Caldesmon inhibition of actin-tropomyosin activation of myosin MgATPase activity was investigated. >90% inhibition of ATPase activation correlated with 0.035–0.1 caldesmon bound per actin monomer over a wide range of conditions.

Caldesmon inhibited sheep aorta actin-tropomyosin activation of skeletal muscle heavy meromyosin (HMM) by 85%, but had no effect on the binding affinity of HMM-ADP-Pi to actin. At ratios of 2 and 0.12 subfragment 1 (S1):1 actin, addition of caldesmon inhibited the ATPase activation by up to 95%, but did not alter the fraction of S1-ADP-Pi associated with actin-tropomyosin. We concluded that caldesmon inhibited actomyosin ATPase by slowing the rate-limiting step of the activation pathway.

At concentrations comparable to the ATPase measurements, S1 displaced caldesmon from native thin filaments both in the absence (rigor) and the presence of MgATP. We therefore concluded that caldesmon could displace S1-ADP-Pi from actin-tropomyosin only under exceptional circumstances.

An expressed mutant of caldesmon comprising just the C-terminal 99 amino acids bound actin 10 times weaker than whole caldesmon but otherwise inhibited actin-tropomyosin activation with the same potency and same mechanism as intact caldesmon. Thus, the entire inhibitory function of caldesmon resides in its extreme C terminus.

Ca2+ regulates smooth muscle actomyosin ATPase by mechanisms associated with both the thick and thin filaments (1, 2). Ca2+ controls myosin filament activity via the Ca2+-calmodulin-dependent myosin light chain kinase which activates myosin by phosphorylation. In addition, the thin filaments, which contain actin, tropomyosin, calponin, caldesmon, and a calcium binding protein, are also Ca2+-regulated (2, 3). It has been demonstrated, both by reconstitution of thin filaments from purified components (4–6) and directly by the effect of anti-caldesmon antibodies on native thin filaments (7), that caldesmon plays a central role in this Ca2+-dependent control mechanism.

A physiological role for Ca2+-control of the thin filaments in smooth muscle has not yet been demonstrated; nevertheless, in vitro, both native thin filaments (3, 8) and synthetic systems consisting of actin, tropomyosin, caldesmon, and calmodulin at ratios similar to native thin filaments (4, 9) are effectively regulated by Ca2+ in a manner formally analogous to the regulatory function of troponin in striated muscles. Caldesmon is a very potent inhibitor of actomyosin ATPase activity; inhibition of actin activation of myosin MgATPase activity by over 90% is commonly observed with fewer than 1 caldesmon molecule bound to every 10 actin monomers (4, 10). Ca2+ and calmodulin, or a calcium binding protein, can reverse this inhibition (4, 9).

In a previous paper, we investigated the mechanism by which caldesmon inhibits actin-tropomyosin activation of smooth muscle HMM MgATPase activity (11). It was observed that at a molar ratio of caldesmon to actin monomer of 0.08:1 there was greater than 80% inhibition of ATPase activation by actin-tropomyosin and yet the binding equilibrium between HMM-ADP-Pi and actin-tropomyosin was unaltered. This result paralleled the results of similar experiments performed on the actin-tropomyosin-troponin system (12), and we reached the same conclusion, namely that caldesmon inhibits by slowing the rate-limiting step of the ATPase mechanism, since it did not affect the association equilibrium of the actin-tropomyosin-HMM-ADP-Pi complex.

An alternative mechanism has been suggested by Chalovich and his colleagues (10, 13–15) who observed that at higher ionic strengths caldesmon inhibition of actin-tropomyosin activation of S1 MgATPase was associated with a reduction of the binding of S1-ADP-Pi to actin-tropomyosin which paralleled or even correlated with inhibition. The ability of high concentrations of caldesmon or active fragments of caldesmon to apparently compete with S1-ADP-Pi for sites on actin-tropomyosin has been exploited in a number of investigations of the mechanism of force generation in striated muscle (16).

A mechanism in which caldesmon inhibits by displacing S1-ADP-Pi from actin-tropomyosin is directly contradicted by our earlier experiments (11). It, therefore, seems important to reinvestigate the mechanism of caldesmon regulation under a wider variety of conditions. The displacement model for control predicts that caldesmon inhibition would be favored under conditions where caldesmon could easily displace S1 (e.g. at high ionic strength where S1-ADP-Pi affinity for actin-tropomyosin is relatively weak or where there is a large molar excess of actin, tropomyosin, and caldesmon over S1-ADP-Pi). On the other hand, a mechanism acting on the rate-limiting step ought not to be dependent on any factors other than caldesmon binding to actin-tropomyosin.

