Smooth-muscle caldesmon phosphatase is SMP-I, a type 2A protein phosphatase

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Caldesmon phosphatase was identified in chicken gizzard smooth muscle by using as substrates caldesmon phosphorylated at different sites by protein kinase C, Ca^{2+} /calmodulin-dependent protein kinase II and cdc2 kinase. Most (~90%) of the phosphatase activity was recovered in the cytosolic fraction. Gel filtration after $(\text{NH}_4)_2\text{SO}_4$ fractionation of the cytosolic fraction revealed a single major peak of phosphatase activity which co-eluted with calponin phosphatase [Winder, Pato and Walsh (1992) *Biochem. J.* **286**, 197–203] and myosin LC_{20} phosphatase. Further purification of caldesmon phosphatase was achieved by sequential chromatography on columns of DEAE-Sephacel, ω -amino-octyl-agarose, aminopropyl-agarose and thiophosphorylated myosin LC_{20} -Sephacel. A single peak of caldesmon phosphatase activity was detected at each step of the purification. The purified phosphatase was identified as SMP-I [Pato and Adelstein (1980) *J. Biol. Chem.* **255**, 6535–6538] by subunit composition (three subunits, of 60, 55 and 38 kDa) and Western blotting using antibodies against the holoenzyme which recognize all three subunits and antibodies specific for the 38 kDa catalytic

subunit. SMP-I is a type 2A protein phosphatase [Pato, Adelstein, Crouch, Safer, Ingebritsen and Cohen (1983) *Eur. J. Biochem.* **132**, 283–287; Winder et al. (1992), cited above]. Consistent with the conclusion that SMP-I is the major caldesmon phosphatase of smooth muscle, purified SMP-I from turkey gizzard dephosphorylated all three phosphorylated forms of caldesmon, whereas SMP-II, -III and -IV were relatively ineffective. Kinetic analysis of dephosphorylation by chicken gizzard SMP-I of the three phosphorylated caldesmon species and calponin phosphorylated by protein kinase C indicates that calponin is a significantly better substrate of SMP-I than are any of the three phosphorylated forms of caldesmon. We therefore suggest that caldesmon phosphorylation *in vivo* can be maintained after kinase inactivation due to slow dephosphorylation by SMP-I, whereas calponin and myosin are rapidly dephosphorylated by SMP-I and SMP-III/SMP-IV respectively. This may have important functional consequences in terms of the contractile properties of smooth muscle.

INTRODUCTION

Caldesmon is an actin-filament-associated protein which has been implicated in the regulation of smooth-muscle contraction (Walsh, 1990; Marston and Redwood, 1991; Sobue and Sellers, 1991). The isolated protein interacts with actin (Sobue et al., 1981), tropomyosin (Graceffa, 1987; Fujii et al., 1988; Horiuchi and Chacko, 1988; Watson et al., 1990), myosin (Hemric and Chalovich, 1988, 1990; Ikebe and Reardon, 1988; Katayama et al., 1989; Sutherland and Walsh, 1989) and Ca^{2+} /calmodulin (Sobue et al., 1981). Smooth-muscle caldesmon can be divided into three domains: the N-terminal domain contains the myosin-binding site, the central repeat domain rich in charged residues has no known function, and the C-terminal domain contains the sites of interaction with actin, tropomyosin and Ca^{2+} /calmodulin (Marston and Redwood, 1991; Sobue and Sellers, 1991). Caldesmon and fragments of caldesmon containing the actin-binding site inhibit smooth-muscle actin-activated myosin MgATPase activity (Ngai and Walsh, 1984; Szpacenko and Dabrowska, 1986).

Smooth-muscle caldesmon is phosphorylated *in vitro* by at least four protein kinases: Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) (Abougou et al., 1989; Ikebe et al.,

