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The effects of caldesmon on the ATPase activities of rabbit skeletal-muscle myosin

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We studied the effects of caldesmon, a major actin- and calmodulin-binding protein found in a variety of muscle and non-muscle tissues, on the various ATPase activities of skeletal-muscle myosin. Caldesmon inhibited the actin-activated myosin Mg^{2+}-ATPase, and this inhibition was enhanced by tropomyosin. In the presence of the troponin complex and tropomyosin, caldesmon inhibited the Ca^{2+}-dependent actomyosin Mg^{2+}-ATPase; this inhibition could be partly overcome by Ca^{2+}/calmodulin. Caldesmon, phosphorylated to the extent of $\sim$ 4 mol of P_i/mol of caldesmon, inhibited the actin-activated myosin Mg^{2+}-ATPase to the same extent as did non-phosphorylated caldesmon. Both inhibitions could be overcome by Ca^{2+}/calmodulin. Caldesmon also inhibited the Mg^{2+}-ATPase activity of skeletal-muscle myosin in the absence of actin; this inhibition also could be overcome by Ca^{2+}/calmodulin. Caldesmon inhibited the Ca^{2+}-ATPase activity of skeletal-muscle myosin in the presence or absence of actin, at both low (0.1 M-KCl) and high (0.3 M-KCl) ionic strength. Finally, caldesmon inhibited the skeletal-muscle myosin K^+/EDTA-ATPase at 0.1 M-KCl, but not at 0.3 M-KCl. Addition of actin resulted in no inhibition of this ATPase by caldesmon at either 0.1 M- or 0.3 M-KCl. These observations suggest that caldesmon may function in the regulation of actin–myosin interactions in striated muscle and thereby modulate the contractile state of the muscle. The demonstration that caldesmon inhibits a variety of myosin ATPase activities in the absence of actin indicates a direct effect of caldesmon on myosin. The inhibition of the actin-activated Mg^{2+}-ATPase activity of myosin (the physiological activity) may not be due therefore simply to the binding of caldesmon to the actin filament causing blockage of myosin-cross-bridge–actin interaction.

INTRODUCTION

Caldesmon is a major protein component of smooth muscle which interacts *in vitro* with F-actin in a Ca^{2+}-independent manner and with calmodulin in a Ca^{2+}-dependent manner (Sobue *et al.*, 1981, 1982; Ngai *et al.*, 1984; Bretscher, 1984). Actin and calmodulin compete for binding to caldesmon only in the presence of micromolar concentrations of Ca^{2+}. Immunofluorescence studies (Ban *et al.*, 1984; Owada *et al.*, 1984; Bretscher & Lynch, 1985) and characterization of isolated smooth-muscle thin filaments (Marston & Lehman, 1985) suggest that caldesmon is associated with actin filaments *in vivo*. The tissue content of caldesmon has been estimated to be 1 mol/42 actin monomers (Bretscher, 1984) or 11.1 μM (Ngai & Walsh, 1985*a*) in chicken gizzard and 1 mol/26 actin monomers in chicken gizzard, rabbit stomach, sheep aorta and sheep trachea (Marston & Lehman, 1985).

The fact that caldesmon interacts with F-actin and can associate reversibly with calmodulin as a function of Ca^{2+} concentration raised the possibility that this protein may function in the Ca^{2+}-mediated regulation of actin–myosin interaction. In support of this concept, Sobue *et al.* (1982) demonstrated that caldesmon inhibited superprecipitation of a desensitized actomyosin preparation from chicken gizzard, an effect which could be overcome by excess Ca^{2+}/calmodulin. We showed that caldesmon isolated from either chicken gizzard (Ngai & Walsh, 1984) or bovine aorta (Clark *et al.*, 1986) inhibited the actin-activated Mg^{2+}-ATPase activity of smooth-muscle myosin. Furthermore, caldesmon could be phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase and dephosphorylated by a protein phosphatase (Ngai & Walsh, 1984, 1985*a*). Marston *et al.* (1984) and Marston & Smith (1984) isolated Ca^{2+}-regulated thin filaments from vascular smooth muscle which conferred Ca^{2+}-dependence on skeletal-muscle myosin. This regulation was accomplished by a M_r-120000–140000 protein [later identified as caldesmon (Marston & Lehman, 1985)] and a calmodulin-like Ca^{2+}-binding protein.

Caldesmon has been identified in a wide variety of muscle and non-muscle tissues. For example, we used Western immunoblotting with polyclonal antibodies raised in rabbits against chicken gizzard caldesmon to demonstrate the presence of caldesmon in gizzard, oesophagus, duodenum, small intestine, lung, aorta, heart, skeletal muscle, kidney, trachea, brain and liver of the chicken (Ngai & Walsh, 1985*b*). We have observed a similar broad distribution of caldesmon among bovine tissues (Clark *et al.*, 1986) and have identified caldesmon in isolated rat cardiac myocytes (P. K. Ngai & M. P. Walsh, unpublished work). Caldesmon may therefore be involved in regulating actin–myosin interactions in a variety of contractile systems. With a view to gaining some insight into the regulation by caldesmon of actin–myosin interaction in striated muscle, we have studied the effects of caldesmon on a variety of ATPase activities associated with skeletal-muscle myosin: actin-

