The effects of phosphorylation of smooth-muscle caldesmon

Philip K. NGAI and Michael P. WALSH

Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Caldesmon is a major calmodulin- and actin-binding protein of smooth muscle which interacts with calmodulin in a Ca^{2+} -dependent manner or with actin in a Ca^{2+} -independent manner. Isolated caldesmon is capable of inhibiting the actin-activated Mg^{2+} -ATPase of smooth-muscle myosin, suggesting a possible physiological role for caldesmon in regulating the contractile state of smooth-muscle. Caldesmon can be phosphorylated *in vitro* by a co-purifying Ca^{2+} /calmodulin-dependent protein kinase and dephosphorylated by a protein phosphatase, both of which are present in smooth muscle. We investigated further the phosphorylation of caldesmon and the effects which phosphorylation has on the functional properties of the protein. The kinetics of caldesmon phosphorylation were similar whether the caldesmon substrate was free or bound to actin, actin/tropomyosin or thin filaments. Caldesmon containing endogenous kinase activity was rapidly phosphorylated (to approx. 1 mol of P_i /mol of caldesmon in 5 min) when reconstituted with actin, myosin, tropomyosin, calmodulin and myosin light-chain kinase in the presence of Ca^{2+} and MgATP^{2-} . Under conditions in which unphosphorylated caldesmon showed substantial inhibition of the actin-activated myosin Mg^{2+} -ATPase, no inhibition was observed with phosphorylated caldesmon. This was the case whether caldesmon was phosphorylated before addition to the actomyosin Mg^{2+} -ATPase system, or phosphorylation was allowed to take place during the ATPase reaction. Binding studies revealed maximal binding of 1 mol of unphosphorylated caldesmon/9.5 mol of actin and 1 mol of phosphorylated caldesmon/11.7 mol of actin. All the bound phosphorylated caldesmon could be released by Ca^{2+} /calmodulin, with half-maximal release at $0.11 \mu\text{M}-\text{Ca}^{2+}$, whereas only 62% of the bound unphosphorylated caldesmon could be removed, with half-maximal release at $0.16 \mu\text{M}-\text{Ca}^{2+}$. However, under conditions in which inhibition of actomyosin Mg^{2+} -ATPase activity by non-phosphorylated but not by phosphorylated caldesmon was observed, both forms of caldesmon would remain bound to the thin filament. These observations suggest a possible mechanism whereby caldesmon phosphorylation may prevent its inhibitory action on the actomyosin Mg^{2+} -ATPase.

INTRODUCTION

Smooth-muscle contraction is regulated primarily by fluxes in sarcoplasmic $[\text{Ca}^{2+}]$ via the reversible phosphorylation of myosin (Adelstein & Eisenberg, 1980; Kamm & Stull, 1985; Walsh & Hartshorne, 1982). Physiological (Aksoy *et al.*, 1982, 1983, 1986; Butler *et al.*, 1983; Chatterjee & Murphy, 1983; Dillon *et al.*, 1981; Gerthoffer & Murphy, 1983; Silver & Stull, 1982) and biochemical (Marston & Lehman, 1985; Marston & Smith, 1984; Marston *et al.*, 1980) studies have suggested that regulation of the contractile state of smooth muscle may involve additional Ca^{2+} -dependent mechanisms. A potential candidate for such a control system is the protein caldesmon, a major actin- and calmodulin-binding protein, operating in conjunction with a Ca^{2+} -binding protein, possibly calmodulin (Marston & Smith, 1985). Caldesmon 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; Ngai & Walsh, 1985). Immunocytochemistry (Bretscher & Lynch, 1985; Fürst *et al.*, 1986) and isolation of native thin filaments (Marston & Lehman, 1985; Lehman, 1986) support the localization of caldesmon on the actin filament. Binding studies led Sobue *et al.* (1982) to suggest that caldesmon can

'flip-flop' between F-actin and calmodulin as a function of the sarcoplasmic free Ca^{2+} concentration. However, studies with smooth-muscle thin filaments indicate that, even in the presence of high concentrations of Ca^{2+} /calmodulin, only up to 40% of the caldesmon can be removed from the actin filament (Marston & Smith, 1985).

Isolated caldesmon is capable of inhibiting superprecipitation (Sobue *et al.*, 1982) and actin-activated myosin Mg^{2+} -ATPase activity (Ngai & Walsh, 1984) in a system *in vitro* reconstituted from smooth-muscle contractile and regulatory proteins. We observed that caldesmon purified by a method using calmodulin-Sepharose affinity chromatography contained an endogenous protein kinase activity which catalysed the phosphorylation of caldesmon in a Ca^{2+} /calmodulin-dependent manner (Ngai & Walsh, 1984, 1985). The same smooth muscle (chicken gizzard) was observed to contain a protein phosphatase capable of dephosphorylating caldesmon. Preliminary observations suggested that phosphorylation of caldesmon may abolish its inhibitory effect on the actin-activated myosin Mg^{2+} -ATPase (Ngai & Walsh, 1984). We have extended this earlier work to a more detailed analysis of the effects of caldesmon phosphorylation on its ability to inhibit actin-activated myosin Mg^{2+} -ATPase activity and its interactions with F-actin and calmodulin.

MATERIALS AND METHODS

Materials

[γ - 32 P]ATP (10–40 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). Electrophoresis reagents and M_r marker proteins were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). General laboratory reagents used were of analytical grade or better and were purchased from Fisher Scientific (Calgary, Alberta, Canada).