1990; Scott-Woo et al., 1990), protein kinase C (Umekawa and Hidaka, 1985; Vorotnikov et al., 1988; Tanaka et al., 1990), cdc2 kinase (Mak et al., 1991a) and casein kinase II (Vorotnikov et al., 1988; Wawrzynow et al., 1991). CaM kinase II phosphorylates three sites in the N-terminal domain [Ser-26, Ser-59 and Ser-73, using the numbering system of Bryan et al. (1989)] and five sites in the C-terminal domain (Thr-469, Ser-475, Ser-587, Ser-620 and Ser-726). The preferred site of phosphorylation by this kinase is Ser-73, followed by Ser-26, Ser-726 and Ser-587 (Ikebe and Reardon, 1990). Phosphorylation by CaM kinase II blocked the interaction of caldesmon with myosin (Sutherland and Walsh, 1989) and decreased inhibition of the actin-activated myosin MgATPase (Ngai and Walsh, 1984, 1987). Protein kinase C phosphorylates two sites (Ser-587 and Ser-726), both located in the C-terminal domain, and results in decreased inhibition of acto-heavy meromyosin and actomyosin ATPase activities due to a decrease in the affinity of caldesmon for actin (Tanaka et al., 1990; Ikebe and Hornick, 1991). cdc2 kinase phosphorylates five sites in smooth-muscle caldesmon (Ser-582, Ser-667, Thr-673, Thr-696 and Ser-702) and inhibits actin binding (Mak et al., 1991a,b). Casein kinase II phosphorylates Ser-73 of caldesmon, but the effects of this phosphorylation have not been examined (Wawrzynow et al., 1991).

Abbreviations used: CaM kinase II, Ca^{2+} /calmodulin-dependent protein kinase II; MAP kinase, microtubule-associated protein kinase or mitogen-activated protein kinase; myosin LC_{20} , the 20 kDa light chain of smooth-muscle myosin; SMP, smooth-muscle phosphatase.

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Caldesmon phosphorylation has also been observed in a variety of intact smooth-muscle fibres in response to several contractile stimuli (Park and Rasmussen, 1986; Adam et al., 1989, 1990; Pohl et al., 1991; Bárány et al., 1992a,b). Phosphopeptide mapping of caldesmon immunoprecipitated from pig carotid arteries treated with KCl or phorbol 12,13-dibutyrate suggested that the sites phosphorylated *in vivo* are distinct from those phosphorylated *in vitro* by protein kinase C or CaM kinase II (Adam et al., 1989). The sites phosphorylated in intact dog aortic smooth muscle treated with phorbol ester were identified as two serine residues, both located in the C-terminal domain of caldesmon (Adam et al., 1992). One corresponds to Ser-702 of chicken gizzard caldesmon, whereas the other is unique to mammalian caldesmon and is located four residues from the C-terminus. Each of these serine residues is immediately N-terminal to a proline, suggesting that the kinase is a proline-directed kinase, i.e. a member of the cdc2 kinase or MAP kinase (microtubule-associated or mitogen-activated protein kinase) family. MAP kinase was recently identified in vascular smooth-muscle tissues by Western blotting (Adam and Hathaway, 1992).

The evidence is therefore strongly supportive of a physiological role(s) for caldesmon phosphorylation, probably involving different kinases with different site specificities and having different functional effects. This implies that dephosphorylation catalysed by protein serine/threonine phosphatase(s) is required to reverse the functional effects of phosphorylation. Caldesmon dephosphorylation after its phosphorylation has been observed in intact smooth-muscle strips (Adam et al., 1989; Pohl et al., 1991). Furthermore, we have demonstrated caldesmon phosphatase activity in a partially purified preparation of myosin light-chain phosphatase from chicken gizzard smooth muscle (Ngai and Walsh, 1984, 1985). We report here the isolation and identification of the principal caldesmon phosphatase from chicken gizzard using as substrates caldesmon phosphorylated by protein kinase C, CaM kinase II or cdc2 kinase. We also compared the dephosphorylation of these three phosphorylated caldesmons by four well-characterized protein phosphatases [smooth-muscle phosphatase (SMP) I–IV] isolated from turkey gizzard (Pato, 1985).

MATERIALS AND METHODS

Materials

[γ - 32 P]ATP (20–40 Ci/mmol and 3000 Ci/mmol) was purchased from ICN Biomedicals (St. Laurent, Québec, Canada), Sephacryl S-300, DEAE-Sephacel and CNBr-activated Sepharose 4B were from Pharmacia (Baie d'Urfe, Québec, Canada), aminopropyl-agarose, ω -amino-octyl-agarose and 1,3-diolein were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, Ontario, Canada) and L- α -phosphatidyl-L-serine (bovine brain) was from Serdary Research Laboratories (London, Ontario, Canada). General laboratory reagents used were purchased from CanLab (Calgary, Alberta, Canada). The following proteins were purified by published methods: bovine brain calmodulin (Walsh et al., 1984), chicken gizzard calponin (Winder and Walsh, 1990), caldesmon (Sutherland and Walsh, 1989), caldesmon containing contaminating CaM kinase II (Scott-Woo and Walsh, 1988) and the 20 kDa light chain of smooth-muscle myosin (myosin LC₂₀; Hathaway and Haerberle, 1983); rat brain protein kinase C (Wolf et al., 1984); cdc2 kinase (Mak et al., 1991a) was generously provided by Dr. Jerry Wang, Department of Medical Biochemistry, University of Calgary. Turkey gizzard smooth-muscle phosphatases were purified as previously described: SMP-I and

SMP-II (Pato and Adelstein, 1982), SMP-III (Tulloch and Pato, 1991) and SMP-IV (Pato and Kerc, 1985).