In this paper, we demonstrate that caldesmon inhibition of actin-tropomyosin ATPase can occur over a very wide range of conditions, that the relationship between caldesmon inhibition and binding to actin-tropomyosin is independent of conditions, and that the binding of S1-ADP-Pi to actin-
tropomyosin is usually completely independent of inhibition of activation of the ATPase by caldesmon. Displacement of S1-ADP-Pi from actin by caldesmon probably occurs only under restricted sets of conditions which are not likely to occur in vivo.

**Materials and Methods**

The high molecular weight isoform of caldesmon was isolated from sheep aorta and chicken gizzard as described by Taggart and Marston (17), with final purification by ion exchange chromatography on Q-Sepharose (18).

Caldesmon of gizzard caldesmon (668C) comprising the amino acids 658 to 756 (the C terminus) was made by site-directed mutagenesis of a caldesmon cDNA clone (19, 20) which was inserted in the vector MTW172 and expressed in *Escherichia coli* (21, 22). Purification of 668C followed previous protocols for expressed gizzard caldesmon (22).

Sheep aorta thin filaments, actin and tropomyosin, were isolated as described by Marston and Smith (8) and rabbit skeletal muscle F-actin according to Straub (23). Skeletal muscle myosin and chymotryptic S1 and HMM were prepared as described by Margossian and Lowey (24).

Caldesmon was covalently labeled with [14C]iodoacetamide using our published procedure, this does not alter caldesmon inhibition capacity (4). All experiments were done in 5 mM Pipes, pH 7.0, 2.5 mM MgCl2, 5 mM KCl (unless stated otherwise), 1 mM dithiothreitol, and 0.5 μM chloroacetamide. Reaction mixtures (200 μl) were made up and split into two parts: a 100-μl portion was used to measure MgATPase activity at 25 °C and the reaction was initiated by adding MgATP to 7.5 mM and terminated after 10 min with 0.5 ml of 10% trichloroacetic acid. The amount of P1 released by hydrolysis was measured by the method of Taussky and Schorr (25). The remaining 150-μl portion was used for binding measurements; MgATP was added to 7.5 mM, and a 25-μl aliquot of the mixture was taken. The rest of the mixture was immediately centrifuged for 20 min in a Beckman Airfuge at 30 p.s.i. A 25-μl aliquot of the supernatant was taken after sedimentation of actin and actin-bound proteins, then the remainder of the supernatant was carefully removed and the pellet dissolved in 40 μl of 5% SDS, 5% mercaptoethanol, 50 mM Tris, pH 8, 10% glycerol.

14C-Labeled caldesmon binding to actin-tropomyosin was determined by comparing the 14C radioactivity in aliquots of the supernatant before and after centrifugation of the actin-tropomyosin + 14C-caldesmon + S1-ADP-Pi mixture as previously described (4). S1-ADP-Pi, binding to actin-tropomyosin was determined by analysis of the protein content of the pellets using SDS-gel electrophoresis (Fig. 3).

Skeletal muscle HMM-ADP-Pi binding to actin-tropomyosin was determined by assay of the EDTA-ATPase activity in the supernatant before and after sedimentation of the actin-tropomyosin and actin-tropomyosin-HMM-ADP-Pi complexes, according to previous protocols (11, 26).

**Results and Discussion**

Caldesmon Inhibits without Changing K₀ of Actin-HMM-ADP-Pi, Binding—We have shown that low doses of caldesmon (i.e. 0.08 caldesmon/actin) inhibit actin-tropomyosin activation of smooth muscle HMM MgATPase activity and that the affinity of HMM-ADP-Pi for actin-tropomyosin, K₀, was not altered when the ATPase activity was inhibited by caldesmon (11). The subsequent identification of a strong binding affinity between caldesmon and smooth muscle myosin or HMM could have provided an alternative mechanism linking actin to HMM which might explain the lack of an effect of caldesmon on K₀ (15, 21). We have therefore repeated the study using skeletal muscle HMM, which we have shown by direct measurement does not bind to caldesmon in native or synthetic thin filaments (28, 29). Fig. 1 shows actin-tropomyosin activation of skeletal muscle HMM MgATPase activity in low ionic strength buffer. Activation fitted a Michaelis-Menten relationship, and the addition of caldesmon at a fixed ratio of 0.08 caldesmon/actin monomer caused at least 85% inhibition of activation. Simultaneous assay of the association between HMM-ADP-Pi and actin-tropomyosin showed that it was identical in the presence and absence of caldesmon. Thus, under the conditions of this experiment, caldesmon inhibition can be explained only by the slowing down of a rate-limiting process.

