Purification and characterization of calponin phosphatase from smooth muscle

Effect of dephosphorylation on calponin function

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Calponin, a thin-filament protein of smooth muscle, has been implicated in the regulation of smooth-muscle contraction, since *in vitro* the isolated protein inhibits the actin-activated myosin MgATPase. This inhibitory effect, and the ability of calponin to bind to actin, is lost after its phosphorylation by protein kinase C or Ca^{2+} /calmodulin-dependent protein kinase II [Winder & Walsh (1990) *J. Biol. Chem.* **265**, 10148–10155]. If this phosphorylation reaction is of physiological significance, there must be a protein phosphatase in smooth muscle capable of dephosphorylating calponin and restoring its inhibitory effect on the actomyosin MgATPase. We demonstrate here the presence, in chicken gizzard smooth muscle, of a single major phosphatase activity directed towards calponin. This phosphatase was purified from the soluble fraction of chicken gizzard by $(\text{NH}_4)_2\text{SO}_4$ fractionation and sequential chromatography on Sephacryl S-300, DEAE-Sephacel, ω -amino-octyl-agarose and thiophosphorylated myosin 20 kDa light-chain-Sepharose columns. The purified phosphatase contained three polypeptide chains of 60, 55 and 38 kDa which were shown to be identical with the subunits of SMP-I, a smooth-muscle phosphatase capable of dephosphorylating the isolated 20 kDa light chain of myosin but not intact myosin [Pato & Adelstein (1983) *J. Biol. Chem.* **258**, 7047–7054]. Consistent with its identity with SMP-I, calponin phosphatase was classified as a type-2A protein phosphatase. Of several potential phosphoprotein substrates examined, calponin proved to be kinetically the best, suggesting that calponin may be a physiological substrate for this phosphatase. Finally, dephosphorylation of calponin which had been phosphorylated by protein kinase C restored completely its ability to inhibit the actin-activated MgATPase of smooth-muscle myosin. These observations support the hypothesis that calponin plays a role in regulating the contractile state of smooth muscle and that this function in turn is controlled by phosphorylation–dephosphorylation.

INTRODUCTION

Calponin is a major protein component of smooth muscle where it is found at the same molar concentration as tropomyosin and is associated with the thin filaments (Takahashi *et al.*, 1986). Purified calponin inhibits the actin-activated MgATPase activity of myosin (Abe *et al.*, 1990; Winder & Walsh, 1990a,b; Horiuchi & Chacko, 1991; Winder *et al.*, 1991); this inhibition can be reversed by stoichiometric phosphorylation of calponin catalysed by either protein kinase C (PKC) or Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) (Winder & Walsh, 1990a,b; Winder *et al.*, 1991). The functional consequence of calponin phosphorylation is to prevent it from binding to actin, thus allowing phosphorylated myosin heads free access to the actin filament with consequent activation of the MgATPase. On the basis of this evidence obtained *in vitro*, we have proposed that calponin may act as a thin-filament-linked secondary regulatory mechanism in smooth muscle, and that its activity in turn is regulated by phosphorylation (Winder & Walsh, 1990a,b; Winder *et al.*, 1991). For this mechanism to be of physiological significance (Krebs & Beavo, 1979), however, a phosphatase must be present in smooth muscle in order to regenerate unphosphorylated calponin. Using ^{32}P -labelled calponin as a substrate, we have purified and characterized calponin phosphatase from chicken gizzard smooth muscle. The phosphatase

is shown to be identical with SMP-I, a smooth-muscle phosphatase originally purified as a phosphatase capable of dephosphorylating the isolated 20 kDa light chain of myosin, but not intact myosin (Pato & Adelstein, 1983). Of several potential phosphoprotein substrates examined, calponin proved to be kinetically the best, suggesting that the principal physiological substrate for this phosphatase is likely to be calponin. Furthermore, we have used calponin phosphatase to demonstrate that the functional effect of calponin phosphorylation on the actomyosin MgATPase is reversible.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (20–40 Ci/mmol) was purchased from ICN Biomedicals (St. Laurent, Quebec, Canada). Sephacryl S-300, DEAE-Sephacel and CNBr-activated Sepharose 4B were purchased from Pharmacia (Baie d'Urfe, Quebec, Canada), histone 2A, histone 3S, CM-Sephadex, ω -amino-octyl-agarose (AOA), potato acid phosphatase and alkaline phosphatase from Sigma Chemical Co. (St. Louis, MO, U.S.A.), dithiothreitol and adenosine 5'-[γ -thio]triphosphate (ATP[S]) from Boehringer Mannheim (Laval, Quebec, Canada) and electrophoresis reagents from Bio-Rad Laboratories (Mississauga, Ontario, Canada). General laboratory reagents used were of analytical grade or

