Caldesmon is a Ca\(^{2+}\)-regulatory component of native smooth-muscle thin filaments

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Thin-filament preparations from four smooth muscle types (gizzard, stomach, trachea, aorta) all activate myosin MgATPase activity, are regulated by Ca\(^{2+}\), and contain actin, tropomyosin and a 120000–140000-\(M_r\) protein in the molar proportions 1:4:4. The 120000–140000-\(M_r\) protein from all sources is a potent inhibitor of actomyosin ATPase activity. Peptide-mapping and immunological evidence is presented showing that it is identical with caldesmon. Quantitative immunological data suggest that caldesmon is a component of all the thin filaments and that the thin-filament-bound caldesmon accounts for all the caldesmon in intact tissue. The myosin light-chain kinase content of thin-filament preparations was found to be negligible. We propose that caldesmon-based thin-filament Ca\(^{2+}\) regulation is a physiological mechanism in all smooth muscles.

INTRODUCTION

There is increasing evidence that Ca\(^{2+}\) controls the activity of the contractile machinery of smooth muscle by modulating proteins associated with both the myosin-containing thick filaments and the actin-based thin filaments (summarized by Marston, 1983). Evidence for myosin regulation, by means of a Ca\(^{2+}\)-dependent phosphorylation mechanism, is well established (Sobieszek & Small, 1977; Adelstein, 1978; Hartshorne & Siemankowski, 1981). In contrast, there has been disagreement on the existence and nature of a thin-filament-linked Ca\(^{2+}\)-regulatory system. Reasons for the latter controversy involved both the lack of a common approach and the use of different smooth muscle tissues by various investigators.

Initial studies by Sobieszek & Small (1976) showed that visceral smooth-muscle thin filaments consist only of actin and tropomyosin, indicating a complete lack of possible Ca\(^{2+}\)-dependent regulatory proteins associated with such thin filaments. This conclusion now appears to be tenuous, since we have found that the proteins interacting with smooth-muscle thin filaments, including the tropomyosin component, are labile and tend to dissociate in standard thin-filament extraction buffers (Marston & Smith, 1984). In fact, several groups have suggested the presence of thin-filament-based regulatory proteins in smooth muscle (Driska & Hartshorne, 1975; Ebashi et al., 1977; Marston et al., 1980; Kakiuchi & Sobue, 1983). Moreover, one of us (S.B.M.) has isolated Ca\(^{2+}\)-regulated thin filaments from vascular smooth muscle which confer Ca\(^{2+}\)-dependence on Ca\(^{2+}\)-insensitive myosin (Marston et al., 1980; Marston & Smith, 1984). Disassembly and reconstitution studies on this system have shown that regulation is achieved via a 120000–140000-\(M_r\) protein, possibly caldesmon, which inhibits actin–tropomyosin, plus a Ca\(^{2+}\)-binding protein, similar to calmodulin, which reverses the inhibition when Ca\(^{2+}\) is bound (Marston et al., 1984; Smith & Marston, 1985). In the present study, we demonstrate that the 120000–140000-\(M_r\) protein is caldesmon and is responsible for the observed Ca\(^{2+}\)-sensitivity of all smooth-muscle thin filaments.

Caldesmon, first identified by Kakiuchi and associates, is a major calmodulin-binding protein found in a variety of smooth muscle tissues in appreciable quantity (Sobue et al., 1982; Kakiuchi et al., 1983; Ngai et al., 1984; Bretscher, 1984). Experimentation in vitro, using purified proteins, shows caldesmon to have a high affinity for actin in the absence of calmodulin and Ca\(^{2+}\). These studies have also shown that caldesmon binding to actin can both inhibit actomyosin ATPase and cause lateral cross-linking of actin filaments. To date, however, the identification of caldesmon as a putative actin-linked regulatory protein is based almost entirely on binding studies in vitro using purified proteins. Such methods have shown that many non-contractile proteins can associate with actin (Clark et al., 1983; Stewart et al., 1983), an effect having unknown physiological significance. It is possible that caldesmon may spuriously bind to purified actin and likewise may be a contaminant in native smooth muscle thin filament preparations. This, however, does not seem to be the case, and evidence is presented here showing that caldesmon is a component of the smooth-muscle thin filament in vivo.

