Caldesmon Inhibits Skeletal Actomyosin Subfragment-1 ATPase Activity and the Binding of Myosin Subfragment-1 to Actin*

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Smooth muscle contraction is controlled in part by the state of phosphorylation of myosin. A recently discovered actin and calmodulin-binding protein, named caldesmon, may also be involved in regulation of smooth muscle contraction. Caldesmon cross-links actin filaments and also inhibits actin-activated ATP hydrolysis by myosin, particularly in the presence of tropomyosin. We have studied the effect of caldesmon on the rate of hydrolysis of ATP by skeletal muscle myosin subfragment-1, a system in which phosphorylation of the myosin is not important in regulation. Caldesmon is a very effective inhibitor of ATP hydrolysis giving up to 95% inhibition. At low ionic strength (~20 mM) this effect does not require smooth muscle tropomyosin, whereas at higher ionic strength (~120 mM) tropomyosin enhances the inhibitory activity of caldesmon at low caldesmon concentrations. Cross-linking of actin is not essential for inhibition of ATP hydrolysis to occur since at high ionic strength there is very little cross-linking as determined by a low speed sedimentation assay. Under all conditions examined, the decrease in the rate of ATP hydrolysis is accompanied by a decrease in the binding of myosin subfragment-1 to actin. Furthermore, caldesmon weakens the equilibrium binding of myosin subfragment-1 to actin in the presence of pyrophosphate. We conclude that caldesmon has a general weakening effect on the binding of skeletal muscle myosin subfragment-1 to actin and that this weakening in binding may be responsible for inhibition of ATP hydrolysis.

The force producing interaction between myosin and actin can be inhibited by modification of either the myosin filaments or the actin filaments. Activation of vertebrate striated muscle is mediated through the actin filaments. Binding of calcium to the tropo-calcium complex causes a change in position of tropomyosin molecules on the actin filaments (2-4) which has the effect of increasing the rate of a process in the ATPase cycle, probably P, release, that occurs after the binding of myosin to actin (5-9). Regulation of vertebrate smooth muscle contraction is primarily controlled by the state of the myosin filament. Many studies have shown that phosphorylation of myosin by the Ca\(^{2+}\)-calmodulin-dependent enzyme, myosin light chain kinase, is essential for smooth muscle contraction (see, for example, Refs. 10-13). The effect

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1 The abbreviations used are: HMM, heavy meromyosin; S-1, myosin subfragment-1; EGTA, [ethyleneglycol(oxyethylene)nitrilo]etraacetic acid; AMP-PNP, adenylyl-5'-yl imidodiphosphate.
myosin enhances the activity of caldesmon at high ionic strength. Under all conditions the decreased ATPase rate is associated with a decreased association between S-1-ATP and actin. Finally, caldesmon also inhibits the equilibrium binding of S-1 to actin in the presence of pyrophosphate. This weakening in the binding of S-1 to actin by caldesmon may be the cause of the decreased rate of ATP hydrolysis.

MATERIALS AND METHODS

Myosin was isolated from the back and leg muscles of rabbits by standard procedures (33). S-1 was prepared by chymotryptic digestion of myosin (34). Actin was isolated by the procedure of Spudich and Watt (35) as modified by Eisenberg and Kielley (36). Tropomyosin was isolated from turkey gizzards by the method of Bretsch (31). S-1 was radioactively labeled by reaction with [\(^{14}C\)]iodoacetamide (37) and applied to a small affinity column of ATP attached to agarose through C-8 (Sigma) equilibrated with 1 mM MgCl\(_2\), 1 mM dithiothreitol. The column was washed with the same buffer and the \(^{14}C\)-labeled S-1 was eluted with 0.1 M KCl. Radioactively labeled S-1 was used only for binding studies performed in the absence of ATP. Calmodulin was isolated from porcine brain by the method of Yawaza et al. (38). Caldesmon was prepared by a method involving modification of several published procedures (19, 21, 39). Fresh turkey gizzards were cleaned, minced, and homogenized on the middle setting of a Polytron homogenizer in 4 volumes of buffer C containing a total of 1 M KCl. Fractions containing the high molecular weight contaminant located between caldesmon and S-1 in lane A arise from myosin digestion. The heavily overloaded sample of pure caldesmon shown in lane B is virtually free of viable contaminants. Caldesmon isolated by this procedure does not become phosphorylated upon incubation with \([\gamma-\text{P}]\)ATP and calmodulin.