Relationship between Caldesmon Inhibition and Its Binding to Actin-Tropomyosin—We next investigated whether caldesmon inhibition varied with the conditions of the experiment. Simultaneous measurements were made of the actinomyosin ATPase activity and of the amount of 14C-labeled caldesmon bound to aorta actin-tropomyosin. ATPase relative to the uninhibited activation is plotted against mol of caldesmon bound per mol of actin monomer. Aorta caldesmon was used unless otherwise indicated. ©, 1 μM S1 + 20 μM Atm, 5 mM KCl, 25 °C, uninhibited rate 4.2 s⁻¹. ©, 6 μM S1 + 3 μM Atm, 5 mM KCl, 25 °C, uninhibited rate, 1.4 s⁻¹. ©, 1 μM S1 + 10 μM Atm, 658C fragment, 5 mM KCl, 25 °C, uninhibited rate, 4.2 s⁻¹. ©, 0.5 μM myosin + 10 μM Atm, 50 mM KCl, 4 °C, uninhibited rate, 0.07 s⁻¹. ©, 0.5 μM myosin + 10 μM Atm, 70 mM KCl, 37 °C, uninhibited rate, 4.5 s⁻¹. ©, 1 μM S1 + 20 μM Atm, gizzard caldesmon, 5 mM KCl, 25 °C, uninhibited rate 4.2 s⁻¹. ©, data from Ref. 4. Atm, aorta actin-tropomyosin (1:0.35 w/w).
skeletal or smooth muscle and with caldesmon from chicken gizzard and sheep aorta (4) (Fig. 2). In addition, the relationship of inhibition to caldesmon binding was nearly the same when actin-tropomyosin was greatly in excess of S1, as in most previously reported experiments, or when S1 was present in excess over actin-tropomyosin (Fig. 2).

It will be noted that these changes are likely to have considerable effects on the efficiency of any competition between caldesmon and S1-ADP-P, binding. For instance, caldesmon displacement of S1-ADP-P would be favored by higher ionic strengths, weakening Kₙ, and by a large excess of actin-tropomyosin and caldesmon over S1, whereas at low ionic strength and with an excess of S1-APP-P over actin-tropomyosin and caldesmon, one might rather expect S1-ADP-P to displace caldesmon (see for instance Fig. 5). Nevertheless, caldesmon inhibits actin-tropomyosin activation with a very high and invariant potency: 90% inhibition of activation always correlated with the binding of 0.035–0.1 caldesmon per actin, an observation that is compatible with a mechanism in which caldesmon controls a rate process rather than changing an equilibrium.

It is interesting to note that the C-terminal 99-amino-acid caldesmon fragment, 658C, contained the inhibitory function and bound to actin with an affinity about 10-fold less than native caldesmon (Figs. 3 and 4B). Nevertheless, complete inhibition of activation still required only 0.08 658C fragment bound per actin monomer (Fig. 2).

**Caldesmon Inhibition and S1-ADP-P-Actin Binding When [S1] Exceeds [Actin-Tropomyosin]—**We investigated the relationship between caldesmon inhibition and the binding of S1-ADP-P, in the presence of S1 in excess of actin monomer, since this represents a situation where S1-ADP-P displacement by caldesmon is least likely. It is possible to accurately determine S1-ADP-P, associated with actin-tropomyosin by analysis of the pellet following centrifugation of S1, actin-tropomyosin, and caldesmon in 7.5 mM MgATP.

Fig. 3 shows typical results. There was essentially no S1-ADP-P pelleted except in the presence of actin-tropomyosin; the amount co-sedimented was only about 5% of total S1. The S1 binding was reversible since it could be fully dissociated by increasing [KCl] to 100 mM. Addition of caldesmon or the fragment 658C inhibited the actin activation; caldesmon and 658C were observed to be co-sedimented with actin and tropomyosin while the amount of S1-ADP-P, associated with actin tropomyosin appeared constant.

