The Mechanism of Ca\(^{2+}\) Regulation of Vascular Smooth Muscle Thin Filaments by Caldesmon and Calmodulin*

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Contraction of smooth muscles is controlled by the Ca\(^{2+}\) concentration in the vicinity of the contractile proteins (1). The action of Ca\(^{2+}\) is mediated by regulatory mechanisms which are associated with both the contractile proteins, actin and myosin (2, 3). The control of smooth muscle myosin by a Ca\(^{2+}\)-dependent myosin light chain kinase and a Ca\(^{2+}\)-independent phosphatase has been thoroughly investigated during the last 10 years (4), and there is also evidence for Ca\(^{2+}\) acting directly on the myosin molecule (5). In contrast, Ca\(^{2+}\) control of the actin-based thin filaments has received rather little attention until recently (2, 3, 6, 7, 37). We have isolated Ca\(^{2+}\)-regulated thin filaments from a number of vertebrate smooth muscles and have found that they have a constant protein composition of 1 actin:½ tropomyosin dimer:½ of a M, 120,000 protein which is identical to caldesmon (6, 7, 8). Caldesmon has been shown to be exclusively located in the thin filaments of many smooth muscles (7) and in a number of non-muscle motile cells where it is sometimes present as a M, 77,000 isoform (9, 10, 11). Its regulatory role has been demonstrated by biochemical studies in which the thin filament was dismantled into its components and then reassembled (12). Actin and actin-tropomyosin activated skeletal muscle myosin independent of the Ca\(^{2+}\) concentration. Addition of small amounts of caldesmon strongly inhibited this activation independent of Ca\(^{2+}\) concentration. The further addition of bovine brain calmodulin resulted in removal of the inhibition in the presence of Ca\(^{2+}\) (12-15, 17).

It has been suggested that the inhibitory activity of caldesmon may also be modified by a phosphorylation reaction (31, 33). In our work caldesmon was isolated from native thin filaments which have no caldesmon kinase activity (46), consequently caldesmon phosphorylation plays no role in the regulatory system which we are investigating. In this paper we describe measurements of the interactions between the thin filament protein components under varying conditions from which we have proposed a mechanism for the control of smooth muscle thin filaments by Ca\(^{2+}\).

**MATERIALS AND METHODS**

All proteins were prepared according to previously published procedures. Sheep aorta actin, thin filaments, and tropomyosin were prepared according to Ref. 6. Aorta caldesmon (M, 120,000 protein) was prepared by the method of Smith and Marston (12). It was stored at 4° C in a buffer containing 10\(^{-3}\) M dithiothreitol and protease inhibitors (2 µg/ml chymostatin and leupeptin) and was used within 48 h of preparation. During this time no changes were noted in its actin binding, calmodulin binding, or inhibitory properties. Bovine brain calmodulin was prepared as described by Gopalakrishna and Anderson (18). Rabbit skeletal muscle myosin was prepared by the methods of Perry (19) or Margossian and Lowey (20) and actin by the methods of Spudich and Watt (21). Rabbit skeletal muscle tropomyosin was prepared by the same method as aorta tropomyosin (6). Thio-phosphorylated smooth muscle myosin was prepared as described by Hesapil and Chacko (5). Calmodulin was cross-linked to cyanogen bromide-activated Sepharose CL-4B (Pharmacia) by the method of Grund and Perry (22). Aorta caldesmon was covalently linked to Sepharose in the same way except that the coupling buffer also contained 0.1 M NaCl.

Synthetic actomyosins were reconstituted from pure actin and myosin by mixing the individual proteins in 0.6 M KCl and then dialyzing overnight against the desired buffer. The ATPase buffer used in most experiments contained 60 mM KCl, 5 mM MgCl\(_2\), 10 mM Na\(_2\)ATP, 1 mM dithiothreitol, 10 mM Tris-MES, pH 7.0, at 25°C. In some experiments the KCl concentration was varied.