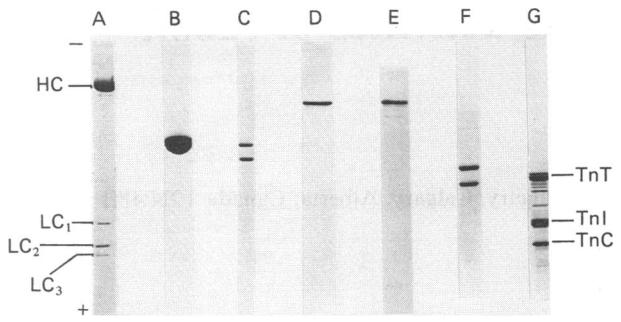

Fig. 1. Purity of protein preparations used in this study

Proteins purified as described in the Materials and methods section were examined by 0.1%-SDS/7.5–20%-polyacrylamide-gradient slab-gel electrophoresis. Key to lanes: A, rabbit skeletal-muscle myosin (10 μg) showing the heavy chain (HC) and three light chains (LC_1, LC_2, LC_3); B, rabbit skeletal-muscle actin (40 μg); C, chicken gizzard tropomyosin (5 μg); D, chicken gizzard caldesmon purified by the first method, involving heat treatment (5 μg); E, chicken gizzard caldesmon purified by the second method, avoiding heat treatment (contains caldesmon kinase) (5 μg); F, rabbit skeletal-muscle tropomyosin (5 μg); G, rabbit skeletal-muscle troponin (10 μg): TnC, troponin C; TnI, troponin I; TnT, troponin T).

activated Mg^{2+}-ATPase in the presence and absence of troponin/tropomyosin, Mg^{2+}-ATPase in the absence of actin, Ca^{2+}-ATPase and K^+/EDTA-ATPase.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (10–40 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). CNBr-activated Sepharose 4B was purchased from Pharmacia Chemical Co. (Mississauga, Ontario, Canada). M_r marker proteins for SDS/polyacrylamide-gel electrophoresis were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). General laboratory reagents used were analytical grade or better and were purchased from Fisher Scientific (Calgary, Alberta, Canada).

Calmodulin was purified from frozen bovine brains by a modification of the method of Gopalakrishna & Anderson (1982), as described in detail by Walsh *et al.* (1984), and coupled to CNBr-activated Sepharose 4B as previously described (Walsh *et al.*, 1982). The following proteins were purified by previously described methods: rabbit skeletal-muscle actin (Zot & Potter, 1981), myosin (Persechini & Rowe, 1984) and tropomyosin (Smillie, 1982), and chicken gizzard tropomyosin (Smillie, 1982). Rabbit skeletal-muscle troponin was purified as described by Potter (1982) up to and including the $(NH_4)_2SO_4$-fractionation (40–60%-satn.) step. Laser-densitometric scanning (with an LKB 2202 Ultroscan laser densitometer equipped with an HP3390A integrator) of a Coomassie-Blue-stained gel of the troponin preparation indicated 90.7% purity. The purity of these protein preparations is shown in Fig. 1. Caldesmon was prepared by two methods. The first included a heat-treatment step described by Bretscher (1984) and yielded a preparation which was 97% pure by SDS/polyacrylamide-gradient slab-gel electrophoresis (Fig. 1, lane D) and laser densitometry, and lacked caldesmon kinase activity. The details of this purification are described elsewhere (Clark *et al.*, 1986). The second method of purification of caldesmon avoided heat treatment and yielded a product which was 88% pure (Fig. 1, lane E) and contained caldesmon kinase activity (Ngai *et al.*, 1984). Caldesmon purified by the second method was used in the experiments of Tables 3 and 4. Caldesmon purified by the first method was used in all other experiments.

Enzyme assays

Actin-activated myosin Mg^{2+}-ATPase activities in the absence of troponin were measured at 30 °C in reaction volumes of 1.1 ml under the following conditions: 25 mM-Tris/HCl (pH 7.5), 20 mM-KCl, 3.5 mM-$MgCl_2$, 0.2 mM-EGTA or 0.1 mM-$CaCl_2$, 1 mM-[γ-^{32}P]ATP (~ 5000 c.p.m./nmol), 0.57 μM-myosin, 3.6 μM-actin. Other additions are indicated in the text. Reactions were initiated by addition of [γ-^{32}P]ATP. Samples (0.2 ml) of reaction mixtures were withdrawn at $t = 1$, 2, 3, 4 and 5 min for quantification of ATP hydrolysis as described by Ikebe & Hartshorne (1985). Rates of ATP hydrolysis (nmol of P_i released/min per mg of myosin) were calculated by linear-regression analysis of the linear ATPase time-course data.

Myosin Mg^{2+}-ATPase activities were also measured in the absence of actin under otherwise identical conditions and in the absence and presence of 1.8 μM-calmodulin. The specific radioactivity of [γ-^{32}P]ATP in these assays was ~ 15000 c.p.m./nmol.