The relationship between caldesmon inhibition and S1-ADP-P binding to actin-tropomyosin was determined quantitatively by scanning densitometry of SDS gels illustrated in Fig. 3. Each track was scanned at 3 positions and S1 quantity in the pellet fraction was normalized by the equation (area SI heavy chain + area S1 light chain)/(area actin + area tropomyosin) to eliminate dilution and pipetting errors. Pellet volume was measured and a correction was applied for free S1 trapped within the pellet. O, ATPase activation expressed as percent uninhibited; ●, S1-ADP-P, bound, percent of value in the absence of caldesmon. A, 6 μM S1 and 3 μM actin-tropomyosin inhibited by caldesmon. Uninhibited rate, 0.44 s⁻¹. B, 8 μM S1 and 4 μM actin tropomyosin inhibited by 658C. Uninhibited rate, 0.28 s⁻¹. C, 2 μM S1 and 15 μM actin-tropomyosin inhibited by caldesmon. Uninhibited rate, 0.64 s⁻¹.

**FIG. 4. Effect of caldesmon and 658C on actin-tropomyosin activation of S1 MgATPase activity and S1-ADP-P binding to actin tropomyosin.** Conditions as for Fig. 1. S1-ADP-P binding was determined by scanning densitometry of the SD8 gels illustrated in Fig. 3. Each track was scanned at 3 positions and S1 quantity in the pellet fraction was normalized by the equation (area SI heavy chain + area S1 light chain)/(area actin + area tropomyosin) to eliminate dilution and pipetting errors. Pellet volume was measured and a correction was applied for free S1 trapped within the pellet. O, ATPase activation expressed as percent uninhibited; ●, S1-ADP-P, bound, percent of value in the absence of caldesmon. A, 6 μM S1 and 3 μM actin-tropomyosin inhibited by caldesmon. Uninhibited rate, 0.44 s⁻¹. B, 8 μM S1 and 4 μM actin tropomyosin inhibited by 658C. Uninhibited rate, 0.28 s⁻¹. C, 2 μM S1 and 15 μM actin-tropomyosin inhibited by caldesmon. Uninhibited rate, 0.64 s⁻¹.

We concluded that caldesmon inhibition does not require S1-ADP-P, displacement from actin-tropomyosin, but that some S1 displacement can occur where actin and caldesmon concentrations are high. At a high ratio of actin and caldesmon to S1 (e.g. 125 actin/S1, 25 caldesmon/S1 in low ionic strength buffer or 250 actin-tropomyosin/S1, 15 caldesmon/S1 in 45 mM NaCl buffer (13)), we observed the same displacement of S1-ADP-P, from actin as Chalovich reported (13). It might appear that S1 displacement was responsible for inhibition in these particular conditions since it is self-
evident that if S1 ⋅ ADP ⋅ Pi is displaced from actin-tropomyosin actin activation will be reduced.

Displacement of Caldesmon from Thin Filaments by S1.—In order to assess the likely significance of such displacements in vivo, we investigated the effect of S1 on caldesmon binding in native thin filaments from sheep aorta. Native thin filaments contain around 0.07 caldesmon per actin monomer (30, 33, 34) as demonstrated by the properties of 658C (Figs. 3 and 4B). Binding of S1 ⋅ ADP ⋅ Pi to the other 14–15 actins which are not as tightly associated with caldesmon in the thin filament would not lead to displacement.

The addition of MgAMP-PNP or MgATP weakens the affinity of S1 for actin-tropomyosin (Kd) by 1000-fold and 5000-fold, respectively (35, 36), despite which S1 was still able to displace caldesmon, although a 3-fold excess of S1 ⋅ ADP ⋅ Pi over actin was required for complete displacement (Fig. 5). We noted already (Fig. 4A) that, where S1 was in excess over actin-tropomyosin, caldesmon did not bind as strongly as when actin was in excess (Fig. 4C); this is probably at least in part a consequence of S1 ⋅ ADP ⋅ Pi competing for caldesmon sites and displacing caldesmon. There is evidently no scope for caldesmon displacing S1 ⋅ ADP ⋅ Pi under those conditions.

