Caldesmon Reduces the Apparent Rate of Binding of Myosin S1 to Actin−Tropomyosin†

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ABSTRACT: Equilibrium measurements of the rate of binding of caldesmon and myosin S1 to actin-tropomyosin from different laboratories have yielded different results and have led to different models of caldesmon function. An alternate approach to answering these questions is to study the kinetics of binding of both caldesmon and S1 to actin. We observed that caldesmon decreased the rate of binding of S1 to actin in a concentration-dependent manner. The inhibition of the rate of S1 binding was enhanced by tropomyosin, but the effect of tropomyosin on the binding was small. Premixing actin with S1 reduced the amplitude (extent) of caldesmon binding in proportion to the fraction of actin that contained bound S1, but the rate of binding of caldesmon to free sites was not greatly altered. No evidence for a stable caldesmon−actin−tropomyosin−S1 complex was observed, although S1 did apparently bind to gaps between caldesmon molecules. These results indicate that experiments involving caldesmon, actin, tropomyosin, and myosin are inherently complex. When the concentration of either S1 or caldesmon is varied, the amount of the other component bound to actin−tropomyosin cannot be assumed to remain fixed. The results are not readily explained by a mechanism in which caldesmon acts only by stabilizing an inactive state of actin−tropomyosin. The results support regulatory mechanisms that involve changes in the actin−S1 interaction.

Caldesmon is an actin binding protein present in smooth muscle and nonmuscle cells that inhibits actin-activated ATPase activity of myosin (*1*) and force production in a muscle fiber system (*2*−*4*). The mechanism by which caldesmon functions is an unsettled issue, but there is agreement on several issues. (1) The effectiveness of caldesmon as an inhibitor of ATPase activity is enhanced by tropomyosin. (2) Tropomyosin enhances the inhibitory activity of caldesmon and reduces by $\sim^2/_3$ the amount of bound caldesmon required to give 50% inhibition of ATPase activity (*5*, *6*). (3) Caldesmon inhibits the binding of myosin S1[1] to actin−tropomyosin (*7*, *8*). Despite agreement on these points, there is no consensus on the manner in which actin, myosin, and caldesmon interact, on the relevancy of the competition of binding to the regulation of ATPase activity, or on the mechanism by which caldesmon inhibits ATPase activity (*9*−*12*).

Several factors contribute to the complexity of the actin−myosin−caldesmon interaction and possibly to the difference in interpretation of caldesmon function (*10*). One problem is that in some types of binding studies it is impossible to determine which protein is bound to which. Caldesmon binds to myosin (*13*, *14*) as well as to actin (*1*), so a number of complexes are possible (*15*, *16*). The relative stabilities of the complexes depend on several factors, including the ionic strength and the nucleotide bound to S1. As a result, large differences have been reported in the observed relationship between bound S1 and caldesmon bound to actin.

In the study presented here, fluorescent probes were placed on actin, S1, and caldesmon so that it was possible to monitor each protein−protein interaction in the S1−actin−caldesmon complex. Pyrene-actin was responsive to S1 binding but not to caldesmon binding. Fluorescein- and SAL-S1 were sensitive to actin binding but not to caldesmon binding. NBD-caldesmon monitored actin binding but not S1 binding. The use of fluorsecent probes permitted the time course of the various interactions to be observed. Caldesmon was found to substantially reduce the rate of binding of rigor S1 to actin in the presence and absence of tropomyosin. The reduction in the rate of binding did not occur with a lag phase under the observed conditions. The binding of S1 to actin-tropomyosin resulted in the rapid displacement of caldesmon from actin−tropomyosin. When actin was saturated with S1,

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; fluorescein-S1, S1 modified with 5-iodoacetamidofluorescein; IANBD, *N*-{[(2-iodoacetoxy)ethyl]methyl}amino-7-nitrobenz-2-oxa-1,3-diazole; ISAL, 4-(iodoacetamido)salicylic acid; NBD-caldesmon, caldesmon modified with IANBD; NEM, *N*-ethylmaleimide; NEM-S1, S1 modified with NEM; pyrene iodoacetamide, *N*-(1-pyrene)iodoacetamide; pyrene-actin, actin modified with pyrene iodoacetamide; SAL-S1, S1 modified with ISAL; S1, myosin subfragment 1.

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caldesmon did not remain bound to the actin in a way that involved the normal actin binding site on caldesmon. Thus, in any experiment designed to study the interaction of actin with caldesmon and S1, it is necessary to monitor the binding of both caldesmon and S1. The results obtained here help to define the mechanism by which caldesmon inhibits the activation of ATP hydrolysis by myosin.

EXPERIMENTAL PROCEDURES

Protein Purification. Skeletal muscle actin (*17*) and myosin (*18*) were isolated from the back and leg muscles of rabbits. S1 was prepared by chymotryptic digestion of myosin (*19*). Turkey gizzard tropomyosin was prepared both with (*20*) and without a heat treatment step (*21*). Caldesmon was purified from turkey gizzards by a modification of the heat treatment method of Bretscher (*20*) as described in ref *6* or by a method that avoids heat treatment (*6*).

Tropomyosin and caldesmon concentrations were determined by the Lowry assay (*22*) with a BSA standard. Actin and S1 concentrations were determined by absorbance at 280 nm with a scattering correction made at 340 nm. The extinction coefficients that were used were 1.15 mL mg^{-1} cm^{-1} for actin and 0.75 mL mg^{-1} cm^{-1} for S1. The molecular weights that were used were as follows: 120 000 for S1, 42 000 for actin, 68 000 for tropomyosin, and 87 000 for caldesmon. The purity of all proteins was verified by polyacrylamide gel electrophoresis in the presence of SDS (*23*).