Myosin Ca^{2+}-ATPase activities were measured at 30 °C in reaction volumes of 1.1 ml under the following conditions: 30 mM-Tris/HCl (pH 7.5), 7 mM-$CaCl_2$, 0.1 M- or 0.3 M-KCl, 1 mM-[γ-^{32}P]ATP (~ 5700 c.p.m./nmol), 0.57 μM-myosin in the absence and presence of 3.6 μM-actin. ATPase rates were determined as described above.

Myosin K^+/EDTA-ATPase activities were measured at 30 °C in reaction volumes of 1.1 ml under the following conditions: 30 mM-imidazole/HCl (pH 6.8), 0.1 M- or 0.3 M-KCl, 2 mM-EDTA, 1 mM-[γ-^{32}P]ATP (~ 5700 c.p.m./nmol), 0.57 μM-myosin in the absence and presence of 3.6 μM-actin.

Actin-activated myosin Mg^{2+}-ATPase activities in the presence of troponin were measured at 30 °C in reaction volumes of 1.1 ml under the following conditions: 25 mM-Tris/HCl (pH 7.5), 20 mM-KCl, 3.5 mM-$MgCl_2$, 0.2 M-EGTA or 0.1 mM-$CaCl_2$, 2 mM-[γ-^{32}P]ATP (~ 2700 c.p.m./nmol), 0.57 μM-myosin, 3.6 μM-actin, 1 μM-tropomyosin, 1 μM-troponin. Reactions were initiated by addition of [γ-^{32}P]ATP. Samples (0.2 ml) of reaction mixtures were withdrawn at $t = 0.5$, 1, 1.5, 2 and 2.5 min for quantification of ATP hydrolysis as described above. The Ca^{2+}-sensitivity of actomyosin Mg^{2+}-ATPase in the presence of troponin/tropomyosin is defined as follows:

$$Ca^{2+}\text{-sensitivity}\ (\%) = \frac{(\text{ATPase rate with } Ca^{2+}) - (\text{ATPase rate without } Ca^{2+})}{(\text{ATPase rate with } Ca^{2+})} \times 100\%$$

where the ATPase rates have been corrected for the low myosin Mg^{2+}-ATPase rates observed in the absence of actin.

Electrophoresis

Electrophoresis was performed in 7.5–20%-polyacrylamide-gradient slab gels (1.5 mm thick), with a 5%-acrylamide stacking gel, in the presence of 0.1% SDS at 36 mA in the discontinuous buffer system of Laemmli (1970). Gels were stained in 45% (v/v) ethanol/10% (v/v) acetic acid containing 0.14% (w/v) Coomassie Brilliant Blue R-250 and diffusion-destained in 10% acetic acid. Gels were dried in a Pharmacia GSD-4 gel slab drier and autoradiographed by using Kodak X-Omat AR film in a Kodak X-Omat AR cassette equipped with an intensifying screen.

Other methods

Protein concentrations were determined by the Coomassie Blue dye-binding assay (Spector, 1978), with dye reagent purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.) and γ-globulin as the standard.

Caldesmon containing endogenous caldesmon kinase was phosphorylated by incubation at a protein concentration of 0.175 mg/ml at 30 °C in 20 mM-Tris/HCl (pH 7.5)/5 mM-$MgCl_2$/0.2 mM-$CaCl_2$ or 2 mM-EGTA/6 μM-calmodulin/0.5 mM-[γ-^{32}P]ATP (~ 15000 c.p.m./nmol) for 60 min. Phosphate (^{32}P) incorporation into caldesmon was measured essentially as described for myosin phosphorylation by Walsh *et al.* (1983). Samples were immediately diluted into ATPase-assay systems to determine their effects on the actin-activated myosin Mg^{2+}-ATPase. In these experiments, the ATPase reactions were initiated by addition of myosin.

Caldesmon containing caldesmon kinase was heat-treated to denature the kinase by immersion in a boiling-water bath for 5 min. The sample was cooled on ice and centrifuged at 45000 *g* for 15 min to remove denatured proteins, and the supernatant (heat-treated caldesmon) was used in subsequent experiments (Table 3).

RESULTS

Inhibition of skeletal-muscle actin-activated myosin Mg^{2+}-ATPase by caldesmon

Fig. 2 shows the inhibition of skeletal-muscle actin-activated myosin Mg^{2+}-ATPase activity by increasing concentrations of caldesmon. Half-maximal inhibition occurred at ~ 0.42 μM-caldesmon, i.e. a caldesmon/actin molar ratio of 1:8.6. This inhibition was prevented by Ca^{2+}/calmodulin. Indeed, in the presence of Ca^{2+}/calmodulin, caldesmon had an activating effect on the myosin Mg^{2+}-ATPase activity, perhaps reflecting a direct effect of the calmodulin–caldesmon complex on the myosin molecule.

The data in Table 1 demonstrate the following: (1) inhibition of the actin-activated myosin Mg^{2+}-ATPase is observed in the presence and absence of tropomyosin, but is more pronounced in the presence of tropomyosin (at 0.57 μM-caldesmon, 47.7% inhibition was observed in the absence of tropomyosin and 75.0% inhibition in the presence of tropomyosin); (2) inhibition in either the presence or the absence of tropomyosin can be partially prevented by 1.8 μM-calmodulin in the presence, but not in the absence, of Ca^{2+}.