MATERIALS AND METHODS

Aorta tropomyosin, F-actin and skeletal-muscle myosin were prepared as described by Marston & Smith (1984). Thiophosphorylated aorta myosin was prepared as described by Chacko & Rosenfeld (1982) and Heaslip & Chacko (1985). Smooth-muscle thin filaments from a variety of tissues were isolated as described by Marston & Smith (1984), and 120000–140000-\(M_r\) protein was purified directly from such preparations by the method of Smith & Marston (1985). A new method was developed for isolating chicken gizzard thin filaments of high purity. The salient features

Abbreviation used: SDS, sodium dodecyl sulphate.
of the technique involve precise pH measurement and adjustment (to eliminate myosin contamination) and thin-filament extraction in the presence of ATP and EGTA at 25 °C. This prevents tropomyosin dissociation, which otherwise occurs at 4 °C. The pH of all buffers was adjusted at 25 °C, and they were used at either 25 °C or 4 °C as indicated. In the 4 °C steps, gizzard smooth-muscle tissue was homogenized (40 s each time, in a Sorvall Omni-Mixer), and washed by sedimentation (18000 g for 5 min) in a buffer consisting of 100 mM-NaCl, 10 mM-sodium phosphate (pH 7.0), 5 mM-MgCl₂, 1 mM-EGTA, 1 mM-Na₂SO₄ and 1 mM-dithioerythritol (Sol. A). This process was repeated four times; the first two washes contained additional 1% Triton X-100. In the 25 °C steps, the resultant white pellets (‘washed muscle’) were rehomogenized in Sol. A, then 100 mM-ATP (pH 7.0) was added (final concn. 5 mM), and the suspension sedimented (12000 g for 5 min). The pellets were then resuspended by gentle stirring in fresh Sol. A + 5 mM-ATP and resedimented. These pellets were subsequently homogenized in Sol. A + 5 mM-ATP, and particulate material was removed by centrifugation at 12000 g for 10 min. Thin filaments were collected by centrifugation at 120000 g for 150 min. They were resuspended in Sol. A. Adjusted to pH 6.0, and ATP was added (final concn. 5 mM). The pH of the suspension was decreased to pH 5.75 with 1 mM-HCl, and contaminating myosin removed by centrifugation at 40000 g for 10 min. The birefringent supernatant was dialysed overnight (at 4 °C) against Sol. A adjusted to pH 6.25, and precipitated material removed by centrifugation at 120000 g for 10 min. Pure thin filaments were collected by centrifugation at 120000 g for 150 min (at 25 °C) and resuspended in Sol. A. Before use, small amounts of aggregated material were removed by centrifugation at 40000 g for 10 min. This method was used ten times without variation in the purity of the thin filaments obtained (Figs. 2a and 2b). The yield was 6–8 mg/g wet wt. of tissue.

Caldesmon was prepared by several procedures. Chicken gizzard caldesmon, prepared as described by Ngai et al. (1984), was given by Dr. Clive Sanders (University of Alberta); chicken gizzard caldesmon, prepared as described by Sobue et al. (1981), was given by Dr. Renata Dabrowska (Nencki Institute, Warsaw, Poland). Sheep aorta caldesmon was prepared by a modification of Bretschger’s (1984) method. A heat-treated extract of homogenized aorta was obtained as described by Bretschger (1984), and caldesmon was purified from the extract by isoelectric fractionation at pH 3.0 (where caldesmon alone is soluble) and (NH₄)₂SO₄ fractionation (40–50% saturation) as described by Smith & Marston (1985).

Gizzard caldesmon used for antibody production (Fig. 2c) was prepared directly from gizzard thin filaments.
Table 1. Comparison of yield, protein content and Ca$^{2+}$-dependent activation of myosin MgATPase by thin-filament preparations from four smooth muscles

