The Effects of Caldesmon on Smooth Muscle Heavy Actomeromyosin ATPase Activity and Binding of Heavy Meromyosin to Actin*

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Joseph A. Lash¶, James R. Sellers†, and David R. Hathaway§

From the ‡Department of Medicine and the Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana 46223 and the $Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Caldesmon was purified to homogeneity from both chicken gizzard and bovine aortic smooth muscles. Caldesmon purified from bovine aorta was slightly larger than caldesmon purified from chicken gizzards (Mr = 140,000) when the two were compared electrophoretically. Caldesmon bound tightly to actin saturating at a molar ratio of 1 caldesmon monomer per 7-10 actin monomers. Ca²⁺-calmodulin appeared to reduce the affinity of caldesmon for actin. Caldesmon was also a potent inhibitor of heavy actomeromyosin ATPase activity producing a maximal effect at a ratio of 1 caldesmon monomer per 7-10 actin monomers. This effect was also antagonized by Ca²⁺-calmodulin. While caldesmon inhibited heavy actomeromyosin ATPase activity, it greatly enhanced binding of both unphosphorylated and phosphorylated heavy meromyosin to actin in the presence of MgATP, reducing the Kd for binding by a factor of 40 for each form of heavy meromyosin. Although we did identify a Ca²⁺-calmodulin-stimulated "caldesmon kinase" activity in caldesmon preparations purified under nondenaturing conditions, we observed no effect of phosphorylation (2 mol of PO₄/mol of caldesmon) on the capacity to inhibit heavy actomeromyosin ATPase activity. Our results suggest that caldesmon could serve some role in smooth muscle function by enhancing cross-bridge affinity while inhibiting actomyosin ATPase activity.

Caldesmon is an actin- and Ca²⁺-calmodulin-binding protein present in relatively high content in smooth muscle (1-4). Published studies to date have suggested that caldesmon from chicken gizzard smooth muscle is a dimer of two 140,000-dalton subunits (1, 3) that binds to actin, achieving saturation at a molar ratio of 1 caldesmon monomer per 7 actin monomers (3, 5). Binding has been shown by several investigators to be either completely (1, 3) or partially (6) reversible upon addition of Ca²⁺ and calmodulin. In addition, caldesmon has been shown to be a potent inhibitor of smooth muscle actomyosin ATPase activity (7). At least one group has suggested that the inhibitory effect of caldesmon requires the presence of tropomyosin (7, 8) while other studies have not confirmed this observation (5). More recently, Nagi and Walsh (5) have presented evidence that caldesmon can be phosphorylated by a Ca²⁺-calmodulin-dependent protein kinase. Phosphorylation to a stoichiometry of 2 mol of PO₄/mol of caldesmon monomer was shown to reverse the inhibitory effect of caldesmon on smooth muscle actomyosin ATPase activity (5). Thus, in addition to the earlier proposal that caldesmon may function as an actin bundling protein (3, 8), it has been suggested that caldesmon may serve to reversibly regulate smooth muscle actomyosin ATPase activity (3, 4, 7). Moreover, certain analogies to the striated muscle actomyosin ATPase inhibitor, troponin, have been made (4, 6).

In the present study, we have examined the effects of caldesmon both on smooth muscle acto-HMM ATPase activity and upon binding of HMM to actin. Our results confirm previous published data which have shown that caldesmon inhibits actomyosin ATPase activity (4, 5, 7) and that inhibition does not require the presence of tropomyosin (5). In contrast to the reported effects of troponin on the binding of skeletal muscle HMM to actin, we report that caldesmon greatly enhances the binding of smooth muscle HMM to actin. These effects occurred maximally at concentrations of caldesmon that did not promote bundling of actin filaments suggesting that caldesmon, like filamin (9), may have distinctly separable capacities to bundle actin filaments and to serve as an actomyosin ATPase inhibitor. Finally, while we identified a Ca²⁺-calmodulin-dependent phosphorylation of caldesmon, we did not find that phosphorylation reverses the inhibitory effect of caldesmon on acto-HMM ATPase activity.