MgATPase activities were assayed by the liberation of P from MgATP. The reaction was started by the addition of MgATP to 2 mM, and aliquots were taken end quenched in an equal volume of 5% trichloroacetic acid. The phosphate released was assayed by the

1. The abbreviations used are: MBS, 4-morpholinonephensulfonic acid; dansyl, 5-dimethylaminoanophthalene-1-sulfonyl; VSM, vascular smooth muscle; EGTA, [ethylenbis(oxyethyl)enitrilo]tetraacetic acid; 1,5-IAEDANS, N-iodoacetyl-N'- (5-sulfo-1-naphthyl) ethylenediamine; Atm, actin-tropomyosin complex; CM, calmodulin; CD, caldesmon.

*This work was supported by a grant from the British Heart Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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method of Taussky and Schorr (23). The rate of phosphate liberation was constant over the time course of the assay (10 min).

For the measurement of caldesmon binding to actin the caldesmon was first labeled with [1-14C]iodoacetamide (Amer sham Corp., 50–60 mCi/mmol). Caldesmon was mixed with a 2-fold molar excess of iodoacetamide in 10 mM Tris-MES, pH 7.0, 150 mM KC1, and left for 10–18 h at 4 °C. Excess unreacted iodoacetamide was removed by dialysis. The level of labeling was 0.57 ± 0.05 mol/mol (mean ± S.E., n = 12). Binding of 14C-labeled caldesmon was determined by the cosedimentation of caldesmon (0.01–5 μM) with actin or actin-tropomyosin (3–12 μM) at 49,000 × g for 2 h. Aliquots were taken before and after (± free caldesmon) and after (± free caldesmon) sedimentation, and radioactivity was determined by liquid scintillation counting.

Actin-bound caldesmon was equated with the difference between total and free caldesmon (24). Values for bound and free caldesmon were corrected for the proportion of caldesmon which sediments in the absence of actin (15 ± 11%, mean ± S.E., n = 10) and the amount which did not sediment even in 1,000-fold molar excess of actin (32 ± 8%, mean ± S.E., n = 10). In control experiments, the binding of labeled caldesmon to actin was determined with varying ratios of labeled and unlabeled caldesmon, but with a constant total caldesmon concentration. The extent of binding measured was directly proportional to the amount of labeled caldesmon in the mixture, indicating that the labeled and unlabeled caldesmon have identical actin binding properties.

Binding of caldesmon to calmodulin was studied by a fluorescence assay using calmodulin labeled with 1,5-IAEDANS (38). Calmodulin (100 μM) was allowed to react with 10 mM 1,5-IAEDANS overnight at 4 °C in 0.2 M sodium acetate, 2 mM CaCl2. Unbound label was removed by gel filtration on Sephadex G-25. Dansyl fluorescence was excited at 340 nm, and emission was measured at 475 nm. In the absence of Ca2+, caldesmon enhanced fluorescence by up to 20%; at these wavelengths background caldesmon fluorescence did not exceed 5% of the total.

RESULTS

Caldesmon Binding to Actin—Caldesmon bound to actin and the actin-tropomyosin complex, although only the binding to actin-tropomyosin was associated with inhibition of actomyosin MgATPase activity. Binding curves were measured over a range of different conditions with 5–1000 nM free caldesmon (Table I, Fig. 1). In most cases the binding could not be accounted for by a single class of binding site. All the data could be accounted for on the basis of two independent classes of binding site which we have termed “tight” and “weak,” although the data probably do not rule out more complex situations such as negative cooperativity or more than two classes of site.

Analysis in terms of the two-site model indicated that a small quantity of caldesmon, amounting to 1 per 20–30 actins bound to the tight sites (affinity range 2–40 × 106 M−1), whereas binding to the weak sites (K = 1–3 × 105 M−1) saturated at a stoichiometry of 1 caldesmon bound per 4–8 actin monomers (Fig. 1). Under many conditions the binding parameters of two classes of sites could be clearly distinguished (e.g. Fig. 1), and a full analysis was possible. Under some conditions (notably higher temperature and [KCl]), where the affinity of the tight sites approached that of the weak sites, only estimates could be made (see Table I).