Labeling with Fluorescent Probes. Actin was modified with pyrene iodoacetamide as described previously (*24*, *25*). The extent of labeling, determined using an extinction coefficient for the pyrene of 2.8×10^4 M^{-1} cm^{-1} at 344 nm, was generally 85%. Modification of the reactive thiol SH1 (Cys 707) of S1 with fluorescent probes was carried out in a method similar to that described previously (*26*, *27*). Labeling was carried out in a buffer composed of 60 mM KCl, 30 mM Tris (pH 7.5), and 0.1 mM dithiothreitol. Either a 3-fold molar excess of 5-iodoacetamidofluorescein or a 10-fold excess of ISAL was added from a 25 mM stock in dimethylformamide. Following incubation for 18 h at 4 °C in the dark, the reaction was quenched with an excess of dithiothreitol and the mixture sedimented to remove particulate matter and dialyzed to remove excess probe. The degree of labeling was determined by absorption measurements: $\epsilon_{494} = 7.13 \times 10^4$ M^{-1} cm^{-1} for fluorescein (*28*) and $\epsilon_{304} \approx 9.5 \times 10^3$ M^{-1} cm^{-1} for ISAL (from the manufacturer). The degree of SH1 labeling with these probes were typically found to be in the range of 0.9−0.95. In some cases, unmodified S1 was removed after the labeling reaction by passing the S1 through a 6 mL column of ATP attached to agarose through C-8 (Sigma) equilibrated with 10 mM imidazole, 2 mM $MgCl_2$, and 1 mM dithiothreitol. S1 that bound to this column was not used for actin binding studies. Note that 5-(iodoacetamido)salicylic acid used by Aguirre et al. (*29*) to monitor S1 binding is no longer commercially available. The 4-(iodoacetamido)salicylic acid in our studies had properties somewhat different than those reported for the 5-isomer.

Modification of the thiol residues of caldesmon was carried out after first reducing the caldesmon [30 min at 37 °C in 0.1 M NaP_i (pH 6.0), 5 mM EDTA, and 10 mM dithiothreitol]. Dithiothreitol was removed by gel filtration on a 1.5 cm × 15 cm column of ACA 202 (Spectrum) equilibrated with 100 mM KCl, 50 mM Tris-HCl (pH 7.5), and 1 mM EDTA. To the caldesmon was added a 5-fold molar excess of IANBD (Molecular Probes) from a concentrated stock in dimethylformamide. The reaction was quenched with dithiothreitol after the mixture had been incubated in the dark for 18 h at 4 °C. The protein was clarified by centrifugation and dialyzed against 84 mM NaCl, 2 mM $MgCl_2$, and 10 mM imidazole-HCl (pH 7.0). The concentration of labeled caldesmon was determined by the Lowry assay, and the extent of labeling was determined using an extinction coefficient of 2.6×10^4 M^{-1} cm^{-1} at 495 nm for the probe (*28*). The molar ratio of probe to caldesmon was typically found to be in the range of 0.9−1. In some cases, the modified caldesmon was further purified by sedimentation in the presence of a 4-fold molar excess of F-actin (final actin concentration = 15 μM) in a pH 7.0 buffer at an ionic strength of 60 mM. The pellet was gently homogenized in a buffer containing 500 mM NaCl so that the final actin concentration was <15 μM. Following centrifugation at 45 000 rpm for 1 h, the caldesmon-enriched supernatant was concentrated on an Amicon concentrator. Traces of actin were removed by size exclusion chromatography on a 1.5 cm × 95 cm column of Aca 54 (Spectrum, Los Angeles, CA) equilibrated with 0.5 M KCl, 10 mM imidazole, 1 mM EGTA, and 1 mM EDTA.

Time Course of Binding. Measurements were taken on a DX17.MV/2 sequential stopped flow spectrophotometer (Applied Photophysics, Leatherhead, U.K.) equipped with a thermostated water bath and an excitation monochromator. Emission wavelengths were determined by long-pass filters. Fluorescein and IANBD were assessed with an excitation wavelength of 490 nm and a 515 nm long-pass filter. Light scattering was measured with excitation at 550 nm and a 515 nm long-pass filter. SAL fluorescence was measured with excitation at 302 nm with a 336 nm long-pass filter. Pyrene fluorescence was measured with excitation at 344 nm and a 366 long-pass filter. All experiments were carried out at 15 °C unless otherwise stated. Actin was normally diluted from a 20 μM stock and used immediately. To facilitate mixing at high actin concentrations, the actin was incubated with cytochalasin D (1 mol/100 mol of actin) for 30 min prior to the measurement. No difference was observed between the traces obtained with and without treatment with cytochalasin D. All proteins, other than actin and tropomyosin, were clarified by ultracentrifugation, and all buffers were passed through 0.2 μm filters prior to the experiment. Most data are the average of three to five traces. Equations for single-, double-, or triple-exponential functions were fit to the data with the software provided in the Applied Photophysics package (Levenberg−Marquardt algorithm). To ensure that the changes in fluorescein fluorescence were not due to changes in pH, controls were run both at a high buffer concentration (30 mM at pH 7) and at higher pH (pH 8.0). Similar results were observed under all conditions.

