Role of ATP in the Binding of Caldesmon to Smooth Muscle Myosin†

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ABSTRACT: We have reported earlier that ATP causes both an increase in the affinity of caldesmon for smooth muscle myosin and a change in stoichiometry from 2 caldesmon molecules per myosin to 1:1 (Hemric & Chalovich, 1990). We now show that this ATP effect does not occur with skeletal muscle myosin, indicating that ATP has a specific effect on the structure of filamentous smooth muscle myosin. This ATP effect does not appear to be due to stabilization of a 10S type of filamentous smooth muscle myosin like that reported earlier (Ikebe & Hartshorne, 1984) since neither phosphorylaton nor extensive modification of myosin with MalNEt (both which stabilize the 6S state of monomeric myosin) eliminates the effect of ATP. Caldesmon does bind more tightly to a form of smooth muscle myosin which is resistant to papain digestion. These results suggest that the ATP effect is due to stabilization of a local conformation of smooth muscle myosin which is independent of the larger 10S/6S conformational change (Suzuki *et al.*, 1988). In the presence of ATP, the two heads of smooth muscle myosin and the S-2 region form a single, higher affinity binding region for caldesmon.

Smooth muscle contraction is thought to be regulated primarily by phosphorylation of myosin light chains by the enzyme myosin light chain kinase [for reviews, see Murphy (1989), Stull *et al.* (1989), and Trybus *et al.* (1991)]. However, numerous studies indicate that additional regulatory processes may also operate in smooth muscle [for example, see Gerthoffer *et al.* (1987), Fisher and Pfitzer (1989), and Tansey *et al.* (1990)]. The protein caldesmon is particularly interesting in this respect since smooth muscle actin filaments, which contain caldesmon, have been shown to stimulate the ATPase activity of skeletal muscle myosin in a Ca^{2+}-dependent manner (Marston & Smith, 1984; Marston & Lehman, 1985). Caldesmon has also been shown to alter the contractility of smooth muscle in experiments using an inhibitory peptide to compete with the action of native caldesmon (Katsuyama *et al.*, 1992) and in experiments where caldesmon was added back to smooth muscle fibers (Pfitzer *et al.*, 1993). In the later study, caldesmon was observed to lower the force produced at a given level of myosin phosphorylation, suggesting cooperation between myosin phosphorylation and the putative regulatory protein.

While the inhibition of actomyosin interactions by caldesmon is associated with the COOH-terminal actin binding region (Szpacenko & Dabrowska, 1986; Fujii *et al.*, 1987; Yazawa *et al.*, 1987), caldesmon has also been shown to bind to myosin in solution (Hemric & Chalovich, 1988, 1990; Hemric *et al.*, 1993; Ikebe & Reardon, 1988; Sutherland & Walsh, 1989; Marston *et al.*, 1992; Mani & Kay, 1993) and in native thin filaments (Marston & Redwood, 1991). The NH_2-terminal region of caldesmon binds to the S-2[1] region of myosin (Hemric & Chalovich, 1988, 1990; Ikebe & Reardon, 1988) and with lower affinity to the S-1 region (Hemric & Chalovich, 1988). Caldesmon binds more strongly to smooth muscle myosin than to skeletal muscle myosin (Hemric & Chalovich, 1988). Since caldesmon is not present in skeletal muscle, the stronger binding to smooth muscle may be of physiological significance. Another interesting feature of the binding of caldesmon to smooth muscle myosin is that this interaction is nucleotide-sensitive. Thus, the strength of binding increases and the stoichiometry changes from 2 caldesmon molecules per myosin molecule to 1 caldesmon per myosin molecule in the presence of 1 mM ATP (Hemric & Chalovich, 1990).

Our present results suggest that the stimulatory effect of ATP on the binding of caldesmon to myosin is due to a conformational change in the myosin molecule that is independent of the 10S/6S conformational change. ATP appears to stabilize a local conformation of smooth muscle myosin which is independent of the larger 10S/6S conformational change (Suzuki *et al.*, 1988). In the absence of ATP, each myosin molecule binds with low affinity to two caldesmon molecules. ATP appears to make the two S-1 groups and the S-2 region of myosin act in a concerted manner to form a single, higher affinity binding site.

MATERIALS AND METHODS

Protein Preparation. Smooth muscle myosin was prepared from chicken gizzards as described by Persechini and Hartshorne (1983) with minor modifications (Hemric & Chalovich, 1988). Smooth muscle rod was prepared by the method of Margossian and Lowey (1982). Skeletal muscle myosin was isolated from the back and leg muscles of rabbits (Kielley & Harrington, 1960). Caldesmon was purified from turkey gizzards by a modification of the method of Bretscher (1984) described previously (Velaz *et al.*, 1989). The myosin

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[1] Abbreviations: S-2, myosin subfragment 2; S-1, myosin subfragment 1; Ap5A, P^1,P^5-di(adenosine-5′) pentaphosphate; HMM, heavy meromyosin; MalNEt, *N*-ethylmaleimide; TNBS, 2,4,6-trinitrobenzenesulfonate; MOPS, 4-morpholinepropanesulfonic acid.