EXPERIMENTAL PROCEDURES

Chemicals

Most reagents were purchased from Sigma. [³²P]ATP was obtained from New England Nuclear. ATPyS was purchased from Boehringer Mannheim.

Protein Preparations

Myosin and HMM—Myosin was prepared from fresh turkey gizzards by methods described previously (10). HMM was prepared by chymotryptic digestion of myosin (11, 12).

Caldesmon—Caldesmon was prepared by two methods. The first method was identical to that described by Bretcher (3) except for addition of protease inhibitors as described below and involved a boiling step. The second method was used to prepare caldesmon that also contained a Ca²⁺-calmodulin-dependent protein kinase activity. This method was somewhat similar to that of Nagi et al. (13) which utilizes MgCl₂ extraction of caldesmon from myofibrils. Briefly, approximately 500 gm of fresh chicken gizzards or 1 kg of bovine aortas was ground in a meat grinder and then homogenized at 4 °C in 3 M 4-morpholinepropanesulfonic acid; EGTA, [ethylenebis(oxyethyl-enenitrilo)]tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ATPyS, adenosine 5'-O-(thiotri-phosphate).

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† To whom correspondence should be addressed.

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1 The abbreviations used are: HMM, heavy meromyosin; MOPS, 4-morpholinepropanesulfonic acid; EGTA, [ethylenebis(oxyethyl-enenitrilo)]tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ATPyS, adenosine 5'-O-(thiotri-phosphate).
volumes of 40 mM MOPS, pH 7.2, 5 mM EGTA, 50 mM MgCl₂, and 1 mM dithiothreitol (Buffer A). A mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na-p-tosyl-l-lysine chro- momethyl ketone, 0.1 mM L-1-tosylamide 2-phenylethyl chloromethyl ketone, and 10 μM leupeptin) was included in all buffers throughout the purification. Buffer B (20 mM MOPS, pH 7.0, 1 mM EGTA, 1 mM EDTA, and 1 mM dithiothreitol) was used to reduce the conductivity to 5 mM. Following dilution, 350 μl of moist DEAE-Sephacel, pre-equilibrated in Buffer B, was added and stirred gently for 3 h at 4 °C. At the end of this period, the DEAE- Sephacel was allowed to settle and the supernatant was decanted. The resin was packed into a 20 × 5 cm diameter column and washed with 2 liters of Buffer B. The column was then eluted with a linear, 4-liter gradient of NaCl in Buffer B (0-0.5 M) and 17-mL fractions were collected. Caldesmon was identified by SDS-PAGE as a 140,000-dalton protein (gizzard) or 150,000-dalton protein (aorta) eluting between 0.08 and 0.16 M NaCl. Fractions were pooled, concentrated under nitrogen in an Amicon ultrafiltration apparatus to 200 ml, and the pool was then subjected to chromatography on Sephacryl S-400 as described above for actin-activated ATPase measurements. Assays were conducted at room temperature (21-24 °C) and started by the addition of HMM. Following vortexing, samples were immediately sedimented at 130,000 × g for 20 min. At no time did ATP consumption exceed 40-50% of the total. Fractional binding was determined by the ratio of actin-activated ATPase activity remaining in the supernatant of samples sedimented in the presence of caldesmon to a sample sedimented in the absence of actin. Fatty acid and gamma globulin-free bovine serum albumin (Sigma) was included in the zero actin sample at a concentration of 1 mg/ml to reduce nonspecific binding of HMM to the centrifuge tubes. Nonspecific sedimentation was estimated to be 10–15% of total HMM added to tubes.

**Phosphorylation Assays**—Turkey gizzard HMM (8-15 mg/ml) was phosphorylated at 25 °C for 30 min in a mixture containing 10 mM MOPS, pH 7.0, 30 μg/ml myosin light chain kinase, 30 μg/ml calmodulin, 5 mM MgCl₂, 5 mM ATP-S, and 1 mM dithiothreitol. The phosphorylation reaction was terminated by adding 10 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 0.1 mM EDTA. Phosphorylation stoichiometries varied between 0.9-1.0 mol of PO4/mole of 20,000-dalton myosin light chain as determined from glycerol-urea gels. Caldesmon was phosphorylated by incubation (4-19 mg/ml) at 25 °C in a mixture of 20 mM MOPS, pH 7.0, 10 mM ATP (500 cpm/pmol), 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM CaCl₂, 50 μM calmodulin, and 50 mM NaCl. Reactions were terminated either by boiling or by freezing at −70 °C.

