The Mechanism of Smooth Muscle Caldesmon-Tropomyosin
Inhibition of the Elementary Steps of the Actomyosin ATPase*

Mustapha Alahyan†, Martin R. Webb§, Steven B. Marston¶, and Mohammed EL-Mezgueldi†††

From the †Myocardial Systems Biology Group, National Heart and Lung Institute, Imperial College, London SW3 6LY and the ‡Medical Research Council, National Institute for Medical Research, London NW7 1AA, United Kingdom

Caldesmon is a component of smooth muscle thin filaments that inhibits the actomyosin ATPase via its interaction with actin-tropomyosin. We have performed a comprehensive transient kinetic characterization of the actomyosin ATPase in the presence of smooth muscle caldesmon and tropomyosin. At physiological ratios of caldesmon to actin (1 caldesmon/7 actin monomers) actomyosin ATPase is inhibited by about 75%. Inhibitory caldesmon concentrations had little effect upon the rate of S1 binding to actin, S1 dissociation by ATP, and dissociation of ADP from actin-S1-ADP; however the rate of phosphate release from the actin-S1-ADP-Pi complex was decreased by more than 80%. In addition the transient of phosphate release displayed a lag of up to 200 ms. The presence of a lag phase indicates that a step on the pathway prior to phosphate release has become rate-limiting. Premixing the actin-tropomyosin filaments with myosin heads resulted in the disappearance of the lag phase. We conclude that caldesmon inhibition of the rate of phosphate release is caused by the thin filament being switched by caldesmon to an inactive state. The active and inactive states correspond to the open and closed states observed in skeletal muscle thin filaments with no evidence for the existence of a third, blocked state. Taken together these data suggest that at physiological concentrations, caldesmon controls the isomerization of the weak binding complex to the strong binding complex, and this causes the inhibition of the rate of phosphate release. This inhibition is sufficient to account for the inhibition of the steady state actomyosin ATPase by caldesmon and tropomyosin.

Smooth muscle contractility and actomyosin ATP hydrolysis are regulated by Ca2+ at two levels. The first involves phosphorylation of the myosin light chain by myosin light chain kinase upon stimulation by Ca2+-calmodulin. The second occurs via the thin filament-associated protein caldesmon. Caldesmon (CaD)2 binds to actin, calmodulin, and tropomyosin (1) and inhibits the actin activation of both smooth and skeletal muscle myosin ATPase in vitro (2) and force production when added to smooth and skeletal muscle fibers (3–5). Caldesmon also regulates the actin activation of myosin I ATPase in smooth muscle (6).

The smooth muscle thin filament is Ca2+-regulated and is made up of actin, tropomyosin, caldesmon, and a Ca2+-binding protein in molar ratios ~14:2:1:1 (7). Previous studies of the molecular mechanism of caldesmon inhibition of the actomyosin ATPase have focused on its effect on the ATPase steady state parameters K_m and V_max (8) and on the equilibrium binding of myosin to actin filaments (9–12). A detailed mechanism by which caldesmon regulates the actin activation of myosin ATPase and force production has not been demonstrated by such experiments but two potentially conflicting models have been developed.

A competition model proposes that ATPase inhibition by caldesmon is caused by a reduction in the formation of the weakly bound actomyosin complex as a result of caldesmon binding to the same site on actin as S1-ADP-Pi, (13). This is supported by the inability of caldesmon to inhibit the cross-linked actin-S1 complex, which does not dissociate on ATP binding (14) and the reduction of the K_m of the steady state ATPase (8). However this model assumes that the thin filament is static during the activation-relaxation process and the role of tropomyosin in this model is not clear.

In contrast Marston et al., (15) developed a cooperative-allosteric model for caldesmon inhibition of actin-tropomyosin similar to skeletal muscle troponin inhibition of actin-tropomyosin (16). This model is based on the observation that caldesmon at physiological ratio to actin, does not affect the initial weak binding of S1 to actin-tropomyosin but rather the subsequent step of S1 strong binding to actin-tropomyosin (12). Like troponin-tropomyosin, it is proposed that the thin filament exists in two states, an OFF state where only weak actin-S1 binding complexes can be formed (which is unable to activate the S1 ATPase) and an ON state, which permits both weak and strong binding of S1 to actin and actin activation of the S1 ATPase. Caldesmon is the allosteric inhibitor responsible for switching the thin filament from the ON state to the OFF state and tropomyosin propagates the signal along the thin filament. This model is supported by the ability of caldesmon to inhibit the ATPase activity of the cross-linked actin-S1 complex in the presence of tropomyosin (14) and to reduce the V_max rate in the hyperbolic fit; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol.
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SCHEME 1

of the steady state ATPase (8). Furthermore, caldesmon inhibits the cooperative turning ON of the smooth muscle heavy meromyosin ATPase by actin-tropomyosin (17) and the cooperative binding of S1 to actin-tropomyosin (12). Finally we have recently demonstrated a direct correlation between caldesmon inhibition of the actin-S1 ATPase and the conformational change accompanying the switch of the actin-tropomyosin complex to the OFF state, measured by the eximer fluorescence of pyrene iodoacetamide-labeled tropomyosin (18).

Transient kinetic studies of the effect of Ca²⁺ on thin filament activation of the S1 ATPase in skeletal muscle has been successful in probing the effect of the skeletal muscle regulatory protein complex, troponin-tropomyosin on the rate of the elementary steps of the actin-S1 ATPase (19–24). They led to the proposition of a cooperative-allosteric model as a mechanism of regulation of skeletal muscle actin-S1 ATPase. This model proposes that the thin filament exists in three states: a blocked state unable to bind S1, a closed state that binds S1 weakly but is unable to activate its ATPase, and an open state that binds S1 strongly and activates the S1 ATPase. Both the blocked and closed states have low steady state ATPase activity; therefore, description of the ATPase activity requires only a two-state model. Heeley et al. (24) have demonstrated that troponin-tropomyosin inhibits the rate of Pᵢ release. This step immediately follows the conformational transition of actin-S1-ADP-Pᵢ, that is believed to be the force-generating step in muscle and which is a major determinant of the overall ATP turnover rate by the actomyosin complex. Numerous studies of the effect of caldesmon on the binding of S1 to actin have been made (10, 12) although with few exceptions (25, 26) analysis of the kinetics has been restricted to the steady state behavior. In most cases skeletal muscle myosin was used instead of smooth muscle myosin. Using probes on caldesmon and S1, Chalovich and co-workers (25) have shown that caldesmon inhibits the rate of S1 binding to actin without affecting the amount of S1 bound to actin at equilibrium. However interpretation of these experiments is complicated by the use of high caldesmon to actin ratios. The effect of caldesmon on subsequent steps of the actomyosin ATPase has not been investigated.

In this study we analyzed in depth the effect of caldesmon on the elementary steps of the actomyosin ATPase. Scheme 1 represents the generally accepted mechanism of actin activation of the S1 ATPase. ATP binds to the actin-S1 complex and induces its dissociation. S1 hydrolyzes ATP, and the resulting complex S1-ADP-Pᵢ rebinds actin. The binding to actin accelerates the rate of product dissociation (phosphate release followed by ADP release). We analyzed the effect of caldesmon on the rate of S1 and S1-ADP binding to actin-tropomyosin (steps 7 and 8). We found that at physiological concentration, caldesmon moderately reduced the rate of S1 binding. This reduction was caused by a decrease in the rate of an isomerization reaction between two actin-S1 complexes. We also measured the effect of caldesmon on the rate of ATP-induced dissociation of S1 from actin-tropomyosin (steps 1 and 5), the rate of phosphate release (step 3), and the rate of ADP release (step 4). The rate of phosphate release was the only step substantially decreased by caldesmon-tropomyosin.

MATERIALS AND METHODS

Reagents—N-Methylnicotinoyl nucleotides (mant-ATP, mant-ADP) were purchased from Molecular Probes. 3-Mant-2’-deoxy-ATP (mant-dATP) was kindly provided by Prof. H. White (East Virginia Medical School, Norfolk, VA). The concentrations of nucleotides (ATP and ADP) and their derivatives (mant-ATP, mant-dATP, and mant-ADP) were determined by absorbance at 259 nm for ADP and ATP (ε₂₅₉ = 15,400 M⁻¹ cm⁻¹) and 255 nm for the mant nucleotides (ε₂₅₅ = 23,300 M⁻¹ cm⁻¹) (27). All chemicals were of the highest analytical grade.

Proteins—Caldesmon and tropomyosin from chicken gizzard were prepared by our established procedures (28–30).

Rabbit skeletal muscle filamentous actin (referred to as actin in the text) was prepared according to Ref. 31, and monomeric actin was purified by gel filtration following a polymerization–depolymerization cycle of filamentous actin (27). Skeletal muscle S1 was obtained by chymotryptic digestion of myosin (32). S1A1 was separated from S1A2 by chromatography as described by Weeds and Taylor (33) using Q-Sepharose. Crude chicken gizzard myosin was prepared as described by Sellers et al. (34). Smooth muscle S1 was prepared from chicken gizzard myosin by chymotryptic digestion and further purified over a Q-Sepharose column (35). Actin was labeled with pyrene iodoacetamide and purified by gel filtration (36).