Preparation of 32 P-labelled substrates

Caldesmon was phosphorylated by three protein kinases individually as follows. Protein kinase C: caldesmon (2.3 μ M) was incubated at 30 °C for 60 min with 2 μ g of rat brain protein kinase C/ml in 20 mM Tris/HCl (pH 7.5)/5 mM MgCl₂/0.1 mM CaCl₂/48.4 μ M phosphatidylserine/12.3 μ M 1,3-diolein/0.2 mM [γ - 32 P]ATP ($\sim$ 170 c.p.m./pmol). CaM kinase II: caldesmon (2.3 μ M) containing contaminating CaM kinase II was incubated at 30 °C for 60 min in 20 mM Tris/HCl (pH 7.5)/5 mM MgCl₂/0.1 mM CaCl₂/2.5 μ M calmodulin/0.2 mM [γ - 32 P]ATP ($\sim$ 430 c.p.m./pmol). cdc2 kinase: caldesmon (5.7 μ M) was incubated at 30 °C for 2 h with 30 μ g of cdc2 kinase/ml in 20 mM Mops (pH 7)/1 mM EGTA/40 mM KCl/2 mM MgCl₂/0.1 mM [γ - 32 P]ATP ($\sim$ 170 c.p.m./pmol). Calponin was phosphorylated by protein kinase C as follows: calponin (6.25 μ M) was incubated at 30 °C for 60 min with 2 μ g of rat brain protein kinase C/ml in 20 mM Tris/HCl (pH 7.5)/5 mM MgCl₂/0.1 mM CaCl₂/48.4 μ M phosphatidylserine/12.3 μ M 1,3-diolein/1 mM [γ - 32 P]ATP ($\sim$ 230 c.p.m./pmol). Reactions were quenched and the respective kinases inactivated by immersion in a boiling-water bath for 2 min; caldesmon is heat-stable (Bretscher, 1984). Samples (0.1 ml) of reaction mixtures were withdrawn at the end of each reaction for quantification of protein-bound [32 P]P_i as previously described (Walsh et al., 1983). The following phosphorylation stoichiometries were determined (values given as means $\pm$ S.D.): 2.09 $\pm$ 0.50 mol of P_i/mol (n = 35) for caldesmon phosphorylated by protein kinase C, referred to as caldesmon (PKC); 1.20 $\pm$ 0.31 mol of P_i/mol (n = 28) for caldesmon phosphorylated by CaM kinase II, referred to as caldesmon (CaM kinase II); 0.74 $\pm$ 0.11 mol of P_i/mol (n = 12) for caldesmon phosphorylated by cdc2 kinase, referred to as caldesmon (cdc2); and 2.24 $\pm$ 0.66 mol of P_i/mol (n = 22) for calponin phosphorylated by PKC, referred to as calponin (PKC). 32 P-labelled myosin LC₂₀ was prepared by phosphorylation with purified myosin light-chain kinase (Walsh et al., 1983).

Phosphatase assays

Two phosphatase assay methods were used in this study. In assay method 1, 32 P-labelled protein substrates ($\sim$ 100 000 c.p.m./ml), prepared as described above, were incubated at 30 °C with phosphatase in 20 mM Tris/HCl (pH 7.5)/10 mM EDTA containing 1 mg of carrier BSA/ml. Samples (0.1 ml) of reaction mixtures were withdrawn at selected times for quantification of protein-bound [32 P]P_i by Čerenkov counting (Walsh et al., 1983). In assay method 2, 32 P-labelled protein substrates, prepared as described above, were dialysed against 4 $\times$ 500 ml of 1 M KCl/20 mM Tris/HCl (pH 7.4)/1 mM dithiothreitol, followed by 2 $\times$ 500 ml of 20 mM KCl/20 mM Tris/HCl (pH 7.4)/1 mM dithiothreitol/3 mM NaN₃. Substrate proteins (2 μ M) were incubated at 30 °C with phosphatase in 50 mM Tris/HCl (pH 7.4)/1 mM dithiothreitol. Samples (40 μ l) of reaction mixtures were withdrawn at selected times and added to 0.1 ml of 17.5% trichloroacetic acid. BSA (0.1 ml of a 6 mg/ml solution) was added to each sample, which was then chilled and centrifuged (15 000 g for 1 min). The supernatant (0.2 ml) was added to scintillation fluid and counted for radioactivity. In some instances (indicated in the text) 10 mM MgCl₂ was included in phosphatase assay media. For kinetic measurements, protein substrate concentrations were varied (Table 4).