Table I summarizes our measurements of caldesmon binding to the tight sites on actin and actin-tropomyosin. There was considerable variability between binding constants measured under identical conditions with different preparations. It is unlikely that the variability in the measured binding parameters is related to the extent of iodoacetamide labeling, since control experiments with a single preparation established that labeling had no effect upon the actin binding or inhibitory properties of caldesmon. Differences between preparations in the correction factors for caldesmon which failed to sediment alone or which sedimented in the absence of actin probably underlie a large amount of the variation. These corrections effectively limit the accuracy of the determined binding parameters. The fact that the binding curves had to be fitted for two classes of binding site was a further contributing factor to limiting the accuracy of determining the binding constants. Caldesmon is known to be susceptible to both proteolytic degradation and to the formation of multimeric aggregates (5, 39) either of which could provide a physical explanation for the caldesmon fractions which did not interact correctly with actin. The binding constants also depended on the temperature and [KCl] (ionic strength) as determined with any single preparation. With actin-tropomyosin the affinity decreased by a mean 3.5-fold between 60 and 120 mM KCl; it also decreased slightly with increasing temperature: Q0 = 1.35 ± 0.1 (n = 5), (ΔH = −30 J·K−1·mol−1). With the actin-tropomyosin complex, the affinity for caldesmon did not significantly alter when skeletal muscle proteins were substituted for vascular smooth muscle proteins. Binding was always tighter with actin-tropomyosin, compared to actin alone (mean 3.7-fold difference ± 2.1 S.D. (n = 4)).

The stoichiometry of the tight caldesmon-binding sites was independent of the conditions used for measurement and was also the same for both actin and actin-tropomyosin complexes (0.032 ± 0.003 (n = 4) caldesmon/actin; 0.039 ± 0.011 (n = 26) caldesmon/actin-tropomyosin). This remarkably low stoichiometry—1/28 actins—is the same as the caldesmon content we observed in preparations of native Ca2+-regulated VSM thin filaments (6).

Caldesmon Binds to Tropomyosin—We found that caldesmon bound tighter to actin-tropomyosin than to actin, which suggests that caldesmon may interact directly with tropomyosin as well as with actin. We demonstrated that tropomyosin does bind to caldesmon by means of a column of caldesmon covalently attached to Sepharose CL-4B (35 mg of caldesmon cross-linked to 5 g of CNBr-activated Sepharose, dry weight). When smooth or skeletal muscle tropomyosin (3 mg) was applied to the column in ATPase buffer (60 mM KCl), all the protein bound. It could be eluted by applying ATPase buffer containing 0.6 mM KCl. It was not possible to determine binding affinities or stoichiometries by this procedure, but the extent of tropomyosin retention suggested a binding constant of at least 105 M−1.

Inhibitory Action of Caldesmon—When caldesmon was added to actin-tropomyosin it always inhibited its activation of myosin MgATPase activity. In contrast, the effect of caldesmon in the absence of tropomyosin was more variable, with slight inhibition, activation, and no effect all being observed with different preparations. In those cases where inhibition was observed with actin alone, however, it was always less than in the presence of tropomyosin, even at caldesmon concentrations which produced full occupancy of the tight binding sites (Fig. 2, Fig. 3). Thus at 25 °C, 60 mM KCl, the effect of caldesmon added to a molar ratio of 1 per 20 actins varied from 38% inhibition to 43% activation in the absence of tropomyosin, compared to inhibition of 78 ± 16% (S.E., n = 6) in the presence of smooth muscle tropomyosin. The only set of conditions at which we consistently observed inhibition in the absence of tropomyosin was at 4 °C, 50 mM KCl (13 ± 4% inhibition, S.D., n = 3 at 1 caldesmon per 20 actins; see Ref. 25). The presence of tropomyosin therefore appears to be obligatory for the inhibitory action of caldesmon.

Fig. 2 shows typical caldesmon inhibition curves with actin-tropomyosin. Two features are apparent. First, inhibition can take place with low stoichiometries of caldesmon to actin-
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**TABLE I**

**Caldesmon binding to actin tight sites**

Binding was measured by sedimentation assay (see "Materials and Methods") and analyzed on the basis of two classes of independent sites. An initial fit of the data onto a Scatchard plot was obtained by a visual iterative process. The contribution of the weak sites was calculated and subtracted from total binding and the residual, tight binding was further analyzed by fitting to the equation for a single class of binding sites (41). Results are given as binding constant, $K$, and maximum binding, $B_{max}$ ± S.E. The estimated weak site parameters were in the range $K = 1 - 5 \times 10^5 M^{-1}$; $B_{max} = 1/5 - 1/8$ per actin. In the experiments marked * there were insufficient data at high caldesmon concentrations to fit the weak sites, so weak site parameters were interpolated from comparable experiments. CD, caldesmon.