The purified proteins were dialyzed against 10 mM PIPES, 10 mM KCl, 2.5 mM MgCl₂, 0.1 mM DTT, pH 7.2 and stored at 4 °C. Caldesmon and S1 were filtered through a 2-μm filter (Millipore). Protein concentrations were determined by the Lowry method. Pyrene concentration was measured by absorbance at 344 nm (ε₄₄₄ = 21,970 M⁻¹ cm⁻¹ or 45.5 μM pyrene OD). Protein purity and activity were analyzed by SDS-polyacrylamide gel electrophoresis and steady state Mg²⁺-ATPase measurements. Caldesmon and actin used in these studies were shown to be free of any tropomyosin contaminant.

Phosphate-binding protein was prepared and labeled with the fluorescent dye, N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) (37) to give the phosphate sensor, MDCC-PBP as described (38, 39).

Steady State ATPase—The actin activation of skeletal and smooth muscle S1 Mg²⁺-ATPase was determined in a medium (100 μl) containing 10 mM PIPES, 10 mM KCl, 2.5 mM MgCl₂, 0.1 mM DTT, pH 7.2. The reaction was started by adding Mg²⁺-ATP to 5 mM, and terminated after 10 min (unless otherwise stated) by adding 0.5 ml of 10% trichloroacetic acid. Pᵢ was determined colorimetrically (40).

Stopped-flow Experiments—All transient kinetic measurements were performed on a Hi-Tech Scientific SF-61 double mixing stopped flow system using a 100-watt Xe/Hg lamp and a monochromator for excitation wavelength selection. Pyrene, mant-ATP, and mant-ADP fluorescence were excited at 364 nm, and emission was monitored through a 400-nm cut-off filter. Light scattering was observed at 90° to
the incident beam using $\lambda_{ex} = 405$ nm and a 400-nm cut-off filter. Experiments measuring the rate of $P_i$ release were made using the same stopped-flow machine in the double mixing configuration. Transient $P_i$ release was measured using the phosphate sensor, MDCC-PBP as described previously (41, 42). Changes in MDCC fluorescence were measured using $\lambda_{ex} = 436$ nm and a 455-nm cut-off filter for emission. Contaminant $P_i$ was removed by incubating the stopped-flow instrument and all solutions with 7-methylguanosine (0.1 mM) and purine nucleoside phosphorylase (0.02 units/ml). The measurements were carried out in ATPase buffer at 25 °C unless otherwise stated. MDCC-PBP was included with S1, ATP, and actin-tropomyosin to eliminate the contribution of residual phosphate to the signal. Usually four to nine transients were averaged for analysis. The data were then fitted to one or two exponentials by a non-linear least square curve fit using the software provided by Hi-Tech. The stated concentrations of reactants are those after mixing in the stopped-flow observation cell.

Kinetic Modeling—With the exception of ADP release, all steps measured in this article showed a hyperbolic dependence of the observed rate constants on the concentration of the component in excess, indicating a two-step mechanism. A general mathematical treatment will be presented here, which is applicable to all steps (except ADP release, which is considered separately at the end).

Scheme 2 represents a two-step mechanism of a binding of a ligand L to a macromolecule A. In the context of the present article, the ligand is S1 myosin head or S1·ADP or S1·ADP-Pi, while the macromolecule is actin-tropomyosin in S1 binding experiments; the ligand is ATP while the macromolecule is the actin-tropomyosin-S1 complex in the actomyosin ATP-induced dissociation. The first step corresponds to the formation of a complex, whereas the second step is an isomerization reaction.

According to this scheme the time course of the reaction is described by a double exponential equation as shown in Equation 1 for all intermediates (43–45),

$$[\text{Intermediate}] = C_0 + C_1e^{-\lambda_1t} + C_2e^{-\lambda_2t} \quad (\text{Eq. 1})$$

where $C_0$ is the concentration of the intermediate at equilibrium, $C_1$ is the amplitude of the fast exponential described by the rate $\lambda_1$, $C_2$ is the amplitude of the slow exponential described by the rate $\lambda_2$. If the experiment monitors the transient approach to equilibrium of AL1 (as may be the case in S1 binding monitored by light scattering), $C_1$ and $C_2$ have the same negative signs. If the reaction is following AL2 (as is the case in S1 binding monitored by actin pyrene fluorescence or phosphate release monitored by the phosphate-binding protein) C1 and C2 have opposite signs, and the fast exponential is displayed as a lag in the time course of the reaction. The exponential rate constants are the roots of the quadratic Equation 2,

$$\lambda_{1,2} = \left((a \pm (a^2 - 4b)^{1/2})/2\right)$$

where $a = (k_{+1}[X] + k_{-1} + k_{+2} + k_{-2})$ and $b = k_{+1}(k_{+2} + k_{-2})[X] + k_{-1}k_{-2}[X]$ is the concentration of the component in excess. The exact solutions are complex; however, the square root approximation yields the approximate rates shown in Equations 3–5,

$$\lambda_1 = k_{+1}[X] + k_{-1} + k_{+2} + k_{-2} \quad (\text{Eq. 3})$$

where Equation 3 is a linear equation.

$$\lambda_2 = k_{+1}(k_{+2} + k_{-2})[X]/(k_{-1}[X] + k_{+1} + k_{+2} + k_{-2}) \quad (\text{Eq. 4})$$

$$\lambda_3 = (k_{+2} + k_{-2})[X]/((k_{+1} + k_{+2} + k_{-2})/k_{+1} + [X]) \quad (\text{Eq. 5})$$

where Equation 5 is the equation for a rectangular hyperbola. If the concentration of the component in excess is increased, the fast rate increases linearly from a minimum value given by $(k_{-1} + k_{+2} + k_{-2})$ where the slope of the line is $k_{+1}$ (from reported values in the literature for the single rate constants for actin-S1 ATPase the minimum value of the fast rate for all steps measured here varies from 300 s$^{-1}$ to more than 5000 s$^{-1}$ and may therefore be over in the dead time of the apparatus). The rate of the slow component varies hyperbolically with the concentration of the component in excess. The hyperbola is described by 3 parameters: the apparent second order rate constant is given by the value from Equation 6,

$$k_{+1}(k_{+2} + k_{-2})/(k_{+1} + k_{+2} + k_{-2}) = k_{+1}k_{+2}/(k_{-1} + k_{+2}) \quad (\text{Eq. 6})$$

if $k_{-2} \ll k_{+2}$. The apparent dissociation constant obtained from the value at half-saturation is given by Equation 7,

$$(k_{+1} + k_{+2} + k_{-2})/k_{+1} \approx k_{-2}/k_{+1} \quad (\text{Eq. 7})$$

(This is the case for the measurement of the rate of phosphate release) or Equation 8,

$$k_{-1} + k_{+2} \approx k_{+1} \quad (\text{Eq. 8})$$

if $k_{-2} \ll k_{+2}$ and $k_{-2} \approx k_{+2}$, and the maximal observed rate constant is given by Equation 9,

$$(k_{+2} + k_{-2}) \approx k_{+2} \quad (\text{Eq. 9})$$

if $k_{-2} \ll k_{+2}$ (46).

The reaction of various actin-S1 complexes with mant-ADP showed a linear relationship with [mant-ADP] and could be modeled by Scheme 3. According to this scheme, the time course of the reaction is described by the single exponential Equation 10 for all intermediates,

$$\text{Fluorescence} = C_0 + C_k e^{-k_{obs}t} \quad (\text{Eq. 10})$$

where $C_0$ is the fluorescence at equilibrium, $C_k$ is the amplitude of the exponential change in the fluorescence signal, and $k_{obs}$ is the rate constant.

$k_{obs}$ is related to the intrinsic rate constants by Equation 11.
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FIGURE 1. Effect of caldesmon on the rate of skeletal S1 binding to actin-TM monitored by actin pyrene fluorescence. A, time course of 0.5 μM S1 binding to pyrene-labeled actin-tropomyosin (2 μM actin, 0.4 μM smooth muscle tropomyosin) in the absence (curve a) and the presence (curve b) of 0.28 μM smooth muscle caldesmon. The solid lines on top of the curves represent a single exponential fit with $k_{obs} = 78$ s$^{-1}$ (curve a) and 50 s$^{-1}$ (curve b). The concentrations quoted here refer to those after mixing in the reaction chamber. Buffer: 10 mm PIPES pH 7.2, 10 mM KCl, 2.5 mM MgCl$_2$, 1 mM DTT, 1 mM NaN$_3$, at 20°C. B, observed pseudo-first order rate constants ($k_{obs}$) were obtained by fitting the transients at each actin concentration to a single exponential. Conditions were as follows: actin (2–9 μM), 0.5 μM skeletal muscle S1A1, smooth muscle tropomyosin 1/5 of actin, caldesmon 1/7 of actin. The solid lines represent a fit of the observed rates to the equation for a hyperbola: $k_{obs} = k_{+8b}[A]/[A] + [A]$ in which $k_{+8a} = 5.7$ μM$^{-1}$s$^{-1}$ and $k_{+8b} = 385$ s$^{-1}$ in the absence of caldesmon (open circles) and $k_{+8a} = 10.49$ μM$^{-1}$s$^{-1}$ and $k_{+8b} = 338$ s$^{-1}$ in the presence of caldesmon (open circles).

$$k_{obs} = k_{+}[\text{mant-ADP}] + k_{-1} \quad \text{(Eq. 11)}$$

The slope of this line gives $k_{+1}$ whereas the intercept gives $k_{-1}$.