RESULTS

Fluorescein-S1. Probes were placed on both S1 and caldesmon so that the binding of both proteins to actin could be monitored. Several different fluorescent probes were used to distinguish events that are due to binding from those that

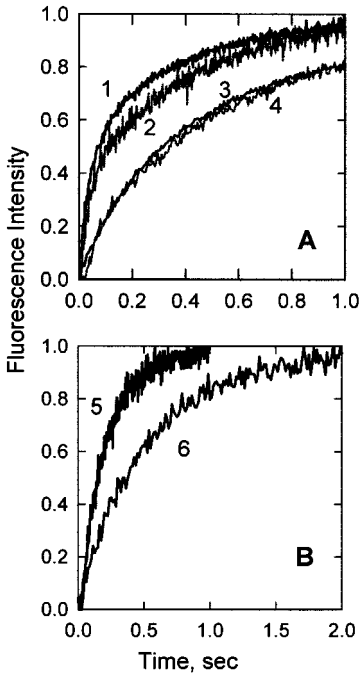


FIGURE 1: Time course of binding of fluorescein-labeled S1 to actin−tropomyosin in the absence and presence of caldesmon. (A) Both fluorescein fluorescence (curves 1 and 3; black lines) and light scattering (curves 2 and 4; gray lines) were monitored. The addition of caldesmon to a final concentration of 0.1 μM (curves 3 and 4) reduced the rate of binding compared to curves 1 and 2. The concentration of both fluorescein-S1 and actin−tropomyosin was 0.75 μM after mixing in the stopped flow. (B) The binding of fluorescein-labeled S1 to actin in the absence (curve 5) and presence (curve 6) of caldesmon under pseudo-first-order conditions. The concentrations of proteins after mixing were 1 μM actin−tropomyosin, 0.071 μM fluorescein-S1, and either 0 or 0.1 μM caldesmon. Conditions were as follows: 15 °C in a buffer containing 34 mM potassium propionate, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM dithiothreitol.

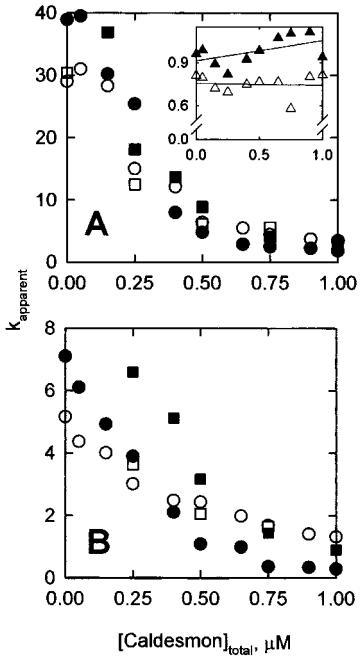


FIGURE 2: Caldesmon reduces the rate of binding of fluorescein-labeled S1 to actin in the absence (white symbols) and presence (black symbols) of smooth muscle tropomyosin. Squares and circles represent different protein preparations. The kinetic transients were analyzed with a double-exponential model: (A) fast process and (B) slow process. The inset of panel A shows the total fluorescence amplitude, a measure of the amount of fluorescein-S1 bound to actin at equilibrium. The protein concentrations after mixing were 3.5 μM actin−tropomyosin and 0.6 μM fluorescein-S1. The buffer is the same as described in the legend of Figure 1.

occur after binding. Light scattering was also used because it is sensitive only to the mass on the actin filament and not to small conformational changes that may occur after binding. Curve 2 of Figure 1A shows the time course of light scattering that occurred when 1.5 μM fluorescein-labeled S1 was mixed with either 1.5 μM actin−tropomyosin (giving a final concentration of 0.75 μM for S1 and actin) or 1.5 μM the actin−tropomyosin−caldesmon complex (final caldesmon concentration after mixing of 0.1 μM). Caldesmon reduced the rate of binding appreciably (curve 4) but did not alter the extrapolated end point of the reaction. An additional slow increase in light scattering occurred after the changes shown here even in the absence of caldesmon. By monitoring fluorescence, we eliminated the slow process, and there was an improvement in the signal-to-noise ratio. Curves 1 and 3 of Figure 1A were obtained under the same conditions as curves 2 and 4 except that a fluorescein probe on S1 was monitored. As in the case of light scattering measurements, caldesmon caused a reduction in the rate of binding but not a large reduction in the extrapolated amplitude of binding. The similarity of the fluorescence measurements and light scattering measurements indicates that caldesmon caused a real reduction in the rate of binding. No fluorescence change occurred when fluorescein-S1 was mixed with a large excess (10 μM) of caldesmon, so the observed changes were due to S1−actin binding.

Figure 1B shows the effect of caldesmon on the fluorescence of fluorescein-S1 under pseudo-first-order conditions. The binding of fluorescein-S1 to actin−tropomyosin could be well described by a double-exponential function. The presence of caldesmon reduced the apparent rates of both the fast and slow phases of the reaction.

The caldesmon concentration dependence of the apparent rate constants for fluorescein-S1 binding to actin is plotted in panels A and B of Figure 2. Caldesmon reduced the apparent rate constant for both the fast process (Figure 2A) and the slow process (Figure 2B). The rate of binding of S1 to actin was slightly faster in the presence of tropomyosin particularly at low caldesmon concentrations (compare the white and black symbols of Figure 2A at caldesmon concentrations of <0.25 μM). The rate of the fast phase of the reaction decreased ~12-fold in the presence of tropomyosin (from 40 to 3 s^{-1}) and 10-fold (from 30 to 3 s^{-1}) in the absence of tropomyosin. The slow phase of the reaction in Figure 2B is similarly affected by tropomyosin. Tropomyosin appears to enhance the competition between S1 and caldesmon for binding to actin. A similar conclusion was reached in equilibrium studies of the binding of the S1−AMP-PNP complex and caldesmon to actin (*15*).