Caldesmon phosphatase assays in cytosolic and particulate fractions

Chicken gizzard smooth muscle (2 g), which had been snap-frozen in liquid N_2 while fresh and stored at -80°C , was homogenized for 2×10 s with a Polytron in 4 vol. of 20 mM Tris/HCl (pH 7.5)/5 mM EDTA containing 5 mM dithiothreitol, 10 μg of leupeptin/ml and 10 μg of pepstatin/ml (Buffer A) and centrifuged at 26000 g for 15 min. The supernatant (S1) was stored on ice and the pellet redissolved in 4 vol. (8 ml) of Buffer A, homogenized and centrifuged as above. The supernatant (S2) was stored on ice and the pellet was washed once more and centrifuged. The supernatant (S3) was stored on ice and the final pellet (P) was resuspended by homogenization in 8 ml of Buffer A. Caldesmon phosphatase activity was assayed in each of the three cytosolic fractions (S1–S3) and the resuspended particulate fraction (P), at 10 μl /0.4 ml reaction volume with caldesmon (PKC) and caldesmon (CaM kinase II) as substrate or 60 μl /0.4 ml reaction volume with caldesmon (cdc2) as substrate, by incubation at 30°C with 30 μg of [^{32}P]caldesmon (PKC)/ml (2.3 mol of P_i /mol; 4058 c.p.m./ μg of caldesmon), 40 μg of [^{32}P]caldesmon (CaM kinase II)/ml (1.75 mol of P_i /mol of caldesmon; 1542 c.p.m./ μg of caldesmon) or 150 μg of [^{32}P]caldesmon (cdc2)/ml (0.76 mol of P_i /mol of caldesmon; 2594 c.p.m./ μg of caldesmon) in 20 mM Tris/HCl (pH 7.5)/10 mM EDTA. Reactions were started by addition of ^{32}P -labelled substrate, and samples (0.1 ml) were withdrawn at selected times, added to an equal volume of SDS-gel sample buffer and boiled before SDS/PAGE (50 μl /lane) and autoradiography. Quantitative data were obtained by scanning laser densitometry of the autoradiographs using an LKB model 2202 Ultrosan laser densitometer equipped with a Hewlett–Packard model 3390A integrator.

Purification of caldesmon phosphatase

All procedures were carried out at 4°C or on ice. Frozen trimmed chicken gizzards (100 g) were partially thawed, homogenized in 4 vol. of Buffer A for 2×30 s with a Waring blender and centrifuged at 31000 g for 30 min. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant slowly with stirring to a final concentration of 30% saturation, stirred for 30 min and centrifuged as above. Additional $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 60% saturation, stirred and centrifuged as above. The pellet was dissolved in 20–30 ml of 20 mM Tris/HCl (pH 7.5)/0.5 M NaCl/1 mM EDTA/1 mM dithiothreitol (Buffer B) and dialysed overnight against 2×5 litres of Buffer B. The dialysed sample was applied to a column (6 cm $\times$ 90 cm) of Sephacryl S-300 previously equilibrated with Buffer B. Fractions were assayed for caldesmon, calponin and myosin LC_{20} phosphatase activities. Peak activity fractions were pooled, dialysed overnight against 2×4 litres of 20 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM dithiothreitol (Buffer C) and applied to a column (2.5 cm $\times$ 40 cm) of DEAE-Sephacel previously equilibrated with Buffer C. Bound proteins were eluted with a 700 ml linear gradient of NaCl (0–0.5 M in Buffer C). Fractions were assayed for caldesmon phosphatase activity, and peak fractions were pooled, dialysed overnight against 2×4 litres of Buffer C and applied to a column (1.5 cm $\times$ 15 cm) of ω -amino-octyl-agarose previously equilibrated with Buffer C. The column was then washed with Buffer C containing 0.4 M NaCl and finally with Buffer C containing 2 M NaCl. Peak fractions of caldesmon phosphatase activity were pooled, dialysed overnight against 2×4 litres of Buffer C and applied to a column (1 cm $\times$ 15 cm) of aminopropyl-agarose previously equilibrated with Buffer C. The column was washed as described for the ω -amino-octyl-agarose column, and peak

fractions of caldesmon phosphatase activity were pooled, dialysed overnight against 2×4 litres of Buffer C and applied to a column (1 cm $\times$ 10 cm) of thiophosphorylated myosin LC_{20} -Sephacryl previously equilibrated with Buffer C. Bound protein (purified caldesmon phosphatase) was eluted with Buffer C containing 1 M NaCl, dialysed against 2×4 litres of Buffer C containing 50 mM NaCl, and stored at -80°C .

