = 10$). Addition of caldesmon in the presence of Ca^{2+} resulted in an increase in probe anisotropy, tending to a maximum of 0.0857 ± 0.0099 (S.D.; $n = 10$), which we presume to be a result of complex-formation. In 1 mM-EGTA, there was no net change in CMAEDANS fluorescence properties (results not shown). The ratio of final to initial emission intensities was determined from the limits of each titration, and lay between 0.91 and 1.19. Net anisotropy changes could be well fitted to a rectangular hyperbola. The fitted binding curve (Fig. 1) shows that CMAEDANS binds to caldesmon in solution with an affinity in the range of 10^6 M^{-1} (Table 1). Binding is approx. 2-fold weaker when temperature is increased from 25 °C to 27 °C. Raising salt concentration from 60 to 120 mM-KCl in conjunction with increased temperature weakened binding affinity nearly 8-fold overall.

Reversal of caldesmon inhibition by calmodulin

Maximally effective concentrations of caldesmon produced an inhibition usually of 75–80% of the actin–tropomyosin-activated myosin MgATPase; addition of

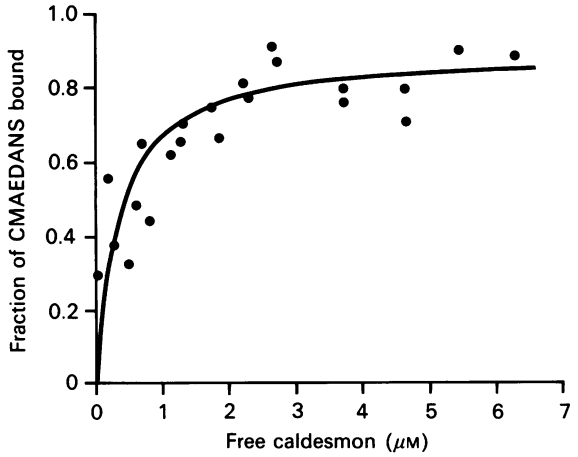

Fig. 1. Binding of CMAEDANS to caldesmon

'ATPase' buffer containing 60 mM-KCl and 0.1 mM-$CaCl_2$ was used; temp. 25 °C, $n = 3$; initial concentration of CMAEDANS was 0.1, 0.5 or 0.9 μM, and data points from independent experiments were overlapping under any particular set of experimental conditions.

Table 1. Effects of conditions on calmodulin–caldesmon interactions

For binding measurements, fluorescence titrations were performed with four preparations of caldesmon (A, B, C, D); *n* is in this case the number of titrations. The concentration of CMAEDANS was 0.1, 0.5, 0.7 or 1.0 μM, and sets of data points of individual titrations under any one condition were overlapping. For reversal of caldesmon inhibition, EC_{50} is the concentration of calmodulin required for 50% neutralization of the inhibition of actin–tropomyosin-activated skeletal-muscle myosin or smooth-muscle heavy-meromyosin MgATPase by a maximally effective concentration of caldesmon; *n* refers to numbers of separate experiments. At least seven calmodulin concentrations over the range 0.05–180 μM were tested under each set of conditions.

Conditions		Caldesmon–CMAEDANS binding affinity		Ca^{2+}–calmodulin reversal of inhibition			
				Skeletal-muscle myosin		Vascular-smooth-muscle heavy meromyosin	
Temp. (°C)	[KCl] (mM)	$10^{-6} \times K$ (M^{-1})	*n*	EC_{50} (μM)	*n*	EC_{50} (μM)	*n*
25	60	2.7±0.7	3 (A,D)	> 300	3	> 300	1
37	70	1.4±0.4	3 (A,B)	14±1	4	2	1
37	120	0.35±0.07	4 (B,C)	4±2	1		

calmodulin in the presence of 0.1 mM-$CaCl_2$ reversed this inhibition in a dose-dependent manner. In the absence of Ca^{2+} (1 mM-EGTA) calmodulin had no effect. With either skeletal-muscle myosin or smooth-muscle heavy meromyosin, the effect of any particular concentration of calmodulin in reversing caldesmon inhibition was critically dependent on experimental conditions (Table 1). At 37 °C, restoration of the myosin MgATPase to non-inhibited values was observed, when using calmodulin concentrations of 60–100 μM or above (in buffer containing 70 mM-KCl) with either myosin type, or by only 30 μM-calmodulin, when using skeletal-muscle myosin with 120 mM-KCl-containing buffer. At 25 °C, the effectiveness of calmodulin was so low that complete disinhibition of the MgATPase rate could not usually be achieved, even with 180 μM-calmodulin.

