Properties of caldesmon isolated from chicken gizzard

Philip K. NGAI and Michael P. WALSH

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

(Received 18 March 1985; accepted 21 May 1985)

Chicken gizzard smooth muscle contains two major calmodulin-binding proteins: caldesmon ($11.1\ \mu\text{M}$; M_r 141 000) and myosin light-chain kinase ($4.6\ \mu\text{M}$; M_r 136 000), both of which are associated with the contractile apparatus. The amino acid composition of caldesmon is distinct from that of myosin light-chain kinase and is characterized by a very high glutamic acid content (25.5%), high contents of lysine (13.6%) and arginine (10.3%), and a low aromatic amino acid content (2.4%). Caldesmon lacked myosin light-chain kinase and phosphatase activities and did not compete with either myosin light-chain kinase or cyclic nucleotide phosphodiesterase (both calmodulin-dependent enzymes) for available calmodulin, suggesting that calmodulin may have distinct binding sites for caldesmon on the one hand and myosin light-chain kinase and cyclic nucleotide phosphodiesterase on the other. Consistent with the lack of effect of caldesmon on myosin phosphorylation, caldesmon did not affect the assembly or disassembly of myosin filaments *in vitro*. As previously shown [Ngai & Walsh (1984) *J. Biol. Chem.* **259**, 13656–13659], caldesmon can be reversibly phosphorylated. The phosphorylation and dephosphorylation of caldesmon were further characterized and the Ca^{2+} /calmodulin-dependent caldesmon kinase was purified; kinase activity correlated with a protein of subunit M_r 93 000. Caldesmon was not a substrate of myosin light-chain kinase or phosphorylase kinase, both calmodulin-activated protein kinases.

Caldesmon is a major calmodulin- and actin-binding protein of smooth muscle (Sobue *et al.*, 1981). Whereas the calmodulin–caldesmon interaction is Ca^{2+} -dependent, the actin–caldesmon interaction is not (Sobue *et al.*, 1981, 1982). Furthermore, in the presence of Ca^{2+} , actin and calmodulin compete for binding to caldesmon. Since calmodulin binds to caldesmon at micromolar concentrations of Ca^{2+} (Sobue *et al.*, 1981), Sobue *et al.* (1982) have suggested that caldesmon is capable of ‘flip-flopping’ between actin and calmodulin as a function of the prevailing sarco-plasmic $[\text{Ca}^{2+}]$. The possibility that caldesmon may play a role in regulating actin–myosin interactions in smooth muscle was originally suggested by Sobue *et al.* (1982), who found, using a desensitized actomyosin preparation from chicken gizzard, that caldesmon inhibited superprecipitation, at least at low concentrations of calmodulin. More recently, using a system reconstituted from the purified contractile and regulat-

ory proteins, we have shown that highly purified caldesmon inhibits both superprecipitation and the actin-activated myosin Mg^{2+} -dependent ATPase activity at high concentrations of calmodulin without affecting myosin phosphorylation (Ngai & Walsh, 1984). This suggests that caldesmon may indeed play an important physiological role in the regulation of smooth-muscle contraction. Furthermore, we identified Ca^{2+} /calmodulin-dependent caldesmon kinase and a caldesmon phosphatase activity in smooth muscle and provided evidence suggesting that the inhibitory effects of caldesmon described above may be abolished by phosphorylation of caldesmon (Ngai & Walsh, 1984). Subsequently, we obtained evidence from immunoblotting experiments that caldesmon is widely distributed among chicken tissues, being found in all tissues examined, which included smooth muscles, skeletal and cardiac muscles and various non-muscle tissues (Ngai & Walsh, 1985). Caldesmon may therefore play a role in regulating actin–myosin interactions in both muscle and non-muscle motile systems.

Abbreviation used: SDS, sodium dodecyl sulphate.

In this paper we describe a new procedure for the purification of caldesmon, which provides a highly purified product free of contaminating caldesmon kinase. Some structural and functional properties of this caldesmon are described, including further characterization of caldesmon phosphorylation and dephosphorylation.

Materials and methods

[γ - 32 P]ATP (10–40 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). CNBr-activated Sepharose 4B and 5'-nucleotidase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Affi-Gel Blue was from Bio-Rad (Richmond, CA, U.S.A.) and DEAE-Sephacel from Pharmacia Chemical Co. (Mississauga, Ontario, Canada). M_r markers for SDS/polyacrylamide-gel electrophoresis were obtained from Sigma. General laboratory reagents used were analytical grade or better and were purchased from Fisher Chemical Co.

Protein purifications

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 by previously described methods: rabbit skeletal-muscle actin (Pardee & Spudich, 1982; Zot & Potter, 1981), chicken gizzard tropomyosin (Smillie, 1982), myosin (Persechini & Hartshorne, 1981) and myosin light-chain kinase (Ngai *et al.*, 1984). A typical preparation of myosin exhibited the following ATPase activities at 30°C: actin-activated myosin Mg^{2+} -ATPase (at 80 mM-KCl), 70.0 nmol of P_i /min per mg of myosin in the presence of Ca^{2+} , and 4.0 nmol/min per mg in its absence; K_2 EDTA-ATPase (at 0.5 M-KCl in the absence of actin), 845.8 nmol of P_i /min per mg of myosin; Ca^{2+} -ATPase (at 0.5 M-KCl in the absence of actin), 394.6 nmol of P_i /min per mg of myosin. These values are close to most published values (see, e.g., Sherry *et al.*, 1978; Sellers *et al.*, 1981, 1983; Bailin & Lopez, 1982; Chacko & Rosenfeld, 1982; Nag & Seidel, 1983). A typical preparation of myosin light-chain kinase exhibited a specific activity of 3.3 μ mol of P_i incorporated/min per mg of enzyme with intact gizzard myosin as the substrate. A partially purified preparation of myosin phosphatase was prepared by a modification of the procedures of Pato & Adelstein (1980, 1983) and Werth *et al.* (1982), as follows. Frozen chicken gizzards (100 g) were minced, homogenized in 4 vol. (400 ml) of 50 mM-Tris/HCl (pH 7.5)/10 mM-MgCl₂/1 mM-dithiothreitol/5 mM-EGTA in a Waring blender for 3 $\times$ 5 s and centrifuged at 10000g