RESULTS

Studies of the effect of regulatory proteins on the kinetics of the actomyosin ATPase in skeletal muscle have been extensive and led to a fairly detailed mechanism in which the inhibited steps have been characterized. With the recent development of probes to monitor most of the elementary steps of the actomyosin ATPase, it is possible to attempt a detailed investigation of the effect of smooth muscle regulatory proteins on these steps. As a preliminary to the transient investigations, steady state ATPases were measured in every experiment. At 10 mM KCl, 25°C, 14 μM skeletal muscle actin activation of 2 μM skeletal muscle S1 ATPase activity varied slightly between 2.1 s$^{-1}$ and 3.1 s$^{-1}$ in the presence of saturating concentration of tropomyosin (1 mol of TM/5 mol of actin monomers). Addition of smooth muscle caldesmon at 1 mol of caldesmon/7 mol of actin inhibited the ATPase by 70–85%.

I-Effect of Caldesmon on the Interaction of Smooth and Skeletal Muscle S1 to Actin-Tropomyosin Filaments—The binding of myosin to actin is routinely studied by monitoring the increase in light scattering upon complex formation or decrease in the fluorescence of pyrene iodoacetamide, covalently attached to actin resulting from the formation of a strong actin-S1 complex (47). The association of S1 with actin was studied under pseudo-first order kinetics. Pseudo-first order kinetics could be achieved by [actin] >> [S1] or [S1] >> [actin]. We have chosen the first possibility for 3 reasons: 1) the kinetics of S1 binding to actin in the case where [S1] >> [actin] is more complex to analyze. As S1 binds to the actin filament in the OFF state, S1 will switch the filament to the ON state, and the kinetics of binding may change during the course of the reaction. This binding reaction cannot be described by an analytical solution. 2) It is known that S1 concentrations higher than actin will displace caldesmon. 3) Conditions in vivo correspond to [actin] >> [S1].

Fig. 1A (curve a) shows the time course of the change in the pyrene fluorescence upon mixing 0.5 μM skeletal muscle S1 with 2 μM skeletal muscle actin in the presence of 0.4 μM smooth muscle tropomyosin. The fluorescent transient was best fit with a single exponential function with an observed rate constant of 78 s$^{-1}$. Addition of an inhibitory concentration of caldesmon (0.28 μM) slightly decreased the observed rate constant (50 s$^{-1}$) (Fig. 1A, curve b). The amplitude of the fluorescence signal (reflecting the amount of S1 bound at equilibrium) was however not affected by the presence of either tropomyosin or caldesmon or both. The observed rate constant increased with the actin concentration (Fig. 1B), and the data deviated from linearity and could be fit well to a rectangular hyperbola. The data were modeled using a two-step reaction (step 8 in Scheme 1, subdivided to 2 steps: $k_{-8a}$ and $k_{-8b}$ shown in Scheme 4.

$$A-TM + M \rightleftharpoons A-TM-M \rightleftharpoons A-TM-M$$

$\text{SCHEME 4}$

At 20°C, the apparent second order rate constant, corresponding to $k_{-8a}$ ($k_{-8b}$) obtained from the linear fit of the data up to 2 μM is 40 μM$^{-1}$s$^{-1}$ for pure actin (data not shown), which is in good agreement with the value of 60 μM$^{-1}$s$^{-1}$ reported by E. Taylor (48) given the slightly higher ionic strength we used. The presence of smooth muscle tropomyosin seems to slightly increase the apparent second order rate constant (46 μM$^{-1}$s$^{-1}$) whereas caldesmon-tropomyosin decreased the apparent second order rate constant (26
The apparent dissociation constant obtained at half-saturation is given by \( \frac{k_{-a} + k_{-ab}}{k_{+a}} \) and is increased by a factor of 2 in the presence of caldesmon (5.4 \( \mu M \) for actin-tropomyosin and 10.6 \( \mu M \) for actin-tropomyosin-caldesmon). The maximum rate given by \( k_{+b} \) could not be accurately defined because at actin concentrations higher than 9 \( \mu M \) the fluorescence change becomes very small in comparison to the total signal. Extrapolation by curve fitting gives \( k_{+b} = 385 \) s\(^{-1}\) in the absence of caldesmon and 338 s\(^{-1}\) in the presence of caldesmon.

To define precisely the effect of caldesmon on the maximum rate, we used a different experimental design. The binding of skeletal muscle S1 to actin-tropomyosin was examined by varying the actin-tropomyosin concentration at a fixed actin/S1 ratio of 5:1. The observed amplitude of the fluorescence signal decreased slightly with increasing rate constants as predicted from signal loss during the dead time of the apparatus.

## Table 1

| Condition                        | Apparent second order rate constant \( \mu M^{-1} \) s\(^{-1}\) | Dissociation constant \( \mu M \) | Maximum rate \( s^{-1} \) |
|----------------------------------|---------------------------------------------------------------|----------------------------------|--------------------------|
| Actin-TM                         | 56 ± 9.6                                                      | 2.88 ± 0.22                     | 287 ± 6.6                |
| Rigor skeletal muscle S1         |                                                               |                                  |                          |
| Pyrene fluorescence              |                                                               |                                  |                          |
| Actin-TM-CaD (1 CaD/7 actin)     | 36 ± 3.9                                                      | 4.58 ± 0.61                     | 262 ± 10.9               |
| Rigor skeletal muscle S1         |                                                               |                                  |                          |
| Pyrene fluorescence              |                                                               |                                  |                          |
| Actin-TM                         | 47.7 ± 1.8                                                    | 5.3 ± 0.78                      | 398 ± 26                 |
| Rigor skeletal muscle S1         |                                                               |                                  |                          |
| Light scattering                 |                                                               |                                  |                          |
| Actin-TM-CaD                     | 35 ± 2.2                                                      | 6.9 ± 1                         | 327 ± 23                 |
| Rigor skeletal muscle S1         |                                                               |                                  |                          |
| Light scattering                 |                                                               |                                  |                          |
| Actin-TM                         | 10.2 ± 1.6                                                    | 12.3 ± 2.5                      | 194 ± 19                 |
| Rigor smooth muscle S1           |                                                               |                                  |                          |
| Pyrene fluorescence              |                                                               |                                  |                          |
| Actin-TM-CaD (1 CaD/7 actin)     | 4.4 ± 0.2                                                     | 19 ± 6.2                        | 155 ± 28                 |
| Rigor smooth muscle S1           |                                                               |                                  |                          |
| Pyrene fluorescence              |                                                               |                                  |                          |
| Actin-TM                         | 4.7 ± 0.13                                                    | 12.3 ± 2.8                      | 95 ± 10.4                |
| Skeletal muscle S1 + ADP         |                                                               |                                  |                          |
| Pyrene fluorescence              |                                                               |                                  |                          |
| Actin-TM-CaD                     | 2.4 ± 0.08                                                    | 4.56 ± 1.1                      | 23 ± 1.87                |
| Skeletal muscle S1 + ADP         |                                                               |                                  |                          |
| Pyrene fluorescence              |                                                               |                                  |                          |

FIGURE 2. Effect of caldesmon on the observed rate of S1 binding to actin-TM as a function of actin concentration monitored by pyrene fluorescence and light scattering. A shows the plot of the observed rate constants of skeletal muscle S1 binding to actin-tropomyosin in the absence (closed circles) and presence (open circles) of caldesmon monitored by the fluorescence of actin-pyrene against increasing concentration of actin. B shows the plot of the observed rate constants of skeletal muscle S1 binding to actin-tropomyosin in the absence (closed circles) and presence (open circles) of caldesmon monitored by light scattering as a function of actin concentration. C shows the plot of the observed rate constants of smooth muscle S1 binding to actin-tropomyosin in the absence (closed circles) and presence (open circles) of caldesmon monitored by the actin-pyrene fluorescence against increasing concentrations of the pyrene-labeled actin. The solid lines represent a fit of the observed rates to a hyperbola as in Fig. 1 (see Table 1 for the derived kinetic parameters). Conditions were as follows: actin as indicated in the x abscissa, skeletal or smooth muscle S1 and smooth muscle tropomyosin 1 mol/5 mol actin, caldesmon 1 mol/7 mol of actin in 10 mM PIPES, pH 7.2, 10 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 1 mM NaN₃, at 20 °C.
results are presented in Fig. 2A. Again the transients were well fitted by a single exponential and there was a hyperbolic dependence of the observed rate constant on actin-tropomyosin concentration. The values of the apparent second order rate constant, the apparent association constant, and the maximum observed rate constant are summarized in Table 1. At this concentration of caldesmon (albeit giving 75% inhibition of the actomyosin ATPase) there was only a moderate decrease in the rates describing the kinetics of skeletal muscle S1 association with actin. We found that caldesmon reduced the apparent second order rate constant to nearly 50% and increased the dissociation constant by two times. The maximum rate was reduced by about 9% (from 287 s\(^{-1}\) to 262 s\(^{-1}\)) (Table 1).