Protein purification methods

Calmodulin was purified from frozen bovine brains by a modification of the procedure of Gopalakrishna & Anderson (1982), as described in detail by Walsh *et al.* (1984). The following proteins were purified from chicken gizzard smooth muscle by methods described previously: actin (Ngai *et al.*, 1986), myosin (Persechini & Hartshorne, 1981), tropomyosin (Bretscher, 1984), myosin light-chain kinase (Ngai *et al.*, 1984). Caldesmon containing endogenous caldesmon kinase activity was purified from chicken gizzard as described by Ngai *et al.* (1984). Thin filaments were isolated from chicken gizzard as described by Marston & Lehman (1985) and shown, by SDS/polyacrylamide-gradient-gel electrophoresis, to consist almost exclusively of actin, tropomyosin, caldesmon (identified by immunoblotting) and an unidentified M_r -32000 polypeptide. Laser densitometry of such gels revealed a typical actin content in thin filaments of 65% (w/w).

Heat-inactivation of caldesmon kinase

Caldesmon (2.37 mg/ml; 6.7 ml) containing endogenous caldesmon kinase activity in 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/0.1 mM-dithiothreitol/1 mM-EGTA was heated on a boiling-water bath to 90 °C; the temperature was maintained at 85–90 °C for 2 min before cooling on ice and centrifugation at 45000 *g* for 15 min. Densitometric scanning of a Coomassie Blue-stained SDS/polyacrylamide-gel electrophoretogram of the preparation before heat-treatment and of the supernatant after heat-inactivation, with the use of an LKB model 2202 Ultrosan laser densitometer equipped with a Hewlett-Packard model 3390A integrator, revealed the caldesmon band (M_r 141000) to constitute 85.6% and 90.1%, respectively, of total protein (see Fig. 1).

Enzymic assays

[32 P]Phosphate incorporation into substrate proteins (caldesmon and myosin) was quantified as described by Walsh *et al.* (1983). Actin-activated myosin Mg^{2+} -ATPase activities were measured as described by Ikebe & Hartshorne (1985). Details of reaction conditions for individual experiments are given in the appropriate Figure legends.

Electrophoretic procedures

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% (w/v) 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% (v/v) acetic acid. Destained gels were sealed in

plastic bags and autoradiographed by using Kodak X-Omat AR film in Kodak X-Omat AR cassettes fitted with intensifying screens.

Large-scale phosphorylation of caldesmon

Caldesmon (0.7 μ M) containing caldesmon kinase activity was incubated in 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/5 mM-MgCl₂/0.1 mM-dithiothreitol/0.1 mM-CaCl₂/0.6 μ M-calmodulin/1 mM-ATP at 30 °C for 90 min. The reaction mixture was then heated to 85–90 °C, and the temperature maintained for 2 min before cooling the sample on ice. Denatured proteins were removed by centrifugation at 45000 *g* for 15 min. Control non-phosphorylated caldesmon was prepared in an identical manner, except that 1 mM-EGTA replaced 0.1 mM-CaCl₂. The caldesmon concentrations of the resulting samples were re-determined before use in binding studies.

Binding of caldesmon to thin filaments

Various amounts of caldesmon (0–1.4 μ M; 0–0.2 mg/ml) containing caldesmon kinase activity were incubated with thin filaments (11.9 μ M-actin; 0.5 mg/ml) in 20 mM-sodium phosphate (pH 6.25)/0.2 M-NaCl/10 mM-MgCl₂/2 mM-EGTA/2 mM-dithiothreitol/2 mM-NaN₃ at 30 °C for 30 min in a total volume of 1.0 ml. Samples were cooled on ice and centrifuged at 120000 *g* for 150 min to sediment actin filaments and bound proteins. Supernatants and pellets were separated, supernatants were freeze-dried, and the pellets and freeze-dried supernatants were redissolved in 0.5 ml of SDS-gel sample buffer and immersed in boiling water for 2 min before SDS/PAGE (20 μ l sample/lane). The amounts of caldesmon in the supernatants and pellets were quantified by scanning laser densitometry of the Coomassie Blue-stained gels.

Binding of caldesmon to F-actin and F-actin/tropomyosin

Various amounts of phosphorylated and non-phosphorylated caldesmon (0–1.2 μ M) were incubated at 30 °C for 30 min in 5 mM-Tris/HCl (pH 7.5)/0.1 M-KCl/0.1 mM-dithiothreitol/2.5 mM-MgCl₂/1 mM-ATP/1 mM-EGTA with 11.9 μ M-actin in the absence and presence of 2.0 μ M-tropomyosin in a total volume of 1.0 ml. Samples were chilled on ice and centrifuged at 200000 *g* for 90 min. The protein contents of the separated supernatants and pellets were examined by SDS/PAGE and quantified by laser densitometry (see above).

Protein concentration determinations

Protein concentrations were determined by the Coomassie Blue dye-binding assay (Spector, 1978), with dye reagent and γ -globulin standard purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), or by spectrophotometric measurements using the following absorption coefficients: calmodulin, $A_{277}^{1\%} = 1.9$ (Klee, 1977); myosin, $A_{280}^{1\%} = 5.6$ (Greene *et al.*, 1983); gizzard caldesmon, $A_{280}^{1\%} = 5.0$ (Clark *et al.*, 1986).