for 30 min. To the homogenate supernatant, solid $(NH_4)_2SO_4$ was added slowly with stirring to 30% saturation, and the mixture centrifuged at 22000g for 15 min. The pellet was discarded; additional $(NH_4)_2SO_4$ was added to the supernatant to make it 60% saturated, and the mixture was centrifuged as above. The supernatant was discarded, and the pellet was dissolved in a minimum volume of 20 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-EGTA/phenylmethanesulphonyl fluoride (75 mg/l)/1 mM-dithiothreitol and dialysed overnight against two changes (10 litres each) of this buffer. The dialysed sample was loaded on a column (1.5 cm $\times$ 30 cm) of DEAE-Sephacel previously equilibrated with dialysis buffer and the column was washed with this buffer until A_{280} reached baseline. Bound proteins (including myosin phosphatase) were then eluted with a linear 0–0.6 M-NaCl gradient generated from 250 ml each of dialysis buffer and buffer containing 0.6 M-NaCl. Selected fractions were assayed for myosin phosphatase activity as previously described (Ngai & Walsh, 1984). Phosphatase-containing fractions were pooled and stored in 1 ml batches at $-80^\circ C$. Rabbit skeletal-muscle phosphorylase kinase was given by Dr. R. K. Sharma, Department of Medical Biochemistry, University of Calgary. Caldesmon was purified from chicken gizzards by a modification of the procedure of Ngai *et al.* (1984). The initial stages of the purification, up to the DEAE-Sephacel chromatography step, were performed as described previously (Ngai *et al.*, 1984). For the further purification of caldesmon, the flow-through column fractions from this ion-exchange column which contained caldesmon (as revealed by electrophoresis in 0.1% SDS/7.5–20% polyacrylamide-gradient slab gel) were pooled, dialysed overnight against two changes (10 litres each) of 20 mM-K₂HPO₄ (pH 8.0)/1 mM-EGTA/1 mM-EDTA/1 mM-dithiothreitol/0.02% NaN₃ (buffer A) and loaded on a column (1.5 cm $\times$ 20 cm) of Affi-Gel Blue previously equilibrated with buffer A. The column was then washed with buffer A until A_{280} returned to baseline, and bound proteins were eluted with a linear gradient of NaCl made from 200 ml each of buffer A and buffer A containing 2 M-NaCl. Selected column fractions were analysed by SDS/polyacrylamide-gel electrophoresis, using authentic caldesmon as a marker to reveal the caldesmon-containing fractions. These fractions were pooled, dialysed overnight against two changes (10 litres each) of 20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/0.5 mM-dithiothreitol/0.1 M-NaCl (buffer B) and applied to a column (1 cm $\times$ 40 cm) of DEAE-Sephacel previously equilibrated with buffer B. After washing of the column with buffer B to remove all unbound protein, bound proteins were eluted with a linear

NaCl gradient (0.1–0.2M) generated from 200ml each of buffer B and buffer B containing 0.2M-NaCl. Again selected fractions were examined for caldesmon content by SDS/polyacrylamide-gradient-slab-gel electrophoresis. Caldesmon-containing fractions were pooled and stored at -80°C in 1ml batches. Caldesmon was very stable when stored in this way for at least 6 months and was unaffected by freezing and thawing at least twice.

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.), or by spectrophotometric measurements for calmodulin ($A_{277}^{1\%} = 1.9$; Klee, 1977) and myosin ($A_{280}^{1\%} = 5.6$; Greene *et al.*, 1983).

Electrophoresis was performed in 7.5–20% polyacrylamide gradient slab gels (1.5 mm thick) in the presence of 0.1% SDS at 36mA 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 destained in 10% acetic acid. Reagents for electrophoresis were purchased from Bio-Rad. Gels were scanned with an LKB 2202 Ultrosan laser densitometer equipped with an HP3390A integrator. Two-dimensional isoelectric focusing/SDS/polyacrylamide-gel electrophoresis was carried out as described by O'Farrell (1975).

The tissue concentrations of caldesmon and myosin light-chain kinase were determined as follows. Samples of chicken gizzard smooth muscle (approx. 100mg each) were homogenized with a Brinkmann Polytron fitted with a PT10ST probe generator, in 25vol. of SDS-gel sample buffer [50mM-Tris/HCl (pH 6.8)/1% SDS/30% (v/v) glycerol/0.01% Bromophenol Blue/1% 2-mercaptoethanol] diluted 1:1 with deionized distilled water, immersed in a boiling-water bath for 2 min and cooled on ice. Large particulate material was allowed to settle under gravity, and samples of the supernatant were subjected to SDS/polyacrylamide-gradient-slab-gel electrophoresis and protein-concentration determination. After electrophoresis, the amounts of caldesmon and myosin light-chain kinase were determined as percentages of total protein by densitometric scanning. These values, together with the calculated tissue protein concentrations, enabled determination of the tissue concentrations of the two calmodulin-binding proteins, assuming intracellular water to be 80% of tissue weight. Alternatively, tissue concentrations were determined on the basis of the known tissue concentration of actin (0.83mM) by expressing caldesmon and myosin light-chain kinase as percentages of actin after gel scanning.

Calmodulin was coupled to CNBr-activated Sepharose 4B as previously described (Walsh *et al.*,

1982). The amount of bound calmodulin was determined to be 1.0mg/ml of packed resin.

Enzyme assays

Phosphate (^{32}P) incorporation into substrate proteins was measured essentially as described for myosin phosphorylation by Walsh *et al.* (1983). Details of reaction conditions for individual experiments are given in the text. Cyclic nucleotide phosphodiesterase activity was assayed as described by Wang *et al.* (1972). One unit of cyclic nucleotide phosphodiesterase activity is defined as the amount of enzyme catalysing the hydrolysis of 1 μmol of cyclic AMP in the presence of excess Ca^{2+} and calmodulin under standard conditions (Wang *et al.*, 1972).