Other procedures

Thiophosphorylated LC_{20} -Sephacryl was prepared as previously described (Pato and Kerc, 1990). Protein concentrations were determined by the Coomassie Blue dye-binding assay (Spector, 1978) with the dye reagent purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.) or by spectrophotometric measurements (Winder and Walsh, 1990). Electrophoresis was performed as previously described (Winder and Walsh, 1990). Western blotting was carried out essentially as described by Towbin et al. (1979), using polyclonal antibodies raised in rabbits against the holo-enzyme form of turkey gizzard SMP-I or the isolated catalytic subunit of SMP-I, which was prepared from SMP-I by ethanol precipitation as described by Brandt et al. (1975). The secondary antibody used was anti-(rabbit IgG)-alkaline phosphatase conjugate and the substrate was 5-chloro-4-bromo-3-indolyl phosphate/Nitro Blue Tetrazolium.

RESULTS

Demonstration of caldesmon phosphatase activity in the cytosolic fraction of chicken gizzard smooth muscle

Cytosolic (S1–S3) and particulate (P) fractions were prepared from chicken gizzard smooth muscle as described in the Materials and methods section. Dephosphorylation by these fractions of caldesmon phosphorylated by protein kinase C is shown in Figure 1. Most of the activity was recovered in S1, less in S2 and less again in S3. Relatively little phosphatase activity was detected in the particulate fraction. Similar results were obtained with caldesmon phosphorylated by CaM kinase II or by cdc2 kinase as substrates. Results for all three substrates are combined in quantitative form in Table 1. Most ($\sim 90\%$) of the phosphatase activity towards caldesmon (PKC), caldesmon (CaM kinase II) and caldesmon (cdc2) is therefore recovered in the cytosolic fraction.

Purification of caldesmon phosphatase

Trial experiments indicated that 30–60%-(NH_4) $_2\text{SO}_4$ fractionation provided a suitable initial purification step. After dialysis, this fraction was subjected to gel filtration on a column of Sephacryl S-300. A coincident major peak of caldesmon (PKC), caldesmon (CaM kinase II) and caldesmon (cdc2) phosphatase activity was observed which was well separated from the bulk of protein eluted from the column (Figure 2a). Similar results (not shown) were obtained when phosphatase activity was assayed in the presence of 10 mM Mg^{2+} ; SMP-II requires Mg^{2+} (or Mn^{2+}) for activity (Pato and Adelstein, 1983). This caldesmon phosphatase activity co-eluted with calponin (PKC) phosphatase and myosin LC_{20} phosphatase (Figure 2b).

We have previously shown that calponin phosphatase is identical with SMP-I (Winder et al., 1992). We proceeded therefore to purify caldesmon phosphatase as described in the Materials and methods section. At each stage of the purification procedure, column fractions were assayed with ^{32}P -labelled caldesmon (PKC), caldesmon (CaM kinase II), caldesmon (cdc2) and calponin (PKC) as substrates. One major peak exhibiting

Figure 1 Demonstration of caldesmon phosphatase activity in the cytosolic fraction of chicken gizzard smooth muscle

The cytosolic (S1–S3) and particulate (P) fractions were prepared from chicken gizzard smooth muscle and assayed for phosphatase activity with as substrate caldesmon (PKC) as described in the Materials and methods section. Samples of reaction mixtures were analysed at the indicated times by SDS/PAGE and autoradiography. Abbreviations: CaD, caldesmon; P-CaD, phosphorylated caldesmon. The positions of molecular-mass marker proteins are indicated in kDa. Similar results were obtained with caldesmon (CaM kinase II) and caldesmon (cdc2) as substrates (see Table 1).

