Calponin phosphorylation *in vitro* and in intact muscle

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Calponin, a thin-filament-associated protein implicated in the regulation of smooth-muscle contraction, is phosphorylated *in vitro* by protein kinase C and Ca^{2+} /calmodulin-dependent protein kinase II [Winder and Walsh (1990) *J. Biol. Chem.* **265**, 10148–10155] and dephosphorylated by a type 2A protein phosphatase [Winder, Pato and Walsh (1992) *Biochem. J.* **286**, 197–203]. Unphosphorylated calponin binds to actin and inhibits the actin-activated myosin MgATPase; these properties are lost on phosphorylation. Although both serine and threonine residues in calponin are phosphorylated, the major site of phosphorylation by either kinase is Ser-175. Calponin also undergoes phosphorylation when bound to actin in synthetic thin filaments, in a reconstituted actomyosin system, in washed myofibrils and in tissue extracts; this results in dissociation of calponin from actin.

Tryptic phosphopeptide mapping indicates that the same sites are phosphorylated in the bound as in the isolated protein. Toad stomach calponin exists in at least three isoforms which differ in charge but exhibit the same molecular mass on SDS/PAGE. In a toad stomach extract, all three isoforms are phosphorylated by protein kinase C or Ca^{2+} /calmodulin-dependent protein kinase II as shown by two-dimensional gel electrophoresis (non-equilibrium pH-gradient gel electrophoresis and SDS/PAGE). Calponin phosphorylation also occurs in intact toad stomach smooth-muscle strips metabolically labelled with $^{32}\text{P}_i$ and stimulated to contract with carbachol. These results support the hypothesis that calponin may be regulated *in vivo* by phosphorylation–dephosphorylation.

INTRODUCTION

Calponin is an actin-, tropomyosin- and Ca^{2+} /calmodulin-binding protein (Takahashi et al., 1986, 1988; Winder and Walsh, 1990a) which is localized to the thin filaments of smooth-muscle cells (Gimona et al., 1991; Takeuchi et al., 1991; Winder et al., 1992a; Walsh et al., 1993) and is present in smooth muscle at a molar ratio to actin of 1:7 (Takahashi et al., 1986). Purified calponin inhibits the actin-activated MgATPase activity of smooth- or skeletal-muscle myosin (Abe et al., 1990; Nishida et al., 1990; Winder and Walsh, 1990a; Horiuchi and Chacko, 1991; Makuch et al., 1991; Miki et al., 1992; Winder et al., 1992b), and the movement of fluorescently labelled actin filaments over immobilized skeletal-muscle heavy meromyosin (Shirinsky et al., 1992). Calponin is stoichiometrically phosphorylated *in vitro* by two protein serine/threonine kinases, protein kinase C (PKC) and Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) (Winder and Walsh, 1990a) and dephosphorylated by a type 2A protein phosphatase (Winder et al., 1992c). Phosphorylation disrupts the calponin–actin interaction and results in loss of actomyosin ATPase inhibition; these properties are restored after dephosphorylation of calponin (Winder and Walsh, 1990a; Winder et al., 1992c). On the basis of these observations, we have suggested that calponin is a thin-filament-associated regulatory protein which modulates actin–myosin interaction, and therefore contraction, and that this function in turn is regulated by phosphorylation–dephosphorylation (Winder et al., 1991).

Our studies of calponin phosphorylation and dephosphorylation reported to date have been carried out with isolated calponin. However, calponin is physically associated with the

thin filaments *in situ* (Gimona et al., 1991; Takeuchi et al., 1991; Winder et al., 1992a; Walsh et al., 1993). In order to evaluate the potential physiological significance of calponin phosphorylation, we have therefore investigated the phosphorylation of calponin bound to actin in: synthetic thin filaments, a reconstituted contractile system, washed myofibrils, tissue extracts and intact smooth-muscle strips. Our results support the hypothesis that calponin-mediated inhibition of actin–myosin interaction in smooth muscle is regulated by phosphorylation–dephosphorylation.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (20–40 and > 5000 Ci/mmol) and $^{32}\text{P}_i$ (approx. 285 Ci/mg of P) were purchased from Amersham (Oakville, Ontario, Canada) and ICN Biomedicals (St. Laurent, Quebec, Canada) respectively. Electrophoresis reagents were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Tos-Phe- CH_2Cl ('TPCK')-treated trypsin (255 units/mg) was purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). General laboratory reagents used were of analytical grade or better and were purchased from CanLab (Edmonton, Alberta, Canada). Turkey gizzard smooth-muscle phosphatase-I (SMP-I), purified as described by Pato and Adelstein (1983), was generously provided by Dr. Mary Pato, University of Saskatchewan.