Although caldesmon decreased the rate constant for binding, the effect of caldesmon on the amount of S1 bound at equilibrium under these conditions was much lower in magnitude. The total fluorescence amplitude (sum of the amplitudes of the fast and slow phases) did not change appreciably with increasing caldesmon concentrations (see the inset of Figure 2A). Over that same range of caldesmon concentrations, the apparent rate constants for binding

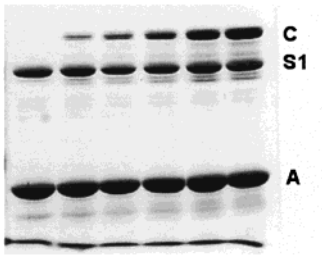


FIGURE 3: Polyacrylamide (8%)−SDS gel showing the effect of increasing caldesmon concentrations on the amount of fluorescein-labeled S1 bound to actin−tropomyosin at equilibrium under the conditions described in the legend of Figure 1. The gels were run on the actin pellets following high-speed sedimentation. The concentrations of S1, actin, and tropomyosin were 0.6, 3.5, and 0.75 μM, respectively. Caldesmon "C" concentrations were as follows from left to right: 0, 0.1, 0.2, 0.3, 0.5, and 1.0 μM. The amount of S1 that sedimented in the absence of actin was insignificant compared to the amounts bound to actin.

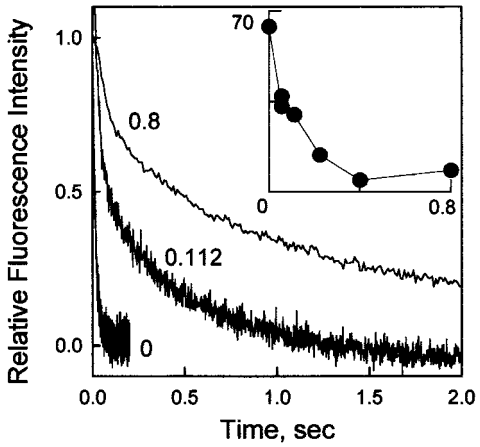


FIGURE 4: Effect of caldesmon on the apparent rate constant of pyrene-actin fluorescence change upon binding to rigor S1. Conditions were as follows: 0.25 μM actin−tropomyosin and 2.5 μM S1 at 15 °C and the same buffer as described in the legend of Figure 1. The caldesmon concentration was 0, 0.11, or 0.8 μM as indicated in the figure. The inset shows the apparent rate constant for the fast rate process as a function of caldesmon concentration. The apparent rate constant decreased from 64 s^{-1} without caldesmon to 8.2 s^{-1} with 0.8 μM caldesmon.

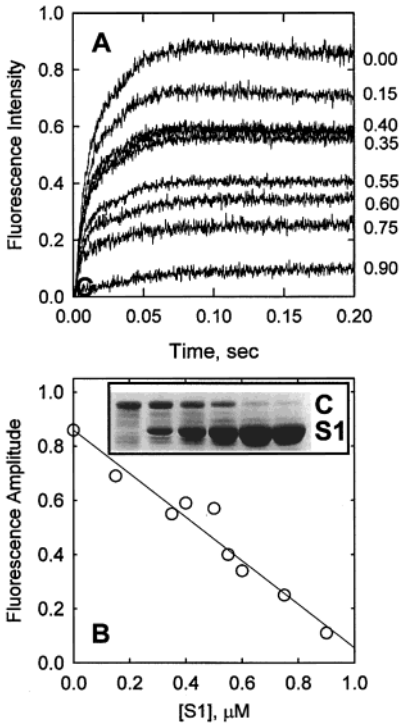


FIGURE 5: Preloading actin−tropomyosin with S1 reduces the amount of caldesmon that can bind to actin. (A) Time course of binding of NBD-caldesmon to actin−tropomyosin at different levels of saturation of actin with S1. The increase in fluorescence resulted from the binding of caldesmon to actin. The concentrations after mixing in the stopped flow were 1 μM actin−tropomyosin and 0.4 μM caldesmon. The concentration of S1 after mixing is shown beside the appropriate transient. All curves can be represented with double-exponential functions with varied amplitudes but with constant apparent rate constants (k_1 = 240 s^{-1} and k_2 = 40 s^{-1}). (B) Fluorescence amplitudes plotted as a function of the concentration of S1. The amplitude was reduced to 6% of the initial value when [S1] = [actin]. The temperature was 15 °C, in the same buffer described in the legend of Figure 1. The inset shows an 8% polyacrylamide−SDS gel showing the displacement of caldesmon by S1 in sedimentation assays. The mixture contained 3.5 μM actin, 0.75 μM smooth tropomyosin, 0.5 μM caldesmon, and 0, 0.5, 1, 2, 3, and 3.5 μM S1 (from left to right). The amount of caldesmon present in the pellet at the two highest S1 concentrations is similar to that observed for the sedimentation of caldesmon in the absence of actin (not shown).

decreased markedly. The lack of an effect of caldesmon on the amount of S1 bound to actin at equilibrium was confirmed, in Figure 3, by analysis of the amount of S1 that cosedimented with actin following ultracentrifugation. Increasing concentrations of caldesmon had little effect on the amount of S1 that cosedimented with actin−tropomyosin. In this experiment, one S1 molecule was bound per six actin monomers so there was sufficient open space on the actin filament for caldesmon to bind to actin (*11*). When the actin was more fully saturated with S1, binding of caldesmon could not occur.