Abbreviations used: AOA, ω -amino-octyl-agarose; ATP[S], adenosine 5'-[γ -thio]triphosphate; CaM kinase II, Ca^{2+} /calmodulin-dependent protein kinase II; DTT, dithiothreitol; LC_{20} , the 20 kDa light chain of smooth muscle myosin; MLCK, myosin light-chain kinase; PKC, protein kinase C; SMP, smooth-muscle phosphatase.

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better and purchased from Canlab (Edmonton, Alberta, Canada). Okadaic acid was a gift generously provided by Dr. Alastair Aitken (NIMR, London, U.K.).

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: chicken gizzard actin (Ngai *et al.*, 1986), tropomyosin (Smillie, 1982), myosin (Persechini & Hartshorne, 1981), myosin light-chain kinase (MLCK; Ngai *et al.*, 1984), calponin (Winder & Walsh, 1990a), caldesmon (Sutherland & Walsh, 1989), caldesmon containing endogenous CaM kinase II (Scott-Woo & Walsh, 1988), isolated 20 kDa light chains of myosin (LC₂₀) (Hathaway & Haerberle, 1983), bovine cardiac catalytic subunit of type-II cyclic AMP-dependent protein kinase (Demaille *et al.*, 1977), rabbit skeletal-muscle inhibitor-1 and inhibitor-2 (the heat-stable inhibitors of type-I protein phosphatase) (Nimmo & Cohen, 1978), rat brain protein kinase C (Woodgett & Hunter, 1987), turkey gizzard smooth-muscle phosphatase I (SMP-I) (Pato & Adelstein, 1983). CaM kinase II (bovine brain) was generously provided by Dr. R. K. Sharma, University of Calgary, rabbit skeletal-muscle phosphorylase kinase by Dr. J. H. Wang, University of Calgary, and type-I protein phosphatase from rabbit liver by Dr. R. L. Khandelwal, University of Saskatchewan.

Preparation of ³²P-labelled substrates

The following substrates were phosphorylated by published methods using [γ -³²P]ATP (approx. 1000 c.p.m./pmol) and the indicated protein kinases: histone 2A, phosphorylase kinase and MLCK by cyclic AMP-dependent protein kinase (Demaille *et al.*, 1977), myosin and LC₂₀ by MLCK (Walsh *et al.*, 1983), caldesmon by co-purifying CaM kinase II (Scott-Woo & Walsh, 1988), calponin by CaM kinase II (Winder & Walsh, 1990a), and histone 3S by PKC (Woodgett & Hunter, 1987). Calponin was phosphorylated by PKC under the following optimal conditions using the liposomal assay (Kikkawa *et al.*, 1982): 25 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/0.4 mM-CaCl₂/40 μ g of L- α -phosphatidyl-L-serine/ml/0.8 μ g of 1,3-diolein/ml/1 mM-[γ -³²P]ATP/5.9 μ M-calponin/8 μ g of rat brain PKC/ml at 30 °C. Phosphorylated calponin was repurified on a column (1 cm $\times$ 10 cm) of CM-Sephadex: the reaction mixture was applied to the column and washed extensively with column equilibration buffer [20 mM-Tris/HCl (pH 7.5)/1 mM-dithiothreitol (DTT)/50 mM-KCl] to remove ATP, phospholipids and PKC. Phosphorylated calponin was eluted in concentrated form with 20 mM-Tris/HCl (pH 7.5)/1 mM-DTT/1 M-KCl and dialysed against 20 mM-Tris/HCl (pH 7.5)/1 mM-DTT/100 mM-KCl before storage at -20 °C.