**Other Methods**

**Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on the buffer system described by Porzio and Pearson (20) with modifications to improve gel stability (10). Glycerol-urea-urea gel electrophoresis was performed as described by Perrie and Perry (22), with slight modification (23).

**Data Analysis**—Binding data were analyzed by double-reciprocal plots and lines were fitted by least squares analysis.

**RESULTS**

**Isolation of Caldesmon**—Caldesmon was isolated under nondenaturing conditions from both chicken gizzard as well as bovine aortic smooth muscle. The bovine aortas purified from both species appeared as single bands by SDS-PAGE. As shown in Fig. 1, bovine aortic caldesmon was slightly larger (Mᵣ = 145,000) than the gizzard protein (Mᵣ = 140,000) when isolated by identical methods. Although calmodulin affinity chromatography was not routinely employed in the purification scheme, we found that caldesmon could reversibly bind to calmodulin-Sepharose. Caldesmon obtained by affinity chromatography on calmodulin-Sepharose could not be distinguished from caldesmon isolated by denaturing methods. An important exception was the presence of Ca²⁺-calmodulin-dependent protein kinase activity and phosphatase activity in caldesmon prepared by nondenaturing methods. The presence of phosphatase activity greatly complicated experiments requiring that HMM be thio-phosphorylated with ATP-S to prevent inhibition of ATPase activity due to HMM dephosphorylation. Moreover, "pseudoATPase" activity probably due to phosphorylation/dephosphorylation of both caldesmon and actin was quite apparent when working with HMM, Ca²⁺-calmodulin, and myosin light chain kinase were incubated together in the presence of ATP.

**Binding of Caldesmon to Actin**—As shown in Fig. 2, varying amounts of caldesmon were added to 20 μM actin and the amount of caldesmon bound was determined by sedimentation analysis. In the presence of EGTA, caldesmon binding to actin was tight as evidenced by complete (i.e. 100%) binding at less than saturating concentrations of caldesmon. Saturation was observed at 3.0 μM caldesmon giving a binding ratio of approximately 1 caldesmon (i.e. assuming caldesmon is a monomer, Mᵣ = 140,000) per 7 actin monomers. Similar saturation data were obtained at higher and lower actin concentrations. Addition of Ca²⁺-calmodulin decreased binding of caldesmon to actin but did not completely inhibit binding.
Caldesmon and Smooth Muscle Acto-HMM ATPase Activity

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified chicken gizzard and bovine aortic caldesmons. Three micrograms of caldesmon from either chicken gizzard (CG) or bovine aorta (BA) were subjected to electrophoresis on 5% sodium dodecyl sulfate polyacrylamide gels as described under "Experimental Procedures."

FIG. 2. Binding of caldesmon to actin. The binding of purified chicken gizzard caldesmon to rabbit skeletal muscle actin was determined by sedimentation as described under "Experimental Procedures." ○, binding in the presence of 1 mM EGTA; ○—○, binding in the presence of 0.5 mM CaCl2 and a 10-fold molar excess of calmodulin over caldesmon.

In fact, a tendency toward saturation binding was observed in the presence of Ca2+-calmodulin as the caldesmon concentration was increased. Increasing the concentration of calmodulin beyond a molar ratio of 10:1 (i.e. calmodulin to caldesmon) did not further enhance dissociation. These results suggested that at 25 °C and relatively low ionic strength, the principal effect of Ca2+-calmodulin is to decrease the affinity of caldesmon for actin.