Pyrene-Actin. A pyrene probe attached to the penultimate thiol residue of actin is sensitive to the binding of S1 to actin (*25*). If caldesmon reduces the rate of binding of fluorescein-S1 to actin, then this effect should also be visible in changes in the fluorescence of pyrene-labeled actin. Figure 4 shows the time courses of fluorescence changes that occur upon binding of S1 to pyrene-labeled actin under pseudo-first-order conditions. The curves could be well described by a double-exponential function indicating that the binding reaction occurred in at least two steps. The apparent rate constants for both the fast rate process and the slow rate process were reduced in the presence of caldesmon. The inset of Figure 4 shows that the apparent rate constant for the fast rate process decreased from 64 s^{-1} without caldesmon to 8.2 s^{-1} with 0.8 μM caldesmon.

NBD-Caldesmon. If S1 and caldesmon are competitive toward binding to actin, then S1 should inhibit the binding of caldesmon to actin. The caldesmon−actin interaction was monitored by the change in fluorescence of an NBD probe on caldesmon. Since NBD and fluorescein have similar excitation and emission spectra, transients observed for the binding of NBD-caldesmon to actin decorated with fluorescein-S1 were complex. The initial decrease in fluorescence due to dissociation of NBD-caldesmon was followed by a much larger increase in fluorescence as fluorescein-S1 bound to actin (not shown). A more convincing demonstration of the effect of S1 on NBD-caldesmon binding was obtained by mixing NBD-caldesmon with actin containing varying amounts of unlabeled S1. Figure 5 shows the effect of rigor S1 on the binding of NBD-caldesmon to actin−tropomyosin.

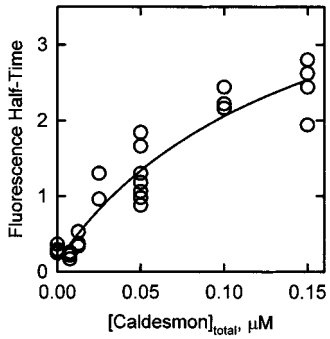


FIGURE 6: Dependence of the time for 50% completion of the binding of ISAL-labeled S1 to actin−tropomyosin with varied caldesmon concentrations. The concentrations of the proteins after mixing were 0.25 μM actin, 0.07 μM smooth muscle tropomyosin, and 0.25 μM SAL-S1. The caldesmon concentration plotted is that at the beginning of the reaction after mixing. The temperature was 15 °C, in the same buffer described in the legend of Figure 1.

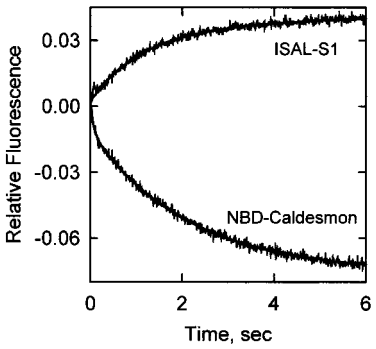
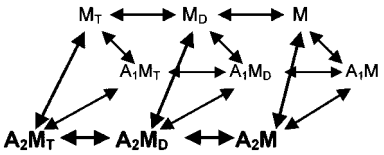


FIGURE 7: Kinetics of NBD-caldesmon detachment and SAL-S1 attachment to actin−tropomyosin when 0.5 μM ISAL-labeled S1 was rapidly mixed with 0.5 μM actin−tropomyosin containing 0.1 μM NBD-caldesmon. The fluorescence of each probe was measured separately by changing the wavelengths for excitation and emission in the stopped-flow apparatus. Note that one caldesmon molecule was initially bound per 11−12 actin monomers, which was calculated from the equation of McGhee and von Hipple (*30*) and the following parameters: $K = 5 \times 10^6$ M^{-1}, $n = 7$, and $\omega = 7$.

In Figure 5A, the NBD fluorescence is shown as a function of time for a series of reactions in which the S1 concentration was increased from 0 to 0.9 μM and the fraction of saturation of actin increased from 0 to 0.9. Because the binding of rigor S1 to actin was of a much higher affinity than the binding of NBD-caldesmon and due to the slow dissociation rate constant for rigor S1, increasing the amount of bound S1 had the effect of decreasing the total amount of actin available for binding. The amplitude of the fluorescence change decreased linearly with the extent of saturation of actin with S1 (Figure 5B). This observation was confirmed by SDS gel electrophoresis (inset of Figure 5B). Therefore, caldesmon and S1 have reciprocal effects on binding to actin, although differences in affinity and rates of association and dissociation cause these effects to be manifested in different ways.

SAL-S1 and NBD-Caldesmon. Because the affinity of both caldesmon and S1 may be changed by chemical modification, it is preferable to have a probe on both proteins in competition experiments. This was made possible by changing the probe on S1 to SAL. The results of an experiment in which equimolar amounts of SAL-S1 and actin−tropomyosin were mixed in the presence of variable amounts of caldesmon are shown in Figure 6. The results are shown as a plot of the time required to reach 50% of the maximum fluorescence amplitude as a function of the total caldesmon concentration after mixing. The time for 50% completion increased by 16-fold as the caldesmon concentration was increased to 0.15 μM. Therefore, NBD-labeled caldesmon inhibited the rate of SAL-S1 binding just as unlabeled caldesmon inhibited the rate of fluorescein-S1 binding to actin. Time courses for changes in the fluorescence of probes on both S1 and caldesmon are shown in Figure 7. The binding of SAL-S1 to actin−tropomyosin occurred with an increase in fluorescence. In contrast to the 4-(iodoacetamido)salicylic acid used here, the fluorescence of 5-(iodoacetamido)salicylic acid-labeled S1 has been reported to decrease upon binding to actin (*29*). During the period over which the SAL-S1 bound to actin, there was a decrease in fluorescence due to the detachment of caldesmon from actin−tropomyosin. If there is a ternary complex in which both S1 and caldesmon are bound directly to the same unit of actin−tropomyosin, this complex must have a very short half-life.