Table 1 Caldesmon phosphatase activities in the cytosolic and particulate fractions of chicken gizzard smooth muscle

Values were calculated from initial rates of dephosphorylation by densitometric scanning of autoradiograms similar to those shown in Figure 1. Results are given as means $\pm$ S.D., *n* indicating the number of experiments. The cytosolic (S1, S2 and S3) and particulate (P) fractions were prepared as described in the Materials and methods section

Substrate	Activity (% of total)				<i>n</i>
	S1	S2	S3	P	
Caldesmon (PKC)	49.1 $\pm$ 13.2	26.9 $\pm$ 5.8	12.6 $\pm$ 4.5	11.4 $\pm$ 6.0	7
Caldesmon (CaM kinase II)	48.2 $\pm$ 9.4	23.6 $\pm$ 6.6	16.1 $\pm$ 3.6	12.1 $\pm$ 6.9	5
Caldesmon (cdc2)	43.5 $\pm$ 3.3	34.1 $\pm$ 5.0	15.3 $\pm$ 6.2	7.2 $\pm$ 2.7	3

phosphatase activity towards all four substrates was detected at each stage of the purification. The final column chromatographic step involved affinity chromatography on thiophosphorylated myosin LC₂₀-Sephacryl (Figure 3). Caldesmon (PKC) phosphatase activity bound to the affinity resin and was eluted at high ionic strength. Similar results (not shown) were obtained with caldesmon (CaM kinase II), caldesmon (cdc2) and calponin (PKC) as substrates.

Figure 4(a) shows the Coomassie Blue-stained SDS/PAGE analysis of the affinity-purified phosphatase preparation. Three polypeptide chains, of 60, 55 and 38 kDa, are apparent. This pattern is reminiscent of the subunit composition of turkey gizzard SMP-I (Pato and Adelstein, 1980), consistent with our previous demonstration that calponin phosphatase is the chicken counterpart of turkey SMP-I (Winder et al., 1992). The Western

Figure 2 Co-elution of caldesmon, calponin and myosin LC₂₀ phosphatase activities from a gel-filtration column

The S1 fraction, prepared from chicken gizzard as described in the Materials and methods section, was fractionated with (NH₄)₂SO₄, and the 30–60% satd. (NH₄)₂SO₄ fraction was subjected to gel filtration on a column of Sephacryl S-300. Fractions covering the entire chromatogram were assayed for caldesmon phosphatase activity with ³²P-labelled caldesmon (PKC) as substrate by assay method 1. A single major peak of phosphatase activity was detected, with a maximum at fraction 33 (results not shown). Fractions in the region of this caldesmon (PKC) phosphatase peak were then assayed for caldesmon (PKC) phosphatase (○), caldesmon (CaM kinase II) phosphatase (●), caldesmon (cdc2) phosphatase (△), (▲), or calponin (PKC) phosphatase (○) and myosin LC₂₀ phosphatase (●) (b) activities by assay method 1 as described in the Materials and methods section. 100% phosphorylation refers to the level of [³²P]P_i in caldesmon incubated under identical conditions but with column equilibration buffer replacing column fraction (assayed in triplicate). The bar in (b) indicates those fractions that were pooled for further purification.

blots in Figures 4(b) and 4(c), with anti-(SMP-I holoenzyme) and anti-(SMP-I catalytic subunit), respectively, confirm that the isolated caldesmon phosphatase is the type 2A protein phosphatase, SMP-I.

Table 2 compares the specific activities of the Sephacryl S-300 column load and the purified phosphatase with the three different phosphorylated forms of caldesmon and phosphorylated calponin as substrates. The activities of both the crude enzyme and the purified phosphatase towards calponin are substantially greater than towards any of the three phosphorylated caldesmon species, in agreement with our earlier conclusion that calponin is the best substrate of SMP-I among a variety of phosphoproteins tested, including caldesmon (CaM kinase II) (Winder et al., 1992).

Caldesmon phosphatase activities of purified SMP-I, -II, -III and -IV

Table 3 compares the rates of dephosphorylation of myosin LC₂₀, caldesmon (PKC), caldesmon (CaM kinase II), caldesmon (cdc2) and calponin (PKC), at a fixed protein substrate concentration, by turkey gizzard smooth-muscle phosphatases SMP-I, -II, -III and -IV. SMP-II and -IV exhibited no significant dephosphorylation of any of the three phosphorylated forms of caldesmon, whereas all three were effectively dephosphorylated by SMP-I at rates comparable with the dephosphorylation of myosin LC₂₀ and significantly slower than the dephosphorylation of calponin (PKC). SMP-III was inactive towards caldesmon (CaM kinase II) and caldesmon (cdc2), but dephosphorylated caldesmon (PKC) at a rate approximately one-third the rate of dephosphorylation of myosin LC₂₀.

Kinetic parameters were determined for the dephosphorylation of the three phosphorylated forms of caldesmon and calponin (PKC) (Table 4). Comparison of the k_{cat}/K_m values verified that phosphorylated calponin is a significantly better substrate than any of the phosphorylated caldesmon species.