RESULTS

Heat-inactivation of caldesmon kinase

Caldesmon is a heat-stable protein (Bretscher, 1984), a property that is exploited in subsequent experi-

Fig. 1. Heat-inactivation of caldesmon kinase

Caldesmon containing endogenous caldesmon kinase activity was heat-treated as described in the Materials and methods section. Untreated (○) and heat-treated (□) caldesmon preparations (0.3 mg/ml) were incubated at 30 °C in 20 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/0.1 mM-CaCl₂/calmodulin (60 μg/ml)/0.5 mM-[γ-³²P]-ATP (4500 c.p.m./nmol) in reaction volumes of 1.5 ml. Reactions were initiated by addition of [γ-³²P]ATP, and samples (0.15 ml) were withdrawn at the indicated times for quantification of protein phosphorylation. At 60 min, an equal volume (0.15 ml) of SDS/gel sample buffer [50 mM-Tris/HCl (pH 6.8)/1% (w/v) SDS/30% (v/v) glycerol/0.01% Bromophenol Blue/1% (v/v) 2-mercaptoethanol] was added to the remainder of each reaction mixture and immersed in boiling water for 2 min before SDS/PAGE and autoradiography. Insets: (a) Coomassie-Blue-stained gel; (b) autoradiogram. Key to lanes: 1, untreated caldesmon containing endogenous caldesmon kinase (10 μg of protein); 2, heat-treated supernatant (10 μg of protein). Abbreviations: CaD, caldesmon; CaM, calmodulin.

ments. The results depicted in Fig. 1 indicate the heat-lability of caldesmon kinase activity. Incubation of untreated caldesmon containing caldesmon kinase activity with Ca²⁺/calmodulin and Mg[γ-³²P]ATP resulted in stoichiometric phosphorylation. SDS/PAGE and autoradiography established that P_i incorporation was specifically into the M_r-141 000 caldesmon polypeptide. Phosphorylation was calmodulin-dependent, since omission of calmodulin from the reaction mixture resulted in the incorporation of only 0.06 mol of P_i/mol of caldesmon in 60 min (results not shown). If caldesmon containing caldesmon kinase activity was heat-treated before the phosphorylation assay, no P_i incorporation into caldesmon was observed (Fig. 1).

Phosphorylation of free caldesmon and caldesmon bound to actin, actin/tropomyosin and thin filaments

The phosphorylation time-course assays depicted in Fig. 2 indicate that the rate and stoichiometry of caldesmon phosphorylation are similar whether caldes-

Fig. 2. Phosphorylation of free caldesmon and caldesmon bound to actin, actin/tropomyosin and thin filaments

Reaction conditions were as described in the legend to Fig. 1 with the following additions, as appropriate: actin (0.25 mg/ml), tropomyosin (75 μg/ml), thin filaments (0.41 mg/ml). Key to symbols in (a): ○, free caldesmon (lane 5); □, caldesmon+actin (lane 4); △, caldesmon+actin+tropomyosin (lane 3); ▽, caldesmon+thin filaments (lane 2); ●, thin filaments alone (no added caldesmon) (lane 1). (b) Coomassie-Blue-stained gel; (c) autoradiogram. Abbreviation: Tm, tropomyosin β-subunit (the γ-subunit co-migrates with actin in this electrophoretic system). Untreated caldesmon containing caldesmon kinase activity was used in these experiments. Loads on gels: lane 1, 4.2 μg of actin; lanes 2–5, 5 μg of caldesmon.

mon is free or bound to F-actin, F-actin/tropomyosin or thin filaments. Phosphorylation of caldesmon was verified in each case by SDS/PAGE and autoradiography (Fig. 2). A very low degree of actin phosphorylation was observed (Fig. 2c, lane 4), which was decreased in the

Fig. 3. Binding of caldesmon to thin filaments

The interaction of exogenous caldesmon with thin filaments was quantified by an ultracentrifugation procedure described in the Materials and methods section in which F-actin plus bound proteins were sedimented, leaving unbound soluble proteins in the supernatant. The protein compositions of separated supernatants and pellets were analysed by SDS/PAGE, and amounts of bound and free caldesmon were quantified by laser densitometry of Coomassie-Blue-stained gels such as that shown in the inset. The quantitative data obtained are plotted as mol of caldesmon bound/mol of actin versus mol of caldesmon added/mol of actin. The gel shows alternating supernatants and pellets with the following concentrations of exogenous caldesmon: 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.4 μM , reading from left to right. The extreme right-hand lane contains the exogenously added caldesmon preparation.

presence of tropomyosin (lane 3) and further decreased when caldesmon was combined with the thin filaments (lane 2). Binding of additional caldesmon to isolated thin filaments was verified by an ultracentrifugation technique (see the Materials and methods section) in conjunction with SDS/PAGE and laser densitometry (Fig. 3). No caldesmon phosphorylation was detected when isolated thin filaments were incubated with Ca^{2+} /calmodulin and $\text{Mg}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 2). This may be due to the low amount of caldesmon in the thin filaments (1 mol/26 mol of actin, based on scanning densitometry) or the absence of caldesmon kinase activity from the thin-filament preparation.