Phosphatase assays

Phosphatase assays were carried out at 30 °C in the presence of 20 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-DTT/1 mg of carrier BSA/ml. Phosphatase activity was quantified by liquid-scintillation counting of the remaining ³²P-labelled protein as previously described (Walsh *et al.*, 1983). In some cases, the remainder of each reaction mixture was added to an equal volume of SDS/polyacrylamide-gel sample buffer and boiled before SDS/PAGE and autoradiography.

Dephosphorylation of calponin

Calponin previously stoichiometrically phosphorylated by PKC using [γ -³²P]ATP (as described above) was dephosphorylated under the following conditions: 20 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-DTT/5 μ M-³²P-labelled calponin/

1.7 μ g of calponin phosphatase/ml. Dephosphorylated calponin was repurified as described above for phosphorylated calponin and used in functional assays.

Actin-activated thiophosphorylated myosin MgATPase assay

Myosin was thiophosphorylated by using ATP[S] under the following conditions: 4.1 μ M-myosin/74 nM-MLCK/0.6 μ M-calmodulin/20 mM-Tris/HCl (pH 7.5)/60 mM-KCl/4 mM-MgCl₂/0.1 mM-CaCl₂/1 mM-ATP[S] for 15 min at 30 °C. ATPase activities were measured as previously described (Ikebe & Hartshorne, 1985) under the following conditions: 20 mM-imidazole (pH 7)/4 mM-MgCl₂/80 mM-KCl/1 mM-DTT/1 mM-EGTA/6 μ M-actin/2 μ M-tropomyosin/1 μ M-thiophosphorylated myosin, in the presence or absence of calponin as indicated in Table 2. Reactions (150 μ l) were carried out at 30 °C and started by the addition of [γ -³²P]ATP (1 mM). For the determination of ATPase rates, samples (25 μ l) of reaction mixtures were withdrawn at 1 min intervals after the addition of ATP up to 5 min. Rates were calculated by linear-regression analysis of the time-course data.

Other procedures

Thiophosphorylated LC₂₀-Sephacrose was prepared as previously described (Pato & Kerc, 1990). Protein concentrations were determined by the Coomassie Brilliant Blue dye-binding assay (Spector, 1978) using dye reagent purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), or by spectrophotometric measurements using the following molar absorption coefficients: calmodulin $\epsilon_{277}^{1\%}$ 1.9 (Klee, 1977), myosin $\epsilon_{280}^{1\%}$ 4.5 (Okamoto & Sekine, 1978), tropomyosin $\epsilon_{278}^{1\%}$ 2.9 (Eisenberg & Kielley, 1974), caldesmon $\epsilon_{280}^{1\%}$ 3.3 (Graceffa *et al.*, 1988) and calponin $\epsilon_{289}^{1\%}$ 11.3 (Winder & Walsh, 1990a). 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 using 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, if necessary, using Kodak X-Omat AR film in DuPont-Cronex cassettes fitted with DuPont Quanta III intensifying screens. Films were allowed to develop for 3 days or less at room temperature. Densitometry of destained gels and autoradiograms was carried out in an LKB model 2202 Ultrosan laser densitometer equipped with a Hewlett-Packard model 3390A integrator.

Preparation of calponin phosphatase

All procedures were carried out at 4 °C unless otherwise stated. Frozen trimmed chicken gizzards (100 g) were ground in a meat grinder and homogenized in 4 vol. of 20 mM-Tris/HCl (pH 7.5)/5 mM-EDTA/5 mM-DTT/0.25 mM-phenylmethanesulphonyl fluoride/10 μ g of leupeptin/ml/10 μ g of pepstatin/ml. After centrifugation at 30 100 g for 30 min, the supernatant was subjected to (NH₄)₂SO₄ fractionation. The 30–55%-satd.-(NH₄)₂SO₄ fraction was dialysed against 20 mM-Tris/HCl (pH 7.5)/500 mM-NaCl/1 mM-EDTA/1 mM-DTT (buffer A) before chromatography on a Sephacryl S-300 column (5 cm $\times$ 90 cm) equilibrated with buffer A. The column fractions were assayed for phosphatase activity toward phosphorylated calponin as described above. In one series of experiments, designed to compare the elution profiles of calponin phosphatase and LC₂₀ phosphatase activities from gel-filtration columns on an analytical scale, a sample (150 μ l) of the dialysed (NH₄)₂SO₄ fraction was chromatographed in buffer A on Zorbax GF-450 and GF-250 columns (9.4 mm $\times$ 25 mm; DuPont) in series on a Beckman 340 h.p.l.c. system. Column fractions were assayed for