Scheme 1



DISCUSSION

A reasonable starting point for a discussion of muscle regulation is the model that was proposed by Hill et al. (*31*, *32*) for striated muscle regulation. An abbreviated form of that model is shown in Scheme 1. Three of the possible chemical states of myosin are shown in this scheme: myosin bound to ATP (M_T), bound to ADP (M_D), or without bound nucleotide (M). In this model, A_1 and A_2 are different states of actin. State A_2 is more active in stimulating ATPase activity and is stabilized by the binding of rigor S1 to actin. In the case of troponin-tropomyosin, state A_1 has very low activity in the absence of Ca^{2+} and a higher, but not maximum, activity in the presence of Ca^{2+}; this effect of Ca^{2+} will not be considered in the case of caldesmon. Myosin can bind to A_1 but with perhaps a lower affinity than A_2.

This model is appropriate here because smooth muscle contains tropomyosin, and there is evidence for a cooperative transition between two smooth muscle actin−tropomyosin states (*33*−*36*). Smooth muscle tropomyosin increases the k_{cat} of actin-activated ATP hydrolysis by 3-fold compared to actin alone (*37*). High concentrations of tight binding but inactive S1 (NEM-S1) stabilize a more active form of the actin filament characterized by a further 1.3-fold increase in k_{cat} and a reduction of the actin concentration required to reach 50% of the k_{cat} to $^1/_7$ of the value in the absence of the high-affinity S1. This "potentiation" by rigor-type S1 binding is another characteristic of cooperativity.

In the model shown in Scheme 1, inhibition of ATPase activity could be caused by preventing myosin from binding to both forms of actin, by stabilizing the inactive form of actin, or by inhibiting the transition between two forms of myosin such as the transition between M_T and M_D. It is known that caldesmon reduces the level of binding of

myosin-bound ATP (M_T) to the pure actin and to actin-tropomyosin (*7*, *8*). We proposed that this inhibition of binding is largely responsible for the inhibition of ATPase activity by caldesmon (*7*). However, other workers have reported that the inhibition of binding requires higher concentrations of caldesmon than required to inhibit the ATPase activity (*11*, *12*). Our subsequent work confirmed our earlier data and indicated that the reduction in the level of binding of myosin to actin played a significant role in the inhibition of ATPase activity (*16*, and references therein). We will present additional supportive evidence elsewhere that caldesmon inhibits the binding of S1-labeled ATP to actin−tropomyosin.

The results presented in this paper support the general concept of competition of binding between caldesmon and S1. Thus, caldesmon inhibited the rate of binding of rigor S1 to actin−tropomyosin, and rigor S1 prevented caldesmon from binding to actin in proportion to the fraction of actin covered by S1. We had shown earlier that caldesmon more effectively inhibits the equilibrium binding of M_T to actin than the binding of M_D or M to actin (*13*). This occurs because the affinity of caldesmon for actin is much stronger than the affinity of S1-labeled ATP for actin, but it is weaker than the affinity of rigor S1 for actin. It is likely that caldesmon also inhibits the rate of binding of S1-labeled ATP to actin−tropomyosin, but we were unable to measure the rate of S1-labeled ATP binding because of the high rate of that reaction.

The effect of caldesmon on the rate of nucleotide free S1 binding to actin−tropomyosin is large. In the absence of caldesmon, the apparent rate of fluorescein-labeled S1 binding was observed to be more than 16-fold greater than that at high caldesmon concentrations (Figure 2). Even subsaturating caldesmon conentrations reduced the apparent rate of binding (increased the half-life) of unmodified S1 to pyrene-labeled actin by more than 8-fold (Figure 4). To provide perspective, troponin-tropomyosin reduces the rate of binding of S1 to actin−tropomyosin by ∼4−5-fold (Figure 1 of ref *38* and Figure 4 of ref *39*). One interpretation of the troponin data is that the ratio of rates of binding reflects a change in the number of sites available for binding (*40*). In the caldesmon case, the data would be interpreted as >95% of the actin sites blocked by the tropomyosin−caldesmon complex. An alternative explanation is that caldesmon stabilized actin into an inactive state (A_1) that does not bind to S1 as readily as in the active state. Such a model was proposed for regulation by troponin (*41*). A third possibility is that that caldesmon but not tropomyosin overlaps the S1 binding site and reduces the rate of binding. That is, caldesmon itself inhibits the binding of S1 to actin without the necessity of moving the tropomyosin.

It is most likely that caldesmon inhibits the binding of S1 by a mechanism that is largely independent of tropomyosin and changes in the distribution between A_1 and A_2. Thus, the observed magnitude of the inhibition of the rate of S1 binding to actin and the caldesmon concentration dependence of inhibition were not greatly different in the presence and absence of tropomyosin (Figure 1). In the presence of tropomyosin, the total decrease in the rate of S1 binding to actin was ≈20% greater than in the absence of tropomyosin. This 20% difference could be due to stabilization of the A_1 state, but this is obviously not the major reason for the inhibition of the rate of S1 binding. This augmentation by tropomyosin is consistent with an earlier observation made at equilibrium that caldesmon more effectively competed with the binding of the S1−AMP-PNP complex in the presence of tropomyosin (*15*).