Fig. 4. Phosphorylation of caldesmon in a reconstituted actomyosin system

Caldesmon (50 $\mu\text{g}/\text{ml}$) containing caldesmon kinase activity was incubated at 30 $^{\circ}\text{C}$ in 25 mM-Tris/HCl (pH 7.5)/60 mM-KCl/10 mM- MgCl_2 /0.1 mM- CaCl_2 /calmodulin (20 $\mu\text{g}/\text{ml}$)/myosin light-chain kinase (10 $\mu\text{g}/\text{ml}$)/tropomyosin (50 $\mu\text{g}/\text{ml}$)/actin (0.25 mg/ml)/myosin (0.44 mg/ml)/1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (8500 c.p.m./nmol) in a reaction volume of 0.5 ml. Reaction was initiated by addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and was quenched at 5 min by addition of 0.5 ml of SDS-gel sample buffer and boiling before SDS/PAGE and autoradiography. A sample of reaction mixture containing 5 μg of actin was loaded on the gel. (a) Coomassie-Blue-stained gel; (b) autoradiogram. Key: HC, myosin heavy chain; CaD, caldesmon; A, actin; Tm, β -subunit of tropomyosin; LC_{20} , M_r -20000 light chain of myosin; LC_{17} , M_r -17000 light chain of myosin.

Rapid phosphorylation of caldesmon in a reconstituted actomyosin system

When caldesmon containing caldesmon kinase activity was reconstituted with purified actin, myosin, tropomyosin, calmodulin and myosin light-chain kinase, rapid phosphorylation of caldesmon and the M_r -20000 light chain of myosin was observed (Fig. 4). Laser densitometry of autoradiograms similar to that shown in Fig. 4(b), together with quantification of total protein phosphorylation as described in the Materials and methods section, enabled calculation of the stoichiometry of caldesmon phosphorylation: 1.0 mol of P_i was incorporated/mol of caldesmon during the 5 min

Table 1. Effect of caldesmon phosphorylation on inhibition of smooth-muscle actin-activated myosin Mg^{2+} -ATPase and superprecipitation

The effect of caldesmon (CaD) containing endogenous caldesmon kinase activity on the actin-activated Mg^{2+} -ATPase of gizzard myosin was compared with that of heat-treated caldesmon either without preincubation or immediately after preincubation at 30 °C for 60 min under the following conditions: 20 mM-Tris/HCl (pH 7.5)/5 mM- $MgCl_2$ /0.1 mM- $CaCl_2$ /calmodulin (60 μ g/ml)/0.5 mM- $[\gamma\text{-}^{32}P]$ ATP (2000 c.p.m./nmol) in the absence and presence of 0.3 mg of caldesmon/ml (caldesmon containing caldesmon kinase activity, either untreated or heat-treated to inactivate the kinase). ATPase assay conditions were: 25 mM-Tris/HCl (pH 7.5)/60 mM-KCl/10 mM- $MgCl_2$ /0.1 mM- $CaCl_2$ or 1 mM-EGTA/calmodulin (20 μ g/ml)/myosin light-chain kinase (10 μ g/ml)/tropomyosin (50 μ g/ml)/actin (0.25 mg/ml)/myosin (0.44 mg/ml)/1 mM- $[\gamma\text{-}^{32}P]$ ATP (2000 c.p.m./nmol) in the presence or absence of caldesmon (0.1 mg/ml). ATPase rates were calculated by linear-regression analysis of the time-course assays obtained by withdrawing samples (0.25 ml) of reaction mixtures (total volume 2.0 ml) at 1, 2, 3, 4, 5, 6 and 7 min for quantification of $[\text{P}_i]$ release. The occurrence of superprecipitation was determined by visual inspection.

Conditions	ATPase rate				Super- precipitation
	(nmol of P _i /min per mg of myosin		(% of control)		
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	
1. No preincubation					
+Ca ²⁺	89.0	79.9	100	100	+
No Ca ²⁺	2.3	5.0	2.6	6.3	—
+CaD/CaD kinase +Ca ²⁺	89.3	76.0	100.3	95.1	+
+heat-treated CaD +Ca ²⁺	57.6	30.4	64.7	38.0	—
2. Preincubated					
+Ca ²⁺	92.1		103.5		+
+CaD/CaD kinase +Ca ²⁺	83.2		93.5		+
+heat-treated CaD +Ca ²⁺	46.8		52.6		—

reaction. A significant degree of caldesmon phosphorylation is therefore achieved during the course of a typical actomyosin Mg^{2+} -ATPase reaction *in vitro* (see below).

Comparison of the effects of phosphorylated and non-phosphorylated caldesmons on the actin-activated myosin Mg^{2+} -ATPase

The effects on the actin-activated myosin Mg^{2+} -ATPase of untreated caldesmon containing caldesmon kinase activity and material heat-treated to inactivate the caldesmon kinase were compared by two experimental protocols (Table 1). Caldesmon samples were either preincubated for 60 min with Ca^{2+} /calmodulin and $Mg[\gamma\text{-}^{32}P]$ ATP before reconstitution with the actomyosin ATPase system and measurement of the ATPase rates, or caldesmon samples were reconstituted directly with the actomyosin ATPase system. In the former case, caldesmon phosphorylation was quantified after the 60 min preincubation: 2.0 mol of P_i was incorporated/mol of untreated caldesmon, compared with 0.06 mol of P_i /mol of heat-treated caldesmon, consistent with the results in Fig. 1. In the latter case, SDS/PAGE and autoradiography verified that caldesmon phosphorylation occurred during the ATPase reaction containing the active caldesmon kinase (consistent with the data in Fig. 4), but not during the ATPase reaction containing the heat-inactivated kinase.