phosphatase activity toward phosphorylated calponin and phosphorylated LC₂₀ in the presence of 10 mM-MgCl₂. [MgCl₂ was included in the assay medium because the second peak of LC₂₀ phosphatase eluted from the gel-filtration column, SMP-II, is Mg²⁺-dependent (Pato & Adelstein, 1983)]. Calponin phosphatase was eluted as a single peak which correlated with the first peak of LC₂₀ phosphatase activity (Fig. 1). The calponin phosphatase peak eluted from the Sephacryl S-300 column was pooled and dialysed against 20 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-DTT (buffer B). The dialysed material was chromatographed on a DEAE-Sephacel column (2.5 cm × 40 cm) equilibrated with buffer B. Bound proteins were eluted with a 750 ml linear gradient of NaCl (0–500 mM in buffer B) (Fig. 2). The pooled calponin phosphatase activity was dialysed against buffer B and applied to an AOA column (1.6 cm × 20 cm) previously equilibrated with buffer B. After application, the column was washed first with 100 ml of buffer B containing 500 mM-NaCl and then with 100 ml of buffer B containing 2 M-NaCl. Calponin phosphatase activity was eluted during the 2 M-NaCl wash. Pooled phosphatase activity was dialysed and applied to a thiophosphorylated LC₂₀-Sephacryl affinity column (1 cm × 10 cm). After the column had been washed with buffer B, bound protein was eluted with buffer B containing 1 M-NaCl (Fig. 3). The purified calponin phosphatase was stored at –20 °C after further dialysis against buffer B containing 20 mM-NaCl. In one case the pooled activity from the AOA column was used to prepare the catalytic subunit of the phosphatase as previously described (Pato & Kerc, 1990).

RESULTS

Purification of calponin phosphatase

Phosphatase activity toward phosphorylated calponin was recovered predominantly (> 90%) in the soluble fraction obtained by centrifugation of a homogenate of chicken gizzard. Gel-filtration h.p.l.c. of the 30–55%-satd.-(NH₄)₂SO₄ fraction revealed a single peak of calponin phosphatase activity but, as expected (Pato & Adelstein, 1983), two major peaks of LC₂₀ phosphatase activity (Fig. 1, peaks A and B). Calponin phosphatase activity was co-eluted with the first peak of LC₂₀ phosphatase activity (Fig. 1, peak A) which contains SMP-I and SMP-IV (Pato & Adelstein, 1983; Pato & Kerc, 1988). This suggested that the calponin phosphatase may be identical with SMP-I or SMP-IV or, alternatively, may be a co-purifying but distinct phosphatase. In order to facilitate identification of the calponin phosphatase, subsequent purification was carried out by previously published protocols for turkey gizzard SMP-I and SMP-IV (Pato & Adelstein, 1983; Pato & Kerc, 1988). Calponin phosphatase activity was eluted from DEAE-Sephacel at 150–180 mM-NaCl (Fig. 2), consistent with the behaviour of both SMP-I and SMP-IV; DEAE-Sephacel chromatography does not resolve these two phosphatases (Pato & Adelstein, 1983; Pato & Kerc, 1988). They are, however, readily separated by AOA chromatography, SMP-IV being eluted below 0.5 M-NaCl and SMP-I between 0.5 and 2 M-NaCl (Pato & Kerc, 1988). Calponin phosphatase activity bound to AOA at 0.5 M-NaCl and was eluted with 2 M-NaCl (results not shown), suggesting that calponin phosphatase and SMP-I might be the same enzyme. Final purification of calponin phosphatase was achieved by affinity chromatography using thiophosphorylated LC₂₀-Sephacryl (Fig. 3). This was a very effective purification step (Table 1) and yielded a preparation containing three polypeptides of 60, 55 and 38 kDa when examined by SDS/PAGE (Fig. 4a, lane 3). This pattern is very similar to the subunit composition of SMP-I (Pato & Adelstein, 1980). Electrophoresis of authentic