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<th>$B_{max}$ (CD/actin)</th>
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Tropomyosin, and second, inhibition saturates at less than 100%. The greatest percentage inhibition was obtained when ATPase activation was highly potentiated by tropomyosin. Thus at 25 °C, 60 mM KCl; 4:1 actin:myosin (w/w) (see Fig. 2) actin tropomyosin activation was potentiated by up to 300% of actin activation and maximal inhibition was 90–95%. However, potentiation was not necessary for inhibition since caldesmon was still an inhibitor under conditions where actin-tropomyosin activated myosin MgATPase less than actin (e.g. if skeletal muscle tropomyosin was used in place of VSM tropomyosin or at 4 °C (25)). In these cases the percentage inhibition at a given level of bound caldesmon was less because the basal level of actin activation with full caldesmon inhibition remained the same as it was when tropomyosin potentiated the activity (Fig. 2). What is the origin of this basal ATPase activation? By examining all our experimental results we found that the basal activation was a consistent fraction of the activation by actin alone (26 ± 14%, $n = 29$). This was found over a very wide range of values for actin activation (22–1100 nmol of P$_i$/mg of myosin-min) obtained by varying [KCl] and temperature, and thus suggests that the basal activation could be a property of the actin.

The quantity of caldesmon required to inhibit actin-tropomyosin activation was found to vary with temperature and [KCl], but this was merely a reflection of the variation of the binding affinity of caldesmon for actin-tropomyosin (Table I). We determined the relationship between inhibition by caldesmon and calmodulin binding under many different conditions and found that inhibition to the basal level consistently correlated with the binding of 1 caldesmon per 29 actins (0.034 ± 0.005 S.D. caldesmon per actin, $n = 26$) (Fig. 3). Thus inhibition correlated with occupation of tight binding sites. This correlation was independent of whether the actin and tropomyosin used were from skeletal or smooth muscle (Fig. 3).

**Caldesmon Binding to Ca$^{2+}$-Calmodulin**—Caldesmon was originally identified as a Ca$^{2+}$-calmodulin binding protein (8), and our previous approximate measurements of the affinity of caldesmon for calmodulin immobilized on Sepharose CL-4B indicated a binding constant in the 10$^5$ M$^{-1}$ range (12, 14, 32). We found that the fluorescence responses of IAEDANS-calmodulin provided more reliable measurements of the binding to caldesmon. When excited at 340 nm, the fluorescence emission at 475 nm was enhanced by 10–20% in the presence of Ca$^{2+}$ and caldesmon. Net fluorescence increases were well fitted by a hyperbola (Fig. 4), and binding constants were obtained (41) with standard deviations not exceeding 25%. Replicate titrations, using any one preparation of proteins,
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0
0.1
0.2
0.3
Caldesmon bound (mol/mol actin)

FIG. 1. Caldesmon binding to sheep aorta actin and actin-tropomyosin. 6 or 12 μM aorta actin (O) or actin saturated with tropomyosin (●) were mixed with 50–6000 nM [14C]iodoacetamide-labeled caldesmon in ATPase buffer (60 mM KCl) at 25 °C. Binding was determined by a sedimentation assay. Results were plotted as a Scatchard plot and analyzed on the basis of two classes of binding sites. Solid lines represent the best fits obtained by a visual iterative procedure using estimates of binding constant and maximum binding for the two classes of sites. In this experiment the best fit was obtained with a tight binding site affinity of 4 × 10⁷ M⁻¹ for actin-tropomyosin, 10⁷ M⁻¹ for actin, and maximum binding of 1 per 28 actins. The weak sites had an affinity of 5 × 10⁶ M⁻¹ for actin-tropomyosin, 1–2 × 10⁶ M⁻¹ for actin, and maximum binding of 1 per 7 actins.