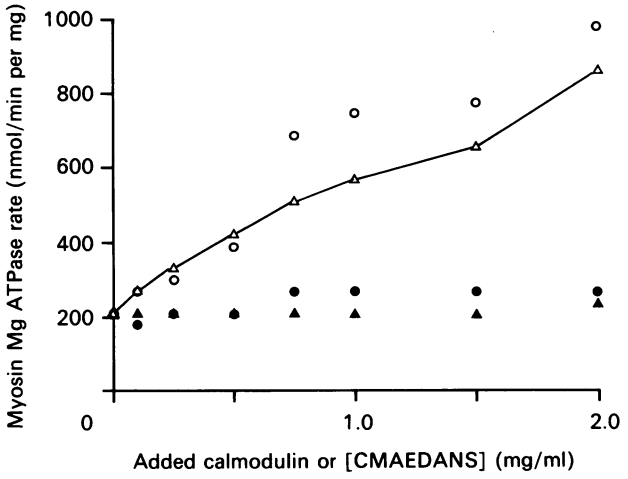

Fig. 2. Functional comparison of calmodulin and calmodulin covalently labelled with 1,5-AEDANS

The MgATPase activity of skeletal-muscle myosin (1.1 μM) was stimulated to 860 nmol/min per mg of myosin by 21 μM aorta actin plus 3.5 μM aorta tropomyosin. Maximal inhibition (down to 210 nmol/min per mg of myosin) was observed after addition of 2.1 μM aorta caldesmon. With 0.1 mM-$CaCl_2$ (○, △) at 37 °C in buffer containing 70 mM-KCl, subsequent addition of labelled (○) or unlabelled (△) calmodulin reversed caldesmon inhibition, seen as an increase in ATPase rate. There was no effect on ATPase rate in the presence of 1 mM-EGTA (●, ▲).

Functional comparison of calmodulin and CMAEDANS

CMAEDANS was fully effective in reversing caldesmon inhibition of myosin MgATPase activated by actin–tropomyosin (Fig. 2). The fluorescently labelled calmodulin produced ATPase rates which were not significantly lower than rates produced by unlabelled calmodulin ($P > 0.1$, paired-sample *t* test; $n = 7$). This result was replicated in three independent experiments.

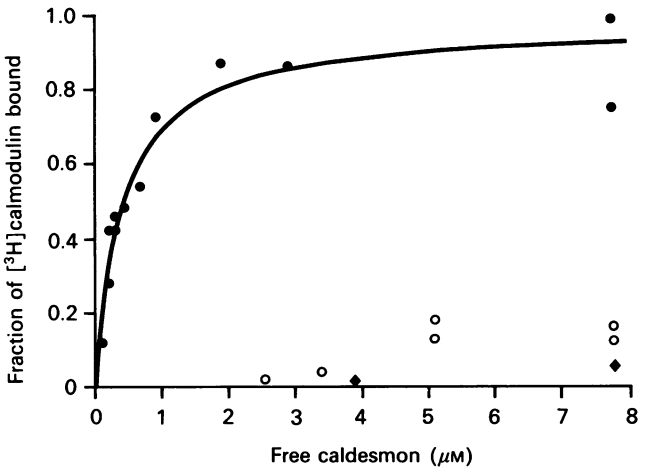

Fig. 3. Binding of [^{3}H]propionyl-labelled calmodulin to actin–tropomyosin–caldesmon

Binding of calmodulin to caldesmon bound to tropomyosin-saturated actin in 120 mM-KCl-containing 'ATPase' buffer, 37 °C, with: 0.1 mM-$CaCl_2$ (●); 1 mM-EGTA (○). Binding in 60 mM-KCl-containing buffer at 25 °C with 0.1 mM-$CaCl_2$ (◆). Samples (150 μl) contained 160 μM-actin, 40 μM-tropomyosin and 0.5 μM-[^{3}H]propionyl-calmodulin.