Several reports suggest the possibility that caldesmon functions by altering the distribution of actin−tropomyosin such as between the A_2 and A_1 states. For example, caldesmon was found to inhibit the cooperative increase in ATPase activity of HMM caused by the addition of NEM-S1 (*8*). The catalytically inactive NEM-S1 was thought to increase the ATPase activity by binding tightly to and stabilizing the active form of actin−tropomyosin, A_2. In another report, the binding of NEM-S1 to actin−tropomyosin was not cooperative but became cooperative in the presence of either caldesmon or troponin (*42*). However, the binding of NEM-S1 is likely to cause detachment of caldesmon from actin−tropomyosin as reported here for rigor S1. In the limit of saturating S1 concentrations, one might expect virtually complete detachment of caldesmon (Figure 5). Changes in ATPase activity and S1 binding are to be expected from a decrease in the amount of bound caldesmon for two reasons. First, the detachment of each caldesmon molecule opens several actin sites so that they may interact with S1. Second, as S1 binds to actin−tropomyosin, there is likely to be a cooperative transition as the tropomyosin position on actin is changed by the action of S1. The extent to which the effects of caldesmon on the properties of actin−tropomyosin are due to direct effects of caldesmon on the state of actin-tropomyosin must be considered uncertain. Our results indicate that unless the binding of both caldesmon and S1 to actin is assessed under identical conditions, it is impossible to distinguish changes in conformation from changes in composition of the actin filament.

Despite the uncertainties caused by the tendency of caldesmon to displace bound S1, there is reason to believe that caldesmon may alter the distribution of actin states. Caldesmon reduces the k_{cat} for actin-activated ATP hydrolysis, although the extent to which that occurs varies widely among the reports (*7*, *43*, *44*). Nevertheless, a k_{cat} effect does seem to occur to some extent, and this could result either by inhibition of the transition from inactive actin A_1 to active actin A_2 or by inhibition of another transition between two actin−myosin species such as P_i release (A_2M_T to A_2M_D). The 20% increase in the effectiveness of caldesmon in inhibiting the rate of S1 binding to actin that is caused by tropomyosin might result from partial stabilization of the A_1 state. Szczesna et al. (*45*) have reported that caldesmon changes the orientation of S1 bound to actin. This could be interpreted as a change in the distribution of actin states or myosin states. Also, a fragment of caldesmon has been produced that accelerates the actin−tropomyosin-activated ATPase activity of HMM (*46*). This fragment was assumed to function by stabilizing the active state of the actin filament, A_2.

Our present and past binding studies are not inconsistent with an inhibitory effect of caldesmon in addition to that resulting from competitive inhibition of S1 binding. We reported earlier that approximately one ATP-bound S1 molecule may remain bound per seven actin monomers even in the presence of saturating caldesmon (*16*). The ATPase activity of that residual ATP-bound S1 was inhibited, but

the mechanism of that inhibition was not determined. One possibility is that the residual S1 was not bound to actin at all but tethered nonproductively to caldesmon. Alternatively, since caldesmon does not necessarily bind uniformly along the actin surface, S1 may be able to bind to one actin out of a unit of seven actin monomers in a tropomyosin unit. This possibility was considered in an earlier publication (*15*). In the work presented here with specific fluorescent probes on caldesmon, actin and S1, there is no evidence that S1 can bind to actin that has bound caldesmon. This means that the residual bound S1 identified by sedimentation experiments is either bound to caldesmon and not to actin or bound to actin in a very different way so that a fluorescence signal change is not produced in either S1 or actin. In the latter case, the inhibition of the activity of the residual S1 would not be competitive.

The question of whether caldesmon and troponin are mechanistically identical in affecting the binding of S1 to actin may be asked. We consider this to be unlikely. As already discussed, the effect of caldesmon on S1 binding was independent of tropomyosin. Also, as S1 bound to actin-tropomyosin, the caldesmon detached rapidly (Figure 7) and virtually completely (Figure 5). Therefore, changes in the properties of the actin filament that occur with an increasing level of binding of S1 cannot be interpreted simply as changes in the conformation in a caldesmon−actin-tropomyosin complex as they had been in the case of tropomyosin-troponin (*32*, *39*). It is also noteworthy that a lag in binding of excess S1 to the actin−tropomyosin−caldesmon complex was not observed (Figures 1 and 4) in contrast to binding to actin−tropomyosin-troponin at low free Ca^{2+} concentrations (*38*, *39*).

A problem faced by competitive binding mechanisms in cellular systems is that the displaced substrate may diffuse away. In the case of caldesmon, one might imagine that following myosin binding, the rate of inhibition would be slow because of the rate of diffusion of caldesmon to the actin filament. There are several points that need to be considered here. First, myosin dephosphorylation will lead to a decrease in activity in the absence of bound caldesmon. That is, inactivation need not be limited by the rebinding of caldesmon. Second, the displacement of caldesmon from actin binding does not necessarily mean that caldesmon is free to diffuse away. Caldesmon does bind to myosin (*9*, *13*), and this may limit caldesmon diffusion. Furthermore, caldesmon interacts with several actin monomers, and the interaction sites appear to be heterogeneous. It is possible that at low levels of myosin binding to actin, caldesmon might be displaced from some actin monomers but held by others at a lower affinity (*15*).

The results presented here are important for testing models for the binding of caldesmon and S1 to actin such as those developed previously (*15*). An accurate description of this binding, together with more detailed information on possible direct effects of caldesmon on other steps in the ATP hydrolysis pathway (Scheme 1), is needed for a complete description of the mechanism by which caldesmon inhibits ATPase activity. An even more ambitious undertaking is the description of the interrelationship of myosin-based (phosphorylation) and actin-based (caldesmon, tropomyosin, and perhaps other proteins) regulatory systems in smooth muscle. Models of caldesmon and S1 binding to actin and the effect of caldesmon on ATPase activity are being developed and will be presented in future publications.