<table>
<thead>
<tr>
<th>Component</th>
<th>Yield of thin filaments (mg/g wet wt. of tissue)</th>
<th>Quantity of protein relative to actin</th>
<th>Activation of myosin MgATPase activity (nmol/min per mg of myosin)</th>
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<tr>
<td></td>
<td></td>
<td>Tropomyosin</td>
<td>120000–140000-Mr protein</td>
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<tr>
<td>Sheep aorta</td>
<td>2</td>
<td>0.46 ± 0.11</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Rabbit stomach</td>
<td>1.5</td>
<td>0.42 ± 0.06</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>Sheep trachea</td>
<td>0.18</td>
<td>0.39</td>
<td>0.27</td>
</tr>
<tr>
<td>Chicken gizzard</td>
<td>1.8</td>
<td>0.35 ± 0.04</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Aorta actin + tropomyosin</td>
<td>—</td>
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* Owing to low yield, sheep trachea thin filaments were only tested with skeletal-muscle myosin at 25 °C

isolated by our new procedure (above) by the methods of Ngai et al. (1984) or Bretscher (1984). The protein was purified further by preparative alkaline 6 M-urea/polyacrylamide-gel electrophoresis and used to immunize rabbits as described by Lehman et al. (1980). Antibodies to chicken gizzard myosin light-chain kinase were given by Dr. J. F. Head and Dr. B. Kaminer (Boston University). IgG fractions were prepared as described by Lehman et al. (1980).

ATPase assay was performed as described by Marston & Smith (1984), and SDS/polyacrylamide-gel electrophoresis as described by either Marston & Smith (1984) or Weber & Osborn (1969) as indicated. Western immunoblotting to determine antibody specificity was performed by the method of Towbin et al. (1979). Non-competitive enzyme-linked immunoabsorbent assay was carried out (Tsang et al., 1983) with horseradish-peroxidase-conjugated anti-rabbit IgG (Miles-Yeda), $H_2O_2$ as substrate, and a hydrogen donor chromogen, o-phenylenediamine. The reaction was stopped after 3 min with 0.75 M-$H_2SO_4$ and $A_{492}$ was read as described by Engvall (1980).

**RESULTS**

Using the method of Marston & Smith (1984), we obtained native thin filaments from chicken gizzard, rabbit stomach, sheep trachea and sheep aorta (Fig. 1). A new method has also been developed for preparing chicken gizzard thin filaments which produces a product with negligible myosin contamination (Figs. 2a and 2b). All the thin-filament preparations contained three major components: actin, tropomyosin and a 120000–140000-Mr protein, and also minor amounts of filamin (Marston & Smith, 1984). The 120000–140000-Mr protein migrated with different Mr values on different SDS/polyacrylamide-gel systems: Marston & Smith (1984) method, 120000; Weber & Osborn (1969) method, 135000; Laemmli (1970) method, 140000. Denistomometric scans of SDS gels of these thin filaments indicated that the proportions of the three major proteins were about the same in each preparation (Fig. 1, Table 1). Trachea thin filaments were obtained in low yield and contained significant impurities: this may reflect a low muscle content of whole trachea. Some preparations [method of Marston & Smith (1984)] were tested for activation of thio phosphorylated aorta myosin MgATPase: at 37 °C they all activated aorta myosin by 10–30-fold in the presence of 10 μM-Ca$^{2+}$, but activated less than 3-fold in the absence of Ca$^{2+}$ (Table 1). This Ca$^{2+}$-sensitivity is considerably better than for the same preparations tested with skeletal myosin (Marston & Smith, 1984). In
component and purified aorta caldesmon (Fig. 5b). Except for minor differences, the peptide maps of the gizzard and aorta proteins closely resemble each other (Fig. 5a). Further confirmation that the 120000–140000-$M_r$ protein is caldesmon is evident from the observation that some of the protein characteristically dissociates from chicken gizzard and aorta native thin filaments in the presence of Ca$^{2+}$ and added calmodulin, consistent with the results of Sobue et al. (1982), Ngai et al. (1984) and Smith & Marston (1985).

As mentioned, a new method was developed for the preparation of very pure gizzard thin filaments for use in the immunological studies described below. These thin filaments retain a full complement of tropomyosin and are free of myosin contamination (Fig. 2). Antibodies were developed against purified chicken gizzard caldesmon, and immunoblotting shows that they react with the 120000–140000-$M_r$ caldesmon band on chicken gizzard thin filaments and with no other protein in chicken gizzard muscle (Fig. 2). The antibody reacts with the 120000-$M_r$, band of aorta thin filaments, even if not as strongly as with gizzard. Immunoprecipitation studies show that anti-caldesmon antibody not only interacts with caldesmon antigen but precipitates intact thin filaments as well. In fact, at saturating concentrations, the antibody precipitates over 80% of the actin in chicken gizzard thin-filament preparations (Fig. 6). This result indicates that caldesmon is a component bound to thin filaments and not simply a contaminant co-purifying with thin-filament preparations. Furthermore, the antibody was used in quantitative enzyme-linked immunoadsorbent assay, showing that the caldesmon concentration present in gizzard thin-filament preparations is 2.1 times that in unfractionated washed muscle (Fig. 7). Since the actin concentration in such thin-filament preparations is also approximately twice (2.2 times) that in unfractionated muscle, these results suggest that caldesmon must be an actin-bound component in vivo which is retained during isolation.