The concentration of caldesmon was determined by the Lowry method (40) using bovine serum albumin as a standard. Other protein concentrations were determined by their absorption at 280 nm. The molecular mass of caldesmon was assumed to be 140,000 daltons.

ATPase Assays—ATPase rates were measured at 25° C by the liberation of \(^{32}P\), from \([\gamma-\text{P}]\)ATP (6). Each assay consisted of three time points. The conditions for the ATPase assays are given in the figure legends.

Binding Assays—The binding of S-1 to actin in the presence of ATP was measured by sedimenting the acto-S-1 in an ultracentrifuge and determining the free S-1 concentration by an NH\/EDTA/ATPase assay as described earlier (6). Binding assays were performed in 1.0 ml of solution having a composition that is described in the figure legends. Caldesmon was added to actin or actin-tropomyosin and allowed to stir for several minutes prior to the addition of S-1. After a further mixing for 1 min at 4° C, the ATP was added. The solution was stirred for 1 min and placed into centrifuge tubes, brought to 25° C, and centrifuged in a Ti-50 rotor at 40,000 rpm for 20 min. All of the supernatant was removed and an aliquot was used for S-1 determination. Caldesmon had no effect on the NH\/EDTA/ATPase rate of S-1. Binding of \([\gamma-\text{P}]\)-labeled S-1 was done by the method of Greene and Eisenberg (41).

Actin Cross-linking Studies—The cross-linking of actin by caldesmon was estimated by a low speed sedimentation assay under the same conditions used for the binding studies (42).

RESULTS

Fig. 2 shows that the normal actin-activated ATP hydrolysis is greatly inhibited by the addition of caldesmon at low ionic strength in the absence of tropomyosin. At 2.5 \(\mu\)M caldesmon monomer, where the caldesmon to actin ratio is 0.1, the ATPase activity is about 70% inhibited.\(^2\) Even greater inhibition is observed at higher concentrations of caldesmon. In experiments not shown here, we have observed that the addition of smooth muscle tropomyosin does not enhance the inhibitory activity of caldesmon under these conditions.

Since an inhibitor of actomyosin ATPase activity could function either by weakening the binding of myosin to actin in the presence of ATP or by inhibiting the rate of some process in the hydrolysis reaction, we determined the effect of caldesmon on the association of S-1 with actin in the presence of ATP. The results of these binding studies are also shown in Fig. 2. In the absence of caldesmon, roughly 50% of the S-1-ATP was bound to actin. The fraction of S-1-ATP bound decreased with increasing caldesmon concentrations in a manner that paralleled the inhibition of ATPase activity. Once again, when the ratio of caldesmon to actin was 0.1, the binding was decreased to about 30% of the initial level. Therefore, the decreased ATPase activity is most likely the result of a decreased binding of S-1 to actin. To demonstrate that the effects of caldesmon on the binding of S-1-ATP to actin is specific, we attempted to reverse this inhibition of binding by the addition of calmodulin. The inset to Fig. 2 shows that the inhibition of binding by caldesmon is, in fact, reversed by the addition of a large molar excess of calmodulin to the reaction in the presence of 0.5 mM CaCl\(_2\). It is important to note that the curve drawn through the points in the inset is arbitrary.

\(^2\) Since the protein assay of Lowry et al. (40) does not give an absolute protein concentration, the ratios given must be considered approximate.
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**Fig. 2. Effect of caldesmon on the ATPase activity of S-1 and the binding of S-1 to actin in the presence of ATP at low ionic strength.** ATPase activity (O) and binding (●) are expressed relative to the values in the absence of caldesmon, 3 s⁻¹ and 0.5 for the ATPase rate, and fraction of S-1 bound, respectively. The conditions used were: 1 mM ATP, 5 mM MgCl₂, 10 mM imidazole, pH 7.0, 0.5 mM EGTA, 1 mM dithiothreitol, 25 μM S-1 actin, and 0.2 μM S-1 at 25 °C. Inset, reversal of binding inhibition by calmodulin. The actin concentration was 10 μM with 2 μM caldesmon and 0.5 mM CaCl₂ substituted for 0.5 mM EGTA.