Figure 3 Thiophosphorylated myosin LC₂₀-Sepharose affinity chromatography

The final step of purification involved affinity chromatography as described in the Materials and methods section. Bound protein was eluted by application of column equilibration buffer containing 1 M NaCl at the arrow. Protein elution, as indicated by A_{280} , was monitored continuously (—) and fractions were assayed for caldesmon phosphatase activity with ³²P-labelled caldesmon (PKC) as substrate (○) by assay method 1.

Figure 4 SDS/PAGE and Western-blotting analysis of isolated caldesmon phosphatase

The purified phosphatase was examined by SDS/PAGE and Coomassie Blue staining (a): molecular masses (kDa) indicated were calculated from the migration of marker proteins. (b) and (c) show Western blots of caldesmon phosphatase (lane 1) and turkey gizzard SMP-I (lane 2), with anti-(SMP-I holoenzyme) (b) or anti-(SMP-I catalytic subunit) (c).

Table 2 Specific activities of caldesmon phosphatase with different substrates

Values represent the mean $\pm$ S.E.M. of 2–6 determinations. Assay method 1 was used under linear dephosphorylation conditions. 'Purified phosphatase' refers to the final product of the preparation (see Figure 4).

Substrate	Sephacryl S-300 load (pmol of P _i /min per mg)	Purified phosphatase (nmol of P _i /min per mg)
Caldesmon (PKC)	85.1 $\pm$ 24.8	467.1 $\pm$ 50.0
Caldesmon (CaM kinase II)	15.0 $\pm$ 6.2	97.0 $\pm$ 15.4
Caldesmon (cdc2)	86.1 $\pm$ 19.2	706.8 $\pm$ 135.7
Calponin (PKC)	2100.0 $\pm$ 687.6	7160.0 $\pm$ 770.9

Table 3 Dephosphorylation of various phosphoproteins by turkey gizzard SMP I-IV

Phosphatase activities were determined by assay method 2 under linear dephosphorylation conditions. Rates of dephosphorylation by each phosphatase with myosin LC₂₀ as substrate are defined as 100% activity.

Substrate	Relative phosphatase activity (%)			
	SMP-I	SMP-II*	SMP-III	SMP-IV
Myosin LC ₂₀	100	100	100	100
Caldesmon (PKC)	111	3.5	35.1	3.3
Caldesmon (CaM kinase II)	100	2.6	1.7	1.9
Caldesmon (cdc2)	177	0.1	2.0	2.8
Calponin (PKC)	744	47.4	51.4	20

* Assayed in the presence of 10 mM Mg²⁺ (Pato and Adelstein, 1983).

Table 4 Kinetic parameters of SMP-I

Phosphatase activities were determined by assay method 2 under linear dephosphorylation conditions at varying substrate concentrations as follows: caldesmon (PKC): 1, 2, 2.5, 5 and 6.5 μM ; caldesmon (CaM kinase II): 1, 1.5, 2 and 2.3 μM ; caldesmon (cdc2): 1, 1.5, 2.5, 5 and 6.33 μM ; calponin (PKC): 1, 2, 5, 6.67, 10 and 14.6 μM . The concentration of SMP-I in all reactions was 22.4 nM. Values represent the mean of triplicate determinations.

Substrate	K_m (μM)	$V_{\max}$ ($\mu\text{mol of P}_i/\text{min per mg}$)	k_{cat}/K_m
Caldesmon (PKC)	2.2	0.24	4.9
Caldesmon (CaM kinase II)	3.2	0.25	3.5
Caldesmon (cdc2)	1.9	0.4	9.4
Calponin (PKC)	14.3	5.0	15.6

DISCUSSION

Two physiological functions have been proposed for caldesmon: (1) a role in regulation of the contractile state of smooth muscle through its ability to inhibit actomyosin ATPase activity (Marston and Redwood, 1991; Sobue and Sellers, 1991) and (2) an organizational role through its ability to cross-link actin and myosin filaments (Walsh, 1990, 1991). These functions of caldesmon could be regulated by phosphorylation, and several protein kinases have been shown to phosphorylate caldesmon *in vitro*. Furthermore, caldesmon phosphorylation has been demonstrated in several smooth muscles in response to a broad range of contractile stimuli (see the Introduction). All the known phosphorylation sites in caldesmon are located in the N-terminal myosin-binding domain and the C-terminal actin-binding domain, and phosphorylation at the appropriate sites can affect myosin or actin binding.