Amino acid composition analysis

Triplicate samples of chicken gizzard caldesmon (0.21 nmol) and myosin light-chain kinase (0.20 nmol) were hydrolysed *in vacuo* at 110°C in 6M-HCl containing 0.1% (w/v) phenol and 0.2% (v/v) 2-mercaptoethanol for 24, 48 and 72 h before amino acid composition analysis in a Beckman model 121 M amino acid analyser. Tryptophan was determined after methanesulphonic acid hydrolysis as described by Simpson *et al.* (1976), and cysteine after performic acid oxidation as described by Hirs (1967).

Results

Quantification of caldesmon and myosin light-chain kinase

Samples of frozen chicken gizzards were homogenized in SDS-gel sample buffer and prepared for electrophoresis as described in the Materials and methods section. Bands corresponding to caldesmon and myosin light-chain kinase were identified by comparison with the electrophoretic mobilities of the purified proteins and with the aid of antibodies in immunoblotting experiments. Scanning laser densitometry of gels similar to that shown in Fig. 1(a) enabled quantification (Table 1) of the tissue contents of these two calmodulin-binding proteins, which were easily distinguished and integrated separately by scanning the gel at a speed of 6mm/min (Fig. 1). Myosin light-chain kinase and caldesmon were determined to be present in chicken gizzard at concentrations of 4.6 μM and 11 μM respectively. Two-dimensional isoelectric focusing/SDS/polyacrylamide-gel electrophoresis revealed that no proteins co-migrated with either caldesmon or myosin light-chain kinase in the one-dimensional gels of Fig. 1(a) even when up to 500 μg of protein was electrophoresed. Investigation of the staining properties of purified caldesmon and myosin light-chain kinase indi-

Fig. 1. Quantification of myosin light-chain kinase and caldesmon in chicken gizzard

(a) Total gizzard proteins were electrophoresed for determination of the tissue concentrations of myosin light-chain kinase and caldesmon as described in the Materials and methods section; 150 μ g of protein was applied to the gel. (b) Caldesmon (5 μ g) purified as described in the Materials and methods section. (c) Myosin light-chain kinase (5 μ g) purified as previously described (Ngai *et al.*, 1984). Below is shown an actual densitometric scan of the stained gel, with the top of the gel to the left. Caldesmon (CaD) and myosin light-chain kinase (MLCK) peaks are indicated.

Table 1. Tissue concentrations of myosin light-chain kinase and caldesmon

Values represent the means $\pm$ S.E.M. for five determinations. Method 1 used total protein concentration and Method 2 used actin as an internal standard in calculating the tissue concentrations, as described in the Materials and methods section.

	Concn. (μ M)		
	Method 1	Method 2	Average
Myosin light-chain kinase	5.0 $\pm$ 0.4	4.2 $\pm$ 0.3	4.6
Caldesmon	11.4 $\pm$ 0.6	10.7 $\pm$ 0.5	11.1

cated no differential dye-staining. We can therefore be confident of the accuracy of these tissue concentration values.

Purification of caldesmon

The early stages in the purification of caldesmon were as reported previously (Ngai *et al.*, 1984). Subsequent purification steps employed Affi-gel Blue affinity chromatography and a second ion-exchange column rather than affinity chromatography on calmodulin-Sepharose. This modified procedure results in a higher purity of caldesmon (Fig. 1b), and avoids problems of proteolysis,

which are associated with calmodulin-Sepharose affinity chromatography (Ngai *et al.*, 1984). The rationale for the use of Affi-gel Blue, which is relatively specific for proteins containing a dinucleotide fold or related binding-site structure, was based on preliminary experimental results which suggested that caldesmon may be a protein kinase: incubation of caldesmon, purified by calmodulin-Sepharose affinity chromatography as described by Ngai *et al.* (1984), with Mg^{2+} and $[\gamma\text{-}^{32}P]ATP$ led to the incorporation of $[^{32}P]P_i$ into caldesmon, as revealed by gel electrophoresis and autoradiography. More detailed examination, however, indicated that this phosphorylation was due to a contaminating kinase activity rather than autophosphorylation of caldesmon (see below). Nevertheless, Affi-gel Blue chromatography provided a suitable and very reproducible purification step. Caldesmon was tightly bound to the column and was eluted at a high salt concentration, with a peak at approx. 1.5M-NaCl, as revealed by gel electrophoresis. Caldesmon was finally obtained in highly purified form by a second ion-exchange chromatography with a shallow salt gradient (0.1–0.2M-NaCl). Caldesmon was eluted as a sharp peak at 0.12M-NaCl. The electrophoretic purity of the caldesmon preparation is illustrated in Fig. 1(b). The yield of caldesmon, based on seven separate purifications, was 14–20mg/100g of gizzard. This isolated protein was identified as caldesmon by the following criteria, as described in detail elsewhere (Ngai *et al.*, 1984): (1) subunit molecular mass of 141 000 Da; (2) Ca^{2+} -dependent interaction with calmodulin; (3) Ca^{2+} -independent interaction with F-actin; and (4) competition between F-actin and calmodulin for caldesmon binding in the presence, but not in the absence, of Ca^{2+} . Furthermore, specific antibodies against caldesmon cross-reacted with the isolated protein (Ngai & Walsh, 1985).

Amino acid composition of caldesmon

Table 2 shows the complete amino acid compositions of caldesmon and myosin light-chain kinase. These are distinctly different, emphasizing the fact that, although they both bind calmodulin in a Ca^{2+} -dependent manner and F-actin in a Ca^{2+} -independent manner (Sobue *et al.*, 1981; Dabrowska *et al.*, 1982; Ngai *et al.*, 1984), and have similar subunit M_r values, they are distinct proteins. Caldesmon has a very high content of glutamic acid (25.5% of total residues), with a moderate content of aspartic acid (7.3%), and relatively high contents of lysine (13.6%) and arginine (10.3%). On the other hand, caldesmon has very low contents of histidine (0.4%), tyrosine (0.6%), cysteine (0.6%) and tryptophan (0.7%). The ratio of acidic to basic residues would account for

Table 2. Amino acid compositions of caldesmon and myosin light-chain kinase

The analyses were carried out as described in the Materials and methods section. The results are expressed as mol/mol and represent the means of triplicate determinations at three times of hydrolysis (24, 48 and 72h)

	Caldesmon	Myosin light-chain kinase
Lysine	166.6	120.6
Histidine	4.7	15.1
Arginine	125.8	47.7
Aspartic acid	89.8	120.6
Threonine*	50.5	76.2
Serine*	57.6	109.8
Glutamic acid	312.1	169.2
Proline	37.0	65.2
Glycine	51.0	82.4
Alanine	134.6	105.9
Cysteine†	6.6	28.2
Valine	56.0	76.3
Methionine	16.7	26.0
Isoleucine	21.5	46.2
Leucine	60.9	71.7
Tyrosine	6.9	29.2
Phenylalanine	14.5	34.8
Tryptophan‡	9.4	20.5

* Extrapolated to zero time of hydrolysis.