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binding fragment of caldesmon was produced as described by Hemric *et al.* (1993). The concentrations of smooth and skeletal muscle myosin were determined by absorbance at 280 nm, whereas the concentrations of caldesmon and smooth muscle myosin rod were determined by the Lowry assay (1951) with bovine serum albumin as a standard. The molecular weights used for calculation of protein concentrations were myosin (532 000), rod (263 000), and caldesmon (87 000). The purity of all proteins was verified by 7−20% polyacrylamide gel electrophoresis in the presence of SDS (Laemmli, 1970).

Smooth muscle myosin was thiophosphorylated as described by Trybus and Lowey (1984) and Hemric *et al.* (1994) using 5 mM ATPγS (Sigma), 5 mg/mL myosin, 5 μg/mL myosin light chain kinase, and calmodulin (provided by Dr. David J. Hartshorne) and incubated for 12 h at 4 °C. The level of thiophosphorylation was determined to be 100% by urea/glycerol gel electrophoresis (Perrie & Perry, 1970).

In some cases, cysteine residues of smooth muscle myosin were modified. This has been suggested to stabilize the active, 6S conformation (Chandra *et al.*, 1985; Nath *et al.*, 1986). Smooth muscle myosin (8−12 mg/mL) was treated with 0.8 mM MalNEt (Sigma) at 0 °C for 5 min in a solution containing 450 mM NaCl, 40 mM MOPS (pH 7.5), and 0.1 mM dithiothreitol. The reaction was initiated by addition of MalNEt and terminated by addition of dithiothreitol to 10 mM. Two thiol residues, one in the S-2 region (SH-A) and one in the 17 kDa light chain of myosin (SH-B), are modified in the initial phase of the reaction (Nath *et al.*, 1986). MalNEt-labeled myosin rod was prepared by digesting MalNEt myosin.

The lysine groups of smooth muscle myosin were sometimes trinitrophenylated by reaction with TNBS (Sigma) (Srivastava *et al.*, 1985). Smooth muscle myosin (6−7 μM) was modified with 2.5 mM TNBS at 25 °C in 100 mM KCl, 100 mM Tris-HCl (pH 7.5). The reaction was monitored by the change in absorbance at 345 nm, and the number of reacted Lys residues was determined using a molar absorption coefficient at 345 nm of 14 500 M^{-1} cm^{-1}. The reaction was stopped after modification of two Lys residues per myosin by addition of 10 volumes of 10 mM Lys (pH 7.5). Control myosin was treated identically except for the omission of TNBS.

Nucleotide Purity. ATP and ADP were checked for purity by thin-layer chromatography on PEI-Cellulose F (Bodman) in a solvent composed of 0.75 M sodium phosphate, pH 3.4 (Goody & Eckstein; 1971).

Determination of Myosin Conformation. Trybus and Lowey (1988) showed that the folded and extended forms of monomeric smooth muscle myosin could be distinguished by their elution volume from a HPLC gel permeation column. Fifteen micrograms of modified or native myosin was chromatographed on a 7.5 × 600 mm Biosil SEC TSK 400−10 column (Bio-Rad Laboratories) in 10 mM NaP_i (pH 7.3), 1 mM $MgCl_2$, 0.1 mM EGTA, and either 150 or 400 mM NaCl at a flow rate 1 mL/min. The molecular weight size exclusion range of the column is 20 000−1 000 000. The absorbance was monitored at both 214 and 280 nm.

Changes in myosin conformation can also be monitored by changes in the rate of digestion by papain (Ikebe & Hartshorne, 1984; Suzuki *et al.*, 1988). In the present case, papain was activated by incubation with 5 mM cysteine, 1 mM EDTA, and 14.3 mM 2-mercaptoethanol (pH 6) for 30 min and then filtered through a 0.2 μm filter (Margossian & Lowey, 1982). Digestion was carried out at a papain:myosin ratio of 1:400 (w/w) in 30 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7), and 1 mM dithiothreitol. The reaction was terminated by the addition of iodoacetic acid (pH 7.3) to a final concentration of 1 mM, and the products were determined by gel electrophoresis. The gels were stained with Coomassie Brilliant Blue, and the band corresponding to the myosin heavy chain was excised and incubated with 25% pyridine in water (v/v). The protein was quantitated by measuring the absorbance at 605 nm (Fenner *et al.*, 1975).