0
0.02
0.04
0.06
Caldesmon bound (mol/mol actin)

FIG. 2. Inhibition of actin activation of myosin MgATPase activity by caldesmon. MgATPase activity was measured at 25 °C, 60 mM KCl with 0.125 mg/ml skeletal muscle myosin, 0.5 mg/ml (12 μM) aorta actin, or 0.5 mg/ml actin + 0.125 mg/ml tropomyosin (1.8 μM), and 0–0.2 mg/ml (1.7 μM) aorta caldesmon. ▲, actin; ●, actin and aorta tropomyosin; ○, actin and skeletal muscle tropomyosin. Myosin MgATPase activity (12 nmol/mg-min) was subtracted from total MgATPase activity.

usuually agreed within 10%. However, there was a large variability between caldesmon preparations: at 25 °C with 60 mM KCl, the binding constant was 3.7 ± 3.5 × 10⁸ M⁻¹ (mean ± S.D., nine different protein preparations). With any individual preparation, binding was not much affected by increasing temperature from 25 to 37 °C, nor by varying KCl from 60 or 70 mM to 120 mM. Thus, at 37 °C, 60 mM KCl, the binding constant was decreased by 22 ± 39% relative to the constant at 25 °C, 60 mM KCl, whereas the constant at 37 °C, 120 mM KCl was decreased by 52 ± 37% (n = 3 in each case). In the absence of Ca²⁺ (i.e., 1 mM EGTA), no binding of caldesmon to calmodulin could be detected by either the fluorescence assay or with immobilized calmodulin.

Release of Caldesmon Inhibition by Ca²⁺ - Calmodulin—It has been shown by several groups that Ca²⁺ - calmodulin can reverse the inhibitory effect of caldesmon on actin-tropomyosin (12, 14–17, 26). We observed that Ca²⁺ - calmodulin always reduced caldesmon inhibition, but the quantity of Ca²⁺ - calmodulin required to release inhibition was very dependent on the conditions (Fig. 5). Calmodulin never had any effect upon the action of caldesmon in the absence of Ca²⁺. Fig. 5 shows the release of inhibition of Ca²⁺ - calmodulin in four different sets of solution conditions. The comparison shows that Ca²⁺ - calmodulin became more potent at releasing caldesmon inhibition of VSM actin-tropomyosin at higher temperatures and [KCl]. In conditions approximating the physiological (37 °C, 120 mM KCl), 50% release of inhibition required only 2 μM Ca²⁺ - calmodulin, whereas at 25 °C, 60 mM KCl, in excess of 60 μM Ca²⁺ - calmodulin was required. Another factor which
under favorable conditions it was possible to measure the binding of actin-tropomyosin-calmodulin. The ability of Ca\(^{2+}\)-calmodulin to release caldesmon inhibition of actin-tropomyosin-activated myosin MgATPase was measured under a range of conditions. ATPase activities were determined as described under "Materials and Methods" in ATPase buffer at 10\(^{-4}\) m CaCl\(_2\). Protein concentrations were: actin, 0.5 mg/ml (12 \(\mu\)M); skeletal muscle myosin, 0.125 mg/ml (0.27 \(\mu\)M); aorta tropomyosin, 0.125 mg/ml (1.8 \(\mu\)M); calmodulin, 0–10 mg/ml (60 \(\mu\)M). Caldesmon was added to 0.1–0.4 mg/ml (0.8–3.2 \(\mu\)M) to give similar levels of inhibition according to conditions. M, 25 °C 60 mM KCl, skeletal muscle actin, calmodulin 0.2 mg/ml (1.7 \(\mu\)M); N, 25 °C 60 mM KCl, aorta actin, caldesmon, 0.1 mg/ml (0.8 \(\mu\)M); O, 37 °C, 70 mM KCl, aorta actin, caldesmon, calmodulin 0.15 mg/ml (1.25 \(\mu\)M); P, 37 °C, 120 mM KCl, aorta actin, caldesmon, calmodulin, 0.4 mg/ml (3.2 \(\mu\)M). In 1 mM EGTA, calmodulin never had any effect (data not shown).