Modification of actin by labeling with the pyrene fluorophore might affect the association of caldesmon with actin-tropomyosin, and there is a possibility that a proportion of the change in the fluorescence is caused by a change in the actin filament conformation. We have therefore used light scattering as another method to assess the effect of caldesmon and tropomyosin on skeletal muscle S1 binding to actin. The light scattering signal fitted two exponentials of roughly equal amplitudes in the absence and presence of regulatory proteins. The fast observed rate constant showed hyperbolic dependence on the concentration of actin-tropomyosin (Fig. 2B). The presence of caldesmon decreased slightly the maximum rate and the apparent second order rate constant of binding and increased the apparent dissociation constant in agreement with the pyrene fluorescence data. The slow rate constant was independent of the actin concentration.

The effect of caldesmon on the kinetics of S1 binding to actin was also investigated using smooth muscle S1 and pyrene-labeled skeletal muscle actin. The results are presented in Fig. 2C. The fluorescent transients fitted two exponentials in both the absence and presence of caldesmon. The fast component represented around 66%, whereas the slow component represented around 34% of the total fluorescence signal. The slow rate constant scattered between 0.2 s\(^{-1}\) and 3 s\(^{-1}\) and was not affected by the presence of caldesmon. In contrast, the rate of the fast component showed a hyperbolic dependence on the actin concentration. In the absence of caldesmon a fit to a hyperbola gave a maximum rate of 194 s\(^{-1}\) and an apparent second order rate constant of 10.2 \(\mu\text{M}^{-1}\text{s}^{-1}\). The maximum rate was not determined previously for

The mixture was then pushed in a stopped flow apparatus against increasing concentrations of actin-tropomyosin (at a molar ratio 5:1) in the absence (closed circles) or presence (open circles) of caldesmon (1 mol caldesmon/7 mol actin). Each transient is the average of five consecutive shots. The transients were fitted to a two exponential function. Temperature and buffer conditions are the same as in previous figures. A shows the dependence of the fast component of the observed rate of S1-ADP binding to pyrene actin on actin concentration in the absence (closed circles) and presence (open circles) of caldesmon. The solid lines represent a fit of the observed rates to the equation: 

\[
 k_{\text{obs}} = k_{\text{fast}} [A]/(k_{\text{fast}} + [A])
\]

where \( k_{\text{fast}} \) is the maximum rate constant, \( k_{\text{obs}} \) is the observed rate constant, and \([A]\) is the actin concentration. B shows an expansion of the initial part of the graph in A, \( k_{\text{obs}} \) is linearly dependent on the actin concentration up to 4 \(\mu\text{M}\). The slope represents \( k_{\text{obs}} [k_{\text{app}} + k_{\text{app}}] = 4.7 \mu\text{M}^{-1}\text{s}^{-1}\) in the absence of caldesmon and 2.4 \(\mu\text{M}^{-1}\text{s}^{-1}\) in the presence of caldesmon. C shows the dependence of the slow component of the observed rate of S1-ADP binding to pyrene actin on actin concentration in the absence (closed circles) or presence (open circles) of caldesmon. The solid lines represent a fit of the observed rate constant to the equation: 

\[
 k_{\text{obs}} = k_{\text{fast}} [A]/(k_{\text{app}} + [A])
\]

where \( k_{\text{app}} \) is the apparent dissociation constant in agreement with the pyrene fluorescence data. The slow rate constant was independent of the actin concentration.
smooth muscle S1 but an apparent second order rate constant of 
12 μM⁻¹ s⁻¹ was reported (35). Addition of smooth muscle caldesmon reduced the apparent second order rate constant by 57% (4.4 μM⁻¹ s⁻¹ in the presence of caldesmon) and the maximum rate by 20% (155 s⁻¹ in the presence of caldesmon) (Table 1). These results are in agreement with the data obtained with skeletal muscle S1.

The intercepts on the y axis define in theory the apparent dissociation rate; however, they are very close to zero and cannot be determined accurately from these plots. The dissociation rate constant was determined by mixing pyrene-actin-skeletal muscle S1 complex with a 30-fold excess of unlabeled actin. The fluorescent transients were best fit with single exponential functions. The apparent rate of skeletal muscle S1 dissociation from actin was 0.024 s⁻¹ in good agreement with the value 0.025 s⁻¹ reported in the literature (48). Smooth muscle tropomyosin slightly decreased the apparent rate of dissociation 0.02 s⁻¹ as was previously observed for skeletal muscle tropomyosin (48). On the contrary caldesmon and caldesmon-tropomyosin increased this (0.08 s⁻¹ and 0.085 s⁻¹, respectively). Thus it seems that caldesmon weakens the affinity of actin to skeletal muscle S1 both by reducing the rate of association and by increasing the rate of dissociation of skeletal muscle S1 from actin.

Because Kₐₐ and Kₐ₈ are large in the rigor state an effect of caldesmon, although present, may not necessarily be noticed. Therefore, we decided to investigate the effect of caldesmon on the kinetics of skeletal muscle S1-ADP binding to actin. ADP is known to reduce the affinity of skeletal muscle S1 to actin, and this has been attributed to a reduction in the isomerization (49). The binding of skeletal muscle S1-ADP to actin is however still a strong binding state and could therefore be investigated by the fluorescence of pyrene-labeled actin. The dissociation constant of skeletal muscle S1-ADP binding to actin is less than 0.1 μM, whereas the dissociation constant of ADP binding to actin-skeletal muscle S1 is in the range of 50 μM (35, 49). The protein concentrations used were such that both Kₐ₈ and Kₐ₈ might be determined, whereas the ADP concentration used was a compromise between the necessity to have a ternary complex actin-skeletal muscle S1-ADP and the need to prevent substantial rebinding of dissociated ADP to actin-S1. Mixing actin with skeletal muscle S1-ADP resulted in a fluorescence decrease best fit with a double exponential function. The fast component represented 85% while the slow component represented 15% of the total signal. The concentration dependence of the observed rate constant of both the slow and the fast components is shown in Fig. 3. In the absence of caldesmon the rate of the fast component showed a hyperbolic dependence on the actin concentration. The data were again modeled by a two-step binding reaction (step 7 in Scheme 1 subdivided to 2 steps: 7ₐ and 7ₖ) shown in Scheme 4. The observed rate constant of S1 binding to actin is given by Equation 12.

\[ k_{obs} = k_{sₐ₈}[A]/((k_{sₐ₈} + k_{sₐ₉})/k_{sₐ₉}) + [A] \]  

(Eq. 12)

The slope of the initial part of the data is given by \( k_{sₐ₈}k_{sₐ₉}/(k_{sₐ₈} + k_{sₐ₉}) \) and is ~4.7 μM⁻¹ s⁻¹. The maximum rate is given by \( k_{sₐ₈} \) and was obtained from the hyperbolic fit as 95 ± 10.4 s⁻¹. However, this value probably contains a large error because there were not many points at the high actin concentrations to define the plateau accurately. Previously, a value of 150 – 170 s⁻¹ (48) was measured in the absence of tropomyosin. In the presence of caldesmon, the observed rate constant of the fast process was also hyperbolically dependent on the actin concentration. The transients are the average of 4 to 5 shots and were fit to single exponential functions. B represents the effect of increasing caldesmon concentration on the fast rate constant (closed squares) and on the slow rate constant (open squares) of the binding of 0.5 μM smooth muscle S1 to 3.5 μM skeletal muscle actin in the presence of 0.75 μM smooth muscle tropomyosin monitored by pyrene-actin fluorescence (closed circles) and on the actin-TM-S1 ATPase (open circles). The transients are the average of 4 to 5 shots and were fit to single exponential functions. B represents the effect of increasing caldesmon concentration on the fast rate constant (closed squares) and on the slow rate constant (open squares) of the binding of 0.5 μM smooth muscle S1 to 3.5 μM skeletal muscle actin in the presence of 0.75 μM smooth muscle tropomyosin monitored by pyrene-actin fluorescence. The transient of smooth muscle S1 binding to actin were fit to a double exponential. Temperature and buffer conditions are the same as in previous figures.
Effect of Caldesmon on Kinetics of Actomyosin ATPase