SMP-I alongside (Fig. 4a, lane 2) and together with (Fig. 4a, lane 4) calponin phosphatase clearly demonstrated that the component polypeptides of the two enzyme preparations have identical molecular masses. Polyclonal antibodies raised against the SMP-I holoenzyme recognized all three polypeptides in the calponin phosphatase preparation (Fig. 4b, lane 6), as well as the three subunits of SMP-I (Fig. 4b, lane 5) and the isolated catalytic subunit of SMP-I (Fig. 4b, lane 7). Furthermore, polyclonal antibodies raised against the purified catalytic subunit

Fig. 1. Gel filtration of the 30–55% (NH₄)₂SO₄ fraction of a chicken gizzard homogenate

A sample (150 µl) of the (NH₄)₂SO₄ fraction was subjected to h.p.l.c. as described in the Materials and methods section at a flow rate of 1 ml/min. A₂₈₀ was monitored (—) and 0.5 ml fractions were collected for assay of calponin phosphatase (●) and LC₂₀ phosphatase activities (○) as described in the Materials and methods section.

Fig. 2. DEAE-Sephacel chromatography of pooled calponin phosphatase activity from Sephacryl S-300

Bound proteins were eluted with a linear gradient of NaCl from 0 to 250 mM (---). A₂₈₀ (—) was monitored and fractions (10 ml) were assayed for calponin phosphatase activity (●) as described in the Materials and methods section.

Fig. 3. Thiophosphorylated LC₂₀-Sepharose affinity chromatography of the pooled phosphatase activity from AOA chromatography

The sample was applied to the column in the absence of salt, A_{280} (—) was monitored and fractions (4 ml) were collected. At the arrowhead, the buffer was changed to one containing 1 M-NaCl. Calponin phosphatase activity (●) was assayed as described in the Materials and methods section.

of SMP-I recognized the 38 kDa polypeptide in the calponin phosphatase preparation (Fig. 4c, lane 9) and in the SMP-I holoenzyme (Fig. 4c, lane 8), as well as the purified catalytic subunit of SMP-I (Fig. 4c, lane 10). We conclude therefore that calponin phosphatase and SMP-I are identical.

The specific activities of fractions obtained during the early stages of the purification procedure varied from preparation to preparation, perhaps reflecting the presence of interfering proteins such as phosphatase inhibitors. The calculated purification (fold) is therefore a qualitative rather than a truly quantitative

Fig. 4. SDS/PAGE (a) and Western blotting (b, c) of calponin phosphatase and SMP-I

(a) Lane 1, molecular-mass markers (a, phosphorylase b, 97.4 kDa; b, BSA, 66.2 kDa; c, ovalbumin, 45 kDa; d, carbonic anhydrase, 29 kDa; e, soyabean trypsin inhibitor, 20.1 kDa; f, lysozyme, 14.4 kDa); lane 2, SMP-I; lane 3, calponin phosphatase; lane 4, a mixture of SMP-I and calponin phosphatase. (b) Western blotting of SMP-I (lane 5), calponin phosphatase (lane 6) and the catalytic subunit of SMP-I (lane 7) with polyclonal antibodies raised against SMP-I. Numbers indicate the molecular masses (kDa) of the protein subunits. (c) Western blotting of SMP-I (lane 8), calponin phosphatase (lane 9) and the catalytic subunit of SMP-I (lane 10) with polyclonal antibodies raised against the catalytic subunit of SMP-I.

measure of purification of the phosphatase. The data presented in Table 1 are from a representative purification.