We determined the quantity of Ca\(^{2+}\)-calmodulin needed to release inhibition was the origin of the actin: at 25 °C, 60 mM KCl, 7 \(\mu\)M Ca\(^{2+}\)-calmodulin produced a 50% release of inhibition with skeletal muscle actin + VSM tropomyosin compared with over 60 \(\mu\)M for VSM actin + VSM tropomyosin (Fig. 5).

Effect of Ca\(^{2+}\)-Calmodulin on Caldesmon Binding to Actin Tropomyosin—Ca\(^{2+}\)-calmodulin binds to caldesmon (Fig. 4), and when Ca\(^{2+}\)-calmodulin is added to the reconstituted actin-tropomyosin-calmodulin complex this binding is associated with the release of caldesmon inhibition of actomyosin ATPase activity (Fig. 5). It has been proposed that this is due to dissociation of the caldesmon from actin-tropomyosin as it binds preferentially to Ca\(^{2+}\)-calmodulin (14).

When we measured the effect of increasing concentrations of Ca\(^{2+}\)-calmodulin on the binding of caldesmon to actin-tropomyosin we found that the binding was reduced as inhibition was released (12). However, whereas inhibition was fully reversed at high Ca\(^{2+}\)-calmodulin concentrations, the binding of caldesmon to actin-tropomyosin reached a steady level rather than declining to zero. For instance, at 37 °C, 70 mM KCl, the steady level of binding at 50 \(\mu\)M Ca\(^{2+}\)-calmodulin was 60 ± 5% S.D. (n = 4) of the binding in the absence of Ca\(^{2+}\)-calmodulin. Thus Ca\(^{2+}\)-calmodulin weakened the binding of caldesmon to actin tropomyosin but did not abolish it. Calculations indicated that the remaining binding could not all be accounted for by occupancy of the weak binding sites. Therefore the tight binding sites were occupied in the presence of 50 \(\mu\)M Ca\(^{2+}\)-calmodulin.

We determined binding curves for caldesmon binding to actin tropomyosin in the presence of 50 \(\mu\)M Ca\(^{2+}\)-calmodulin (Fig. 6). From these measurements it was clear that caldesmon could still bind to the tight sites on actin tropomyosin, and under favorable conditions it was possible to measure the binding constant. At 37 °C, 70 mM KCl, with VSM actin Ca\(^{2+}\)-calmodulin reduced the binding constant by only 2-fold (three experiments), whereas at 25 °C, 60 mM KCl, with skeletal muscle actin the binding constant was reduced 7-fold (two experiments).

Since we have shown that caldesmon inhibition is fully reversed at high Ca\(^{2+}\)-calmodulin concentrations (Fig. 5), we conclude that caldesmon is not inhibitory when Ca\(^{2+}\)-calmodulin is bound to actin-tropomyosin-caldesmon. Fig. 7 indicates the simplest scheme compatible with our data. Using the binding constants determined in this paper and the observation that occupancy of the tight sites is proportional to the percentage inhibition of actin-tropomyosin (Fig. 3), model calculations were carried out to predict the inhibition by caldesmon and its release by Ca\(^{2+}\)-calmodulin under various conditions. The calculated curves roughly match the experimental data (Fig. 8). A flip-flop model (i.e. assuming \(\text{K}_{\text{Atm-CD}} = \text{K}_{\text{Atm-CD}}\)) would not account for the results at 37 °C; the calculated release of inhibition would need at least 10 times more Ca\(^{2+}\)-calmodulin than was required experimentally. It will be necessary to develop new techniques for measuring the protein-protein interactions in order to determine whether the imperfect match is due to a faulty model or errors in the measurements of the constants.