Binding Assays. [^{14}C]Iodoacetamide-labeled caldesmon was prepared as described by Velaz *et al.* (1989). The binding of [^{14}C]caldesmon to smooth muscle myosin was measured by a low-speed sedimentation assay as described earlier (Hemric & Chalovich, 1990). Binding data were corrected for the fraction of inactive caldesmon after modification, nonspecific sedimentation of caldesmon in the absence of myosin, and the fraction of the myosin which did not sediment. Binding measurements were made in a buffer containing 30 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7.0), and 1 mM dithiothreitol at 25 °C. In general, 98% of the myosin sedimented both in the presence and in the absence of caldesmon or ATP. We note that Shirinsky *et al.* (1993) reported that ATP solubilizes smooth muscle myosin at 10 mM MOPS (pH 7.0), 50 mM NaCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, and 1 mM EGTA. We too observed that ATP solubilized myosin under these same conditions. Furthermore, Ikebe and Hartshorne (1984) observed that smooth muscle myosin is aggregated in the presence of ATP at low ionic strength and 10 mM Mg^{2+}. Thus, it is probably the high Mg^{2+} which ensures that the myosin remains insoluble. Some of the studies done in the presence of ADP were carried out in the presence of a 0.2 mM aliquot of the myokinase inhibitor P^1,P^5-di(adenosine-5′) pentaphosphate.

ATPase Assays. ATPase rates were measured by the rate of liberation of [^{32}P]phosphate from γ-^{32}P-labeled ATP (Chalovich & Eisenberg, 1982). Three to five time points were taken for each determination.

RESULTS

Figure 1A shows that enhanced binding of caldesmon to myosin occurred at low concentrations of ATP; the effect was near-maximal at 0.1 mM ATP. Figure 1B shows that ATP also stimulated the binding of the myosin binding fragment of caldesmon to smooth muscle myosin. ADP enhanced the interaction between caldesmon and myosin, but this effect was not saturated even at 2 mM ADP (Figure 1C). While the experiments shown in Figure 1 were done at different protein concentrations, this does not affect the results. Results similar to those in Figure 1 were observed for ATP and ADP when both were measured at 3.1 μM myosin and 0.5 μM caldesmon (not shown). The effect of ADP on caldesmon binding to myosin could not be attributed to ATP contamination since the ADP purity was checked and in some cases a myokinase inhibitor (Ap5A) was added to prevent regeneration of ATP.

Because caldesmon is found in smooth muscle but not in skeletal muscle, it was of interest to determine if the effect of ATP was specific for smooth muscle myosin. Figure 2 shows the binding of [^{14}C]iodoacetamide-labeled caldesmon

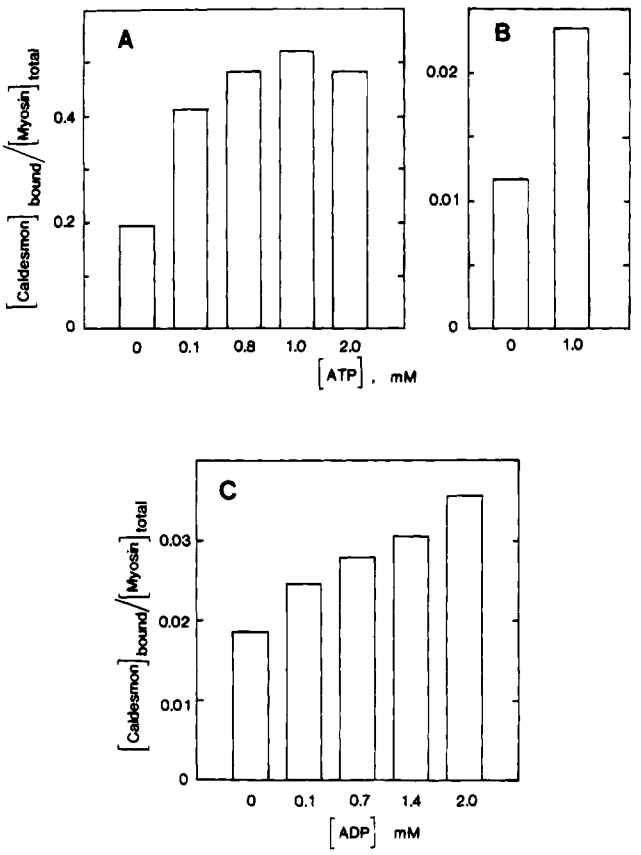

FIGURE 1: ATP and ADP strengthen the binding of caldesmon to smooth muscle myosin. Binding was measured in the presence of 3.1 μM smooth muscle myosin, 5 μM ^{14}C-labeled caldesmon (A), 0.5 μM labeled myosin binding fragment of caldesmon (B), or 0.5 μM labeled caldesmon (C) and varied concentrations of ATP (A, B) or ADP (C). The final conditions were 30 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7.0), and 1 mM dithiothreitol, 25 °C.