† Determined as described by Hirs (1967).

‡ Determined as described by Simpson *et al.* (1976).

the fact that caldesmon is moderately acidic, being eluted from an anion-exchange resin at a lower NaCl concentration (0.12M) than is myosin light-chain kinase (0.25M; Ngai *et al.*, 1984). The low content of aromatic amino acids (tryptophan + tyrosine + phenylalanine = 2.4%) accounts for the fact that caldesmon absorbs poorly at 280nm (results not shown).

Caldesmon lacks myosin phosphatase activity

We considered the possibility that caldesmon may be a myosin phosphatase which is inhibited by Ca^{2+} and calmodulin. This would provide a logical component in the regulation of myosin phosphorylation, and thereby smooth-muscle contraction, since a rise in sarcoplasmic $[Ca^{2+}]$ after stimulation would activate myosin light-chain kinase and simultaneously inactivate myosin phosphatase, by binding of calmodulin, enabling rapid phosphorylation of myosin. Conversely, after sarcoplasmic $[Ca^{2+}]$ decreases to resting values, the myosin light-chain kinase would be inactivated and the myosin phosphatase would be activated, both by dissociation of calmodulin. However, the data presented in Fig. 2 show clearly that caldesmon, whether added before myosin phos-

Fig. 2. Caldesmon lacks myosin phosphatase activity

Smooth-muscle myosin (0.5 mg/ml) was incubated at 30°C in 25 mM-Tris/HCl (pH 7.5)/4 mM-MgCl₂/0.1 mM-CaCl₂/60 mM-KCl with calmodulin (30 µg/ml) and myosin light-chain kinase (10 µg/ml) in the presence (▽) and absence (○, □, △) of caldesmon (50 µg/ml), purified as described in the Materials and methods section. Myosin phosphorylation was initiated by the addition of [γ -³²P]ATP (~4000 c.p.m./nmol; final concn. 0.75 mM). Reaction volumes were 6.5 ml. Samples (0.5 ml) of reaction mixtures were withdrawn at 2, 4, 6, 8, 10 and 12 min for quantification of protein-bound [³²P]P_i as described in the Materials and methods section. At 15 min (indicated by the arrow), EGTA (1 mM final concn.) was added to two reactions (▽, ○), EGTA (1 mM final concn.) plus caldesmon (50 µg/ml) to a third reaction (□), and no addition was made to the fourth reaction (△). Additional samples (0.5 ml) were withdrawn at 20, 25, 30 and 35 min for quantification of protein-bound [³²P]P_i. The inset depicts a gel electrophoretogram of the myosin preparation used in this and other experiments and indicates the 20000-Da heavy chain and the 20000- and 17000-Da light chains.

phorylation or with EGTA after myosin phosphorylation, exhibited no myosin phosphatase activity.

Effect of caldesmon on myosin light-chain kinase and cyclic nucleotide phosphodiesterase activities

As described previously (Sobue *et al.*, 1982; Ngai *et al.*, 1984), caldesmon itself has no myosin light-chain kinase activity. We investigated the possible inhibitory effect of caldesmon on two calmodulin-dependent enzymes, myosin light-chain kinase and cyclic nucleotide phosphodiesterase. In the presence of low concentrations of myosin light-chain kinase (3.8 nM) and calmodulin (12.1 nM), excess caldesmon (70.9 nM) had no effect on the phosphorylation of myosin (Fig. 3a). This was true whether calmodulin, myosin light-chain kinase and caldesmon were incubated in the absence of Ca²⁺ and myosin phosphorylation was initiated by the addition of Ca²⁺, or caldesmon was preincubated at 25°C for 30 min with Ca²⁺ and calmodulin before addition of myosin light-chain kinase and substrates. Similarly, caldesmon did not inhibit another calmodulin-dependent enzyme, bovine brain cyclic nucleotide phosphodiesterase, under similar conditions (Fig. 3b). Caldesmon had no effect on either the calmodulin activation of

cyclic nucleotide phosphodiesterase or the basal, i.e. calmodulin-independent, activity of this enzyme. On the other hand, myosin light-chain kinase inhibited the calmodulin activation of cyclic nucleotide phosphodiesterase, almost to the extent observed in the absence of calmodulin (Fig. 3c), and, conversely, cyclic nucleotide phosphodiesterase inhibited the myosin light-chain kinase (results not shown).

Effect of caldesmon on myosin-filament assembly-disassembly

It is well established that smooth-muscle and non-muscle myosin filaments can be disassembled *in vitro* by the addition of stoichiometric amounts of ATP and induced to re-assemble by Ca²⁺/calmodulin-dependent phosphorylation of the myosin (Suzuki *et al.*, 1978; Kendrick-Jones *et al.*, 1983). The assembled filaments can then be disassembled by dephosphorylation catalysed by myosin phosphatase. Such events are shown for chicken gizzard myosin in Fig. 4, where filament assembly and disassembly was monitored by recording the turbidity (A_{340}) of the solution as described by Kendrick-Jones *et al.* (1983). Myosin filaments were initially disassembled by addition of ATP and induced to re-assemble by the addition

of Ca^{2+} and calmodulin (12 nM), thereby activating the myosin light-chain kinase (39 nM) present in the incubation medium and allowing myosin phosphorylation to occur. Filament disassembly was then induced by the addition of EGTA, to inactivate the myosin light-chain kinase, and a preparation of partially purified myosin phosphatase, which dephosphorylated the myosin. Reassembly was effected by addition of excess Ca^{2+} to a final concentration of 0.1 mM free Ca^{2+} , which re-activated the myosin light-chain kinase and caused myosin phosphorylation to occur once again. The inclusion of excess caldesmon (355 nM) at the outset had no effect on the assembly-disassembly of myosin filaments. No effect of caldesmon was observed either at limiting concentrations of calmodulin (12 nM) (Fig. 4) or in the