TABLE 2
Effect of caldesmon on the observed kinetic constants of ATP-induced actin-S1 dissociation

|                | Apparent second order rate constant $\mu M^{-1} s^{-1}$ | Dissociation constant $\mu M$ | Maximum rate $s^{-1}$ |
|----------------|--------------------------------------------------------|-------------------------------|-----------------------|
| Actin-TM       | 5.18 ± 0.32                                            | 263 ± 90                      | 1588 ± 374            |
| Actin-TM-CaD (1 S1/7 actin) | 5.95 ± 0.26                                            | 306 ± 66                      | 2351 ± 365            |
| Actin-TM       | 4.79 ± 0.06                                            | 209 ± 59                      | 1460 ± 266            |
| Actin-TM-CaD (1 S1/3.5 actin) | 5.82 ± 0.05                                            | 239 ± 51                      | 1914 ± 274            |
| Actin-TM       | 4.59 ± 0.16                                            | 246 ± 29                      | 1396 ± 106            |
| Actin-TM-CaD (1 S1/1 actin) | 5.33 ± 0.08                                            | 279 ± 45                      | 1925 ± 207            |

Because the equilibrium binding of S1 to actin-tropomyosin is known to be reduced by caldesmon at ratios to actin larger than physiological (above 1 caldesmon/7 actins) (Table 1), we assessed the effect of high caldesmon concentrations on the kinetics of skeletal and smooth muscle S1 binding to actin using pyrene actin fluorescence. Fig. 4A shows that increasing concentration of caldesmon led to a reduction in the observed rate of skeletal muscle S1 binding to pyrene actin from about 180 s$^{-1}$ to about 50 s$^{-1}$ at 1 caldesmon/actin. Fig. 4B shows the same experiment using smooth muscle S1. The transients of smooth muscle S1 binding to pyrene-labeled actin show a fast and a slow phase. Increasing concentrations of caldesmon led to a reduction of the fast rate from about 40 s$^{-1}$ to about 4 s$^{-1}$ at 1 caldesmon/actin (Fig. 4B). The rate of the slow phase also decreased from about 2.5 s$^{-1}$ to about 0.5 s$^{-1}$ but the data were more scattered. The amplitude of the pyrene fluorescence signal decreased by about 20% for both skeletal and smooth muscle S1 indicating that at least 80% of S1 was still bound even at the highest caldesmon concentration used. The reduction of the rate of S1 binding was observed with both smooth and skeletal muscle S1 whether S1 or actin was in excess (data not shown).

II-Actin-S1 Dissociation by ATP—Pyrene-actin fluorescence was used to monitor the ATP-induced dissociation of actin-skeletal muscle S1A1. Mixing Mg$^{2+}$-ATP with pyrene-actin-tropomyosin-skeletal muscle S1 in the presence or absence of caldesmon resulted in an increase in the pyrene fluorescence (Fig. 5A). The fluorescence transients were best fit to single exponentials at all Mg$^{2+}$-ATP concentrations used, and the rate of the fluorescence increase was hyperbolically related to the Mg$^{2+}$-ATP concentration. The data were modeled using the two-step reaction shown in Scheme 5,
where $K_1$ is the equilibrium binding constant for the first step and $k_{-5}$ is the rate of actomyosin dissociation. The values for actin-tropomyosin in the presence or absence of caldesmon were not significantly different (Table 2). Thus caldesmon has no significant effect on the slope of the initial part of the curve (Fig. 5C); however, caldesmon increased the maximal rate of $\text{Mg}^{2+}$-ATP induced actin-tropomyosin-skeletal muscle S1 dissociation (Table 2). The experiment was done with skeletal muscle S1 concentration equal to actin, at 1 S1/3.5 actin and 1 S1/7 actin. The experiments at lower concentrations of skeletal muscle S1 were conducted to ensure that caldesmon was not displaced from actin. The results were very similar under the three conditions (Table 2).

III-Effect of Caldesmon on the Rate of Phosphate Release—The rate of phosphate release was measured in single turnover double mixing stopped-flow experiments directly using the fluorescence of MDCC-PBP. Initial experiments were attempted with smooth muscle S1, but were unsuccessful. In this experiment it is necessary to use very low salt to obtain a measurable amount of S1-ADP-P$_i$ bound to actin at concentrations of S1 in the range of 2 $\mu$M to 10 $\mu$M and actin-tropomyosin-caldesmon in the range of 12 $\mu$M to 80 $\mu$M. Higher concentrations could not be used in the stopped-flow apparatus as actin above 50 $\mu$M is very viscous. At this low salt concentration the affinity of skeletal muscle S1 is higher than smooth muscle S1, and therefore the experiment is just about achievable with skeletal muscle S1.

Skeletal muscle S1 was mixed with ATP and held in a delay line for 1 s to generate a steady state mixture of S1-ATP and S1-ADP-P$_i$. This solution was then mixed with an increasing concentration of actin-tropomyosin in the presence of the MDCC-PBP sensor in the presence or absence of caldesmon. S1-ATP and S1-ADP-P$_i$ are in rapid equilibrium with actin, and the corresponding actin-S1-ATP and actin-S1-ADP-P$_i$ are formed in a few milliseconds. The association of phosphate with MDCC-PBP leads to an increase in the fluorescence of the MDCC label.

Fig. 6 shows the results of a typical experiment. Fig. 6A shows the time course of the fluorescence increase upon mixing 16 $\mu$M actin-tropomyosin in the absence (curve a) or presence (curve b) of 2 $\mu$M caldesmon with a mixture of 1 $\mu$M S1 and 1.25 $\mu$M ATP. In the absence of caldesmon the transient was poorly fit by a single exponential function (Fig. 6B). A two exponential function gave a reasonably good fit as estimated visually and by the residuals (Fig. 6C). A fast component representing 25% of muscle S1 with 1.25 $\mu$M ATP in a delay line for 1 s and then mixing this solution with an equal volume of 16 $\mu$M actin and smooth muscle tropomyosin (1T/M/5 actin) in the absence (curve a) or presence (curve b) of 2.3 $\mu$M caldesmon. A fit to a double exponential function is superimposed on the experimental traces. The inset shows an expansion of the initial part of the transient in the presence of caldesmon clearly displaying a lag of 150 ms followed by a slow exponential increase in the fluorescence signal. B, the residuals after fitting curve a to a single exponential function. C, the residuals after fitting curve a to a double exponential function with the same amplitude signs. D, the residuals after fitting curve b to a single exponential function. E, the residuals after fitting curve b to a double exponential function with opposite amplitude signs. Buffer: 5 mM PIPES, pH 7.2, 5 mM KCl, 3 mM MgCl$_2$ at 20 °C.

**FIGURE 6.** Time course of phosphate release measured by the phosphate-binding protein in the absence and presence of caldesmon. A shows the change in the fluorescence of the MDCC-PBP upon premixing 1 $\mu$M skeletal
the fluorescence increase with an observed rate of 30 s\(^{-1}\) was followed by a slow component (amplitude 75% of the total signal, \(k_{\text{obs}}: 6\) s\(^{-1}\)). In the presence of caldesmon, the fluorescence transient has a markedly different shape. After a lag of ~150 ms, there was a slow increase in the fluorescence. The total amplitude of the fluorescence increase was similar to that in the absence of caldesmon. Because of the presence of a large lag, the transient was poorly fit with a single exponential function (Fig. 6D). A fit of the transient to a double exponential function (of opposite amplitude signs) gave a reasonably good fit (Fig. 6E).

The fast exponential component corresponding to the lag has a rate of 2.9 s\(^{-1}\) and an amplitude of ~27, and the slow component has an observed rate of 1.6 s\(^{-1}\) and an amplitude of 47. The dependence of the observed rate constants on actin-tropomyosin concentration is shown in Fig. 7. In the absence of caldesmon the observed rate constant of the fast component (\(k_{\text{obs}}\)) increased with the actin concentration and was fit with a rectangular hyperbola. The data were, therefore, modeled using the two-step reaction shown in Scheme 6,

\[
A + \text{M ADP-P}_i \rightleftharpoons A\cdot \text{M ADP-P}_i \rightarrow A\cdot \text{M ADP} + P_i
\]

where \(K_6\) is the equilibrium constant for rapid actin binding and \(k_{+3}\) is the rate of phosphate release. \(k_{-3} = 0\) because phosphate has a low affinity for actin-S1-ADP (in the millimolar range), and MDCC-PBP has a very high affinity for phosphate (in the range of \(10^{-7}\)) such as any phosphate liberated is sequestered by the phosphate-binding protein. The equation describing the hyperbolic dependence of \(k_{\text{obs}}\) on the actin concentration is shown in Equation 13.

\[
k_{\text{obs}} = k_{+3}[A]/(1/K_6 + [A])
\]

The apparent second order rate constant (\(K_6k_{+3}\)) obtained from the linear fit of the data up to 16 \(\mu\)M is 1.4 \(\mu\)M\(^{-1}\)s\(^{-1}\). At high actin concentration the data deviated from linearity and a maximum rate \(k_{+3} = 74.9\) s\(^{-1}\) was obtained from a hyperbolic fit. The apparent dissociation constant of the hyperbola is 40 \(\mu\)M and represents the dissociation constant of S1-ADP-P\(_i\) to actin (1/\(K_6\)). The rate of the slower component did not vary with actin concentration and stayed constant at around 3 s\(^{-1}\). In the presence of caldesmon, the rate of the exponential increase did not vary with actin concentration and stayed constant at around 2.5 s\(^{-1}\) (Fig. 7A, open circles). The duration of the lag however decreased with actin concentration (Fig. 8A).