Substrate specificity of calponin phosphatase

The ability of calponin phosphatase to dephosphorylate several potential phosphoprotein substrates was investigated as shown in Fig. 5(a). Calponin (phosphorylated by PKC and CaM kinase II) and isolated LC₂₀ (phosphorylated by MLCK) proved to be kinetically the best substrates. MLCK (phosphorylated by cyclic AMP-dependent protein kinase) and caldesmon (phosphorylated by CaM kinase II) were dephosphorylated at a significantly lower rate, and phosphorylase kinase (phosphorylated by cyclic AMP-dependent protein kinase) was an even poorer substrate. Histone 2A (phosphorylated by cyclic AMP-dependent protein kinase) was poorly dephosphorylated by calponin phosphatase, whereas histone 3S (phosphorylated by PKC) was not a

Table 1. Purification of calponin phosphatase

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Purification (fold)
Homogenate	982	4.6	4468	3.2	14298	—
30–55% (NH ₄) ₂ SO ₄	50	42.0	2100	1.8	3864	0.6
Sephacryl S-300	232	4.0	923	6.7	6142	2.1
DEAE-Sephacel	360	1.1	396	25	9979	7.9
AOA	120	0.19	22.6	137	3084	43
LC ₂₀ -Sepharose	90	0.01	0.9	386	347	121

Fig. 5. Substrate specificity of the calponin phosphatase

(a) The time-course of dephosphorylation of various ^{32}P -labelled substrates ($1\ \mu\text{M}$) by calponin phosphatase ($1.33\ \mu\text{g}/\text{ml}$) was examined as described in the Materials and methods section: myosin (□) and LC₂₀ (◇) phosphorylated by MLCK; histone 3S (▲) and calponin (●) phosphorylated by PKC; phosphorylase kinase (▼), histone 2A (■) and MLCK (▽) phosphorylated by cyclic AMP-dependent protein kinase; caldesmon (△) and calponin (○) phosphorylated by CaM kinase II. The myosin data points (□) are partially masked by the histone 2A data points (▲). (b) The time-course of dephosphorylation of calponin ($3.75\ \mu\text{M}$) phosphorylated by PKC (○) and LC₂₀ ($3.75\ \mu\text{M}$) phosphorylated by MLCK (●) by calponin phosphatase ($6.3\ \mu\text{g}/\text{ml}$) in 20 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-DTT/1 mg of BSA/ml. Samples ($20\ \mu\text{l}$) of reaction mixtures were withdrawn at the indicated times, reactions quenched by addition to an equal volume of SDS/polyacrylamide-gel sample buffer and boiling before SDS/PAGE and autoradiography. Phosphorylation levels were determined by laser densitometric scanning of the SDS/polyacrylamide gels and autoradiograms.

Fig. 6. Classification of calponin phosphatase

(a) Dephosphorylation of the α - (△, ▲) and β - (○, ●) subunits of phosphorylase kinase phosphorylated by cyclic AMP-dependent protein kinase in the presence (▲, ●) or absence (△, ○) of calponin phosphatase. Data were quantified by densitometric scanning of SDS/polyacrylamide gels and autoradiograms. (b) Inhibition of dephosphorylation by calponin phosphatase of calponin phosphorylated by PKC (●) and liver type-1 phosphatase dephosphorylation of phosphorylase kinase β -subunit (▲) by okadaic acid. Half-maximal inhibition of calponin phosphatase (21 nM) occurred at 0.6 nM-okadaic acid and half-maximal inhibition of liver type-1 phosphatase (72 nM) occurred at 250 nM-okadaic acid.

substrate for the phosphatase. Intact myosin was also not dephosphorylated by calponin phosphatase. More detailed analysis of the kinetics of dephosphorylation of calponin and myosin LC₂₀ revealed that calponin is actually a significantly better substrate than the light chain (Fig. 5b). A similar result was obtained using purified turkey gizzard SMP-I: calponin was dephosphorylated at a rate 31.5 times greater than LC₂₀ (using the holoenzyme) and 30 times greater than LC₂₀ (using the purified catalytic subunit of SMP-I). These results suggest a high degree of specificity for calponin dephosphorylation by SMP-I. The specificity of calponin phosphatase for smooth-muscle substrates therefore follows the order: calponin > LC₂₀ > MLCK = caldesmon > myosin. It should be noted, however, that these specificity studies were carried out at a single substrate concentration and therefore do not provide comparisons of V_{max} and K_m values. With calponin phosphorylated by PKC as substrate, Lineweaver-Burk plots revealed a K_m for calponin of $5.0\ \mu\text{M}$ and V_{max} of 423 nmol of P_i/min per mg of calponin phosphatase. The enzyme preparation used in these experiments

Table 2. Effects of phosphorylation and dephosphorylation of calponin on inhibition of actin-activated thiophosphorylated myosin MgATPase

Actothiophosphorylated myosin MgATPase rates were measured as described in the Materials and methods section in the absence or presence of 2 μ M- or 3 μ M-calponin. Calponin was either untreated, phosphorylated to 1.66 mol of P_i /mol by PKC, or phosphorylated to 1.66 mol of P_i /mol by PKC and then dephosphorylated to 0.05 mol of P_i /mol by purified calponin phosphatase.