Fig. 3. Effect of caldesmon on myosin light-chain kinase and cyclic nucleotide phosphodiesterase activities

(a) Smooth-muscle myosin (0.5 mg/ml) was incubated at 25°C in 25 mM-Tris/HCl (pH 7.5)/4 mM- MgCl_2 /0.1 mM- CaCl_2 /60 mM-KCl, with calmodulin (0.2 $\mu\text{g}/\text{ml}$) and myosin light-chain kinase (0.5 $\mu\text{g}/\text{ml}$), in the presence (●) or absence (○) of caldesmon (10 $\mu\text{g}/\text{ml}$), purified as described in the Materials and methods section. Myosin phosphorylation was initiated by the addition of [γ - ^{32}P]ATP (~6000 c.p.m./nmol; final concn. 0.75 mM). Reaction volumes were 3.5 ml. Samples (0.45 ml) were withdrawn at the indicated times for quantification of protein-bound [^{32}P]P_i as described in the Materials and methods section. (b) Bovine brain cyclic nucleotide phosphodiesterase (0.32 unit) was incubated at 30°C in 40 mM-Tris/HCl/40 mM-imidazole/HCl (pH 7.5)/3 mM-magnesium acetate, containing 0.11 mM- CaCl_2 (○, ●) or 0.11 mM-EGTA (□, ■), with calmodulin (0.49 $\mu\text{g}/\text{ml}$) and 5'-nucleotidase (1.375 units) in the presence (●, ■) or absence (○, □) of caldesmon (100 $\mu\text{g}/\text{ml}$), purified as described in the Materials and methods section. Reactions were initiated by the addition of cyclic AMP (final concn. 1.2 mM). Reaction volumes were 4.95 ml. Samples (0.45 ml) of reaction mixtures were withdrawn at the indicated times for quantification of cyclic AMP hydrolysis as previously described (Wang *et al.*, 1972). (c) Cyclic nucleotide phosphodiesterase (0.32 unit) was incubated at 30°C in 40 mM-Tris/HCl/40 mM-imidazole/HCl (pH 7.5)/3 mM-magnesium acetate/0.11 mM- CaCl_2 with 5'-nucleotidase (1.375 units) in the presence (○, ●) and absence (□) of calmodulin (0.49 $\mu\text{g}/\text{ml}$) and in the presence (●) and absence (○) of myosin light-chain kinase (25 $\mu\text{g}/\text{ml}$). Reactions were initiated by the addition of cyclic AMP (final concn. 1.2 mM). Reaction volumes were 4.95 ml. Samples (0.45 ml) of reactions mixtures were withdrawn at the indicated times for quantification of cyclic AMP hydrolysis as previously described (Wang *et al.*, 1972).

Fig. 4 Effect of caldesmon on myosin-filament assembly-disassembly

Smooth-muscle non-phosphorylated myosin (0.5mg/ml) was incubated at 22°C in 25mM-imidazole/HCl (pH 7.0)/0.15M-NaCl/10mM-MgCl₂/0.2mM-EGTA/0.25mM-dithiothreitol with myosin light-chain kinase (5µg/ml) in the presence or absence of caldesmon (50µg/ml). The state of myosin-filament assembly was determined by turbidimetric measurements of A₃₄₀, as described by Kendrick-Jones *et al.* (1983). Reaction volumes were 1ml. At 5min, the myosin filaments were disassembled by addition of ATP (final concn. 2.5mM). At 10min, CaCl₂ was added (final concn. 0.1mM excess free Ca²⁺), and at 20min, calmodulin was added (final concn. 0.2µg/ml; CaM). At 45min, myosin light-chain phosphatase (partially purified as described in the Materials and methods section) (50µl/ml) was added. At 50min, EGTA was added (final concn. 0.1mM excess over Ca²⁺). Finally, at 70min, CaCl₂ was again added (final concn. 0.1mM excess free Ca²⁺).

presence of saturating concentrations of calmodulin (0.6µM; results not shown). Therefore, consistent with a lack of effect on myosin phosphorylation, caldesmon was without effect on myosin

Fig. 5. Reversible phosphorylation of caldesmon
Caldesmon (79µg/ml), purified as previously described (Ngai *et al.*, 1984) and containing endogenous caldesmon kinase activity, was incubated at 30°C in 20mM-Tris/HCl (pH 7.5)/5mM-MgCl₂/0.1mM-CaCl₂/0.5mM-[γ-³²P]ATP (~17000 c.p.m./nmol) with calmodulin (10µg/ml). Reaction volumes were 7.0ml. Samples (0.5ml) of reaction mixtures were withdrawn at 5, 10, 15, 20, 25 and 30min for quantification of protein-bound [³²P]P_i as previously described (Walsh *et al.*, 1983). At 35min, EGTA (1mM final concn.) was added to one reaction (○), and EGTA (1mM final concn.) plus partially purified myosin light-chain phosphatase (50µl/ml) to a second reaction (●). Additional samples (0.5ml) were withdrawn at 40, 45, 50, 55, 60, 75 and 90min for quantification of protein-bound [³²P]P_i.

Table 3. Myosin light-chain kinase does not phosphorylate caldesmon

Myosin (0.5mg/ml) or caldesmon (0.25mg/ml), purified as described in the Materials and methods section, was incubated at 30°C in 20mM-Tris/HCl (pH 7.5)/5mM-MgCl₂/60mM-KCl/0.75mM-[γ-³²P]ATP (~5000 c.p.m./nmol), with 0.1mM-CaCl₂ or 1mM-EGTA in the presence or absence of calmodulin (CaM; 30µg/ml) and myosin light-chain kinase (MLCK; 10µg/ml). Samples (0.5ml) of reaction mixtures were withdrawn at the indicated times for quantification of protein-bound [³²P]P_i as previously described (Walsh *et al.*, 1983).