Binding of [^{3}H]propionyl-labelled calmodulin to actin–tropomyosin–caldesmon

In the presence of tropomyosin-saturated actin, with 0.1 mM-$CaCl_2$, addition of caldesmon (max. 8 μM) at 37 °C in buffer containing 120 mM-KCl led to removal of radioactivity from the supernatant after centrifugation. When [^{3}H]propionyl-calmodulin was centrifuged with caldesmon (up to 8 μM) alone, in either the presence or the absence of Ca^{2+}, no radioactivity was removed from the supernatant. Thus Ca^{2+}–calmodulin bound to caldesmon–actin–tropomyosin. The maximum binding reached a plateau of 40–50 % of the total radioactivity added. The affinity of calmodulin–caldesmon binding in the synthetic thin filament was $2.40(\pm 0.36) \times 10^6$ M^{-1} (S.E.M.; 12 data points) in the experiment illustrated (Fig. 3). Two separate experiments on independent preparations of proteins gave a binding constant of $3.33(\pm 0.83) \times 10^6$ M^{-1} (S.E.M. from pooled results; 18 data points).

At 25 °C in buffer containing 60 mM-KCl, radiolabelled calmodulin did not co-sediment with caldesmon bound to actin–tropomyosin in either the presence or the absence of Ca^{2+}.

DISCUSSION

Calmodulin binding to caldesmon

Fluorescence-anisotropy titrations allowed a precise determination of the binding affinity between CMAEDANS and caldesmon in solution. This method has the advantage that the anisotropy depends on the rotational mobility of the probe, a physical property which is directly influenced by binding of calmodulin to the much larger and less mobile caldesmon molecule. The initial anisotropy of the 1,5-AEDANS probe bound to calmodulin was 0.043, as reported in the literature [19]. The maximum anisotropy recorded when the labelled calmodulin was bound to caldesmon (120 kDa) was 0.0857. Reported values for CMAEDANS bound to troponin I (23 kDa) or to myosin light-chain kinase (77 kDa; [20]) are 0.0834 [19] and 0.0832 [21] respectively. As expected from the relationship between anisotropy and Stokes radius [19], maximum anisotropy tends to be greater with binding to target proteins of higher molecular mass. We found that this method provided more consistent data than did titrations of emission intensity, which we have previously employed [8]; however, the estimates of binding constants by the two methods are similar (Table 1). Recently the binding of caldesmon to fluorescently labelled spinach calmodulin has been studied [22]; in buffer containing 90 mM-KCl at room temperature, the reported binding constant was 4×10^6 M^{-1}, similar to the value reported here (Table 1). Caldesmon binding to calmodulin–Sepharose affinity columns has been reported by several groups [3,4,10,23], yielding binding constants of about $(0.5–1) \times 10^6$ M^{-1} [23]. Binding of unlabelled calmodulin to caldesmon in solution has also been detected by monitoring changes in gizzard caldesmon intrinsic tryptophan fluorescence [24]. There is thus a consensus that calmodulin binds to caldesmon with an affinity in the range of 10^6 M^{-1}, in the presence of Ca^{2+}; binding in EGTA has never been reported to occur, and none was detected in the present study.

The fluorescently labelled calmodulin was as effective as an equal concentration of unlabelled calmodulin in reversing caldesmon inhibition of myosin MgATPase activated by actin–tropomyosin (Fig. 2), which implies that the fluorophore had no effect on the affinity or function of calmodulin in its interaction with caldesmon. The tightest binding ($K = 2.7 \times 10^6$ M^{-1}) was observed at 25 °C in 60 mM-KCl-containing buffer. This is much weaker than that between Ca^{2+}–calmodulin and calmodulin-regulated enzymes, where reported binding constants are typically in the range 10^8–10^9 M^{-1} [19,21,25,26]. At near-physiological temperature and ionic strength, Ca^{2+}–calmodulin binding to caldesmon becomes weaker ($K = 3.5 \times 10^5$ M^{-1}), comparable with that of Ca^{2+}-free calmodulin for myosin light-chain kinase (2.2×10^5 M^{-1}; [27]). Hydrophobic interactions are believed to make a large contribution to the high affinity of binding [28], and binding should thus be favoured by increases in ionic strength and temperature [17]. We observed the opposite effect (Table 1). Calmodulin has several modes of binding interaction [28], and possibly an ionic component of the binding site [28–30] is particularly influential in determining the binding affinity to caldesmon.