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REFERENCES

1. Sobue, K., Muramoto, Y., Fujita, M., and Kakiuchi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5652−5655.
2. Brenner, B., Yu, L.C., and Chalovich, J. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5739−5743.
3. Pfitzer, G., Zeugner, C., Troschka, M., and Chalovich, J. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5904−5908.
4. Malmquist, U., Arner, A., Makuch, R., and Dabrowska, R. (1996) *Pfluegers Arch. 432*, 241−247.
5. Smith, C. W. J., Pritchard, K., and Marston, S. B. (1987) *J. Biol. Chem. 262*, 116−122.
6. Velaz, L., Hemric, M. E., Benson, C. E., and Chalovich, J. M. (1989) *J. Biol. Chem. 264*, 9602−9610.
7. Chalovich, J. M., Cornelius, P., and Benson, C. E. (1987) *J. Biol. Chem. 262*, 5711−5716.
8. Horiuchi, K. Y., and Chacko, S. (1989) *Biochemistry 28*, 9111−9116.
9. Chalovich, J. M., and Pfitzer, G. (1997) in *Cellular Aspects of Smooth Muscle Function* (Kao, C. Y., and Carsten, M. E., Eds.) pp 253−287, Cambridge University Press, Cambridge, U.K.
10. Chalovich, J. M., Sen, A., Resetar, A., Leinweber, B., Fredricksen, R. S., Lu, F., and Chen, Y.-D. (1998) *Acta Physiol. Scand. 164*, 427−435.
11. Marston, S. B., and Redwood, C. S. (1992) *J. Biol. Chem. 267*, 16796−16800.
12. Marston, S. B., and Redwood, C. S. (1993) *J. Biol. Chem. 268*, 12317−12320.
13. Hemric, M. E., and Chalovich, J. M. (1988) *J. Biol. Chem. 263*, 1878−1885.
14. Ikebe, M., and Reardon, S. (1998) *J. Biol. Chem. 263*, 3055−3058.
15. Chen, Y.-D., and Chalovich, J. M. (1992) *Biophys. J. 63*, 1063−1070.
16. Sen, A., and Chalovich, J. M. (1998) *Biochemistry 37*, 7526−7531.
17. Eisenberg, E., and Kielley, W. W. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 145−152.
18. Kielley, W. W., and Harrington, W. F. (1960) *Biochim. Biophys. Acta 41*, 401−421.
19. Weeds, A. G., and Taylor, R. S. (1975) *Nature 275*, 54−56.
20. Bretscher, A. (1984) *J. Biol. Chem. 259*, 12873−12880.
21. Graceffa, P., Jansco, A., and Mabuchi, K. (1992) *Arch. Biochem. Biophys. 297*, 46−51.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
23. Laemmli, U. K. (1970) *Nature 227*, 680−685.
24. Brenner, S. L., and Korn, E. D. (1983) *J. Biol. Chem. 258*, 5013−5020.
25. Kouyama, T., and Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33−38.
26. Lin, S.-H., and Cheung, H. C. (1991) *Biochemistry 30*, 4317−4322.
27. Lin, S.-H., Harzelrig, J. B., and Cheung, H. C. (1993) *Biophys. J. 65*, 1433−1444.
28. Trayer, H. R., and Trayer, I. P. (1988) *Biochemistry 27*, 5718−5727.
29. Aguirre, R., Gonsoulin, F., and Cheung, H. C. (1986) *Biochemistry 25*, 6827−6835.
30. McGhee, J. D., and vonHippel, P. H. (1974) *J. Mol. Biol. 86*, 469−489.

31. Hill, T. L., Eisenberg, E., and Greene, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3186−3190.
32. Hill, T. L., Eisenberg, E., and Chalovich, J. M. (1981) *Biophys. J. 35*, 99−112.
33. Graceffa, P. (1999) *Biochemistry 38*, 11984−11992.
34. Sobieszek, A (1982) *J. Mol. Biol. 157*, 275−286.
35. Williams, D. L., Greene, L. E., and Eisenberg, E. (1984) *Biochemistry 23*, 4150−4155.
36. Lehrer, S. S., and Morris, E. P. (1984) *J. Biol. Chem. 259*, 2070−2072.
37. Chacko, S., and Eisenberg, E. (1990) *J. Biol. Chem. 265*, 2105−2110.
38. Trybus, K. M., and Taylor, E. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7209−7213.
39. McKillop, D. F. A., and Geeves, M. A. (1993) *Biophys. J. 65*, 693−701.
40. McKillop, D. F. A., and Geeves, M. A. (1991) *Biochem. J. 279*, 711−718.
41. Chen, Y.-D., Yan, B., Chalovich, J. M., and Brenner, B. (2000) *Biophys. J. 78*, 236A.
42. Marston, S. B., Fraser, I. D. C., and Huber, P. A. J. (1994) *J. Biol. Chem. 269*, 32104−32109.
43. Marston, S. B. (1988) *FEBS Lett. 238*, 147−150.
44. Horiuchi, K. Y., Samuel, M., and Chacko, S. (1991) *Biochemistry 30*, 712−717.
45. Szczesna, D., Graceffa, P., Wang, C.-L. A., and Lehrer, S. S. (1994) *Biochemistry 33*, 6716−6720.
46. Fraser, I. D. C., Copeland, O., Bing, W., and Marston, S. B. (1997) *Biochemistry 36*, 5483−5492.

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