We considered the possibility that the inhibition of binding caused by caldesmon may have been the result of aggregation of actin filaments by caldesmon. We were alerted to this possibility by our observation that addition of caldesmon to actin solutions under the conditions of Fig. 2 caused a large increase in turbidity, suggesting cross-linking of the actin filaments. Also, during the course of this investigation, reports of cross-linking of actin by caldesmon have been published (25, 31, 32). It was important, therefore, to quantitate the degree of cross-linking caused by caldesmon at low ionic strength.

**Fig. 3. Effect of caldesmon on the solubility of actin and on the ATPase activity at low ionic strength.** O, ATPase activity; ●, cross-linking. The ATPase activity in the absence of caldesmon was 1.3 s⁻¹ and the fraction of soluble actin in the absence of caldesmon was >0.98. The conditions were the same as in Fig. 2 except that 4 μM actin and 0.4 μM S-1 were used. The inset shows reversal of the effect of caldesmon on ATPase activity (O) and cross-linking (●) by 1 μM calmodulin when 0.5 mM CaCl₂ was substituted for 0.5 mM EGTA in the mixtures.

ATP hydrolysis and the fraction of soluble actin. To further test this correlation we attempted to dissociate the effect of caldesmon on the cross-linking from the effect on ATPase activity and S-1 binding by varying the ionic strength.

The ionic strength dependence on the amount of actin sedimented at low speed is shown in Fig. 4. In the experiments shown here the caldesmon was dialyzed overnight against 17 mM imidazole-HCl, pH 7.0, 7 mM MgCl₂, 1 mM EGTA, and 1 mM dithiothreitol, sodium chloride was then added to each reaction mixture to give the required ionic strength. Whereas caldesmon is a potent cross-linker at low ionic strength, this activity disappears at ionic strengths greater than 50 mM. It is important to note that caldesmon/actin mixtures must be handled gently since aggregation can occur, even at high ionic strength, following vigorous mixing. Studies on the effect of caldesmon on ATPase activity and binding were repeated at high ionic strength where cross-linking did not occur.

**Fig. 5.** Shows the results of ATPase assays and low speed sedimentation studies at 125 mM ionic strength and 25 μM actin. At these conditions caldesmon remains an effective inhibitor of ATP hydrolysis in both the presence (open circles) and absence (solid circles) of smooth muscle tropomyosin. However, in contrast to low ionic strength experiments, tropomyosin does enhance the inhibitory activity of caldesmon. The ratio of caldesmon to actin required to produce 50% inhibition of ATPase activity is reduced from about 0.08 to 0.02 in the presence of tropomyosin in this experiment. In further contrast to low ionic strength experiments, the inhibition of ATP hydrolysis is not accompanied by aggregation of actin. Even at 5 μM caldesmon (a caldesmon to actin ratio of 0.2) where there is 95% inhibition of ATP hydrolysis, no
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Fig. 4. Effect of ionic strength on the cross-linking of actin filaments by caldesmon. Experiments were performed with 5 μM actin and 0.5 μM caldesmon (○), with 25 μM actin and 1.5 μM caldesmon (□), or with 50 μM actin and 1.5 μM caldesmon (□). The conditions used were: 2 mM ATP, 5 mM MgCl₂, 10 mM imidazole, pH 7, 0.5 mM EGTA, 1 mM dithiothreitol, and variable NaCl, 25 °C.

Fig. 5. Effect of caldesmon on the ATPase activity of S-1 and the cross-linking of actin at high ionic strength. ○, ATPase activity; □, actin cross-linking. Experiments were performed with 25 μM actin in either the presence (○) or absence (□) of 5 μM tropomyosin. The ATPase activities in the absence of caldesmon were 0.56 s⁻¹ and 0.24 s⁻¹ in the presence and absence of tropomyosin, respectively. The conditions used were: 100 mM NaCl, 2 mM ATP, 5 mM MgCl₂, 10 mM imidazole, pH 7, 0.5 mM EGTA, 1 mM dithiothreitol, 25 °C.