Substrate	Phosphate incorporation (mol/mol of substrate)							
	+Ca ²⁺ +CaM		-Ca ²⁺ +CaM		+Ca ²⁺ +CaM+MLCK		-Ca ²⁺ +CaM+MLCK	
	10min	60min	10min	60min	10min	60min	10min	60min
Myosin	0.15	0.15	0.03	0.18	1.40	1.26	0.05	0.15
Caldesmon	0.02	0.04	0.02	0.03	0.01	0.05	0.02	0.05

filament assembly-disassembly, which is dependent on the phosphorylation state of myosin.

Calmodulin-dependent caldesmon kinase

We described previously that caldesmon is a substrate of a protein kinase and that phosphorylated caldesmon could be dephosphorylated by a phosphatase partially purified from chicken gizzard (Ngai & Walsh, 1984). Fig. 5 shows a time course of caldesmon phosphorylation by caldesmon kinase and dephosphorylation by a partially purified myosin phosphatase preparation. The stoichiometry of caldesmon phosphorylation is variable (2–4 mol of P_i /mol of caldesmon), perhaps reflecting variable extents of pre-phosphorylation in different caldesmon preparations. The phosphatase rapidly dephosphorylated caldesmon; the stable low extent of phosphorylation apparent in the presence of phosphatase after 40 min represents Ca^{2+} -independent phosphate incorporation into proteins other than caldesmon which are present in the phosphatase preparation, as revealed by gel electrophoresis and autoradiography.

Caldesmon kinase requires Ca^{2+} and calmodulin for activity, as shown in Fig. 6, which also shows, by gel electrophoresis and autoradiography, that phosphate incorporation in the presence of Ca^{2+} and calmodulin is exclusively into caldesmon.

Table 3 indicates that myosin light-chain kinase, which catalyses the phosphorylation of myosin in a Ca^{2+} /calmodulin-dependent manner, does not utilize caldesmon as a substrate. Hence the calmodulin-dependent caldesmon kinase is a distinct enzyme from myosin light-chain kinase. Similarly, rabbit skeletal-muscle phosphorylase kinase, another calmodulin-activated protein kinase, did not phosphorylate caldesmon.

The experiments indicated in Figs. 5 and 6 were carried out with a preparation of caldesmon which contained caldesmon kinase activity after purification by calmodulin-Sepharose affinity chromatography (Ngai *et al.*, 1984). This is shown in Fig. 7. When this preparation of caldesmon was subjected to ion-exchange chromatography (Fig. 8), the caldesmon kinase was separated from the caldesmon. Recombination of peak fractions from the caldesmon kinase (I) and caldesmon (II) peaks restored caldesmon phosphorylation. The gel inset of Fig. 7 indicates that caldesmon kinase activity correlated with a Coomassie Blue-stained band of M_r 93000. Whether this band truly represents caldesmon kinase requires more rigorous experimentation. The separated caldesmon kinase was found to be extremely labile, and for this reason the experiments depicted in Figs. 5 and 6 were performed with the caldesmon-caldesmon kinase

Fig. 6. Ca^{2+} /calmodulin-dependence of caldesmon kinase

Caldesmon (79 μ g/ml), purified as previously described (Ngai *et al.*, 1984) and containing endogenous caldesmon kinase activity, was incubated at 30°C in 20 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/0.5 mM-[γ - 32 P]ATP (~ 17000 c.p.m./nmol), with 0.1 mM- $CaCl_2$ (○, △) or 1 mM-EGTA (□, ▽) in the presence (○, □) or absence (△, ▽) of calmodulin (CaM; 10 μ g/ml). Reaction volumes were 5.5 ml. Samples (0.5 ml) were withdrawn at the indicated times for quantification of protein-bound [32 P] P_i as previously described (Walsh *et al.*, 1984). To the remainder of each reaction mixture was added an equal volume of SDS-gel sample buffer. Samples for electrophoresis were immersed in a boiling-water bath for 2 min before SDS/polyacrylamide-gradient-slab-gel electrophoresis as described in the Materials and methods section. The stained and destained gel was dried and subjected to autoradiography with Kodak Min R film. (a) Coomassie Blue-stained gel; (b) autoradiogram. The arrows indicate the position of the caldesmon band.

combination eluted from calmodulin-Sepharose; in this state caldesmon kinase activity was quite stable and survived freezing and thawing.

Fig. 7. Co-purification of caldesmon and caldesmon kinase by calmodulin-Sepharose affinity chromatography. Caldesmon was purified as described previously (Ngai *et al.*, 1984) up to the DEAE-Sepharose ion-exchange chromatography stage. The pooled material from this column was dialysed overnight against 2×10 litres of 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/1 mM-EGTA/0.1 mM-dithiothreitol. Solid CaCl_2 was added to the dialysed sample (final concn. 1.2 mM) before loading on two columns (1 cm $\times$ 15 cm each) of calmodulin-Sepharose previously equilibrated with 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/0.2 mM- CaCl_2 /0.1 mM-dithiothreitol. Flow rate was 12 ml/h, and fraction size 4 ml. Unbound protein was thoroughly washed from the column with equilibration buffer before elution of specifically bound proteins with 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/1 mM-EGTA/0.1 mM-dithiothreitol (applied at fraction 130). Eluted protein was monitored by A_{280} (—), and caldesmon kinase activity of selected fractions was assayed under the following conditions: 20 mM-Tris/HCl (pH 7.5), 5 mM- MgCl_2 , 0.1 mM- CaCl_2 (○) or 1 mM-EGTA (□), 10 μg of calmodulin/ml, 50 μl of column fraction/ml, 0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (~ 5000 c.p.m./nmol), 30°C, 60 min incubation time. No kinase activity was detected in the absence of calmodulin and in the presence of either 0.1 mM- CaCl_2 or 1 mM-EGTA. The three peak activity fractions (137, 138 and 139 respectively) were examined by SDS/polyacrylamide-gradient-slab-gel electrophoresis (inset). The arrow indicates the caldesmon band. Fractions 137–139 were pooled for further purification.