Protein purifications

The following proteins were purified by previously described

Abbreviations used: CaM kinase II, Ca^{2+} /calmodulin-dependent protein kinase II; MLCK, myosin light-chain kinase; NEPHGE, non-equilibrium pH-gradient gel electrophoresis; PKC, protein kinase C; PTH, phenylthiohydantoin; SMP-I, smooth-muscle phosphatase-I; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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methods: bovine brain calmodulin (Walsh et al., 1984), chicken gizzard actin (Ngai et al., 1986), tropomyosin (Smillie, 1982), myosin (Persechini and Hartshorne, 1981), myosin light-chain kinase (MLCK) (Ngai et al., 1984), calponin (Winder and Walsh, 1990a), caldesmon containing endogenous CaM kinase II (Scott-Woo and Walsh, 1988), calponin phosphatase (Winder et al., 1992c) and rat brain protein kinase C (PKC) (Wolf et al., 1984). Polyclonal antibodies were raised in rabbits against purified chicken gizzard calponin and affinity-purified on a column of calponin-Sepharose. Antibody specificity was established by the fact that only calponin was recognized by the affinity-purified antibody in Western blots of chicken gizzard homogenates.

Electrophoresis and immunoblotting

SDS/PAGE was carried out 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). Two-dimensional gel electrophoresis comprised non-equilibrium pH-gradient gel electrophoresis (NEPHGE) in the first dimension and SDS/PAGE, as described above, in the second dimension. In the first dimension, electrophoresis was for 1800 Vh in a pH 3.5–10 gradient as described by O'Farrell et al. (1977). Samples for two-dimensional gel electrophoresis were dissolved in 9.5 M urea/2% (w/v) Triton X-100/0.2% Ampholines (pH 3.5–10)/5% 2-mercaptoethanol (NEPHGE buffer). Gels were stained in 45% (v/v) ethanol/10% (v/v) acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue R-250, and diffusion destained in 10% (v/v) acetic acid. Destained gels were sealed in plastic bags and, when necessary, autoradiographed using Kodak X-Omat AR film in DuPont-Cronex cassettes fitted with DuPont Quanta III intensifying screens. Densitometry of autoradiograms was carried out in an LKB model 2202 Ultrosan laser densitometer equipped with a Hewlett-Packard model 3390A integrator. For immunoblotting, proteins separated by two-dimensional gel electrophoresis were transferred to nitrocellulose filters (0.22 µm pore diam.) (Bio-Rad Laboratories) for 1 h at 60 V and 4 °C in buffer containing 25 mM Tris, 192 mM glycine, 20% (v/v) methanol and 0.1% (w/v) SDS using a Bio-Rad Trans-Blot Cell. The filters were blocked by treatment with 3% (w/v) low-fat milk in Tris-buffered saline (TBS) for 2 h at room temperature, incubated overnight at room temperature with affinity-purified rabbit anti-(chicken gizzard calponin) [1 µg/ml in 1% (w/v) BSA in TBS], and washed three times with 0.05% Tween 20 in TBS. Filters were then incubated for 1 h at room temperature with goat anti-(rabbit IgG)-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 1% (w/v) BSA in TBS, and washed three times with 0.05% Tween 20 in TBS. Immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium.