Calponin	Concn. (μ M)	MgATPase rate (nmol of P_i /min per mg of myosin)
None	—	124.1
Untreated	2	25.1
	3	17.1
Phosphorylated	2	140.9
	3	134.5
Phosphorylated and dephosphorylated	2	38.0
	3	12.0

was different from that in Table 1, accounting for the slight difference in velocity.

We found that the supernatant after centrifugation of the gizzard homogenate contained more than 90 % of total calponin phosphatase activity, and no significant calponin phosphatase activity was detected in the 0–30 %-(NH₄)₂SO₄ fraction or the supernatant after 30–55 %-(NH₄)₂SO₄ fractionation. A single peak of calponin phosphatase activity was eluted from the gel-filtration column after (NH₄)₂SO₄ fractionation (Fig. 1). We conclude therefore that calponin was not significantly dephosphorylated by other gizzard protein phosphatases. Furthermore, calponin was not dephosphorylated by acid or alkaline phosphatases (results not shown).

Classification of calponin phosphatase

Calponin phosphatase was classified according to the scheme of Ingebritsen & Cohen (1983). Calponin phosphatase preferentially dephosphorylated the α -subunit of phosphorylase kinase (Fig. 6a), identifying it as a type-2 phosphatase, and was inhibited by low concentrations (IC₅₀ = 0.6 nM) of okadaic acid (Fig. 6b), identifying it as a type-2A phosphatase. Calponin phosphatase was not inhibited by inhibitor-1 or inhibitor-2, the heat-stable inhibitors of type-1 phosphatase (Nimmo & Cohen, 1978), confirming its classification as a type-2 phosphatase (results not shown). The classification of calponin phosphatase as a type-2A phosphatase was supported by the lack of effect of bivalent cations (Ca²⁺, Mg²⁺ or Mn²⁺) or of Ca²⁺/calmodulin (results not shown). The classification of calponin phosphatase as a type-2A protein phosphatase is also consistent with its identity with SMP-I (Pato *et al.*, 1983).

Functional effects of calponin dephosphorylation

Calponin can be phosphorylated by PKC and CaM kinase II (Winder & Walsh, 1990a). In the phosphorylated state, calponin no longer binds to actin and consequently is unable to inhibit the actin-activated MgATPase activity of myosin (Winder & Walsh, 1990a). One would predict therefore that dephosphorylation should reverse these effects of phosphorylation. The effects of calponin, phosphorylated calponin and dephosphorylated calponin on the actothiophosphorylated myosin MgATPase are shown in Table 2. ATPase rates were determined as described in the Materials and methods section. Under control conditions, i.e. without calponin, the actin-activated thiophosphorylated myosin MgATPase rate was 124.1 nmol of P_i /min per mg of

myosin. Consistent with previous observations (Winder & Walsh, 1990a), the addition of calponin caused 80 % inhibition of the ATPase rate at 2 μ M-calponin and 86 % inhibition at 3 μ M-calponin. Prior phosphorylation of the same preparation of calponin by PKC (to 1.66 mol of P_i /mol of calponin) completely blocked the inhibitory effect of calponin on the actomyosin MgATPase, consistent with previous findings (Winder & Walsh, 1990a). The same preparation of phosphorylated calponin, which had no inhibitory effect on the actomyosin MgATPase, was 97 % dephosphorylated (from 1.66 mol of P_i /mol of calponin to 0.05 mol of P_i /mol of calponin) by treatment with purified calponin phosphatase. Addition of purified dephosphorylated calponin to the ATPase assay resulted in 69 % inhibition of the actomyosin MgATPase at 2 μ M-dephosphorylated calponin and 90 % inhibition at 3 μ M-dephosphorylated calponin. Dephosphorylation of calponin therefore completely reverses the functional effect of phosphorylation and restores the inhibitory effect of calponin on the actomyosin MgATPase.