Enzyme-linked immunoadsorbent assay using anti-(myosin light-chain kinase) antibody demonstrates that myosin light-chain kinase is not a constituent of our native thin filaments (Fig. 7). Also, our elution profile of thin-filament extracts separated on a DEAE-Sephacel column (performed as described by Ngai et al., 1984) indicates that the myosin light-chain kinase content of thin filaments is at most 2% that of caldesmon.

**DISCUSSION**

Using our new preparative procedures, we have demonstrated that thin filaments of similar composition can be extracted from any smooth muscle. Activation of smooth-muscle myosin MgATPase by these thin filaments is highly Ca$^{2+}$-dependent. SDS/polyacrylamide-gel electrophoresis shows they all consist of actin, tropomyosin, a single 120000–140000-$M_r$ protein, and small amounts of filament (a cytoskeletal protein) (Fig. 1, Table 1). The thin-filament preparations all contain comparable amounts of the 120000–140000-$M_r$ protein, which when isolated is a potent inhibitor of actomyosin ATPase (Fig. 3) and, as previously shown, is a necessary component of the thin-filament-linked Ca$^{2+}$-regulatory system in vascular smooth muscle (Marston et al., 1984; Smith & Marston, 1985). This protein has many properties in common with Kakiuchi's caldesmon preparation (Sobue et al., 1985).
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Fig. 5. Peptide maps of digests of 120000–140000-Mr protein and caldesmon

Gel pieces containing 10 µg of pure protein (Fig. 4) were cut out and placed on a 20%–polyacrylamide/1%–SDS gel with a 2 cm-wide 4%–polyacrylamide stacking gel (Laemmli, 1970), together with an equal quantity (w/w) of Staphylococcus aureus V8 protease. Gels were run into stacking gel for 1.25 h at 100 V, followed by a wait of 30 min, followed by electrophoresis at 300 V in separating gel (Cleveland et al., 1977). Temperature was maintained at 17 °C. In Fig. 5(a), lanes: a and d, gizzard 120000–140000-Mr protein; b and e, aorta 120000–140000-Mr protein; c, gizzard caldesmon [prepared by Sobue et al. (1981) method]; i, M, (× 10⁻²) markers (Pharmacia). In Fig. 5(b), lanes: g, aorta 120000–140000-Mr protein; h, aorta caldesmon [Bretscher (1984) method]. Owing to differing degrees of digestion, the peptide patterns in Figs. 5(a) and 5(b) are not directly comparable. The band corresponding to V8 protease is indicated.

Fig. 6. Precipitation of chicken gizzard thin filaments by anti-caldesmon antibody

In this experiment, various amounts of IgG prepared from anti-caldesmon serum (○) or from non-immune control serum (■) were mixed with 1 mg of clarified thin filaments, the mixtures were adjusted to 1 ml with phosphate-buffered saline (PBS; 150 mm-NaCl/10 mm-sodium phosphate buffer, pH 7.0), and allowed to react at 25 °C for 1 h; these samples were then centrifuged for 10 min at 9000 rev./min. Pellets were resuspended in 1 ml of buffer and the thin-filament content in pellets and respective supernatants was analysed by measuring their actin content by SDS/polyacrylamide-gel electrophoresis followed by densitometry. Results show that at saturating amounts of anti-caldesmon antibody more than 80% of the actin of thin filaments is precipitated. The experiment was performed on two preparations of thin filaments, and the results were also confirmed, qualitatively, by observing a complete loss of thin-filament birefringence after anti-caldesmon treatment and maintained birefringence with non-immune IgG.