Fig. 6. Caldesmon inhibits the binding of S-1 to actin in the presence of ATP in the absence of cross-linking. A, binding (○) and ATPase rates (△) in the presence of 50 μM actin, 7.1 μM tropomyosin. Each binding point is the average of five measurements shown with the standard error. The fraction bound at 0 caldesmon is about 0.2. ATPase rates are relative to the value in 0 caldesmon (1.8 s⁻¹). B, binding (○) and ATPase rates (△) in the presence of 75 μM actin and 11 μM tropomyosin. The fraction of S-1 bound at 0 caldesmon was 0.29 and the corresponding ATPase rate was 2.4 s⁻¹. The conditions used were: 42 mM NaCl, 2 mM ATP, 5 mM MgCl₂, 10 mM imidazole, pH 7, 0.25 mM EGTA, 1 mM dithiothreitol, 25 °C, 0.2 μM S-1.
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FIG. 7. Caldesmon inhibits the equilibrium binding of S-1 to actin in the presence of Pi. Binding in the presence (○) and absence (○) of tropomyosin are shown. The conditions used were: 42 mM NaCl, 4 mM PPi, 5 mM MgCl2, 10 mM imidazole, pH 7.25 mM EGTA, 1 mM diethiothreitol, 25°C, 0.63 μM 14C-labeled S-1, 10 μM actin, and 1.5 μM tropomyosin.

binding can be accurately measured, the correlation between inhibition of ATPase activity and the binding of S-1-ATP is good. In all cases the degree of binding in the absence of caldesmon is consistent with our earlier results (6) and it is reduced to a very low value at high caldesmon concentrations. The effect of caldesmon on the binding is reversible as demonstrated by the addition of excess calmodulin. The effect of caldesmon on binding does not appear to result from gross aggregation of actin filaments since the aggregation would be seen in low speed sedimentation experiments. In fact, our results strongly indicate that inhibition of ATP hydrolysis and binding are not caused by actin aggregation. While this present work was in progress, Moody et al. (32) also showed inhibition of ATP hydrolysis in the absence of actin cross-linking at low actin concentrations using electron microscopy to estimate actin cross-linking. Our demonstration of the ionic strength dependence of the cross-linking may help to explain why ATPase inhibition may sometimes seem to vary together with the degree of cross-linking.

The second indication that caldesmon functions in this system by weakening the binding of S-1 to actin comes from equilibrium binding studies in the presence of pyrophosphate. It was shown in these studies that caldesmon greatly weakens the binding of S-1-pyrophosphate to actin. There are several advantages to studying the binding in the presence of pyrophosphate. The binding of S-1-substrate complexes is complicated by the presence of intermediate states. A change in the distribution of these intermediate states could cause a change in the observed binding constant. Since pyrophosphate is not a substrate for S-1, no intermediate states are formed and the binding is straightforward. Also, since the binding in pyrophosphate is stronger than in the presence of ATP, it is possible to accurately measure binding at high ionic strengths and low actin concentrations where actin aggregation is not a problem.

Two related, but unanswered questions concern the possibility that a caldesmon-actin-S-1 complex can form and whether such a complex would be catalytically active. Preliminary results indicate that caldesmon can inhibit the ATPase activity of chemically cross-linked acto-S-1 by about 30%. While this is much smaller than the inhibition in binding that we observe, it must be considered a possibility that caldesmon has multiple inhibitory activities.