Effects of Caldesmon on acto-HMM ATPase Activity and on Binding of HMM to Actin—Caldesmon was a potent inhibitor of both acto-HMM and acto-S1 ATPase activities. As shown in Fig. 3, the inhibition of acto-HMM ATPase activity at an actin concentration of 20 µM appeared to achieve a maximal effect at caldesmon concentrations between 2-3 µM. Through a series of experiments in which the concentrations of actin and caldesmon were varied, we found that optimal inhibition was achieved at a caldesmon to actin ratio of between approximately 1:10 and 1:7. Electron microscopy of acto-caldesmon filaments prepared in this proportion did not demonstrate any bundling (data not shown). Although tropomyosin (molar ratio to actin ~1:6) seemed to slightly enhance the effect of caldesmon as we have previously reported (26), it was not required for maximal inhibition of the acto-HMM ATPase activity. Finally, Ca2+-calmodulin antagonized the inhibitory effects of caldesmon (Fig. 4).

The effect of graded concentrations of caldesmon on the binding of both unphosphorylated and phosphorylated HMM to actin is shown in Fig. 4. In both cases, caldesmon enhanced binding of HMM to actin providing a 7-fold stimulation for unphosphorylated HMM binding and 3-fold stimulation for phosphorylated HMM binding. Again, the molar ratio of caldesmon to actin that produced the maximal effect was found to be between 1:10 and 1:7. By maintaining the ratio of caldesmon to actin constant at 1:10, the effect of caldesmon on binding of HMM to actin in the presence of MgATP was...
Caldesmon and Smooth Muscle Acto-HMM ATPase Activity
determined as shown in Fig. 5. In the absence of caldesmon
(Fig. 5A), binding of unphosphorylated and phosphorylated
HMM to actin yielded different results \( K_d = 170.4 \text{ mM versus} \)
33.4 mM, respectively) with the magnitude of the difference
\( (i.e. ~5\text{-fold}) \) similar to that reported by Sellers et al. (11).
When caldesmon was added at a molar ratio of 1:10 (Fig. 5B),
there was enhanced binding of both unphosphorylated and
phosphorylated HMM producing a 40.6- and 35.5-fold de-
crease, respectively, in \( K_d \) values for actin binding. Fractional
HMM binding extrapolated to infinite actin concentrations
\( (B_{\text{actin}}) \) yielded values that were between 0.92 and 1.00, sup-
porting the conclusion that the principal effect of caldesmon
was to enhance affinity of HMM for actin irrespective of the
state of HMM phosphorylation.

The capacity of caldesmon to enhance binding of both
unphosphorylated and phosphorylated HMM to actin could
be partially reversed by Ca\(^{2+}\)-calmodulin as shown in Table I.

**Table I**
Effects of Ca\(^{2+}\)-calmodulin on enhanced binding of unphosphorylated
and phosphorylated HMM to actin

| Caldesmon   | Actin  | HMM bound |
|-------------|-------|-----------|
|             | \( \mu \text{M} \) | \( \mu \text{M} \) | % |
| Unphosphorylated HMM |    |          |   |
| 0           | 10    | 10        |  |
| 0.5         | 10    | 73        |  |
| 0.5 + Ca\(^{2+}\)-Cam | 10 | 15        |  |
| 0           | 20    | 10        |  |
| 1.0         | 20    | 86        |  |
| 1.0 + Ca\(^{2+}\)-Cam | 20 | 39        |  |
| Phosphorylated HMM |    |          |   |
| 0           | 5     | 18        |  |
| 0.25        | 5     | 83        |  |
| 0.25 + Ca\(^{2+}\)-Cam | 5 | 31        |  |
| 0           | 10    | 29        |  |
| 0.5         | 10    | 87        |  |
| 0.5 + Ca\(^{2+}\)-Cam | 10 | 39        |  |

\( \text{Ca}^{2+}\)-Cam = 0.2 mM CaCl\(_2\) and 5-20 \( \mu \text{M} \) cal-
modulin. Cam, calmodulin.

The effect of Ca\(^{2+}\)-calmodulin was more prominent at lower
actin concentrations which would be expected if the effect of
calmodulin is to decrease affinity of caldesmon for actin.