The data in Table 1 indicate that substantial inhibition of the actin-activated myosin Mg^{2+} -ATPase occurred in the presence of caldesmon which had been heat-treated to inactivate the caldesmon kinase, as expected from earlier studies (Ngai & Walsh, 1984). However, no inhibition was observed by caldesmon containing an

active caldesmon kinase. This was the case whether caldesmon was prephosphorylated or caldesmon phosphorylation was allowed to take place during the ATPase reaction. Inhibition of superprecipitation correlated with inhibition of the ATPase rate (Table 1). In the absence of Ca^{2+} , myosin phosphorylation did not occur, the actin-activated myosin Mg^{2+} -ATPase activity was very low and superprecipitation was not observed (Table 1), as expected.

The effects of increasing amounts of phosphorylated and non-phosphorylated caldesmons on the actin-activated myosin Mg^{2+} -ATPase are compared in Fig. 5. Almost complete inhibition was observed at a 1:6.7 molar ratio of non-phosphorylated caldesmon to actin monomers. Very little inhibition was observed at this ratio of phosphorylated caldesmon to actin monomers. At the highest caldesmon concentration used (0.2 mol/mol of actin), the phosphorylated protein inhibited the ATPase rate by only 27.7%, compared with 92.1% inhibition by non-phosphorylated caldesmon.

Effects of caldesmon phosphorylation on binding to actin, actin/tropomyosin and Ca^{2+} /calmodulin

The binding of phosphorylated and non-phosphorylated caldesmons to F-actin in the absence of Ca^{2+} was compared by using the ultracentrifugation method described in the Materials and methods section (Fig. 6). Less phosphorylated caldesmon than non-phosphorylated caldesmon was bound at each caldesmon concentration examined; the relatively small difference observed is reproducible. Tropomyosin had a slight effect, enhancing the binding of both phosphorylated and non-phosphorylated caldesmons to F-actin (Fig. 6).

Fig. 5. Comparison of the effects of phosphorylated and non-phosphorylated caldesmons on the actin-activated Mg^{2+} -ATPase of smooth-muscle myosin

Various amounts of caldesmon containing caldesmon kinase activity, either untreated (○) or heat-treated to inactivate the kinase activity (□), were incubated at 30 °C in 25 mM-Tris/HCl (pH 7.5)/60 mM-KCl/10 mM-MgCl₂/0.1 mM-CaCl₂/calmodulin (20 μg/ml)/myosin light-chain kinase (10 μg/ml)/tropomyosin (50 μg/ml)/actin (0.25 mg/ml)/myosin (0.44 mg/ml)/1 mM-[γ -³²P]-ATP (4000 c.p.m./nmol) in a reaction volume of 2.0 ml. Reactions were initiated by the addition of [γ -³²P]ATP, and samples (0.25 ml) were withdrawn at 1, 2, 3, 4, 5, 6 and 7 min for quantification of ATP hydrolysis. ATPase rates were determined from the linear time-course assays of P_i release by linear-regression analysis.

Similar binding studies revealed that both phosphorylated and non-phosphorylated caldesmons could be released from F-actin/tropomyosin by Ca²⁺/calmodulin (Fig. 7). However, Ca²⁺/calmodulin was more effective at releasing phosphorylated caldesmon.

Finally, the Ca²⁺-dependence of release of phosphorylated and non-phosphorylated caldesmons from actin/tropomyosin by calmodulin was examined (Fig. 8). In the absence of Ca²⁺ (presence of 0.1 mM-EGTA), 72.8% of the phosphorylated caldesmon was bound to F-actin, compared with 89.2% of the non-phosphorylated caldesmon. This corresponded to maximal stoichiometry of binding of 1 mol of phosphorylated caldesmon/11.7 mol of actin and 1 mol of non-phosphorylated caldesmon/9.5 mol of actin. Increasing the free [Ca²⁺] to 0.1 mM resulted in the removal of 97.0% of the bound phosphorylated caldesmon, but only 62.0% of the bound non-phosphorylated caldesmon. Half-maximal removal occurred at 0.11 μM-Ca²⁺ for phosphorylated caldesmon and 0.16 μM-Ca²⁺ for non-phosphorylated caldesmon.

DISCUSSION

Since the demonstration by Sobieszek (1977) that phosphorylation is required for actin activation of the smooth-muscle myosin Mg^{2+} -ATPase, much effort has been devoted to evaluating the physiological importance of myosin phosphorylation (for reviews see Kamm & Stull, 1985; Walsh, 1985). What emerges is a general consensus that myosin phosphorylation is required for

Fig. 6. Binding of phosphorylated and non-phosphorylated caldesmons to F-actin in the presence and absence of tropomyosin

Caldesmon binding to actin was quantified as described in the Materials and methods section. Key to symbols: phosphorylated caldesmon in the presence (○) and absence (●) of tropomyosin; non-phosphorylated caldesmon in the presence (△) and absence (▽) of tropomyosin.