Mechanism of Ca^{2+} regulation by calmodulin

The binding affinity between caldesmon and actin–tropomyosin at 25 °C in 60 mM-KCl-containing buffer is 28×10^6 M^{-1} [8]. Consideration of the equilibria involved, assuming a simple competition between actin–tropomyosin and Ca^{2+}–calmodulin for caldesmon binding ('flip-flop' binding; [9]), would predict that a considerable molar excess of calmodulin would be required to reverse inhibition by caldesmon under these conditions, consistent with our experimental findings. Our binding data (Table 1; [8]) would on this basis lead to the prediction that calmodulin ought to be less effective in reversing caldesmon inhibition at 37 °C in buffer containing 120 mM-KCl than at lower temperature and ionic strength. This prediction conflicts directly with our experimental measurements of ATPase (Table 1; see also [8]). Under these conditions the regulation of caldesmon by calmodulin cannot be explained by a simple competition scheme. At 37 °C caldesmon binds to actin–tropomyosin in the presence of Ca^{2+}–calmodulin [4,8], implying that at 37 °C Ca^{2+}–calmodulin reversed inhibition by binding to caldesmon–actin–tropomyosin [8]. The sedimentation experiment using radiolabelled calmodulin (Fig. 3) demonstrates directly that at 37 °C/120 mM-KCl, under the conditions where calmodulin is most effective in reversing caldesmon inhibition, a quaternary complex of Ca^{2+}–calmodulin–caldesmon–actin–tropomyosin is formed; the affinity of Ca^{2+}–calmodulin for caldesmon in this complex (Fig. 3) is comparable with that for caldesmon alone (Table 1). There was no evidence for the formation of this complex at 25 °C in 60 mM-KCl-containing buffer.

The Ca^{2+} regulation of actin–myosin interaction (ATPase) can thus be accounted for solely on the basis of the measured binding affinities between actin–tropomyosin, caldesmon and calmodulin. This means that there is no significant influence of either smooth-muscle or skeletal-muscle myosin on these equilibria. Evidence has recently been reported that there is a strong binding interaction between smooth-muscle myosin and caldesmon [31,32]. This interaction [5,33] is presumably a quite separate function of caldesmon.

Relevance to Ca^{2+} regulation in intact 'native' thin filaments

Myosin phosphorylation is a pre-requisite for smooth-muscle contraction after stimulation. In this report we have concentrated on the additional actin-based mechanism of regulation. Caldesmon is the basis of the observed Ca^{2+} regulation of the activation of myosin MgATPase by intact 'native' thin filaments [5]. That paper confirms that concentrations of Ca^{2+}–calmodulin similar to those occurring in smooth-muscle tissues (perhaps as high as 30–40 μM; [34,35]) can Ca^{2+}-regulate caldesmon *in vitro*, under specific experimental conditions, which approximate to the physiological. We have shown that the Ca^{2+} regulation of caldesmon, under physiological conditions of temperature and ionic strength, depends on the formation of a non-inhibited quaternary complex (Fig. 3). It seems likely that the observed Ca^{2+} regulation of intact 'native' thin filaments involves an analogous intra-filament mechanism, based on caldesmon.

These studies emphasize several differences between the behaviour of the reconstituted thin-filament system regulated by calmodulin, and the 'native' thin filaments of smooth muscles. Ca^{2+} regulation of the latter is better at lower temperatures and ionic strengths [5,6], yet the 'native' filaments certainly do not contain the very large molar excess of calmodulin which would be required to regulate caldesmon at 25 °C (Table 1; [4,6]). Furthermore, the preparation of Ca^{2+}-sensitive 'native' thin filaments [6] involves washes in EGTA-containing buffers, which would remove calmodulin. These findings support our earlier suggestions that the native smooth-muscle thin filament is regulated not by calmodulin but by a different Ca^{2+}-binding protein, and this possibility is under investigation [36].

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