Caldesmon Phosphorylation and Effects on Acto-HMM
ATPase Activity—In view of the observation that phos-
phorylation of caldesmon by a copurifying Ca\(^{2+}\)-calmodulin-de-
pendent protein kinase could reverse the inhibitory effect of
caldesmon on smooth muscle actomyosin ATPase activity (5),
we prepared caldesmon under nondenaturing conditions
which contained kinase activity. The time course of phos-
phorylation of caldesmon by the endogenous kinase is shown in
Fig. 6. Phosphorylation was entirely dependent on the pre-

![Fig. 5.](image)

**Fig. 5.** Binding of unphosphorylated and phosphorylated
HMM to actin in the absence and presence of caldesmon. Assays
were performed as described under “Experimental Procedures.”
\( K_d \) values were estimated from x-intercepts of the double
reciprocal plots as determined by linear regression analysis. \( \text{P-HMM} \) ——— \( \text{OUP-HMM} \),
unphosphorylated HMM; \( \text{P-HMM} \) ——— \( \text{OUP-HMM} \), phosphorylated HMM.

![Fig. 6.](image)

**Fig. 6.** Phosphorylation of caldesmon. Caldesmon was puri-
ified under nondenaturing conditions and phosphorylation assays
were conducted as described under “Experimental Procedures.”
Phosphorylation levels were determined in the presence of 0.5 mM Ca\(^{2+}\) and a
10-fold molar excess of calmodulin over caldesmon (\( \circ \)—\( \circ \)) or in
the presence of 1 mM EGTA (\( \text{O} \)—\( \text{O} \)).

Attempts to purify the kinase to homogeneity were tharrayed by loss
of all enzyme activity after about two purification steps.
Caldesmon phosphorylation effects on capacity of caldesmon to inhibit acto-HMM ATPase activity

TABLE II

| ATPase activity | 1 mM EGTA | 0.2 mM CaCl₂ |
|-----------------|-----------|--------------|
| 0 caldesmon     | 100       | 100          |
| 2 μM caldesmon  | 33 ± 6    | 49 ± 8       |
| 2 μM phosphorylated caldesmon | 27 ± 3 | 52 ± 4 |

FIG. 7. Scheme for regulation of the smooth muscle acto-HMM ATPase cycle. This figure was adapted and simplified from Ref. 36. Binding states refer to binding of HMM to actin. P, release, indicated as f, is regulated by troponin (17). Inhibition of the cycle associated with high affinity actomyosin binding would be a predictable outcome if ADP release (indicated by 2) was slowed or inhibited. Inhibition at 2 by caldesmon could account for both inhibition of acto-HMM ATPase activity and enhanced binding of HMM to actin.

The effects of caldesmon phosphorylation on inhibition of acto-HMM ATPase activity are summarized in Table II. As inferred from the data, phosphorylation did not alter the inhibitory effect of caldesmon on ATPase activity. Although not shown here, caldesmon dephosphorylation during the assay was never more than 10%. In addition, as shown in Table II, we did not find that phosphorylation of caldesmon affected Ca²⁺-calmodulin reversibility of caldesmon inhibition.

DISCUSSION

Both biochemical (25) and physiological (26-31) data support a major role for phosphorylation of the 20,000-dalton light chains of myosin in the regulation of smooth muscle contraction. On the other hand, force maintenance in the absence of myosin light chain phosphorylation appears to be a reversibly consistent observation in several types of smooth muscle (26, 27, 32). In addition, the unloaded shortening velocity of all smooth muscle types declines during the steady state of an isometric contraction (26, 27, 32). Precisely how unphosphorylated cross-bridges in smooth muscle can maintain high affinity binding in the presence of MgATP that is both Ca²⁺-dependent and characterized by very slow cycling, is not understood at a biochemical level, although some models proposed to explain physiological data have placed emphasis on a myosin-based form of regulation (26). Recently, Sellers (35) has inferred that the rate-limiting step in the smooth muscle acto-HMM ATPase cycle is inorganic phosphate release. Phosphorylation of the 20,000-dalton light chain subunits of smooth muscle HMM accelerates this step nearly 1000-fold (33). Both binding and kinetic data indicate that unphosphorylated HMM remains in a relatively low affinity state (i.e., M·ATP and/or M·ADP·P) for actin and cycling is effectively zero. If in vitro kinetic data are extrapolated to the physiological level, myosin light chain phosphorylation could serve as a switch to generate actively cycling cross-bridges that develop and maintain force by virtue of repeated cycles of low and high affinity binding to actin. This kind of mechanism for the development and maintenance of force in smooth muscle might explain cross-bridge behavior in certain chemically skinned smooth muscles where there is a correlation between steady-state levels of isometric force and myosin light chain phosphorylation (28, 29, 31), but cannot account for force maintenance in intact muscle (26, 27, 32).