Fig. 7. Removal of phosphorylated (○) and non-phosphorylated (△) caldesmons from F-actin/tropomyosin by Ca²⁺/calmodulin

Binding of caldesmon to actin/tropomyosin was quantified as described in the Materials and methods section at various calmodulin concentrations, as indicated. Other conditions: 1.4 μM-caldesmon (phosphorylated or non-phosphorylated), 11.9 μM-actin, 2.0 μM-tropomyosin, 0.1 mM-CaCl₂.

tension development in smooth muscle, and a correlation exists between myosin phosphorylation and the velocity of shortening (Aksoy *et al.*, 1982; Dillon *et al.*, 1981). However, it has not been possible to explain all the biochemical and physiological observations on the basis of myosin phosphorylation. For example, tension in smooth-muscle fibres can be maintained in a Ca²⁺-dependent manner while myosin is dephosphorylated, the so-called 'latch state' (Aksoy *et al.*, 1982, 1983;

Fig. 8. Ca^{2+} -dependence of calmodulin-induced removal of phosphorylated and non-phosphorylated caldesmons from F-actin/tropomyosin

Binding of caldesmon [phosphorylated (○) and non-phosphorylated (□)] to actin/tropomyosin was quantified as described in the Materials and methods section at various free Ca^{2+} concentrations obtained by using a Ca^{2+} /EGTA buffer system and determined by assuming a pK_d (Ca^{2+}) for EGTA of 10.955 (Fabiato, 1981). Conditions: 10 mM-Mops (pH 7.1)/0.1 M-NaCl/0.1 mM-dithiothreitol/0.1 mM-EGTA/2.5 mM- MgCl_2 /1 mM-ATP/11.9 μM -actin/2.0 μM -tropomyosin/1.4 μM -caldesmon/20 μM -calmodulin and various concentrations of CaCl_2 to give the desired free $[\text{Ca}^{2+}]$.

Butler *et al.*, 1983; Chatterjee & Murphy, 1983; Dillon *et al.*, 1981; Gerthoffer & Murphy, 1983; Silver & Stull, 1982). Furthermore, Marston and co-workers (Marston & Lehman, 1985; Marston & Smith, 1984) isolated thin filaments from several smooth muscles which conferred Ca^{2+} -sensitivity on the Mg^{2+} -ATPase activity of skeletal-muscle myosin and thiophosphorylated smooth-muscle myosin, suggesting the existence of a thin-filament-linked Ca^{2+} -dependent regulatory system in smooth muscle. This notion was supported by the results of the myosin competition test (Lehman & Szent-Györgyi, 1975) as applied to vertebrate smooth muscle (Marston *et al.*, 1980), which indicated the existence of thin-filament-linked regulation in this muscle.

Concurrent with these developments was the discovery and characterization of caldesmon. This protein was discovered as a calmodulin-binding protein which also bound to F-actin; the former interaction was Ca^{2+} -dependent, whereas the latter was Ca^{2+} -independent (Sobue *et al.*, 1981). Interest in this protein and its possible role in the regulation of smooth-muscle contraction has developed rapidly (Walsh, 1987). The major observations suggesting that caldesmon may be involved in regulating the contractile state of smooth muscle in a Ca^{2+} -dependent manner may be summarized

as follows. (1) Binding studies *in vitro* (Bretscher, 1984; Ngai *et al.*, 1984; Sobue *et al.*, 1981), immunocytochemical localization (Ban *et al.*, 1984; Bretscher & Lynch, 1985; Fürst *et al.*, 1986) and characterization of isolated thin filaments (Marston & Lehman, 1985) suggest that caldesmon is located on the actin filament. (2) Caldesmon can be dissociated, at least partially, from actin by Ca^{2+} /calmodulin (Clark *et al.*, 1986; Marston & Smith, 1985; Sobue *et al.*, 1981). (3) Isolated caldesmon inhibits both superprecipitation and actin-activation of the myosin Mg^{2+} -ATPase in a system *in vitro* reconstituted from purified contractile and regulatory proteins (Sobue *et al.*, 1982; Ngai & Walsh, 1984). (4) Caldesmon enhances the binding of smooth-muscle heavy meromyosin (phosphorylated and unphosphorylated) to actin by approx. 40-fold (Lash *et al.*, 1986). (5) Native thin filaments, which are composed primarily of actin, tropomyosin and caldesmon, confer Ca^{2+} -sensitivity on the Mg^{2+} -ATPase of both skeletal-muscle myosin and thiophosphorylated smooth-muscle myosin (Marston & Lehman, 1985). (6) Addition of purified caldesmon to contracting chicken gizzard skinned fibres at pCa 5.9 induces relaxation; this can be reversed by a large molar excess of Ca^{2+} /calmodulin (Szpacenko *et al.*, 1985).

We reported previously that caldesmon is the substrate of a co-purifying protein kinase which requires Ca^{2+} and calmodulin for activity (Ngai & Walsh, 1984, 1985). Smooth muscle also contains a protein phosphatase which catalyses the dephosphorylation of caldesmon (Ngai & Walsh, 1985). These observations suggested the possibility that caldesmon function may be regulated by $[\text{Ca}^{2+}]$ fluxes via reversible phosphorylation. We have examined this possibility in detail in the present work, in which phosphorylated and non-phosphorylated forms of caldesmon were compared in terms of their ability to inhibit superprecipitation and actin activation of the myosin Mg^{2+} -ATPase in a reconstituted system, and in terms of their binding characteristics. For these studies, a preparation of caldesmon containing endogenous caldesmon kinase activity was used. This preparation was composed of 85–90% caldesmon and was used because of the extreme lability of the kinase upon further purification (Ngai & Walsh, 1985).