Discussion

The two major calmodulin-binding proteins of chicken gizzard smooth muscle, caldesmon (11.1 μM) and myosin light-chain kinase (4.6 μM), are both associated with the contractile apparatus, specifically actin filaments (Sobue *et al.*, 1981; Dabrowska *et al.*, 1982; Ngai *et al.*, 1984), from which they can be released by treatment with a high concentration (25 mM) of Mg^{2+} . Sobue *et al.* (1981) calculated a tissue content of caldesmon of 8% of total protein (240 mg/100 g), i.e. 20 μM , based on densitometry of SDS/polyacrylamide gels of a 0.3 M-KCl extract of chicken gizzard. We have found that homogenization of the tissue in 0.3 M-KCl results in complete extraction of caldesmon, but incomplete extraction of other proteins, no-

tably actin, leading to an erroneously high estimate of the tissue content of caldesmon. Our procedure described here, using extraction with SDS-gel sample buffer, results in quantitative solubilization of tissue proteins.

Myosin light-chain kinase plays a central role in the regulation of smooth-muscle contraction (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982). The presence in smooth muscle of another calmodulin-binding protein, caldesmon, at such a high concentration (11.1 μM) raised the possibility that caldesmon may inhibit myosin phosphorylation in the presence of Ca^{2+} by competing with myosin light-chain kinase for available calmodulin. Such inhibition could have important implications in the kinetics of myosin phosphorylation *in vivo* and the development of tension in smooth

Fig. 8. Separation of caldesmon kinase from caldesmon by DEAE-Sephacel ion-exchange chromatography. The pooled fractions from calmodulin-Sephacel (Fig. 7) were loaded directly on a column (1 cm $\times$ 40 cm) of DEAE-Sephacel previously equilibrated with 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/1 mM-EGTA/0.5 mM-dithiothreitol. Flow rate was 12 ml/h, and fraction size 4 ml. After washing of the column with equilibration buffer, a linear 0.1–0.2 M-NaCl gradient (400 ml) was applied at the arrow to elute bound proteins. Protein elution was monitored by A_{280} (—), and caldesmon kinase activity in selected fractions (O) was assayed under the following conditions: 20 mM-Tris/HCl (pH 7.5), 5 mM-MgCl₂, 0.1 mM-CaCl₂, 10 μ g of calmodulin/ml, 0.1 mg of caldesmon (purified as described in the Materials and methods section)/ml, 50 μ l of column fraction/ml, 0.5 mM-[γ -³²P]ATP ($\sim$ 5000 c.p.m./nmol), 30°C, 60 min incubation time. Selected fractions were also analysed by SDS/polyacrylamide-gradient-slab-gel electrophoresis (gel inset). Key to lanes: 1, M_r markers (myosin heavy chain, M_r 205 000; β -galactosidase, M_r 116 000; phosphorylase *b*, M_r 97 400; bovine serum albumin, M_r 66 000; ovalbumin, M_r 45 000); 2–20 = fractions 5–15, 30, 35, 40, 45, 50, 55, 60, 65 respectively. The position of the caldesmon band (CaD) is indicated.

muscle. An important consideration in this regard is the tissue concentration of calmodulin. This has been calculated as 31 μ M for chicken gizzard (Grand & Perry, 1979) and 34 μ M for taenia coli (Rüegg *et al.*, 1984), suggesting that there is sufficient calmodulin present to saturate both caldesmon and myosin light-chain kinase in the presence of Ca²⁺. Nevertheless, our experiments *in vitro* with purified calmodulin, caldesmon and myosin light-chain kinase indicate that caldesmon, even when present in excess, does not compete with the kinase for calmodulin. Neither does caldesmon inhibit cyclic nucleotide phosphodiesterase, another calmodulin-activated enzyme. On the other hand, myosin light-chain kinase and cyclic nucleotide phosphodiesterase do compete for available calmodulin, suggesting that they share a common binding site on calmodulin. The lack of inhibition of either myosin light-chain

kinase or cyclic nucleotide phosphodiesterase activities by caldesmon may be due to a much lower affinity of caldesmon for calmodulin than of the kinase or phosphodiesterase for calmodulin. Alternatively, calmodulin may have distinct binding sites, one for myosin light-chain kinase and phosphodiesterase and another for caldesmon; this raises the possibility that the complex caldesmon–calmodulin–myosin light-chain kinase may be formed in the presence of Ca²⁺ and that this complex exhibits normal myosin light-chain kinase activity. Regardless of the mechanism, it is clear that caldesmon does not affect the phosphorylation of smooth-muscle myosin, and hence is unlikely to affect the development of tension induced by myosin phosphorylation *in vivo*. Nairn *et al.* (1980) have observed a lack of inhibition of skeletal-muscle myosin light-chain kinase by three calmodulin-binding proteins, i.e. troponin I,

troponin T and myelin basic protein, suggesting that calmodulin is indeed capable of binding two target proteins simultaneously at different sites.

Our previous observations suggest that caldesmon may function, independently of myosin phosphorylation, in the regulation of smooth-muscle actin–myosin interactions, since it caused a significant inhibition of the actin-activated myosin Mg^{2+} -ATPase activity and superprecipitation in a system reconstituted from the purified contractile and regulatory proteins (Ngai & Walsh, 1984). Preliminary evidence suggested that these inhibitory effects could be abolished by phosphorylation of caldesmon catalysed by a Ca^{2+} /calmodulin-dependent protein kinase (Ngai & Walsh, 1984). We have now succeeded in separating this kinase from caldesmon and shown that kinase activity correlates with a protein of subunit M_r 93000. This kinase is distinct from myosin light-chain kinase and phosphorylase kinase, both known calmodulin-activated kinases. We have observed different degrees of phosphorylation of different preparations of caldesmon by caldesmon kinase (2–4 mol of P_i /mol of caldesmon), perhaps reflecting different extents of pre-phosphorylation. In confirmation of our earlier findings (Ngai & Walsh, 1984), we have identified caldesmon phosphatase activity in a crude preparation of smooth-muscle myosin phosphatase. This phosphatase rapidly dephosphorylates caldesmon. Smooth muscle therefore contains all the components necessary for the regulation of the effects of caldesmon on actin–myosin interactions: caldesmon kinase, which requires Ca^{2+} and calmodulin for activity, and caldesmon phosphatase. Whether or not caldesmon phosphatase activity resides on the same molecule as myosin phosphatase activity remains to be established.