In a preliminary report Lash et al. (29) found that the inhibition of ATPase activity of smooth muscle HMM was associated with an increase in the binding of HMM-ATP to actin. While smooth and skeletal muscle myosins differ quantitatively in their strength of association with actin (46, 47) (smooth muscle myosin binds tighter than skeletal muscle myosin at high ionic strength), qualitatively different effects of caldesmon are unexpected. Both myosins have similar mechanisms of actin-activated ATP hydrolysis (48) and the binding of both myosins to actin is increased as the nucleotide is changed from ATP to AMP-PNP to ADP (47). Based on this knowledge we would expect qualitative similarities in the effect of caldesmon on the binding of smooth and skeletal muscle myosin subfragments. The large difference observed between skeletal S-1 and smooth HMM may be due to: 1) differences between a single-headed subfragment and one with two heads, 2) to two different modes of attachment of the smooth and skeletal myosins to actin, or 3) to a kinetically controlled difference in the population of states between the two myosins. Further exploration of the difference between skeletal and smooth muscle myosins is likely to reveal much.

one might expect that caldesmon would have a similar effect on the equilibrium binding of other forms of S-1. One convenient equilibrium system to study is the S-1-pyrophosphate complex which has a binding constant between that for S-1-ATP and the very tight binding S-1-ADP complex (44). Fig. 7 is a plot of the fraction of S-1-pyrophosphate bound to actin as a function of caldesmon concentration under conditions where actin cross-linking does not occur. In the absence of caldesmon the binding constant of S-1-pyrophosphate to actin is enhanced about 4-fold by smooth muscle tropomyosin, in agreement with a 3.6-fold increase reported by Williams and Greene (45) for skeletal muscle tropomyosin. As the caldesmon concentration is increased, the fraction of S-1-pyrophosphate bound decreases to a low value. It is difficult to determine the absolute degree of inhibition in either case because of the errors associated with measuring very low levels of binding. However, it is clear that marked inhibition of binding occurred in both the presence and absence of tropomyosin. At 1 μM caldesmon, where the caldesmon to actin monomer ratio is 0.1, the binding in both the presence and absence of tropomyosin is reduced to about 58% of the respective original values.

Discussion

Two lines of evidence support the conclusion that the inhibitory effect of caldesmon on skeletal acto-S-1-ATPase activity is primarily the result of weakened binding between S-1-ATP and actin. First, under several different conditions, and whether or not actin aggregates are present, the decreased rate of ATP hydrolysis is accompanied by a decreased association between S-1-ATP and actin. In those cases where the
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about the interaction of these contractile proteins with actin as well as clarify the function of the regulatory protein caldesmon.

A disputed topic is the requirement for tropomyosin in the inhibitory effect of caldesmon. Sobue et al. (28) using a smooth muscle myosin and skeletal actin, have reported that caldesmon functions primarily by reversing the potentiation of ATPase activity that occurs in the presence of tropomyosin. Caldesmon was also reported by Marston and Smith (30) to be relatively ineffective in the absence of tropomyosin when assayed with smooth muscle myosin and actin. In contrast, Ngai and Walsh (27), like Sobue et al. (28), used smooth muscle myosin and skeletal actin but reported significant inhibition by caldesmon in the absence of tropomyosin. Similarly, Dabrowska et al. (25), and Lim and Walsh (24) using skeletal myosin and actin, found that tropomyosin only enhanced the inhibitory activity of caldesmon. Using skeletal muscle actin and myosin S-1 we also find that caldesmon, by itself, is sufficient to inhibit ATP hydrolysis. Caldesmon is an inhibitor of ATPase activity and S-1 binding, whether or not tropomyosin is present. At very low ionic strength we have never observed a difference in the effect of caldesmon upon addition of tropomyosin. At 25 μM actin and the highest ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis.

It therefore appears that caldesmon, whether or not tropomyosin is present, is an inhibitor of ATPase activity and S-1 binding, whether or not tropomyosin is present. At very low ionic strength we have never observed a difference in the effect of caldesmon upon addition of tropomyosin. At 25 μM actin and the highest ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. It therefore appears that caldesmon, whether or not tropomyosin is present, is an inhibitor of ATPase activity and S-1 binding, whether or not tropomyosin is present. At very low ionic strength we have never observed a difference in the effect of caldesmon upon addition of tropomyosin. At 25 μM actin and the highest ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis. At very low ionic strength used, tropomyosin by itself increased the rate of ATP hydrolysis.