If caldesmon phosphorylation is to be of physiological significance, there must exist a phosphatase(s) capable of dephosphorylating caldesmon. We have now purified the major caldesmon phosphatase from chicken gizzard smooth muscle and identified it as the chicken counterpart of SMP-I (Pato and Adelstein, 1980). This enzyme was previously identified as a type 2A protein phosphatase, on the basis of subunit composition, sensitivity to okadaic acid, preferential dephosphorylation of the α -subunit of phosphorylase kinase, lack of inhibition by inhibitor-1 or inhibitor-2, and lack of effect of bivalent cations or of Ca^{2+} /calmodulin (Pato et al., 1983; Winder et al., 1992).

Measurements of the specific activities of the purified phosphatase with different phosphoprotein substrates revealed that calponin (PKC) is a much better substrate than any of the phosphorylated forms of caldesmon (Table 2), consistent with our previous observation that calponin was the best substrate among a wide range of phosphoproteins (Winder et al., 1992). Of the different phosphorylated caldesmon species, caldesmon (cdc2) was a slightly better substrate than caldesmon (PKC), and both were significantly better substrates than caldesmon (CaM kinase II). The data in Table 2 suggest that caldesmon dephosphorylation *in vivo* is likely to take place at a significantly slower rate than calponin dephosphorylation.

We have also extended this work on caldesmon phosphatase by comparing caldesmon dephosphorylation by SMP-I, -II, -III and -IV purified from turkey gizzard smooth muscle (Table 3). Consistent with the results described above for chicken gizzard caldesmon phosphatase, turkey gizzard SMP-I dephosphorylated all three phosphorylated forms of caldesmon, although in this instance caldesmon (CaM kinase II) dephosphorylation was comparable with that of caldesmon (PKC). On the other hand, SMP-II, -III and -IV were ineffective in catalysing the de-

phosphorylation of any of the three phosphorylated caldesmon species, with one exception: SMP-III catalysed significant dephosphorylation of caldesmon (PKC). SMP-III is eluted from a gel-filtration column before SMP-I (Pato, 1985). We observed no significant caldesmon (PKC) phosphatase activity being eluted before the major caldesmon phosphatase peak, indicating that caldesmon (PKC) is a poor substrate of chicken gizzard SMP-III, and/or chicken gizzard contains low amounts of SMP-III. In this context, Tulloch and Pato (1991) have shown that the yield of turkey gizzard SMP-III is lower than that of SMP-I.

Determination of the kinetic parameters for SMP-I-catalysed dephosphorylation of caldesmon (PKC), caldesmon (CaM kinase II), caldesmon (cdc2) and calponin (PKC) (Table 4) confirmed that phosphorylated calponin is the best substrate for this phosphatase. The concentrations of caldesmon and calponin in chicken gizzard smooth muscle are 11 μM (Ngai and Walsh, 1985) and 150 μM (Takahashi et al., 1986; Hartshorne, 1987) respectively. Assuming stoichiometric phosphorylation of calponin and caldesmon *in vivo*, conditions will be close to $V_{\max}$ conditions. The data in Table 4 indicate that the rate of dephosphorylation of calponin will then be 12.5–20 times greater than the rate of dephosphorylation of the various phosphorylated caldesmon species.

In conclusion, we have demonstrated a single major caldesmon phosphatase activity in chicken gizzard smooth muscle identified as SMP-I, a type 2A protein phosphatase. This phosphatase dephosphorylates sites in caldesmon phosphorylated by protein kinase C, CaM kinase II and cdc2 kinase. Activity towards the three phosphorylated forms of caldesmon is significantly lower than that of the best-known smooth-muscle substrate of this phosphatase, i.e. calponin, suggesting that caldesmon phosphorylation *in vivo* may be maintained after inactivation of protein kinases, whereas calponin and myosin are rapidly dephosphorylated by SMP-I (Winder et al., 1992) and SMP-III/IV (Pato, 1985) respectively. If caldesmon serves to organize the contractile filaments via actin–myosin cross-linking in the resting state, its phosphorylation in response to contractile stimuli may disrupt either its interaction with myosin or actin or both, depending on the kinase(s) involved and the sites phosphorylated. The advantage of slow dephosphorylation of caldesmon after kinase inactivation would be that restoration of cross-linking of actin and myosin filaments via unphosphorylated caldesmon would not occur until the resting state of the muscle was re-established after the dephosphorylation of myosin and calponin. Such a mechanism would ensure maintenance of the correct spatial relationship and orientation of the contractile filaments between contractions.

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