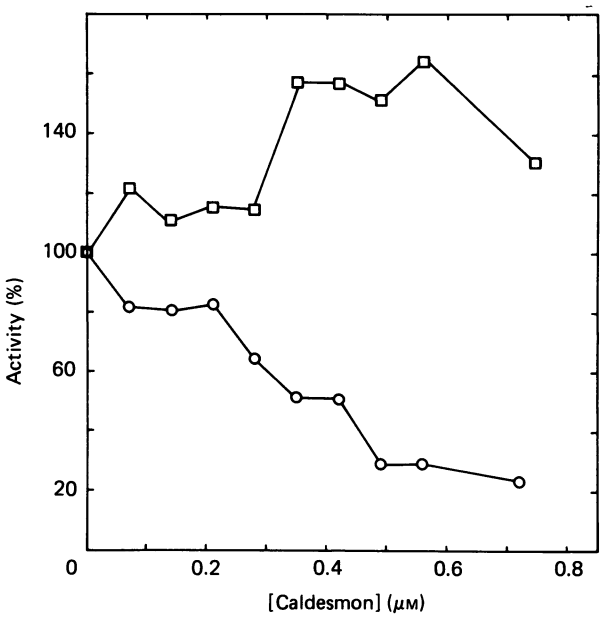

Fig. 2. Inhibition of the actin-activated Mg^{2+}-ATPase activity of skeletal-muscle myosin by caldesmon and reversal by Ca^{2+}/calmodulin

The actin-activated Mg^{2+}-ATPase activity of skeletal-muscle myosin was measured as described in the Materials and methods section in the presence of 0.76 μM-tropomyosin and either 0.2 mM-EGTA (○) or 0.1 mM-$CaCl_2$ and 4.9 μM-calmodulin (□) at the indicated concentrations of caldesmon. The ATPase rates in the absence of caldesmon were (○) 504.2 and (□) 408.5 nmol of P_i/min per mg of myosin. Values represent the means of triplicate determinations.

If caldesmon inhibition of the actomyosin Mg^{2+}-ATPase activity is of physiological significance, it must also occur in a reconstituted system containing troponin as well as tropomyosin, myosin and actin. We therefore examined the effect of caldesmon on the Mg^{2+}-ATPase activity of a system reconstituted from myosin, actin, tropomyosin and the troponin complex (all from rabbit skeletal muscle). In an initial series of experiments, we verified the Ca^{2+}-dependence of the ATPase activity of this reconstituted system. The following ATPase rates (nmol of P_i/min per mg of myosin) were determined as described in the Materials and methods section: 1473.9 ($+Ca^{2+}$) and 124.9 ($-Ca^{2+}$) in the presence of actin, and 35.3 ($+Ca^{2+}$) and 1.5 ($-Ca^{2+}$) in the absence of actin. The Ca^{2+}-sensitivity was calculated from these data (see the Materials and methods section) to be 91.4%. We proceeded therefore to investigate the effect of caldesmon on the actin-activated myosin Mg^{2+}-ATPase activity in this reconstituted system containing tropomyosin and the troponin complex. The data in Table 2 indicate that caldesmon (0.57 μM) inhibited the actomyosin Mg^{2+}-ATPase in the presence of Ca^{2+} by 32.1%. This inhibition was partially overcome by Ca^{2+}/calmodulin (1.8 μM). The low ATPase rates observed in the absence of Ca^{2+} were not significantly affected by caldesmon. The effect of increasing concentrations of caldesmon on actomyosin Mg^{2+}-ATPase activity in the presence of

Table 1. Effects of caldesmon in the absence and presence of Ca^{2+}/calmodulin on the actin-activated Mg^{2+}-ATPase activity of skeletal-muscle myosin

ATPase activities were measured in triplicate as described in the Materials and methods section in the absence and presence of 0.76 μM-tropomyosin, 0.57 μM-caldesmon or 1.8 μM-calmodulin and either 0.1 mM-$CaCl_2$ ($+Ca^{2+}$) or 0.2 mM-EGTA ($-Ca^{2+}$). Abbreviations: M, myosin; A, actin; Tm, tropomyosin; CaM, calmodulin; CaD, caldesmon. Results are means ± S.D.

Conditions	ATPase rate (nmol of P_i/min per mg of myosin)
M+A−Ca^{2+}	320.0±21.7
M+A+Tm−Ca^{2+}	371.9±45.3
M+A+CaM−Ca^{2+}	281.5±41.4
M+A+CaD−Ca^{2+}	167.3± 6.7
M+A+Tm+CaD−Ca^{2+}	80.1±18.8
M+A+CaD+CaM+Ca^{2+}	247.9±44.0
M+A+CaD+CaM−Ca^{2+}	144.7±10.9
M+A+Tm+CaD+CaM+Ca^{2+}	167.6±21.8
M+A+Tm+CaD+CaM−Ca^{2+}	72.3±11.8

Table 2. Effects of caldesmon in the absence and presence of Ca^{2+}/calmodulin on the actin-activated Mg^{2+}-ATPase activity of skeletal-muscle myosin in the presence of troponin/tropomyosin

ATPase activites were measured as described in the Materials and methods section in the presence of 1 μM-troponin/tropomyosin (rabbit skeletal muscle) and in the presence and absence of 0.57 μM-caldesmon (CaD) and 1.8 μM-calmodulin (CaM) and either 0.1 mM-$CaCl_2$ ($+Ca^{2+}$) or 0.2 mM-EGTA ($-Ca^{2+}$). Values in parentheses indicate ATPase activity as a percentage of the maximal rate, i.e. the rate in the absence of caldesmon and calmodulin and in the presence of Ca^{2+}.