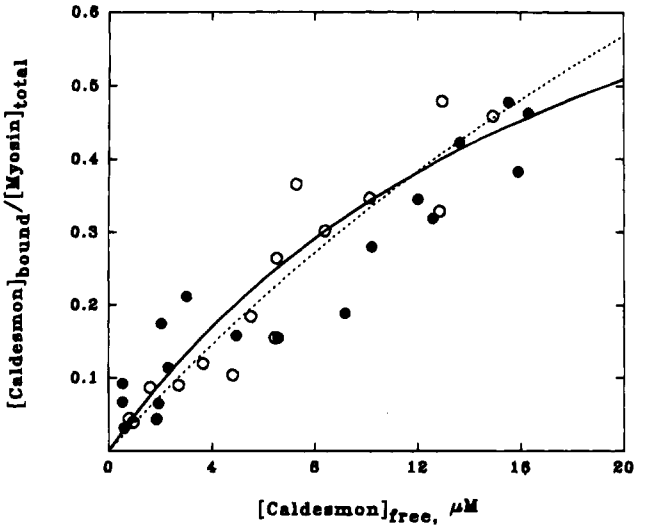

FIGURE 2: Binding of caldesmon to skeletal muscle myosin is independent of ATP. Binding of ^{14}C-labeled caldesmon to 3.1 μM skeletal myosin was measured in the presence (solid circles) and absence (open circles) of 1 mM ATP under the conditions of Figure 1. The solid line is the best fit to the data in the absence of ATP assuming a stoichiometry of 1:1; this gives an association constant of 5×10^4 M^{-1}. The dotted line is the best fit assuming a stoichiometry of 2 caldesmon molecules per myosin molecule; this gives an association constant of 2×10^4 M^{-1}.

Table 1: Effect of ATP on the Binding of Caldesmon to 100% Phosphorylated Myosin

	$caldesmon_{bound}/myosin_{total}$[a]	
	+ATP	−ATP
dephosphorylated myosin	0.31 ± 0.04 (n = 4)	0.13 ± 0.02 (n=3)
phosphorylated myosin	0.34 ± 0.05 (n = 6)	0.14 ± 0.02 (n = 3)

[a] 5 μM myosin, 6 μM caldesmon; other conditions as in Figure 1.

to skeletal muscle myosin in the presence and absence of ATP. These data were from two experiments with different preparations of both skeletal muscle myosin and caldesmon. The binding assays were done over a range of free caldesmon concentrations which would show any effect of ATP on the binding of caldesmon to smooth muscle myosin (see Figures 3 and 4; Hemric & Chalovich, 1990). The curves shown are best fits to the data, in the absence of ATP (open circles), assuming stoichiometries of 1 (solid line) or 2 (dotted line) caldesmon molecules per myosin. While the low affinity prevented unambiguous determination of the stoichiometry of binding, it was possible to determine that ATP did not strengthen the binding of caldesmon to skeletal muscle myosin.

Because ATP strengthened the binding of caldesmon to smooth muscle myosin but not skeletal muscle myosin, the effect of ATP involved a change specific to smooth muscle myosin. Several approaches were used to determine the nature of this change. The rate of digestion of smooth muscle myosin with papain has been shown to be sensitive to conformational changes in myosin (Ikebe & Hartshorne, 1984) and in HMM (Suzuki *et al.*, 1988). Under the conditions used for the present binding assays, the rate of digestion of dephosphorylated smooth muscle myosin was ATP-dependent. Figure 3A (top panel) shows an SDS gel of the products of digestion of dephosphorylated myosin with papain as a function of time. In the absence of ATP, the high molecular weight band corresponding to myosin heavy chain was almost totally depleted while it was clearly visible in the presence of ATP. Note that the material at the top of the gel is most likely denatured myosin which did not enter the gel. The amount of the material is only a small fraction of the total myosin (about 5% of the total protein on the gel) and does not affect the observed pattern of digestion significantly. Plots of the myosin heavy chain intensity against time are shown in Figure 3B (bottom panel) together with first-order decay curves fitted to the data (dotted lines). The rate of digestion in the absence of ATP was 4 times the rate in the presence of ATP.

ATP caused a change both in caldesmon binding to myosin and in the rate of myosin digestion by papain. To determine if these changes were related to the activity of the myosin, we examined the effects of phosphorylation, MalNEt modification, and TNBS modification of smooth muscle myosin. Each of these modifications is thought to stabilize the active or 6S conformation of smooth muscle myosin.