We have previously demonstrated the Ca^{2+} /calmodulin-dependent phosphorylation of isolated caldesmon (Ngai & Walsh, 1984, 1985). It was important to establish that phosphorylation can also occur when caldesmon is in a more physiological environment. The kinetics of caldesmon phosphorylation (rate and maximum stoichiometry of phosphorylation) were similar whether caldesmon was free, or bound to actin, actin/tropomyosin or thin filaments. Furthermore, when reconstituted with actin, tropomyosin, myosin, calmodulin and myosin light-chain kinase, rapid and stoichiometric phosphorylation of caldesmon occurred in the presence of Ca^{2+} /calmodulin and $\text{Mg}[\gamma\text{-}^{32}\text{P}]\text{ATP}$. On the other hand, we observed no phosphorylation of caldesmon when isolated thin filaments (which contain 1 mol of caldesmon/26 mol of actin) were incubated under identical conditions. Perhaps the conditions used in isolation of the thin filaments, in particular the use of detergent (1% Triton X-100) in the initial stages of the preparation or the prolonged exposure to 25 °C temperatures (Marston & Lehman, 1985), caused inactivation of the kinase. This observation is consistent

with the report by Marston (1986) that isolated thin filaments lacked significant caldesmon kinase activity.

Substantial differences in the functional properties of phosphorylated and non-phosphorylated caldesmons were observed. Whereas non-phosphorylated caldesmon effected almost complete inhibition of superprecipitation and the actin-activated Mg^{2+} -ATPase of smooth-muscle myosin in a reconstituted system, the phosphorylated protein was relatively ineffective in this respect. Short time-course experiments (5–7 min) indicated that caldesmon phosphorylation occurred on the same time scale as loss of ATPase-inhibitory activity, an observation which is of importance in terms of the potential physiological relevance of this phosphorylation reaction. On the other hand, Marston (1986) reported that Ca^{2+} regulation of the myosin Mg^{2+} -ATPase by aortic thin filaments did not involve caldesmon phosphorylation. However, we have observed Ca^{2+} regulation of the Mg^{2+} -ATPase of phosphorylated smooth-muscle myosin reconstituted with gizzard thin filaments which does not involve caldesmon, since identical Ca^{2+} -sensitivity was observed with caldesmon-free thin filaments (Ngai *et al.*, 1987). The possibility arises therefore that the Ca^{2+} -sensitivity observed by Marston (1986) was, in fact, independent of caldesmon. The available information is consistent with the existence of two independent Ca^{2+} -mediated control mechanisms associated with the thin filament in smooth muscle, one involving caldesmon and its reversible phosphorylation and the other involving a minor protein component of the thin filaments which may be an actin-binding protein regulating actin filament structure in a Ca^{2+} -dependent manner (Ngai *et al.*, 1987).

Apart from our initial observation (Ngai & Walsh, 1984) that caldesmon can be phosphorylated by a Ca^{2+} /calmodulin-dependent protein kinase, two other reports have investigated the effects of caldesmon phosphorylation on its functional properties. We (Lim & Walsh, 1986) observed no effect of phosphorylation of gizzard caldesmon on its ability to inhibit the actin-activated Mg^{2+} -ATPase of rabbit skeletal-muscle myosin. The reason for this difference is at present unclear. It may be a fundamental difference between smooth- and skeletal-muscle myosins, or may be due to differences in experimental conditions, e.g. the presence of tropomyosin, calmodulin and myosin light-chain kinase in the experiments involving smooth-muscle myosin and their absence from those involving skeletal-muscle myosin. More recently, Lash *et al.* (1986) observed no effect of caldesmon phosphorylation on its ability to inhibit the actin-activated Mg^{2+} -ATPase activity of gizzard heavy meromyosin. They suggest that the kinase present in their caldesmon preparations may be different from the one that we have reported, or there may be important differences in the filamentous form of myosin and/or experimental design between their experiments and ours.

We have also found that caldesmon phosphorylation alters the binding properties of the protein. Phosphorylation caused a decrease, albeit slight, in the affinity of caldesmon for actin or actin/tropomyosin. More interestingly, Ca^{2+} /calmodulin was more effective in removing phosphorylated caldesmon from actin/tropomyosin than its non-phosphorylated counterpart. Ca^{2+} titration of the release of phosphorylated and non-phosphorylated caldesmons from actin/tropomyosin in the presence of

calmodulin revealed half-maximal release of phosphorylated caldesmon at $0.11 \mu M$ - Ca^{2+} and of non-phosphorylated caldesmon at $0.16 \mu M$ - Ca^{2+} , i.e. within the physiological range of $[Ca^{2+}]$ fluxes (Morgan & Morgan, 1982; Williams & Fay, 1986). However, whereas a maximum of only 62% of bound non-phosphorylated caldesmon could be released by Ca^{2+} /calmodulin, all the bound phosphorylated caldesmon could be released under identical conditions.

From a comparison of the reaction conditions in the ATPase studies described herein with the results of the binding studies, it appears that little or no removal from the thin filament of either phosphorylated or non-phosphorylated caldesmon would occur during the ATPase reactions. Phosphorylation can therefore prevent caldesmon-induced inhibition of the actomyosin Mg^{2+} -ATPase without necessarily removing it from the thin filament, i.e. without flip-flop. Our results support the notion that caldesmon may be involved in regulating the contractile state of smooth muscle and that this regulatory function is in turn controlled by reversible Ca^{2+} /calmodulin-dependent phosphorylation of caldesmon.