Conditions	ATPase rate (nmol of P_i/min per mg of myosin)			
	−CaD		+CaD	
−CaM−Ca^{2+}	178.5	(13.3)	149.7	(11.1)
−CaM+Ca^{2+}	1345.8	(100)	914.4	(67.9)
+CaM−Ca^{2+}	215.5	(16.0)	154.0	(11.4)
+CaM+Ca^{2+}	1332.9	(99.0)	1121.8	(83.4)

troponin/tropomyosin is shown in Fig. 3. Half-maximal inhibition was observed at 0.91 μM-caldesmon, i.e. ~ 1 caldesmon:1 tropomyosin.

Effect of phosphorylation of caldesmon on inhibition of the actin-activated myosin Mg^{2+}-ATPase

Caldesmon was purified as described by Ngai *et al.* (1984) by preparation of washed myofibrils, extraction with 25 mM-Mg^{2+}, ion-exchange chromatography and affinity chromatography on a column of calmodulin–Sepharose. This preparation contains minor contaminating proteins (see Fig. 1, lane E). As demonstrated previously, this preparation contains endogenous Ca^{2+}/calmodulin-dependent caldesmon kinase activity (Ngai & Walsh, 1984, 1985*a*). Incubation of this caldesmon preparation with calmodulin and Mg[γ-^{32}P]ATP, as described in the Materials and methods section, led to the incorporation of 3.8 mol of P_i/mol of caldesmon in the presence of Ca^{2+} and 0.3 mol of P_i/mol of caldesmon in the absence of Ca^{2+}. Specific phosphorylation of caldesmon was verified by SDS/polyacrylamide-gradient slab-gel electrophoresis and autoradiography (results not shown). The effects of both phosphorylated (3.8 mol/mol) and non-phosphorylated (0.3 mol/mol) caldesmons on the actin-activated myosin Mg^{2+}-ATPase were examined. In addition, a sample of the caldesmon preparation was heat-treated, as described in the Materials and methods section, to inactivate caldesmon kinase, and denatured proteins were removed by centrifugation. The supernatant (heat-treated caldesmon) was used as a control in the ATPase experiment. The results are shown in Table 3. As shown previously, heat-treated caldesmon inhibited the actin-activated myosin Mg^{2+}-ATPase (to 52.1% of the activity observed in the absence of caldesmon). A similar effect was observed with phosphorylated (3.8 mol/mol) and non-phosphorylated (0.3 mol/mol) caldesmons: inhibition to 45.0% and 41.1%, respectively, of the activity in the absence of caldesmon. Similar inhibition was observed with caldesmon containing endogenous caldesmon kinase without pre-phosphorylation (Table 3). In this series of experiments, the ATPase activity was measured under four sets of conditions: in the presence of 0.2 mM-EGTA, 0.1 mM-$CaCl_2$, 0.2 mM-EGTA + 1.8 μM-calmodulin, and 0.1 mM-$CaCl_2$ + 1.8 μM-calmodulin. Caldesmon phosphorylation was detected only in the presence of Ca^{2+} and calmodulin, and reached 0.9 mol of P_i/mol of caldesmon during the 5 min ATPase time-course assay (results not shown). Inhibition of the actin-activated myosin Mg^{2+}-ATPase by both phosphorylated and non-phosphorylated caldesmons could be prevented by Ca^{2+}/calmodulin (Table 4).

Inhibition of the myosin Mg^{2+}-ATPase by caldesmon in the absence of actin

Fig. 4 demonstrates that caldesmon is also capable of inhibiting the Mg^{2+}-ATPase activity of skeletal-muscle myosin in the absence of actin. Half-maximal inhibition was observed at ~ 0.44 μM-caldesmon, i.e. similar to the concentration required for half-maximal inhibition of the actin-activated myosin Mg^{2+}-ATPase. This concentration represents a caldesmon/myosin molar ratio of 1:1.3. The data in Table 5 indicate that this inhibition of the myosin Mg^{2+}-ATPase could be overcome by calmodulin, but only in the presence of Ca^{2+}.