Following 100% thiophosphorylation of smooth muscle myosin, the actin-activated ATPase rate increased 7-fold compared to unphosphorylated myosin when measured at 37 °C in 20 mM imidazole (pH 7.0), 50 mM NaCl, and 10 mM $MgCl_2$. Table 1 shows that the binding of fully phosphorylated myosin to caldesmon was ATP-dependent. We had shown earlier that phosphorylation of 60% of the myosin light chains did not affect the binding of caldesmon to myosin (Hemric *et al.*, 1993). Since only those myosin

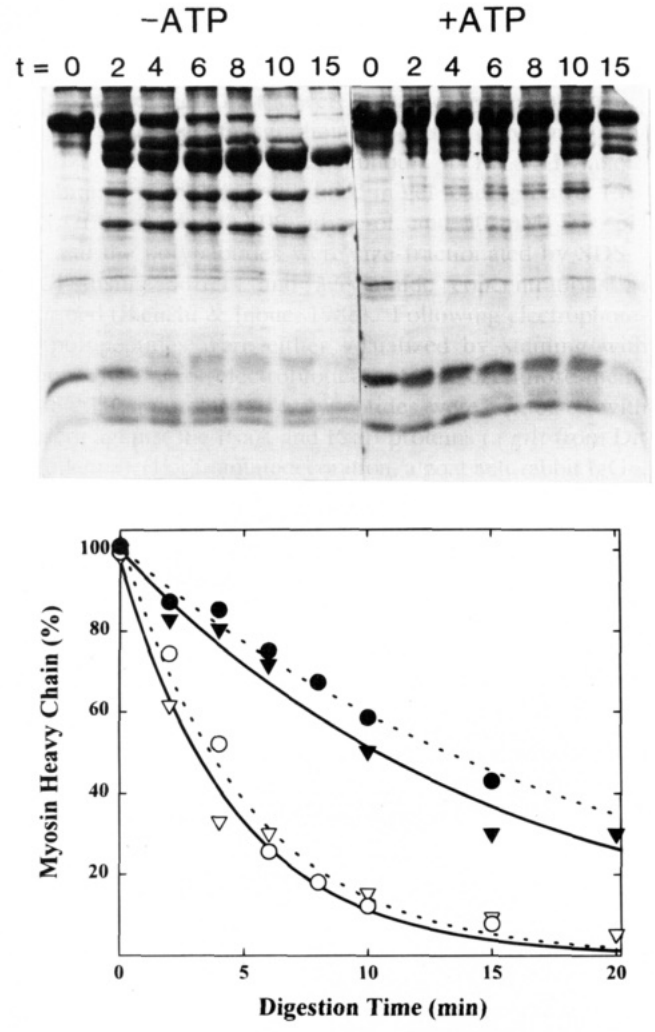

FIGURE 3: Comparison of the rate of digestion of smooth muscle myosin with papain in the presence and absence of 1 mM ATP. (A, top panel) SDS/7−20% polyacrylamide gradient gel of myosin (1.4 μM) digested with papain under the conditions of Figure 1 and terminated at times ranging from 0 to 20 min. 30 μg of myosin was loaded on each lane; the gel was subsequently stained with Coomassie blue. Myosin was digested in both the absence (lanes 1−7) and presence (lanes 8−14) of 1 mM ATP. (B, bottom panel) Plot of the myosin heavy chain intensity versus the digestion time. The curves are best fits of a single-exponential decay to the data in the absence (○, ▽) and presence (●, ▼) of ATP. The dotted curves show that the apparent rate constants of digestion for dephosphorylated myosin (○, ●) are 0.2 s^{-1} (−ATP) and 0.05 s^{-1} (+ATP). In the case of 100% thiophosphorylation of myosin light chains (▽, ▼), solid curves show that the rate constants of digestion are 0.22 s^{-1} (−ATP) and 0.066 s^{-1} (+ATP).

molecules with two phosphorylated light chains may be active (Persichini & Hartshorne, 1981; Sellers *et al.*, 1983), it was possible that we could have missed the effect of phosphorylation. The present results with 100% phosphorylation of myosin confirm that phosphorylation affects neither the affinity to caldesmon nor the effect of ATP on this interaction.

The rate of digestion of phosphorylated myosin was also ATP-dependent. Figure 3B (solid lines) shows that the rate of digestion in the absence of ATP was 3.3 times faster than in the presence of 1 mM ATP. Phosphorylation of smooth muscle myosin is known to enhance the digestion by papain. ATP and phosphorylation have opposite effects, suggesting that ATP caused a change that was independent of that caused by phosphorylation.