It is necessary to consider the origin of these 2 components. White et al. (42) have investigated this extensively using stopped flow and quenched flow techniques with ATP, mant-ATP, aza-ATP, CTP, mant-CTP, and GTP and demonstrated that the fast component corresponds to the rate of phosphate release from actin-S1-ADP-P\(_i\), and the slow rate corresponds to the rate of phosphate release from actin-S1-ATP following hydrolysis (it is limited by the rate of hydrolysis of actin-attached-S1-ATP into actin-attached-S1-ADP-P\(_i\), and therefore the slow rate defines the rate of hydrolysis of ATP by S1 attached to actin). In the presence of caldesmon, the rate of the slow exponential increase probably corresponds to the slow component observed in the absence of caldesmon (around 5 s\(^{-1}\)) and thus represents the rate of ATP hydrolysis on actin-S1. It is not surprising that caldesmon has very little or no effect on the rate of hydrolysis of S1-ATP into S1-ADP-P\(_i\), because caldesmon is an actin-binding protein unlikely to interfere with the rate of ATP hydrolysis at the active site of S1. The lag phase probably corresponds to the switch from the OFF state to the ON state as discussed below.
Effect of Caldesmon on Kinetics of Actomyosin ATPase

TABLE 3

| Effect of caldesmon on the observed kinetic constants of $P_i$ release from actin-S1-ADP-P$_i$ | Apparent second order rate constant | Dissociation constant | Maximum rate |
|---|---|---|---|
| Actin-TM | 1.2 ± 0.77 | 18.7 ± 3.7 | 91 ± 7.2 |
| Mant-dATP | ND* | ND | 10? |
| Actin-TM-CaD (1 CaD/7 actin) | ND | ND | 2.5? |
| Mant-dADP | 1.45 ± 0.44 | 40.7 ± 16.3 | 74.9 ± 10.4 |
| MDCC-PBP | ND | ND | 2.5? |

* Not determined.

Actins led to the disappearance of the lag and the rate of fluorescence increase become biphasic (fast phase: 45 s$^{-1}$ and slow phase: 10 s$^{-1}$) (Fig. 8B).

The rate of phosphate release was also measured indirectly using the fluorescence of mant-ATP. Skeletal S1A1 was mixed with mant-dATP and held in a delay line for 1 s to generate a steady state mixture of S1A1-mant-dATP and S1A1-mant-dADP-P$_i$. This solution was then mixed with an increasing concentration of actin-tropomyosin in the presence or absence of caldesmon. S1A1-mant-dATP and S1A1-mant-dADP-P$_i$ are in rapid equilibrium with actin and the corresponding actin-S1A1-mant-dATP and actin-S1A1-mant-dADP-P$_i$ are formed in few milliseconds. The dissociation of mant-dATP and mant-dADP leads to a decrease in mant fluorescence. The dissociation of ADP is much faster (>600 s$^{-1}$) than the dissociation of phosphate (<100 s$^{-1}$) and so the rate of mant-dADP fluorescence decrease is probably limited by the rate of $P_i$ release. The results are presented in Table 3 and show that caldesmon reduced the rate of phosphate release substantially (from 91 s$^{-1}$ to around 10 s$^{-1}$). These results are in agreement with those obtained directly using the MDCC-PBP method (Figs. 6 and 7 and Table 3).

IV-ADP Binding Kinetics in the Absence and Presence of Caldesmon—Stopped-flow methods were also used to investigate the kinetics of ADP association with and dissociation from actin-S1 complex by monitoring the increase in fluorescence, which occurs upon mant-ADP interaction with S1 myosin head. Experiments were performed with smooth muscle S1 only, because the rate of ADP interaction with skeletal muscle S1 is too fast to be measured by stopped-flow techniques. Fig. 9A shows a representative plot of the rate of the mant fluorescence change upon mixing 4 μM mant-ADP and 1 μM S1. The fluorescent transients were best fit by double exponentials. Fig. 9B shows that the rate of fluorescence change is a linear function of mant-ADP concentration. The data were modeled using the one-step reaction scheme shown in Scheme 7.

$\text{A·M + ADP} \rightleftharpoons \text{A·M·ADP}$

$\text{A·M·ADP}$

$\text{Skeletal S1}$

$\text{Mant-dATP}$

$\text{Actin-TM-CaD (1 CaD/7 actin)}$

$\text{ND}$

$\text{ND}$

$\text{2.5?}$

$\text{Actin-TM}$

$\text{1.2 ± 0.77}$

$\text{18.7 ± 3.7}$

$\text{91 ± 7.2}$

$\text{Mant-dADP}$

$\text{1.45 ± 0.44}$

$\text{40.7 ± 16.3}$

$\text{74.9 ± 10.4}$

$\text{MDCC-PBP}$

$\text{ND}$

$\text{ND}$

$\text{2.5?}$

$\text{Actin-TM-CaD (1 CaD/7 actin)}$

$\text{ND}$

$\text{ND}$

$\text{2.5?}$

$\text{Not determined.}$

$\text{A} \cdot \text{M} + \text{ADP} \rightleftharpoons \text{A} \cdot \text{M} \cdot \text{ADP}$

$\text{Skeletal S1}$

$\text{Mant-dATP}$

$\text{Actin-TM-CaD (1 CaD/7 actin)}$

$\text{ND}$

$\text{ND}$

$\text{2.5?}$

$\text{Actin-TM}$

$\text{1.2 ± 0.77}$

$\text{18.7 ± 3.7}$

$\text{91 ± 7.2}$

$\text{Mant-dADP}$

$\text{1.45 ± 0.44}$

$\text{40.7 ± 16.3}$

$\text{74.9 ± 10.4}$

$\text{MDCC-PBP}$

$\text{ND}$

$\text{ND}$

$\text{2.5?}$

$\text{Actin-TM-CaD (1 CaD/7 actin)}$

$\text{ND}$

$\text{ND}$

$\text{2.5?}$

$\text{Not determined.}$
Effect of Caldesmon on Kinetics of Actomyosin ATPase

Caldesmon inhibition of actin-tropomyosin interaction with myosin is a key component of Ca$^{2+}$-regulation in smooth muscle thin filaments yet equilibrium and steady state methods have not been able to resolve the mechanism of regulation unambiguously. We have therefore used transient kinetic techniques to address this question. In the following discussion we will detail the interpretation of the kinetic experiments and identify the step inhibited by caldesmon-tropomyosin and explain the effect of caldesmon-tropomyosin on the elementary steps by a molecular mechanism of regulation. We will then compare this mechanism with troponin-tropomyosin regulation in skeletal muscle.

**Effect of Caldesmon on the Rate of S1 Binding to Actin**—According to current understanding, the pathway of the actin-S1 ATPase involves at least five processes: ATP binding to actin-S1, ATP-induced actin-S1 dissociation, ADP dissociation from actin-S1, ADP association with and dissociation from actin-tropomyosin and explain the effect of caldesmon-troponin on the rate of ADP release was also measured in a chase experiment where the actin-S1-ADP complex was mixed with a large excess of ATP. The rapid ATP binding to S1 will result in the dissociation of actin from S1 providing a decrease in light scattering with the rate limited by ADP dissociation from S1. The transients fit a single exponential function with rates unaffected by the presence of caldesmon (58 s$^{-1}$ and 67 s$^{-1}$, in the absence and presence of caldesmon, respectively; Fig. 9C and Table 4) and fairly similar to those obtained from the mant-ADP binding experiment (Fig. 9B).

**DISCUSSION**

Caldesmon inhibition of actin-tropomyosin interaction with myosin is a key component of Ca$^{2+}$-regulation in smooth muscle thin filaments yet equilibrium and steady state methods have not been able to resolve the mechanism of regulation unambiguously. We have therefore used transient kinetic techniques to address this question. In the following discussion we will detail the interpretation of the kinetic experiments and identify the step inhibited by caldesmon-tropomyosin and explain the effect of caldesmon-tropomyosin on the elementary steps by a molecular mechanism of regulation. We will then compare this mechanism with troponin-tropomyosin regulation in skeletal muscle.