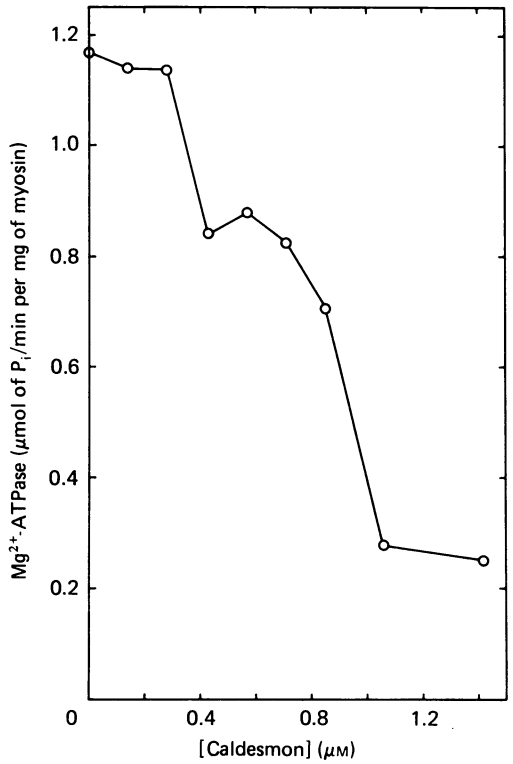

Fig. 3. Inhibition of the actin-activated Mg^{2+}-ATPase activity of skeletal-muscle myosin by caldesmon in the presence of troponin/tropomyosin

The actin-activated Mg^{2+}-ATPase activity of skeletal-muscle myosin was measured as described in the Materials and methods section in the presence of 1 μM-troponin, 1 μM-tropomyosin and 0.1 mM-$CaCl_2$ at the indicated concentrations of caldesmon.

Inhibition of the myosin Ca^{2+}-ATPase by caldesmon in the presence and absence of actin

Caldesmon inhibited the Ca^{2+}-ATPase activity of skeletal-muscle myosin at both low (0.1 M-KCl) and high (0.3 M-KCl) ionic strength, in the presence and absence of actin (Table 6). Actin had no significant effect on the myosin Ca^{2+}-ATPase activity at low ionic strength, but inhibited this activity at 0.3 M-KCl by 58.5%. Caldesmon caused a further inhibition (to 26.7% of the Ca^{2+}-ATPase rate of myosin alone).

Inhibition of the myosin K^+/EDTA-ATPase by caldesmon

Caldesmon inhibited the K^+/EDTA-ATPase activity of skeletal-muscle myosin at low ionic strength (0.1 M-KCl) in the absence of actin to 28.6% of the ATPase rate observed in the absence of caldesmon (Table 7). No significant inhibition of this ATPase rate by caldesmon was observed in the presence of actin at 0.1 M-KCl or in the presence or absence of actin at 0.3 M-KCl. Actin was observed to enhance the myosin K^+/EDTA-ATPase activity at 0.1 M-KCl and to inhibit this activity at 0.3 M-KCl.

DISCUSSION

Caldesmon is broadly distributed among tissues and species (Kakiuchi *et al.*, 1983; Ban *et al.*, 1984; Owada *et al.*, 1984; Bretscher & Lynch, 1985; Marston & Lehman, 1985; Ngai & Walsh, 1985*b*; Clark *et al.*, 1986), including smooth, skeletal and cardiac muscles and a variety of non-muscle tissues. The demonstration of caldesmon in isolated rat cardiac myocytes confirms the presence of this protein in striated-muscle cells, since one can rule out possible contamination by vascular

Table 3. Effects of phosphorylated (P) and non-phosphorylated (nonP) caldesmons on the actin-activated Mg^{2+}-ATPase activity of skeletal-muscle myosin

Caldesmon containing caldesmon kinase (non-heat-treated CaD) was pre-phosphorylated to 3.8 mol of P_i/mol ($+Ca^{2+}$) and 0.3 mol of P_i/mol ($-Ca^{2+}$) as described in the Materials and methods section. Another sample of caldesmon containing caldesmon kinase was heat-treated to denature the kinase as described in the Materials and methods section. The effects of these caldesmons and non-heat-treated caldesmon on the actin-activated myosin Mg^{2+}-ATPase were examined at 0.2 mM-EGTA ($-Ca^{2+}$) or 0.1 mM-Ca^{2+} ($+Ca^{2+}$). Caldesmon, where present in the ATPase assays, was at a concentration of 0.57 μM. Assays were performed in triplicate in the absence of tropomyosin. For abbreviations see Table 1. Results are means ± S.D. Values in parentheses indicate activity as a percentage of that in the absence of caldesmon.

Conditions	ATPase rate (nmol of P_i/min per mg of myosin)	
M+A−Ca^{2+}	304.9±11.4	(100)
M+A+P-CaD (3.8 mol/mol)−Ca^{2+}	137.3±14.0	(45.0)
M+A+nonP-CaD (0.3 mol/mol)−Ca^{2+}	125.2±13.6	(41.1)
M+A+heat-treated CaD−Ca^{2+}	159.0±11.1	(52.1)
M+A+non-heat-treated CaD−Ca^{2+}	105.2±19.6	(34.5)
M+A+non-heat-treated CaD+Ca^{2+}	126.0±12.6	(41.3)
M+A+non-heat-treated CaD+CaM−Ca^{2+}	129.4±10.3	(42.4)
M+A+non-heat-treated CaD+CaM+Ca^{2+}	160.6±24.9	(52.7)

Table 4. Effect of Ca^{2+}/calmodulin on inhibition of the actin-activated myosin Mg^{2+}-ATPase by phosphorylated and non-phosphorylated caldesmons

The actin-activated Mg^{2+}-ATPase activity of skeletal-muscle myosin was measured, as described in the Materials and methods section, in the absence and presence of phosphorylated (P; 3.8 mol of P_i/mol) and non-phosphorylated (nonP; 0.3 mol of P_i/mol) caldesmons at 0, 2 μM- and 5 μM-calmodulin. Where present, caldesmon was at a concentration of 0.57 μM. For abbreviations, see Table 1. Values in parentheses indicate activity as a percentage of that in the absence of caldesmon.