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REFERENCES

- Adelstein, R. S. & Eisenberg, E. (1980) *Annu. Rev. Biochem.* **49**, 921–956
- Aksoy, M. O., Murphy, R. A. & Kamm, K. E. (1982) *Am. J. Physiol.* **242**, C109–C116
- Aksoy, M. O., Mras, S., Kamm, K. E. & Murphy, R. A. (1983) *Am. J. Physiol.* **245**, C255–C270
- Aksoy, M. O., Stewart, G. J. & Harakal, C. (1986) *Biochem. Biophys. Res. Commun.* **135**, 735–741
- Ban, T., Ishimura, K., Fujita, H., Sobue, K. & Kakiuchi, S. (1984) *Acta Histochem. Cytochem.* **17**, 331–338
- Bretscher, A. (1984) *J. Biol. Chem.* **259**, 12873–12880
- Bretscher, A. & Lynch, W. (1985) *J. Cell Biol.* **100**, 1656–1663
- Butler, T. M., Siegman, M. J. & Mooers, S. U. (1983) *Am. J. Physiol.* **244**, C234–C242
- Chatterjee, M. & Murphy, R. A. (1983) *Science* **221**, 464–466
- Clark, T., Ngai, P. K., Sutherland, C., Gröschel-Stewart, U. & Walsh, M. P. (1986) *J. Biol. Chem.* **261**, 8028–8035
- Dillon, P. F., Aksoy, M. O., Driska, S. P. & Murphy, R. A. (1981) *Science* **211**, 495–497
- Fabiato, A. (1981) *J. Gen. Physiol.* **78**, 457–497
- Fürst, D. O., Cross, R. A., DeMey, J. & Small, J. V. (1986) *EMBO J.* **5**, 251–257
- Gerthoffer, W. T. & Murphy, R. A. (1983) *Am. J. Physiol.* **244**, C182–C187
- Gopalakrishna, R. & Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun.* **104**, 830–836
- Greene, L. E., Sellers, J. R., Eisenberg, E. & Adelstein, R. S. (1983) *Biochemistry* **22**, 530–535
- Ikebe, M. & Hartshorne, D. J. (1985) *Biochemistry* **24**, 2380–2387
- Kamm, K. E. & Stull, J. T. (1985) *Annu. Rev. Pharmacol. Toxicol.* **25**, 593–620
- Klee, C. B. (1977) *Biochemistry* **16**, 1017–1024
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lash, J. A., Sellers, J. R. & Hathaway, D. R. (1986) *J. Biol. Chem.* **261**, 16155–16160

- Lehman, W. (1986) *Biochim. Biophys. Acta* **885**, 88–90
- Lehman, W. & Szent-Györgyi, A. G. (1975) *J. Gen. Physiol.* **66**, 1–30
- Lim, M. S. & Walsh, M. P. (1986) *Biochem. J.* **238**, 523–530
- Marston, S. B. (1986) *Biochem. J.* **237**, 605–607
- Marston, S. B. & Lehman, W. (1985) *Biochem. J.* **231**, 517–522
- Marston, S. B. & Smith, C. W. J. (1984) *J. Muscle Res. Cell Motility* **5**, 559–575
- Marston, S. B. & Smith, C. W. J. (1985) *J. Muscle Res. Cell Motility* **6**, 669–708
- Marston, S. B., Trevett, R. M. & Walters, M. (1980) *Biochem. J.* **185**, 355–365
- Morgan, J. P. & Morgan, K. G. (1982) *Pflügers Arch.* **395**, 75–77
- Ngai, P. K. & Walsh, M. P. (1984) *J. Biol. Chem.* **259**, 13656–13659
- Ngai, P. K. & Walsh, M. P. (1985) *Biochem. J.* **230**, 695–707
- Ngai, P. K., Carruthers, C. A. & Walsh, M. P. (1984) *Biochem. J.* **218**, 863–870
- Ngai, P. K., Gröschel-Stewart, U. & Walsh, M. P. (1986) *Biochem. Int.* **12**, 89–93
- Ngai, P. K., Scott-Woo, G. C., Lim, M. S., Sutherland, C. & Walsh, M. P. (1986) *J. Biol. Chem.* **261**, 8028–8035
- Persechini, A. & Hartshorne, D. J. (1981) *Science* **213**, 1383–1385
- Silver, P. J. & Stull, J. T. (1982) *J. Biol. Chem.* **257**, 6145–6150
- Sobieszek, A. (1977) in *the Biochemistry of Smooth Muscle* (Stephens, N. L., ed.), pp. 413–443, University Park Press, Baltimore
- Sobue, K., Muramoto, Y., Fujita, M. & Kakiuchi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5652–5655
- Sobue, K., Morimoto, K., Inui, M., Kanda, K. & Kakiuchi, S. (1982) *Biomed. Res.* **3**, 188–196
- Spector, T. (1978) *Anal. Biochem.* **86**, 142–146
- Szpacenko, A., Wagner, J., Dabrowska, R. & Rüegg, J. C. (1985) *FEBS Lett.* **192**, 9–12
- Walsh, M. P. (1985) in *Calcium and Cell Physiology* (Marmé, D., ed.), pp. 170–203, Springer-Verlag, Berlin
- Walsh, M. P. (1987) in *Smooth Muscle Contraction* (Siegelman, M. J., ed.), Alan R. Liss, New York, in the press
- Walsh, M. P. & Hartshorne, D. J. (1982) in *Calcium and Cell Function* (Cheung, W. Y., ed.), vol. 3, pp. 223–269, Academic Press, New York
- Walsh, M. P., Hinkins, S., Dabrowska, R. & Hartshorne, D. J. (1983) *Methods Enzymol.* **99**, 279–288
- Walsh, M. P., Valentine, K. A., Ngai, P. K., Carruthers, C. A. & Hollenberg, M. D. (1984) *Biochem. J.* **224**, 117–127
- Williams, D. A. & Fay, F. S. (1986) *Am. J. Physiol.* **250**, C779–C791

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