Phosphorylation reaction conditions

Phosphorylation by PKC was carried out using the liposomal method described by Kikkawa et al. (1982) under the following conditions: 20 mM Tris/HCl (pH 7.5), 5 mM MgCl₂, 0.1 mM CaCl₂, 48.4 µM phosphatidylserine, 12.3 µM diolein containing 1 µg of PKC/ml and 1 or 2 mM [γ -³²P]ATP (approx. 100 c.p.m./pmol) at 30 °C. The stoichiometry of phosphorylation of free calponin by PKC was 1.31 ± 0.44 mol of P_i/mol (mean $\pm$ S.D., $n = 69$). Phosphorylation by CaM kinase II was carried out under the following conditions: 20 mM Tris/HCl (pH 7.5), 5 mM

MgCl₂, 0.1 mM CaCl₂ containing 10 µg of calmodulin/ml, 0.2 mg of caldesmon containing endogenous CaM kinase II/ml and 1 mM [γ -³²P]ATP (approx. 100 c.p.m./pmol). The stoichiometry of phosphorylation of free calponin by CaM kinase II was 1.08 ± 0.18 mol of P_i/mol (mean $\pm$ S.D., $n = 23$).

Phosphoamino acid analysis

[³²P]Calponin was hydrolysed in 5.7 M HCl at 110 °C for 2 h, mixed with non-radiolabelled phosphoamino acids and Phenol Red; 20 µl (2000 c.p.m.) was subjected to two-dimensional thin-layer electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension as described by Cooper et al. (1983). Separated phosphoamino acids were visualized with ninhydrin, and radiolabelled phosphoamino acids derived from calponin were identified by autoradiography.

Sequencing of phosphopeptides

Calponin (0.5 mg) phosphorylated by CaM kinase II was dialysed against 4 litres of 50 mM NH₄HCO₃ three times and digested with trypsin at 37 °C for 7 h by the addition of 10 µg of Tos-Phe-CHCl₂-treated trypsin at 0, 2 and 4 h. The digest was dried in a vacuum centrifuge, dissolved in 0.3 ml of aq. 0.1% trifluoroacetic acid (TFA), redried and subjected to reversed-phase h.p.l.c. using an aq. TFA/acetonitrile solvent system (Mahoney and Hermodsen, 1980), as described in Figure 2, on a Vydac C18 column (5 µm, 0.46 cm $\times$ 25 cm) at 30 °C and at a flow rate of 1.0 ml/min, 0.5 ml fractions being collected. Radioactivity in column fractions was determined by Čerenkov counting. Radiolabelled peptides were further purified by reversed-phase h.p.l.c. using a linear gradient over 30 min of 5–25% acetonitrile in 10 mM triethylamine acetate (pH 5.0) (Bennett, 1983). Purified radiolabelled peptides were sequenced using an Applied Biosystems 470A gas-phase protein sequencer with methanolic-HCl conversion chemistry (Hewick et al., 1981). Phenylthiohydantoin (PTH)-amino acids were identified by C18 reversed-phase h.p.l.c. (Zimmerman et al., 1977) with modifications described by McKay et al. (1985). To identify phosphoamino acids in the sequence, purified phosphopeptides were dried, dissolved in aq. 50% acetic acid and covalently attached through the C-terminal carboxy group to Sequelon-AA (Millipore) using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide. The support was washed to remove unattached peptide and reagents and installed in the gas-phase sequencer cartridge. The standard gas-phase degradation cycle for sequencing samples on poly(vinylidene difluoride) was modified by replacing the extraction solvent, 1-chlorobutane, with aq. 90% acetonitrile. Each cycle product was subjected to Čerenkov counting and then used for PTH-amino acid identification by reversed-phase h.p.l.c.

Preparation of synthetic thin filaments

Actin (9 µM), tropomyosin (3 µM) and calponin (3 µM) were combined in a final volume of 1 ml in buffer A [20 mM Tris/HCl (pH 7.5), 0.1 M KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM CaCl₂, 1 mM ATP] and incubated at room temperature for 30 min. Reconstituted synthetic thin filaments were recovered by centrifugation at 50000 *g* and 2 °C for 30 min. The pellet was resuspended in 1 ml of buffer A.

Reconstitution of contractile filaments

Actin (6 µM), myosin (1 µM), tropomyosin (2 µM), calmodulin (0.6 µM), MLCK (0.09 µM) and calponin (2 µM) were combined

on ice in buffer B [20 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 60 mM KCl] and centrifuged at 50000 *g* for 30 min at 4 °C. The pellet was resuspended gently in buffer B.