Effect of Caldesmon on the Rate of S1 Binding to Actin—According to current understanding, the pathway of the actin-S1 ATPase involves at least five processes: ATP binding to actin-S1 mant-ADP with a mixture of actin (1 μM), smooth muscle tropomyosin (0.2 μM), and smooth muscle S1 (0.25 μM) in the absence (lower curve) or presence (top curve) of caldesmon (0.15 μM). The smooth line represents a fit of the transients to a double exponential function with $k_{\text{obs fast}} = 141$ s$^{-1}$ and $k_{\text{obs slow}} = 15$ s$^{-1}$ in the absence of caldesmon and $k_{\text{obs fast}} = 132$ s$^{-1}$ and $k_{\text{obs slow}} = 12$ s$^{-1}$ in the presence of caldesmon. B shows the dependence of both the fast (circles) and slow (squares) processes on the actin concentration in the absence (closed symbols) and presence (open symbols) of caldesmon. The slow phases were independent of the actin concentration. The fast phase increased linearly with actin concentration and was fit to the equation $k_{\text{obs fast}} = k_{-4} + k_{a4} + k_{-4} + k_{a4} = 7.2$ μM$^{-1}$s$^{-1}$ and $k_{-4} = 7.68$ s$^{-1}$ in the absence of caldesmon and $k_{-4} = 16.8$ μM$^{-1}$s$^{-1}$ and $k_{a4} = 81.7$ s$^{-1}$ in the presence of caldesmon. C shows a phase experiment. A mixture of ADP (50 μM) and actin (7 μM) and smooth muscle S1 (1 μM) without (curve a) and with (curve b) 1 μM caldesmon, was mixed with 1 mM Mg$^{2+}$-ATP. The light scattering decrease because of ATP-induced actin-S1 dissociation is limited by ADP dissociation from the actin-S1-ADP complex. The transients were fit to a single exponential with observed rate constant, $k_{\text{obs}} = 58$ s$^{-1}$ in the absence of caldesmon and $k_{\text{obs}} = 67$ s$^{-1}$ in the presence of caldesmon. Buffer: 10 mM PIPES, pH 7.2, 10 mM KCl, 2.5 mM MgCl$_2$, 1 mM DTT, 1 mM NaN$_3$, at 25°C. The concentrations quoted here refer to those after mixing in the reaction chamber.

### Table 4

| S1 complex       | Association rate constant, $k_{a4}$, μM$^{-1}$s$^{-1}$ | Dissociation rate constant, $k_{-4}$, s$^{-1}$ | Dissociation rate constant from chase experiment, s$^{-1}$ |
|------------------|--------------------------------------------------------|-----------------------------------------------|----------------------------------------------------------|
| Actin-TM         | 17.26 ± 3                                              | 76.8 ± 9.9                                    | 58 ± 0.44                                                |
| Actin-TM-CaD     | 16.82 ± 1.92                                           | 81.7 ± 6.3                                    | 67 ± 0.63                                                |

The y intercept yields the rate of ADP release $k_{-4} = 77$ s$^{-1}$. The rate of ADP association with and dissociation from actin-smooth muscle S1 complex is similar in the presence of caldesmon ($k_{-4} = 16.8$ μM$^{-1}$s$^{-1}$, $k_{a4} = 81$ s$^{-1}$).

The effect of the regulatory proteins caldesmon and tropomyosin on the rate of ADP release was also measured in a chase experiment where the actin-S1-ADP complex was mixed with a large excess of ATP. The rapid ATP binding to S1 will result in the dissociation of actin from S1 providing a decrease in light scattering with the rate limited by ADP dissociation from S1. The transients fit a single exponential function with rates unaffected by the presence of caldesmon (58 s$^{-1}$ and 67 s$^{-1}$, in the absence and presence of caldesmon, respectively; Fig. 9C and Table 4) and fairly similar to those obtained from the mant-ADP binding experiment (Fig. 9B).
and dissociation of S1-ATP from actin, ATP hydrolysis by S1 myosin heads, S1-ADP-Pi binding to actin, phosphate release from actin-S1-ADP-Pi, and finally ADP release. Actin activation of the myosin ATPase is believed to occur through the acceleration of the rate of products (ADP and P_i) release. P_i release is also associated with the myosin power stroke and force generation in muscle (50). It is reasonable to assume that a regulatory protein such as caldesmon that inhibits the actin-S1 ATPase through binding to actin is unlikely to affect ATP binding or hydrolysis by S1. The remaining 3 steps namely S1-ADP-Pi binding to actin, phosphate release, and ADP release could potentially be inhibited.

It is technically difficult to assess the effect of caldesmon on the kinetics of S1-ADP-P_i binding to actin because of the weak affinity and the fast rates governing the interaction of S1-ADP-P_i with actin. However reasonable conclusions could be drawn from investigations of the effect of caldesmon on S1-ADP and S1 rigor binding to actin since it is believed that the presence of nucleotides (and their nature) on the myosin head does not change the pathway of S1 binding to actin but rather the rate of transition between the intermediates (51). It is generally accepted today that S1 binding to actin occurs in 2 steps (Scheme 2) (52, 53): the formation of a first complex characterized by a low affinity for actin and by a high affinity for the nucleotides (defined by the equilibrium constant \( K_{a} \), the forward rate constant \( k_{+a} \) and the backward rate constant \( k_{-a} \)) followed by an isomerization to a second complex characterized by a high affinity for actin and a low affinity for the nucleotides (defined by the equilibrium constant \( K_{b} \), the forward rate constant \( k_{+b} \), and the backward rate constant \( k_{-b} \)). The first reaction is ionic strength-dependent whereas the second is affected by the presence and nature of nucleotides (\( K_{b} = 2000 \) in the absence of nucleotides, \( K_{b} = 200 \) for ADP, and \( K_{b} < 1 \) for ATP). In solution biochemistry, the second step can be detected by the quenching of pyrene actin fluorescence whereas light scattering essentially measures the first step (although a contribution from the second step is too small to occur). The data presented in Figs. 1–3 demonstrate the two-step binding mechanism for actin-tropomyosin measured by the quenching of the pyrene fluorescence and by an increase in light scatter. The apparent rate constant reaches a maximum value at high actin concentration indicating that a first order transition, presumably the isomerization is rate-limiting at high actin concentration. In the presence of caldesmon (at 1:7 caldesmon to actin molar ratio, which gives at least 75% inhibition in ATPase assay) the maximum rates, the apparent second order rate constants and the apparent dissociation constants calculated in the absence and presence of caldesmon were quite similar (Table 1). They demonstrate that caldesmon slightly decreased the apparent second order rate constants \( K_{b} k_{+b} \), the dissociation constant \( \left( k_{-a} + k_{-b} \right) k_{+a} \), and the maximum rate \( k_{+b} \). These findings clearly show that inhibitory concentrations of caldesmon only cause a small inhibition of the rate of S1 binding to actin and that inhibition is achieved by a decrease in the rate of the isomerization reaction. At higher caldesmon to actin-tropomyosin ratios a different process is revealed with a substantial decrease in the rate of S1 binding accompanied by a decrease in signal amplitude indicating a decrease in the actin-S1 affinity because of competition between S1 and caldesmon for actin. These observations are similar to those reported by Chalovich and co-workers (25, 26); however, they are not relevant to the regulation of thin filaments, which are inhibited at 1 caldesmon/7–14 actin (11).

Caldesmon Drastically Reduces the Rate of Phosphate Release

The binding of S1-ADP-P_i to actin results in the acceleration of the release of P_i (37). We have directly measured the rate of this step using two different methods. In the absence of caldesmon the transient of phosphate release was biphasic with a fast phase and a slow phase. The rate of the fast phase showed hyperbolic dependence on the actin concentration and represents the rate of phosphate release from S1-ADP-P_i following an actin binding (a two-step reaction where the signal is coming from the second step). The plateau rate represents the isomerization of the actin-S1-ADP-P_i complex, which is considered to be the tension generating step of muscle contraction (50). Such a mechanism predicts a lag because of the formation of the complex actin-S1-ADP-P_i before phosphate is liberated but the lag phase is too short to be observed in these transients. The rate governing the lag is given by Equation 14,

\[
K_{d}\text{[actin]} + K_{-6} + K_{3}
\]

and is very fast because \( K_{-6} \) is about 5000 s\(^{-1}\) (54). The rate of the slow phase was independent of the actin concentration and indicates a first-order transition, which may be attributed to the rate of ATP hydrolysis whereas S1 was bound to actin (step 2 in Scheme 1) (42). Previous measurements of the rate of phosphate release using MDCC-PBP in intact and Triton-permeabilized smooth muscle have reported a biphasic behavior for both thiophosphorylated (1.5 s\(^{-1}\) and 0.27 s\(^{-1}\)) and nonthiophosphorylated (1.0 s\(^{-1}\) and 0.13 s\(^{-1}\)) myosin light chain after a lag of 54 and 100 ms, respectively (55). These measurements are, however, not comparable to our measurements because of the use of smooth muscle tissue (we used skeletal muscle S1), the use of intact muscle (in contrast to isolated proteins) and the initiation of cross-bridge cycling by photolytic release of caged ATP (thus the kinetics of P_i release in this case include ATP binding to actomyosin, actomyosin dissociation, ATP hydrolysis, M-ADP-P_i rebinding to actin and P_i release). In the presence of caldesmon at a ratio of 1 caldesmon/7 actin monomers the shape of the transient was markedly different. After a lag of up to 150 ms, the rate of phosphate release followed a slow
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single exponential increase, the rate of which appeared to be independent of actin concentration and stayed constant at \( \sim 2-3 \text{s}^{-1} \). Thus caldesmon specifically decreases the rate of the isomerization reaction and the decrease correlates with the reduction in ATPase activity. This mode of inhibition is compatible with the observations that physiological caldesmon/actin ratios reduce the \( V_{\max} \) of steady state ATPase rather than increasing \( K_m \) (8, 11). To test whether the lag was related to cooperative switching of actin-tropomyosin activity states, we preincubated the actin-tropomyosin-caldesmon filaments with S1 (1 S1/4 actin monomers), which switches the filaments to the fully active state despite the presence of caldesmon (56). We found that the lag had disappeared even though cosedimentation measurements showed that a quaternary complex actin-tropomyosin-caldesmon-S1 was formed.