Conditions	ATPase rate (nmol of P_i/min per mg of myosin)
M+A−Ca^{2+}	119.0 (100)
M+A+P-CaD−Ca^{2+}	38.9 (32.7)
M+A+nonP-CaD−Ca^{2+}	26.5 (22.3)
M+A+P-CaD+2 μM-CaM+Ca^{2+}	98.2 (82.5)
M+A+nonP-CaD+2 μM-CaM+Ca^{2+}	105.9 (89.0)
M+A+P-CaD+5 μM-CaM+Ca^{2+}	125.8 (105.7)
M+A+nonP-CaD+5 μM-CaM+Ca^{2+}	113.4 (95.3)

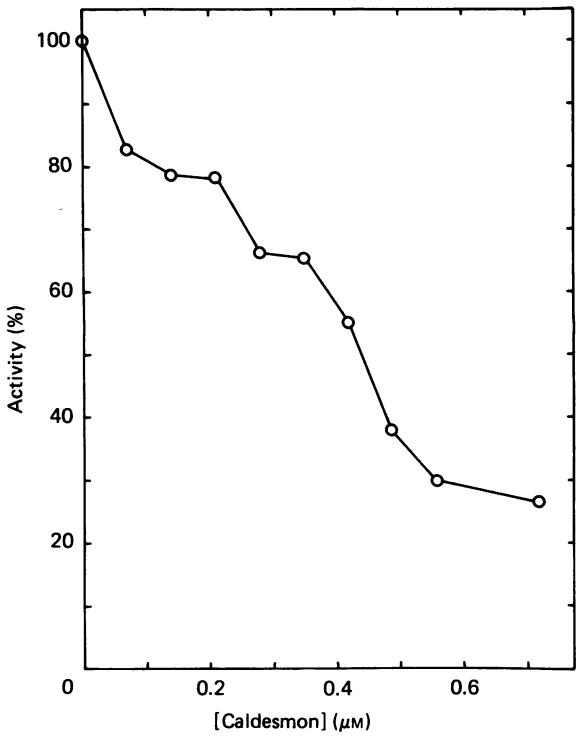

Fig. 4. Inhibition by caldesmon of the Mg^{2+}-ATPase activity of skeletal-muscle myosin in the absence of actin

The Mg^{2+}-ATPase activity of skeletal-muscle myosin was measured in the absence of actin as described in the Materials and methods section. Other conditions: 0.2 mM-EGTA, various caldesmon concentrations as indicated. The ATPase rate in the absence of caldesmon (100% activity) was 24.7 nmol of P_i/min per mg of myosin.

smooth-muscle cells in such a system. In addition, we have partially purified caldesmon from fresh bovine myocardium, identifying the protein by its heat-stability, Ca^{2+}-dependent interaction with calmodulin and immunological cross-reactivity with antibodies against chicken gizzard caldesmon (results not shown). The bovine cardiac protein has a M_r (146000) intermediate between those of chicken gizzard caldesmon (141000; Ngai *et al.*, 1984) and bovine aorta caldesmon (149000; Clark *et al.*, 1986). On the basis of Western immunoblots of tissue homogenates, we estimate the amount of caldesmon in skeletal muscle to be ~ 25% of that in chicken gizzard, assuming the antigen from the two tissues to be equally cross-reactive with anti-(chicken gizzard caldesmon). This would correspond to a concentration in skeletal muscle of ~ 3 μM.

Functionally, caldesmon has been found to inhibit actin-activated myosin Mg^{2+}-ATPase activity (Ngai & Walsh, 1984; Dabrowska *et al.*, 1985; Marston & Lehman, 1985; Clark *et al.*, 1986) and to induce the bundling of actin filaments (Bretscher, 1984; Dabrowska *et al.*, 1985) *in vitro*. In addition, caldesmon can induce polymerization of G-actin (Galazkiewicz *et al.*, 1985). Studies by Moody *et al.* (1985) suggest that caldesmon-induced inhibition of actomyosin ATPase is not related to its ability to bundle actin filaments, but is presumably a direct effect on actin–myosin interaction. In order to understand better the potential role of caldesmon in the regulation of actin–myosin interaction in striated muscles, we have studied the effects of caldesmon (both phosphorylated and non-phosphorylated) on the actin-activated Mg^{2+}-ATPase activity of rabbit skeletal-muscle myosin. As shown by others (Dabrowska *et al.*, 1985; Marston & Lehman, 1985), caldesmon inhibits this activity and inhibition is more potent in the presence than in the absence of tropomyosin. We observed half-maximal inhibition at a caldesmon/actin molar ratio of 1:8.6, compared with 1:12.5 seen by Dabrowska *et al.* (1985). This difference may be due to the higher [Mg^{2+}] (3.5 mM compared with 2 mM) used in our experiments, since Dabrowska *et al.* (1985) have shown diminished inhibition at increasing [Mg^{2+}]. If caldesmon inhibition is of physiological significance, it should also occur in a reconstituted skeletal-muscle system which also contains the troponin complex and tropomyosin. We have clearly demonstrated inhibition by caldesmon of the actomyosin Mg^{2+}-ATPase in a system *in vitro* reconstituted from rabbit skeletal-muscle myosin, actin, tropomyosin and troponin.