Evidence from several laboratories indicates that, although myosin phosphorylation is a prerequisite for smooth-muscle contraction, actin–myosin interactions can be controlled by independent Ca^{2+} -mediated regulatory mechanisms. These may include direct Ca^{2+} binding to myosin (Chacko *et al.*, 1977; Rees & Frederiksen, 1981; Chacko & Rosenfeld, 1982), the leiotonin complex (Ebashi, 1980; Nonomura & Ebashi, 1980), myosin phosphorylation catalysed by the Ca^{2+} - and phospholipid-dependent protein kinase (Endo *et al.*, 1982; Nishikawa *et al.*, 1983), caldesmon (Sobue *et al.*, 1982) and other unidentified calmodulin-dependent systems. The involvement of a secondary Ca^{2+} -dependent regulatory mechanism in controlling actin–myosin interactions in smooth muscle is supported by physiological studies (e.g. Aksoy *et al.*, 1983; Chatterjee & Murphy, 1983). The observations that caldesmon inhibits the actin-activated myosin Mg^{2+} -ATPase activity in a

reconstituted system and that this inhibitory effect is apparently controlled by the Ca^{2+} /calmodulin-dependent phosphorylation of caldesmon suggest that this system may represent a physiologically significant mechanism for controlling actin–myosin interactions in smooth muscle which operates independently of myosin phosphorylation.

This work was supported by grants (to M. P. W.) from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research. P. K. N. is recipient of a studentship from the Alberta Heritage Foundation for Medical Research.

References

- Adelstein, R. S. & Eisenberg, E. (1980) *Annu. Rev. Biochem.* **49**, 921–956
- Aksoy, M. O., Mras, S., Kamm, K. E. & Murphy, R. A. (1983) *Am. J. Physiol.* **245**, C255–C270
- Bailin, G. & Lopez, F. (1982) *J. Biol. Chem.* **257**, 264–270
- Chacko, S. & Rosenfeld, A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 292–296
- Chacko, S., Conti, M. A. & Adelstein, R. S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 129–133
- Chatterjee, M. & Murphy, R. A. (1983) *Science* **221**, 464–466
- Dabrowska, R., Hinkins, S., Walsh, M. P. & Hartshorne, D. J. (1982) *Biochem. Biophys. Res. Commun.* **107**, 1524–1531
- Ebashi, S. (1980) *Proc. R. Soc. London Ser. B* **207**, 259–286
- Endo, T., Naka, M. & Hidaka, H. (1982) *Biochem. Biophys. Res. Commun.* **105**, 942–948
- Gopalakrishna, R. & Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun.* **104**, 830–836
- Grand, R. J. A. & Perry, S. V. (1979) *Biochem. J.* **183**, 285–295
- Greene, L. E., Sellers, J. R., Eisenberg, E. & Adelstein, R. S. (1983) *Biochemistry* **22**, 530–535
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 59–62
- Kendrick-Jones, J., Cande, W. Z., Tooth, P. J., Smith, R. C. & Scholey, J. M. (1983) *J. Mol. Biol.* **165**, 139–162
- Klee, C. B. (1977) *Biochemistry* **16**, 1017–1024
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Nag, S. & Seidel, J. C. (1983) *J. Biol. Chem.* **258**, 6444–6449
- Nairn, A. C., Grand, R. J. A., Wall, C. M. & Perry, S. V. (1980) *Ann. N.Y. Acad. Sci.* **356**, 413–414
- Ngai, P. K. & Walsh, M. P. (1984) *J. Biol. Chem.* **259**, 13656–13659
- Ngai, P. K. & Walsh, M. P. (1985) *Biochem. Biophys. Res. Commun.* **127**, 533–539
- Ngai, P. K., Carruthers, C. A. & Walsh, M. P. (1984) *Biochem. J.* **218**, 863–870
- Nishikawa, M., Hidaka, H. & Adelstein, R. S. (1983) *J. Biol. Chem.* **258**, 14069–14072
- Nonomura, Y. & Ebashi, S. (1980) *Biomed. Res.* **1**, 1–14
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Pardee, J. D. & Spudich, J. A. (1982) *Methods Enzymol.* **85**, 164–181

- Pato, M. D. & Adelstein R. S. (1980) *J. Biol. Chem.* **255**, 6536–6538
- Pato, M. D. & Adelstein R. S. (1983) *J. Biol. Chem.* **258**, 7047–7054
- Persechini, A. & Hartshorne, D. J. (1981) *Science* **213**, 1383–1385
- Rees, D. D. & Frederiksen, D. W. (1981) *J. Biol. Chem.* **256**, 357–364
- Rüegg, J. C., Pfitzer, G., Zimmer, M. & Hofmann, F. (1984) *FEBS Lett.* **170**, 383–386
- Sellers, J. R., Pato, M. D. & Adelstein, R. S. (1981) *J. Biol. Chem.* **256**, 13137–13142
- Sellers, J. R., Boon Chock, P. & Adelstein, R. S. (1983) *J. Biol. Chem.* **258**, 14181–14186
- Sherry, J. M. F., Górecka, A., Aksoy, M. O., Dabrowska, R. & Hartshorne, D. J. (1978) *Biochemistry* **17**, 4411–4418
- Simpson, R. J., Neuberger, M. R. & Liu, T. Y. (1976) *J. Biol. Chem.* **251**, 1936–1940
- Smillie, L. B. (1982) *Methods Enzymol.* **85**, 234–241
- 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
- Suzuki, H., Onishi, H., Takahashi, K. & Watanabe, S. (1978) *J. Biochem. (Tokyo)* **84**, 1529–1542
- 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., Flink, I. L. & Hartshorne, D. J. (1982) *Biochemistry* **21**, 6890–6896
- Walsh, M. P., Hinkins, S., Dabrowska, R. & Hartshorne, D. J. (1983) *Methods Enzymol.* **29**, 279–288
- Walsh, M. P., Valentine, K. A., Ngai, P. K., Carruthers, C. A. & Hollenberg, M. D. (1984) *Biochem. J.* **224**, 117–127
- Wang, J. H., Teo, T. S. & Wang, T. H. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1306–1311
- Werth, D. K., Haeberle, J. R. & Hathaway, D. R. (1982) *J. Biol. Chem.* **257**, 7306–7309
- Zot, H. G. & Potter, J. D. (1981) *Prep. Biochem.* **11**, 381–395