The rate of ADP release was monitored using the mant-ADP analogue. The value of ADP dissociation obtained, 76 s^{-1}, is in agreement with the previously reported value for smooth muscle S1 (20 s^{-1}) (35) given the slightly higher temperature we used and the use of modified ADP (ADP labeled with mant) instead of unlabeled ADP. The presence of caldesmon did not affect the ADP release rate even though a quaternary complex was observed in co-sedimentation experiments. This contrasts skeletal muscle where the rate of ADP release was found to be increased by Ca^{2+} binding to the troponin-tropomyosin complex (20, 57).

Mechanism of Regulation of the Actomyosin ATPase by Caldesmon—It is apparent that there are two classes of caldesmon effects on actomyosin kinetics. At caldesmon:actin-tropomyosin ratios of 1:7 caldesmon mainly reduces the rate of the isomerization of the complexes actin-tropomyosin-S1 (by 8–18%), actin-tropomyosin-S1-ADP (by 80%), and actin-tropomyosin-S1-ADP-P_i (by 95%); however, higher caldesmon/actin ratios, additional effects are observed with reduction of the rates of binding of S1 to actin-tropomyosin (Fig. 4). Previous transient kinetic experiments have largely explored the effects of higher caldesmon:actin ratios that are much higher than present in smooth muscle thin filaments. Our measurements made at 1 caldesmon/7 actin correlate with the amount of caldesmon needed to inhibit actomyosin ATPase in vitro (Fig. 4) and the measured caldesmon content of Ca^{2+}-regulated smooth muscle thin filaments (7). We therefore believe that our measurements can provide insight into the physiological mechanism of caldesmon regulation.

The mechanism of regulation of actomyosin ATPase activity by caldesmon is yet to be settled. It is not clear whether caldesmon inhibits the actomyosin ATPase by a purely competitive mechanism or by an allosteric cooperative mechanism. Chalovich and co-workers (9, 10) consistently found a decrease in the weak binding and argue that caldesmon inhibits the actomyosin ATPase by preventing the initial binding of S1-ADP-P_i to actin (13). In the cooperative allosteric mechanism, it is the state of the actin filament that determines its reactivity and caldesmon binding turns the actin filament to an inactive state. The transition from the inactive state to the active state is necessary before cross-bridge cycling can proceed. Equilibrium binding studies have not proven conclusive and indeed are not mechanistically discriminating as both the competitive and the allosteric models could lead to a weakening of the binding affinity. Studies of the kinetics of the effect of caldesmon on the elementary steps of the actin-S1 ATPase can indicate at least which mechanism is least likely to be valid.

Having investigated the effect of caldesmon on all the elementary steps of the actomyosin ATPase, it is appropriate to look at the predictions of the models on the kinetics of the reactions we assessed. In the competition model caldesmon would prevent the formation of the weak binding complex (first step of lower path in Fig. 10). In a cooperative allosteric mechanism (upper path in Fig. 10) the filament exists in two states: an active state and an inactive state. S1 would switch the filament to the ON state. The presence of caldesmon would make this switch much more difficult. We have used simulations of the rate of P_i release for both models. For a competitive model the reduced isomerization rate would need a reduction of the affinity of S1-ADP-P_i to actin-tropomyosin of at least 20-fold (Fig. 7, curves a and c). However, the maximum reported reduction in the affinity of S1-ADP-P_i is less than 10-fold (9, 10), and in our experience caldesmon (at 1/7 actin) has no effect at all on S1-ADP-P_i affinity for actin-tropomyosin (11, 12). It is very difficult to explain the observed lag with a competition model. To obtain a lag in the range of up to 150 ms in a purely competitive model, a substantial reduction of the \( k_{-\alpha} \) is needed (from 5000 \text{s}^{-1} to less than 10 \text{s}^{-1}). However if caldesmon was competing with S1, one would imagine that caldesmon decreases \( k_{-\alpha} \) (that is the rate of S1-ADP-P_i going on the actin) and not \( k_{-\alpha} \) (the rate of S1 coming off the actin).

On the other hand, a cooperative mechanism can simply account for both the slower isomerization and the lag. The presence of a lag is an indication that a prior slow step on pathway is taking place. In the cooperative allosteric mechanism this step will be the transition between the ON and the OFF states (see Fig 10). Initially the fraction of actin-S1-ADP-P_i in the ON state is very low because of caldesmon binding to the OFF state but as strong binding complexes are formed they will switch the filament to the ON state cooperatively, leading to an acceleration of the reaction. In fitting the rate of phosphate release to this model a change in \( K_T \) from 100 to 0.1 gives the observed reduction in the rate of P_i release without a change in the affinity of S1-ADP-P_i. Our measurements of S1 binding to actin-tropomyosin in the presence of inhibitory caldesmon indicate that \( K_T \) is too small to measure (12) and measurements of caldesmon binding to actin-tropomyosin in the ON state are best simulated with a 10,000-fold change in \( K_T \) (58). We have
recently demonstrated a direct correlation between caldesmon inhibition of the actomyosin ATPase and the conformational change accompanying the switch of the actin-tropomyosin complex to the OFF state, measured by the excimer fluorescence of pyrene iodoacetamide-labeled tropomyosin (18). Thus transient kinetics, equilibrium binding, and direct measurements support the hypothesis that caldesmon inhibits actin-tropomyosin by a cooperative-allosteric mechanism.

Comparison of Smooth and Skeletal Muscle Thin Filament Regulation—The possibility that smooth muscle thin filaments are regulated by the same cooperative-allosteric mechanism as skeletal muscle thin filaments was first proposed in 1985 (59). Many experiments document the relationship between caldesmon and S1 binding to actin-tropomyosin and inhibition of ATPase activation and its similarity to troponin-tropomyosin regulation (15). Recent studies of the effect of troponin-tropomyosin on the transient kinetics of the actomyosin ATPase in skeletal muscle have led to a fairly detailed mechanism in which the inhibited steps have been characterized (24). It is instructive to compare these results with our data on caldesmon inhibition. The most important parallel is that the rate of phosphate release is the main step inhibited in both cases. In this study we have gone further and demonstrated a lack of effect of caldesmon on the rate of the formation of the actin-S1 complex, ATP-induced dissociation and ADP release thus confirming the inference that caldesmon and troponin act at a single step. The lag in the P_i release we observed is direct evidence that a cooperative change of the state of the thin filament precedes the isomerization step and thus controls the rate of phosphate release. The cooperative transition with caldesmon as allosteric inhibitor and S1 as activator has also been inferred from S1 and caldesmon binding measurements, although these have been disputed (12, 60, 61).

This cooperative-allosteric mechanism is well established for the troponin-tropomyosin system; however, there are apparent differences. Current models of troponin regulation propose that the thin filament exists in three states (22, 23, 62): a blocked state unable to bind the myosin head, a closed state that binds myosin but is unable to activate ATPase, and an open state that activates the myosin ATPase. This article has produced evidence for the closed state when caldesmon is bound to actin-tropomyosin, but there is no evidence for a blocked state induced by caldesmon. Indeed in skeletal muscle thin filaments a substantial proportion of actin-tropomyosin-troponin is unable to bind to myosin at low Ca^{2+} levels. This can be most clearly observed as a reduction in the second order rate constant for S1 binding to the thin filament (62); however, as is shown in Fig. 1 and Table 1, inhibitory caldesmon does not affect the rate of actin-tropomyosin binding to S1 significantly. Structural evidence also supports the proposal that caldesmon induces the closed state. In the three-dimensional images of the smooth muscle thin filament, determined from electron microscopy and helical reconstruction, tropomyosin was observed located over the actin inner domain near the junction of inner and outer domains when caldesmon was bound. This location corresponds to the position observed with actin-tropomyosin-troponin in the presence of Ca^{2+} (i.e. the closed state rather than the blocked state) (63, 64).

The physiological role of caldesmon inhibition in regulating smooth muscle contractility has yet to be fully clarified. Smooth muscle is a dual regulated muscle and the primary mechanism of Ca^{2+}-regulation is via the activation of myosin light chain by phosphorylation by myosin light chain kinase. Ca^{2+}-dependent caldesmon inhibition seems to play a secondary role in modulating Ca^{2+} sensitivity (5) by accelerating relaxation (65, 66) and in maintaining basal relaxation (67, 68). The ability of caldesmon to inhibit phosphate release should promote relaxation even if myosin is still phosphorylated.

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