**DISCUSSION**

Caldesmon is known to be a component of Ca\(^{2+}\)-regulated thin filaments in all vertebrate smooth muscles (7), and Ca\(^{2+}\)-regulated thin filaments may be reconstituted from actin, tropomyosin, caldesmon and calmodulin (12-15, 26). Here we have demonstrated the interaction of caldesmon with the...
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other three components of reconstituted VSM thin filaments (actin, tropomyosin, and calmodulin). In the case of actin and calmodulin, we have been able to characterize quantitatively the interaction with caldesmon under a range of conditions and to correlate the interactions with the restoration of Ca\(^{2+}\) sensitivity in the reconstituted thin filaments. The calculations allow us to propose a mechanism for the Ca\(^{2+}\) regulation of VSM thin filaments (summarized in Fig. 7) which may be briefly stated thus: (i) Caldesmon binds to actin with a high affinity at a stoichiometry of 1 per 28 actin monomers. (ii) In the presence of tropomyosin the bound caldesmon inhibits actin activation of myosin MgATPase by up to 95%. Neither the binding nor the inhibition are Ca\(^{2+}\)-sensitive. (iii) In the presence of Ca\(^{2+}\), calmodulin binds to the caldesmon and weakens its interaction with actin-tropomyosin, as a result of which there is a partial dissociation of calmodulin-caldesmon from the actin-tropomyosin. (iv) Ca\(^{2+}\)-calmodulin binding releases the caldesmon inhibition of actomyosin ATPase activity.

Ca\(^{2+}\) regulation of the thin filaments is therefore achieved by the conversion of the inhibited actin-tropomyosin-caldesmon complex to the active complexes of actin-tropomyosin-caldesmon-calmodulin-Ca\(^{2+}\) and actin-tropomyosin.

The key regulatory property of caldesmon is its inhibition of actomyosin MgATPase activity. Inhibition correlated with the occupancy of the high affinity sites on actin-tropomyosin at a stoichiometry of 1 per 28 actin monomers though a similar occupancy of these sites in the absence of tropomyosin had no such inhibitory effect (Figs. 2 and 3). The potency of inhibition varied with assay conditions, but this could be attributed to changes in binding affinity; the relationship between binding and inhibition remained the same under all conditions tested (Fig. 3). Caldesmon did not fully inhibit actomyosin MgATPase activity under any conditions (Fig. 3); a basal activation, amounting to about 1/4 of that by actin alone, persisted even at high caldesmon concentrations. This basal activation may represent a fraction of actin in our preparations which is unable to interact correctly with caldesmon and/or tropomyosin, or it may represent the fully turned off state of the actin-tropomyosin-caldesmon complex.

If so, this may be equivalent to the so-called weak state of actin-tropomyosin filaments (27-29). In general our data are compatible with other reports using caldesmon isolated by different methods and from different smooth muscle sources. The few published actin binding studies show affinities in the 10^-10 M^-1 range saturating at 1 caldesmon bound per 6-10 actins and with the affinity greater in the presence of tropomyosin (16, 17, 26). These reports evidently failed to distinguish the strong and weak sites, and the binding parameters represent the weak noninhibitory sites. Others have reported the inhibitory activity of caldesmon (15, 17, 26, 31) although the inhibition obtained was usually less potent than that reported here. This could be due to assay conditions where caldesmon binding is weak or to partially inactive protein preparations. In these reports the tropomyosin requirement for inhibition was not always absolute, although inhibition was always more potent in the presence of tropomyosin (15, 16, 25, 31).

Tropomyosin is clearly an important component of the VSM thin filament regulatory mechanism. In the reconstituted system it was found that the substitution of skeletal muscle tropomyosin for smooth muscle tropomyosin had no effect upon the maximal inhibition or the actin binding of caldesmon (Figs. 2 and 3, Table 1) even though smooth muscle tropomyosin has a greater tendency to potentiate actomyosin MgATPase and has stronger end to end interactions (Fig. 2) (27, 40). The tropomyosin requirement may help to explain the unusually low stoichiometry of inhibitory interaction of caldesmon with actin. Caldesmon is known to be an extended molecule with a monomer length of 67-90 nm (42). Nevertheless, this is not long enough for caldesmon to interact directly with 28 actin monomers. It has been shown that caldesmon can induce cross-linking of actin filaments (15, 30), but this does not correlate with inhibition (25) and has been shown to be due to the presence of multimeric caldesmon aggregates (39). The inhibitory effect could, however, be envisaged as being propagated by tropomyosin. By binding along the filament, caldesmon could interact directly with 3-4 tropomyosin molecules, in one or both strands of the actin filament. The tropomyosin could then propagate the inhibitory effect over 28 actin monomers.