Caldesmon Inhibition Mechanism.—A general conclusion from all the in vitro studies on the mechanism of caldesmon inhibition (10, 11, 13, 37) would be that two processes may be identified: inhibition of a rate-limiting step of the ATPase and displacement of S1 ⋅ ADP ⋅ Pi from actin-tropomyosin due to a decrease in the affinity of "weak" actin-myosin bonds. In our experience, inhibition of a rate-limiting step may be demonstrated under a wide range of experimental conditions (Figs. 1, 2, and 4) whereas S1 displacement (10, 13) or mixed cases (37) may only be observed at high caldesmon:S1 ratios (>10 caldesmon:S1 (13) compared to 0.1–1 caldesmon:S1 in Fig. 4). S1 displacement also seems to be associated with the absence of tropomyosin or higher ionic strengths (13, 37).

Both tropinin and the smooth muscle myosin phosphorylation regulatory mechanisms act by inhibiting a rate-limiting step (12, 15, 38); we believe there is no evidence that caldesmon regulation is fundamentally different. Regulation of the rate of a biochemical process is the most efficient and widely observed biochemical control mechanism, since it requires no energy expenditure. In contrast, it is necessary to put energy into a system if an equilibrium is to be altered.

Conditions in intact smooth muscles are not likely to favor S1 ⋅ ADP ⋅ Pi, dissociation from actin. The physical excess of actin over myosin molecules in smooth muscles is of the order of 11:1 (39), which is within the range of our experiments (e.g. Figs. 1, 3, and 4C) which showed little or no displacement of S1 ⋅ ADP ⋅ Pi. Furthermore, the thick and thin filaments are located in close proximity such that the apparent concentration of actin and myosin is in the millimolar range; therefore, the equilibrium is strongly in favor of myosin-ADP-Pi association with actin-tropomyosin, and correspondingly more energy input would be required to shift the equilibrium toward dissociation. The experiments of Brenner and co-workers (16) have shown that in striated muscles such a shift in equilibrium can be detected, but it requires energy input in the form of a high concentration of exogenous caldesmon.

Finally, it is known that in smooth muscles, caldesmon binds to the myosin as well as to actin; this binding results in cross-linking of thick and thin filaments (18, 27, 28) and thus increases the probability that myosin and actin remain associated when inhibited by caldesmon. This has been directly demonstrated in an in vitro motility assay (40) where low doses of caldesmon actually promoted actomyosin association and contractility via the cross-linking property.

While this discussion shows that caldesmon could be involved in regulating smooth muscle contractility by the mechanism we observed in vitro, there is as yet little positive evidence that caldesmon does regulate in vivo (2, 17, 41).

Although caldesmon inhibition seems to control the same step in the actomyosin ATPase pathway as tropinin, the mechanism by which it does so may be different. It must involve a propagation of inhibitory conformational changes through the actin filament since each caldesmon has a high affinity binding site for only one actin (30, 33, 34) but inhibits up to 14 (Fig. 2). This is clearly demonstrated by the properties of the C-terminai 99 amino acids contained in our expressed fragment 658C and the "10K" cyagen bromide cleavage fragment studied by Bartegi et al. (34). This part of caldesmon contains actin binding and inhibition sites (30, 34) (see Figs. 3 and 4B), and the binding of one 658C fragment to actin resulted in the inhibition of a total of 14 actins in the presence of tropomyosin (Fig. 2). Since 658C can also inhibit actin activation in the absence of tropomyosin and is not known to contain any tropomyosin binding site (this has been located between amino acids 508 and 565) [19, 22, 30], inhibition must be propagated through the actin filament from a single actin-caldesmon contact to up to 14 other actins. This observation has similarities to a mechanism once proposed for tropinin regulation (42). Much more work is required to explain how caldesmon inhibition works.

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Fig. 5. Displacement of caldesmon from native thin filaments by skeletal muscle subfragment 1. 0.8 μM sheep aorta thin filaments (actin monomer concentration) was incubated with skeletal muscle S1 (0–2 μM) in low ionic strength buffer (see Fig. 1) and in buffer plus 2 mM AMP-PNP or 5 mM MgATP. The filaments were sedimented and the composition of the pellets was determined by gel electrophoresis (see Fig. 3) and densitometric scans. S1 reduced the caldesmon content of pelleted thin filaments while leaving actin and tropomyosin unaffected. Arrow indicates the actin monomer concentration.
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