Table 2: Volume of Elution of Myosin and Modified Myosin from an HPLC Gel Filtration Column[a]

protein	elution volume (mL)	
	0.15 M NaCl	0.4 M NaCl
myosin	13.3	11.1
MalNEt-myosin	11.1	11.1
Myosin + MalNEt-myosin	13.3 + 11.1	11.1
trinitrophenylated myosin	13.3	11.1

[a] Biosil SEC TSK 400−10 (7.5 × 600 mm) equilibrated with 10 mM NaP_i (pH 7.3), 1 mM $MgCl_2$, and 0.1 mM EGTA plus the added NaCl.

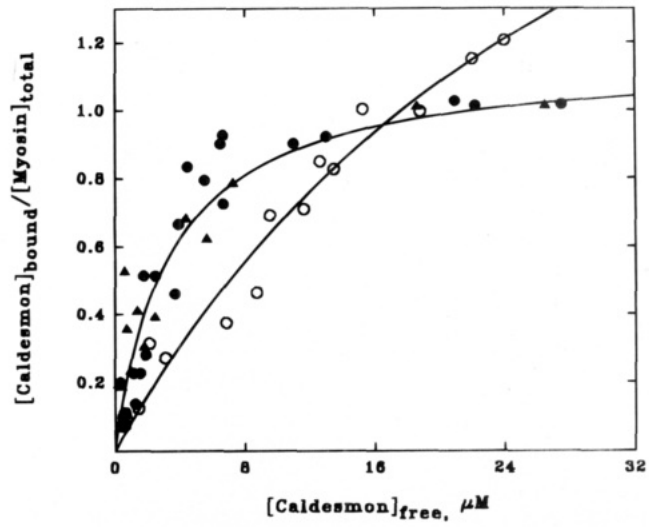

FIGURE 4: Effect of ATP on the binding of caldesmon to MalNEt-modified smooth muscle myosin. Binding of ^{14}C-labeled caldesmon to 3.1 or 8 μM myosin (triangles) and 2.5 μM MalNEt-modified myosin (circles) was measured in the presence (solid symbols) and absence (open symbols) of 1 mM ATP under the conditions of Figure 1. Binding in the presence of ATP is well described by a curve generated with a stoichiometry of 1 caldesmon per myosin and an association constant of 3.9 × 10^5 M^{-1}. In the absence of ATP, the data are best described by a curve generated by assuming a stoichiometry of 2 caldesmon molecules per myosin molecule and an association constant of 5.6 × 10^4 M^{-1}.

Chandra *et al.* (1985) have shown earlier that the active 6S form of myosin could be stabilized by modification of myosin SH groups with MalNEt. We found that MalNEt modification resulted in a 3-fold increase in the Mg-ATPase rate at 25 °C in a buffer containing 10 mM imidazole (pH 7.0), 30 mM NaCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol. We also confirmed that the modified myosin was stabilized in a conformation different from that of native myosin. This was done by examining the ionic strength dependent change in elution time from a gel permeation column. Trybus and Lowey (1988) had shown earlier that the elution time from an HPLC gel permeation column was characteristic of the conformation of myosin. Table 2 shows the results of the present HPLC gel filtration measurements. Unmodified smooth myosin eluted earlier at high ionic strength, where the extended form is favored, than at low ionic strength, where the folded form of smooth muscle myosin dominates. In contrast, MalNEt-modified myosin eluted at the position of the extended myosin form, at both ionic strengths, indicating that the extended active form had been stabilized.

Figure 4 shows the binding of caldesmon to the MalNEt-modified myosin in the presence and absence of ATP. Although the MalNEt myosin was in a different conformation

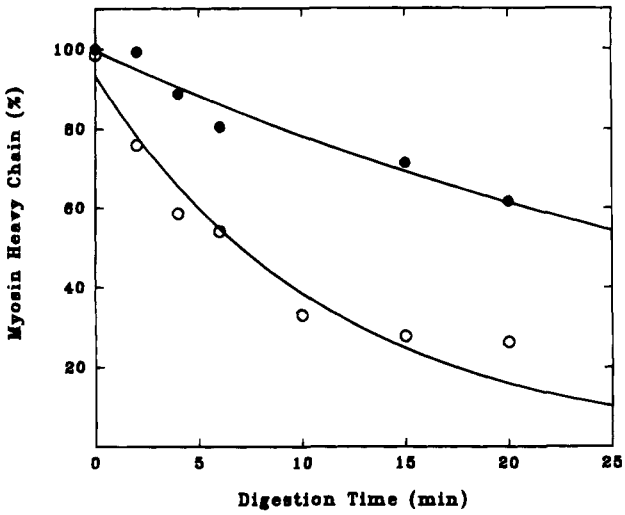

FIGURE 5: Rate of digestion of MalNEt-modified smooth muscle myosin heavy chain by papain in the presence (solid circles) and absence (open circles) of ATP. Digestion was done as described in Figure 3. The rate constants of digestion are 0.088 s^{-1} (−ATP) and 0.024 s^{-1} (+ATP).