DISCUSSION

The major regulatory event in smooth-muscle contraction involves phosphorylation of the 20 kDa light chains of myosin by MLCK (Hartshorne, 1987; Walsh, 1991). Other regulatory systems, however, may also have a role to play in the regulation of smooth-muscle contraction (Kamm & Stull, 1989; Walsh, 1989). The discovery of a new thin-filament-associated protein, calponin (Takahashi *et al.*, 1986, 1987), has led to the suggestion that this protein may function as a thin-filament-linked regulatory system in smooth muscle (Winder & Walsh, 1990a,b; Horiuchi *et al.*, 1991; Winder *et al.*, 1991). We have proposed that calponin regulation of smooth-muscle contraction (inhibition of the actomyosin MgATPase) is itself regulated by phosphorylation (Winder & Walsh, 1990a,b; Winder *et al.*, 1991). The demonstration that chicken gizzard contains a potent calponin phosphatase, identified as the chicken counterpart of SMP-I, and that calponin phosphatase-mediated dephosphorylation of PKC-phosphorylated calponin completely restores the inhibitory effect of calponin on the actomyosin MgATPase, is clear evidence that all the enzymic machinery necessary for the reversible phosphorylation of calponin is present in this smooth muscle. Preliminary studies (Pohl *et al.*, 1991) indicated phosphorylation of calponin in intact canine trachealis during carbachol-stimulated contractions. Furthermore, the time course of changes in calponin phosphorylation was similar to the time course of changes in myosin LC₂₀ phosphorylation and shortening velocity in this tissue (Gerthoffer, 1986; Pohl *et al.*, 1991). Bárány *et al.* (1991), however, did not detect any phosphorylation of calponin in pig carotid arterial smooth muscle at rest or when contracted in response to several stimuli. Further studies are required to establish the physiological relevance of calponin phosphorylation during smooth-muscle contraction in intact systems. On the basis of the evidence obtained *in vitro*, we have proposed a model for the possible role of calponin in regulating smooth-muscle contraction (Winder *et al.*, 1991). In resting smooth muscle, the sarcoplasmic free [Ca²⁺] is low (approx. 130 nM) (Williams *et al.*, 1987), Ca²⁺-dependent kinases (MLCK, CaM kinase II and PKC) are inactive, and myosin and calponin are dephosphorylated. In these conditions, calponin would be bound to the thin filament, the MgATPase activity of myosin would be very low and there would be no cross-bridge cycling or force development. On stimulation, resulting in a rapid rise in sarcoplasmic free [Ca²⁺] to 500–700 nM (Williams & Fay, 1986; Williams *et al.*, 1987), MLCK, CaM kinase II and possibly PKC would be activated leading to concomitant phosphorylation of myosin by MLCK and calponin by CaM kinase II and/or PKC.

Phosphorylated calponin would dissociate from the thin filament and phosphorylated myosin heads would cycle along the actin filament at a maximal rate. As the sarcoplasmic free $[Ca^{2+}]$ begins to fall, the Ca^{2+} -dependent kinases will become inactivated sequentially due to their differing dependencies on $[Ca^{2+}]$. The $[Ca^{2+}]$ required for half-maximal activation of CaM kinase II is higher ($0.4\text{--}0.6\ \mu\text{M}$) than for MLCK [reviewed by Stull *et al.* (1986)] and, similarly, the $[Ca^{2+}]$ required to activate PKC half-maximally is $0.2\text{--}0.4\ \mu\text{M}$ (Huang *et al.*, 1988; Marais & Parker, 1989). This could lead to a situation in which calponin becomes dephosphorylated by calponin phosphatase (SMP-I), causing it to reassociate with the thin filament, whereas myosin heads remain predominantly phosphorylated. Under these conditions, calponin would exert an inhibitory effect on the actin-activated myosin MgATPase, i.e. a decrease in the cross-bridge cycling rate (velocity of shortening). Once sarcoplasmic free $[Ca^{2+}]$ returns to resting concentrations, on removal of the stimulus, myosin would also be dephosphorylated and the muscle would relax.

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