et al., 1981, 1982), and we have shown that the two proteins are indeed identical in gizzard and aorta, although there may be small differences between the tissues detected by peptide mapping and immunological techniques (Figs. 4 and 5). Our data, taken as a whole, suggest that caldesmon is a component of all preparations of native smooth-muscle thin filaments. Furthermore, since gel electrophoresis shows no components other than caldesmon capable of being influenced by Ca²⁺, caldesmon is the only component that could be involved in thin-filament regulation. Our thin filaments contain no detectable troponin-like proteins or leitotonin-A or C (Fig. 1) (Ebashi et al., 1977; Ebashi, 1980). Our studies also indicated that myosin light-chain kinase is not a component of native smooth-muscle thin filaments (Fig. 7), confirming previous results (Marston & Smith, 1984), even though it has been shown capable of binding to actin (Sellers & Paton, 1984).

Immunoprecipitation assays show that caldesmon is not restricted to a small sub-class of smooth-muscle-cell thin filaments, but is present on virtually all thin filaments, indicating that it must be part of the contractile machinery (Fig. 6). Moreover, enzyme-linked immunoabsorbent assay implies that caldesmon cannot be a contaminant of thin-filament preparations and must be a component of the thin filament in vivo, since there are equal caldesmon/actin ratios in unfractionated tissue and in isolated thin filaments (Fig. 7).

The precise mechanism and role of caldesmon in modulating smooth-muscle function cannot be answered at present. It is not certain whether caldesmon effects are influenced directly by Ca²⁺-calmodulin (Sobue et al., 1982; Smith & Marston, 1983) or by Ca²⁺-calmodulin activating a caldesmon kinase (Ngai & Walsh, 1985). It is also not clear whether the influence of caldesmon on actomyosin ATPase and its effect on actin-filament cross-linking are distinct functions (Bretscher, 1984; Goch et al., 1985; C.J. Moody, unpublished work). Finally, assay in vitro of possible function cannot determine if the primary role of caldesmon resides in
regulating shortening velocity or tension in contracting muscle, or in effecting stress maintenance during relaxation (i.e. the ‘latch’ state; Aksoy et al., 1981). In addition to being found in smooth muscle, caldesmon has also been identified in non-muscle cells (Kakiuchi et al., 1983; Owada et al., 1984; Ngai & Walsh, 1985). Hence the caldesmon-based Ca²⁺-dependent regulatory system may be a general mechanism for controlling smooth-muscle and cellular actin–myosin interactions.

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REFERENCES


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Fig. 7. Enzyme-linked immunoabsorbent assay of antigenic activity in chicken gizzard thin filaments and washed muscle preparations

In this experiment, crude antigen, consisting of either gizzard thin filaments (○, □, ---) or washed muscle (●, ■, ▲, ..., -----) suspended in 0.6 m-NaCl/40 mM-Na₂CO₃/NaHCO₃ buffer (pH 9.6), was resuspended in 40 mM-Na₂CO₃/NaHCO₃ buffer (pH 9.6) and used, in concentrations specified on the abscissa, to sensitize polystyrene tubes. The coated tubes were incubated with anti-caldesmon serum (○, ●), anti-(myosin-light-chain kinase) serum (□, ■), or non-immune serum (▲, ▲), each diluted 1/1000 in phosphate-buffered saline, a concentration of antibody that we found sufficient to saturate antigen sites. Antibody binding was used as an indirect measurement to compare the relative amount of caldesmon or myosin light-chain kinase present in thin filaments and washed muscle. This in turn was quantified by reaction of primary antibody with horseradish-peroxidase-conjugated anti-rabbit IgG, with H₂O₂ as substrate and o-phenylenediamine as chromogen. Points plotted represent averages from two experiments on different preparations of thin filaments and dissolved muscle. Straight lines were obtained by linear regression analysis of the data. The value of the slope for anti-caldesmon binding to thin filaments (---) is 2.1 times that for washed muscle (-----), indicating approximately twice the caldesmon content in thin filaments. In contrast, anti-(myosin-light-chain kinase) binding to washed muscle (■) is appreciable, but binding to thin filaments is only slightly higher than background. Corresponding analysis of actin content, by densitometry of actin bands on SDS/polyacrylamide-gels, shows the amount of actin to be (○) approx. 2.2 times as much in thin filaments as in washed muscle.