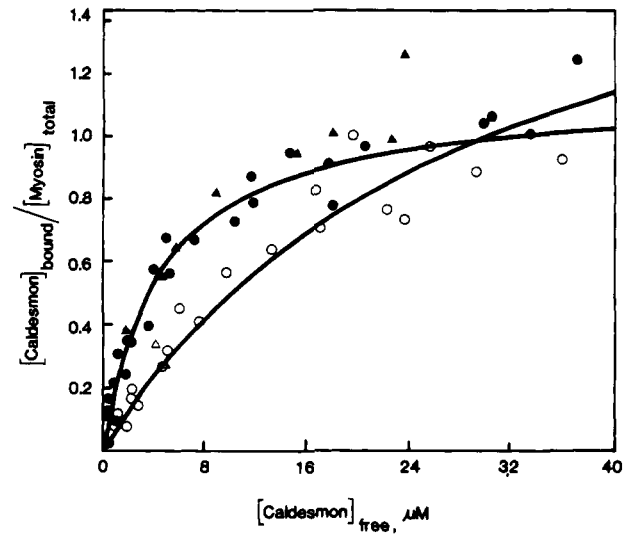

FIGURE 6: Effect of ATP on the binding of caldesmon to trinitrophenylated smooth muscle myosin. Binding of ^{14}C-labeled caldesmon to 6 μM myosin (triangles) and 3.5 or 6 μM trinitrophenylated myosin (circles) was measured in the presence (solid symbols) and absence (open symbols) of 1 mM ATP under the conditions of Figure 1. Binding in the presence of ATP is well described by a curve generated with a stoichiometry of 1 caldesmon per myosin and an association constant of 3.5×10^5 M^{-1}. In the absence of ATP, the data are best described by a curve generated by assuming a stoichiometry of 2 caldesmon molecules per myosin molecule and an association constant of 3.3×10^4 M^{-1}.

from native myosin, its binding to myosin was highly ATP-dependent. The addition of ATP caused both a change in stoichiometry from 2 caldesmon per myosin to 1:1 and a 6.5-fold increase in affinity. The effect of ATP on MalNEt-modified myosin was also apparent in the rate of papain digestion. Figure 5 shows that the rate of digestion with papain was 3.7-fold faster in the absence of ATP.

Trinitrophenylation of the lysine groups with TNBS is another way to stabilize the 6S active state of monomeric myosin (Srivastava *et al.*, 1985). We observed a 3–4-fold increase in the actin-activated ATPase rate following trinitrophenylation. However, as judged by the elution times from a gel filtration column, there did not appear to be the same global change that occurred with MalNEt modification (Table 2). The binding of caldesmon to trinitrophenylated myosin is shown in Figure 6. As in all of the previous cases involving smooth muscle myosin, the binding was ATP-dependent. The addition of ATP changed the stoichiometry form 2 mol of caldesmon per mole of myosin to 1:1 and increased the association constant 10-fold.

DISCUSSION

This report supports our earlier results (Hemric & Chalovich, 1988) which show that caldesmon binds to both skeletal and smooth muscle myosin. The association constant of caldesmon with skeletal myosin was estimated, from Figure 2, to be 2×10^4 M^{-1} assuming a stoichiometry of 2 caldesmon molecules per myosin. This binding was about $^1/_{50}$th as strong as the association of caldesmon with smooth muscle myosin·ATP (Hemric & Chalovich, 1990). The failure of others (Marston *et al.*, 1992) to detect the interaction of caldesmon with skeletal muscle myosin may be due to this low affinity. The observed relative strength of binding of caldesmon to smooth and skeletal muscle myosin is in agreement with our earlier affinity chromatography study (Hemric & Chalovich, 1988).

We have shown earlier that ATP enhances the binding of caldesmon to gizzard smooth muscle myosin (Hemric & Chalovich, 1990). We have confirmed this ATP effect and have shown that ADP also enhances the binding but to a lesser extent. In contrast to gizzard myosin, ATP did not affect the binding of caldesmon to rabbit skeletal muscle myosin. However, smooth muscle myosin is known to undergo conformational changes which are not seen with its skeletal muscle counterpart (Walker *et al.*, 1985). The ATP effect on smooth muscle myosin binding required the presence of the S-1 region since there was no effect of nucleotide on the binding of caldesmon to smooth myosin rod (not shown). The ATP effect did not, however, require the COOH-terminal region of caldesmon. The effects of nucleotide may be attributed to changes in the myosin molecule, particularly myosin isolated from smooth muscle. It is interesting that the enhancement of binding of caldesmon to myosin, by ATP, may be specific for certain types of smooth muscle myosin since no ATP effect was observed for the binding of aorta smooth muscle myosin to caldesmon (Marston *et al.*, 1992).