For the reasons described above, it has been proposed that a regulatory system in addition to myosin light chain phosphorylation/dephosphorylation must be present in smooth muscle (36). The possibility that a similar system might reside on actin has been suggested from several studies with isolated smooth muscle thin filaments (37). In the present study we have shown that the actin-binding protein, caldesmon, both inhibits the MgATPase activity of acto-HMM and greatly increases the binding constant of HMM for actin. This occurs with both phosphorylated and unphosphorylated HMM. Although the biochemical mechanism accounting for these effects has not been elucidated, there are two reasonable possibilities: 1) caldesmon could directly affect the affinity of the HMM-ATP and HMM-ADP-P complexes for acto-caldesmon while inhibiting P, release, or 2) caldesmon could inhibit at a step in the acto-RMM ATPase cycle other than P, release (see Fig. 7). In this regard, inhibition of ADP release could produce a high affinity actomyosin state by making the acto-HMM-ADP complex the steady-state or rate-limiting intermediate as shown in Fig. 7. Thus, if caldesmon were to operate at the level of ADP release in smooth muscle, it could produce a high affinity cross-bridge state, regardless of the state of myosin light chain phosphorylation, while slowing the shortening velocity of the muscle (Fig. 7). While the model proposed is attractive, we have not determined the effect of caldesmon on the rate of ADP release from acto-HMM. Studies currently underway should provide more direct evidence for the site of action of caldesmon in the acto-HMM ATPase cycle. Nevertheless, data presented in this study do not suggest a direct analogy to the troponin-dependent regulation of striated muscle actomyosin (18). The latter, at least conceptually, appears more analogous to myosin light chain phosphorylation/dephosphorylation since both systems confer Ca²⁺-dependence on the P, release step in their respective actomyosin ATPase cycles (18, 35).

Although Ca²⁺-calmodulin appeared to reverse the effects of caldesmon, an all-or-none effect was not observed. Our results suggested, rather, that Ca²⁺-calmodulin decreased the affinity of caldesmon for actin, the magnitude of the effect depending, therefore, upon the concentration of actin used for reversal experiments. What role calmodulin might play in regulation of caldesmon function in normal cell physiology remains speculative at this time since the function of caldesmon is not known. It is significant that we did not observe an effect of caldesmon phosphorylation on capacity to inhibit acto-HMM ATPase activity. While we did identify Ca²⁺-calmodulin-dependent protein kinase activity and measure a maximal phosphorylation stoichiometry of 2 mol of PO₄/mol of caldesmon monomer similar to results reported by Ngai and Walsh (5), we cannot be entirely certain that both kinase activities were the same. It is also noteworthy that our studies were done with thio-phosphorylated HMM rather than with whole myosin to which myosin light chain kinase, caldesmon, and calmodulin were added concurrently. Thus, differences in the filamentous form of myosin and/or experimental design...
could have contributed significantly to the difference in results.

In conclusion, caldesmon inhibits smooth muscle acto-
HMM ATPase activity and enhances the binding of HMM to actin. These results do not suggest an exact analogy to the kind of actomyosin regulation proposed from in vitro studies with skeletal muscle HMM and troponin (18). Although our studies do not directly identify a specific role for caldesmon in regulation of smooth muscle contraction, the observed effects of caldesmon on affinity of HMM for actin raises the possibility that an actin-based protein complex could reversibly modulate cross-bridge affinity in smooth muscle irrespective of the state of myosin light chain phosphorylation.

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