Consistent with the fact that Ca^{2+}/calmodulin competes with F-actin for caldesmon binding (Sobue *et al.*, 1981; Bretscher, 1984; Ngai *et al.*, 1984; Clark *et al.*, 1986), we found that caldesmon inhibition of the actomyosin Mg^{2+}-ATPase could be overcome by Ca^{2+}/calmodulin. Interestingly, we also observed that caldesmon that had

Table 5. Effects of caldesmon in the absence and presence of Ca^{2+}/calmodulin on the Mg^{2+}-ATPase activity of skeletal-muscle myosin in the absence of actin

ATPase activities were measured in triplicate as described in the Materials and methods section in the absence and presence of 0.57 μM-caldesmon and 1.8 μM-calmodulin at 0.1 mM-$CaCl_2$ (+Ca^{2+}) or 0.2 mM-EGTA (−Ca^{2+}). The results of two sets of experiments using two different preparations of myosin are shown. The different ATPase rates of the two preparations probably reflect different ages of the myosins (Persechini & Stull, 1984). For abbreviations see Table 1. Results are means ± S.D.

Conditions	ATPase rate (nmol of P_i/min per mg of myosin) Expt. 1	Expt. 2
M	23.2±2.3	13.6±2.3
M+CaD	13.0±1.7	6.6±0.9
M+CaD+CaM+Ca^{2+}	35.4±4.5	16.0±4.2
M+CaD−CaM−Ca^{2+}	9.3±1.6	6.8±0.7

Table 6. Effects of caldesmon in the absence and presence of actin on the Ca^{2+}-ATPase activity of skeletal-muscle myosin

Ca^{2+}-ATPase activities were measured in triplicate as described in the Materials and methods section in the absence and presence of 3.6 μM-actin and 0.57 μM-caldesmon at 0.1 M-KCl and 0.3 M-KCl. For abbreviations see Table 1. Results are means ± S.D.; values in parentheses indicate activity as a percentage of that of myosin alone.

Conditions	ATPase rate (nmol of P_i/min per mg of myosin) 0.1 M-KCl	0.3 M-KCl
M	46.4±4.0 (100)	337.2±6.4 (100)
M+CaD	20.4±2.7 (44.0)	183.9±10.6 (54.5)
M+A	39.5±3.3 (85.1)	140.0±5.4 (41.5)
M+A+CaD	18.9±1.8 (40.7)	90.2±0.5 (26.7)

Table 7. Effects of caldesmon in the absence and presence of actin on the K^+/EDTA-ATPase activity of skeletal-muscle myosin

K^+/EDTA-ATPase activities were measured in triplicate as described in the Materials and methods section in the absence and presence of 3.6 μM-actin and 0.57 μM-caldesmon at 0.1 M-KCl and 0.3 M-KCl. For abbreviations, see Table 1. Results are means ± S.D.; values in parentheses indicate activity as a percentage of that of myosin alone.

Conditions	ATPase rate (nmol of P_i/min per mg of myosin) 0.1 M-KCl	0.3 M-KCl
M	68.1±12.4 (100)	206.4±30.1 (100)
M+CaD	19.5± 2.0 (28.6)	176.4±17.1 (85.5)
M+A	140.9±18.0 (206.9)	100.7± 2.1 (48.8)
M+A+CaD	133.4±12.2 (195.9)	90.9± 4.7 (44.0)

been pre-phosphorylated to the extent of 3.8 mol of P_i/mol of caldesmon was equally as effective as non-phosphorylated caldesmon in inhibition of the actomyosin Mg^{2+}-ATPase. Again, this inhibition could be overcome by excess Ca^{2+}/calmodulin. Our earlier observations (Ngai & Walsh, 1984) suggested the possibility that phosphorylated caldesmon may be ineffective in inhibition of the actin-activated Mg^{2+}-ATPase activity of smooth-muscle myosin. This is clearly not the case with skeletal-muscle myosin.

We have also examined the effects of caldesmon on the Mg^{2+}-ATPase activity of skeletal-muscle myosin in the absence of actin. This activity was also inhibited by caldesmon, and inhibition could be prevented by Ca^{2+}/calmodulin. Similarly, the Ca^{2+}-ATPase activity of skeletal-muscle myosin was inhibited by caldesmon at low and high ionic strength and in the presence and absence of actin. Caldesmon was also observed to inhibit the K^+/EDTA-ATPase activity of skeletal-muscle myosin, but only at 0.1 M-KCl in the absence of actin. In the presence of actin at 0.1 M-KCl, and in the presence or absence of actin at 0.3 M-KCl, no inhibition was

observed. These results suggest that caldesmon regulation of actin–myosin interaction may occur in skeletal muscle and may involve direct effects on the myosin, rather than simple steric blocking of the actin–myosin interaction owing to association of caldesmon with the thin filament.

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