It is unlikely that ATP increased the affinity of caldesmon for myosin and changed the stoichiometry of binding by causing disassociation of myosin filaments. In our experimental conditions, 98% of myosin was sedimented in both the presence and absence of ATP. The effect of ATP on the monomer–polymer equilibrium of smooth muscle myosin is well-known to be ionic strength dependent. In the physiological ionic strength, unphosphorylated myosin filaments are completely disassembled in the presence of stoichiometric amounts of ATP (Trybus *et al.*, 1982; Craig *et al.*, 1983; Kumon *et al.*, 1984). In contrast, at <100 mM ionic strength, little filament disassemble occurs with ATP (Onishi *et al.*, 1978; Kendrick-Jones *et al.*, 1983; Ikebe & Hartshorne, 1984).

It is also unlikely that the affinity of caldesmon for myosin was low, in the absence of ATP, as a result of aggregation or trapping of the myosin heads. A reversal of trapping of myosin heads by ATP cannot explain how the addition of

ATP causes a *decrease* in stoichiometry from 2 caldesmon molecules per myosin molecule to 1:1. Furthermore, because ATP changed both the affinity and stoichiometry of binding, there was actually more binding of caldesmon to myosin in the absence of ATP at high caldesmon concentrations (Figure 4). ATP appeared to cause a loss of half of the binding sites, perhaps by causing the two myosin heads to bind as a higher affinity unit.

It is interesting to consider the possible nature of the change in smooth muscle myosin that results from nucleotide binding. The observed effect of ATP on caldesmon binding was independent of several types of modification of myosin which stabilize the 6S or active configuration of myosin including phosphorylation (Trybus, 1989), MalNEt modification (Chandra *et al.*, 1985), and trinitrophenylation (Srivastava *et al.*, 1985). In each case, the addition of ATP changed the stoichiometry and affinity for caldesmon and reduced the rate of digestion with papain. Thus, ATP causes changes in the myosin molecule that are independent of the state of activation.

Ikebe and Ogihara (1982) have reported that the addition of ATP to dephosphorylated myosin causes an increase in the periodicity of the myosin heads. In contrast, the heads of phosphorylated myosin remain disordered in either the presence or the absence of ATP. A similar correlation between cross-bridge order and activity has been observed in myosins from tarantula (Craig *et al.*, 1987) and scallop (Vibert & Castallani, 1989; Frado & Craig, 1989). Because ATP had a similar effect on caldesmon binding to dephosphorylated and phosphorylated myosin, in the present study, caldesmon binding may not be directly related to the order/disorder of the myosin cross-bridges.

Suzuki *et al.* (1988) observed that the structure of smooth muscle HMM is also affected by ATP. This was seen as a decrease in the rate of digestion of phosphorylated smooth muscle HMM upon the addition of ATP. This ATP-dependent change, which is independent of myosin phosphorylation, could be similar to that which we observe to bind more tightly to caldesmon. Unfortunately, the nature of this change has not been identified. It is known that while caldesmon binds tightly to the S-2 region of myosin, it also binds to the S-1 region since S-1 can displace caldesmon from smooth muscle myosin (Hemric & Chalovich, 1990). Furthermore, the myosin heads are required for the ATP enhancement of caldesmon binding. Thus, it is possible that ATP causes the myosin heads to interact in such a manner as to create one binding site with a high affinity for caldesmon from two low-affinity sites involving separate interactions with each S-1 head and a single caldesmon molecule. Such a change would have to occur without a change in periodicity as described earlier.

It appears unlikely that the binding of caldesmon to smooth muscle myosin has a direct influence on myosin catalytic activity. However, caldesmon may have either a structural role or an indirect regulatory effect on the myosin by linking the S-2 region of myosin to actin. The binding of caldesmon to myosin might be regulated by Ca^{2+}−calmodulin (Ikebe & Reardon, 1988; Hemric *et al.*, 1993) or phosphorylation of caldesmon (Abougou *et al.*, 1989; Adam *et al.*, 1992, 1993; Bogatcheva *et al.*, 1993; Childs *et al.*, 1992; Hemric *et al.*, 1993; Hettasch & Sellers, 1991; Mak *et al.*, 1991; Sutherland & Walsh, 1989, Yamakita *et al.*, 1992; Yamashiro *et al.*, 1991) but not by nucleotide-dependent changes in the myosin molecule since large changes in cellular nucleotide concentration are unlikely. Caldesmon does not appear to regulate the ATPase rate of myosin through its binding to myosin. Rather, the binding of caldesmon to actin is responsible for inhibition of ATPase activity either by inhibiting the binding of myosin to actin (Hemric & Chalovich, 1988) or in an alternate model by attenuating product release (Marston & Redwood, 1992).

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