In support of this model, electron microscopy of smooth muscle thin filaments aggregated by polyclonal antibody to caldesmon shows no regular periodicity. This suggests that caldesmon extends along the thin filament rather than being located at regular discrete sites (33). We found that Ca\(^{2+}\)-calmodulin was able to release the inhibitory activity of caldesmon (Fig. 5), while reducing, but not abolishing, the binding of caldesmon to actin-tropomyosin (Fig. 6). Thus the mechanism of regulation cannot be a simple competition for inhibition between actin-tropomyosin and Ca\(^{2+}\)-calmodulin (14). An uninhibited ternary complex of Ca\(^{2+}\)-calmodulin-caldesmon-actin-tropomyosin also exists (Fig. 7). The four-state model of Fig. 7 was tested by calculating the expected inhibition and its release by Ca\(^{2+}\)-calmodulin, using the measured binding constants, and was found to account adequately for all the data currently available (compare Fig. 8 to Fig. 5). The value of K_{Atn-CD} relative to K_{Atn-CD-CM} varied a great deal under different assay conditions. At 25 °C, 60 mM KCl, the ratio was large (approximates to flip-flop model) whereas at 37 °C, 120 mM KCl, the ratio was close to 1. A higher value of K_{Atn-CD-CM} and hence a lower ratio of the two constants was able to account for the greater...
efficiency of calmodulin release of inhibition when skeletal muscle actin was used in place of smooth muscle actin (Figs. 5 and 8).

The calcium-dependent phosphorylation of myosin has been demonstrated to be involved in the regulation of smooth muscle contraction (4). However, experiments with intact and chemically skinned smooth muscles have shown that myosin phosphorylation alone cannot adequately explain Ca²⁺ control of contractility (3, 4, 43–45). The work reported here demonstrates that caldesmon, calmodulin, and tropomyosin are able to interact to confer Ca²⁺ sensitivity to the interaction between actin and myosin in vitro. A number of lines of evidence suggest that they may be able to interact similarly in vivo to regulate smooth muscle contraction. The stoichiometry of the tight inhibitory interaction between caldesmon and actin-tropomyosin corresponds well with the content of the proteins in whole smooth muscle and in native thin filaments (6, 7, 35). Moreover, caldesmon has been shown to be present in the same cytoplasmic domains as myosin and tropomyosin and so is correctly located for such a regulatory role (42). The affinity of caldesmon for Ca²⁺-calmodulin, at 10⁻⁶ M⁻¹, is rather weak compared with other calmodulin-binding proteins such as myosin light chain kinase and phosphodiesterase (34). However, the total smooth muscle contents of caldesmon and calmodulin are about 12 and 30 μM, respectively (22, 31, 35–37), and the cytoplasmic concentrations may be considerably higher. Caldesmon is the major calmodulin-binding protein in smooth muscle (31) and so, despite the relatively low affinity of caldesmon for Ca²⁺-calmodulin, it is likely that there is sufficient calmodulin to saturate caldesmon and that the free calmodulin is sufficient for full release of caldesmon inhibition (50% release of inhibition with 2 μM calmodulin at 37°C, 120 mM KCl (Fig. 5). There are, nonetheless, important differences between native and reconstituted thin filaments. Native thin filaments were found to be Ca²⁺-regulated over a wide range of conditions (varying KCl, MgCl₂, pH, and temperature) while having a low content of calmodulin-like protein (0.5–1.0%) (6). In contrast, the Ca²⁺ sensitivity of reconstituted thin filaments is highly dependent upon conditions and has never been obtained with equimolar quantities of caldesmon and calmodulin (Fig. 5) (15, 26). Moreover, Ca²⁺-regulated thin filaments can be isolated from smooth muscle homogenates in the absence of Ca²⁺, although caldesmon would not be expected to co-sediment in these conditions. These discrepancies could be accounted for if the properties of caldesmon are altered during isolation causing, for instance, a weakening of its interaction with calmodulin. Alternatively, a thin filament specific Ca²⁺-binding protein, distinct from calmodulin, may be responsible for releasing caldesmon inhibition in vivo. Future work should be able to distinguish between these possibilities.

Acknowledgment—We thank Ziff Meats Ltd. for supplying the sheep arteries.

REFERENCES