Toad stomach homogenization

Toad stomach smooth muscle was frozen in liquid N_2 , pulverized and homogenized in 3.5 vol. of buffer containing 50 mM imidazole/HCl (pH 7.0), 0.3 M KCl, 1 mM dithiothreitol, 0.5 mM phenylmethanesulphonyl fluoride, 1 mM sodium tetrathionate, 10 μ g of leupeptin/ml and 10 μ g of pepstatin A/ml using a glass/glass tissue grinder. The homogenate was centrifuged at 33000 *g* for 35 min at 5 °C. The supernatant containing thin filaments was used in phosphorylation experiments.

Metabolic labelling of muscle strips with $^{32}P_i$

Toad stomach smooth-muscle strips (approx. 0.5 mm $\times$ 5 mm $\times$ 2 mm) were incubated for 20 h at 5 °C in modified (phosphate-free) amphibian Ringer's solution [115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 4 mM Mops (pH 7.4), 5 mM dextrose] supplemented with carrier-free $^{32}P_i$ (1 mCi/ml) in a total volume of 2 ml. Buffer was then replaced with non-radioactive solution, warmed to 20 °C and the muscle strip was connected to a force transducer. Where indicated, contraction was initiated by the addition of carbachol (1 μ M). Tissue strips were quick-frozen in acetone/10% trichloroacetic acid on solid CO_2 . Trichloroacetic acid was removed from the tissue samples by washing three times with acetone, followed by drying and homogenization in a solution of 0.5% SDS, 9.5 M urea, 5% 2-mercaptoethanol and 0.2% Ampholines (pH 3.5–10). An equal volume of a solution of 1% Triton X-100, 9.5 M urea, 5% 2-mercaptoethanol and 0.2% Ampholines (pH 3.5–10) was added to the homogenate before two-dimensional gel electrophoresis as described above.

RESULTS

We have previously shown that calponin is phosphorylated by PKC at Ser-175 and Ser-254 (Winder and Walsh, 1990b). A third

phosphopeptide sequence (NHSGHVQ) does not appear in the deduced amino acid sequence of calponin (Takahashi and Nadal-Ginard, 1991) and was not found in the protein sequences in the SwissProt data bank. In several subsequent analyses of tryptic phosphopeptides of calponin phosphorylated by PKC, after improvements in calponin purification, we have failed to see this peptide and conclude that it was probably derived from a contaminating protein. N-terminal sequencing of phosphopeptides purified by reversed-phase h.p.l.c. has confirmed phosphorylation of calponin by PKC at Ser-175 and Ser-254. In addition, minor phosphorylations at Ser-215, Thr-180, Thr-184 and Thr-259 were observed. Phosphoamino acid analysis confirmed that both serine and threonine residues were phosphorylated (Figure 1). Scraping of the phosphoamino acids from thin-layer electrophoretograms and scintillation counting revealed that approx. 68% of the remaining radioactivity was in serine residues (mean of two experiments for which the individual values were 67% and 69% phosphoserine).

The profile of phosphorylation by CaM kinase II is simpler and will be presented in detail. Calponin was phosphorylated to 1.0 mol of P_i /mol by CaM kinase II using $[\gamma\text{-}^{32}P]\text{ATP}$ and digested with trypsin. Tryptic phosphopeptides were separated by reversed-phase h.p.l.c. and identified by Čerenkov counting (Figure 2). Peaks X and Y contained free $^{32}P_i$ and $[\gamma\text{-}^{32}P]\text{ATP}$, and undigested or partially digested calponin respectively. Ten fractions, designated A–J, were further purified by reversed-phase h.p.l.c. and subjected to N-terminal sequence analysis (Table 1). In several cases, the release of ^{32}P was quantified at each cycle. In other cases, the phosphorylated amino acid was identified by a precipitous drop in yield (Wang et al., 1988). Table 2 summarizes the sequencing results. Some 74% of the total radioactivity recovered in peptides A–J was on Ser-175. The remainder was divided between Ser-71, Ser-254 and Ser-215. Seven of the ten phosphopeptides contained phosphoserine-175 suggesting that trypsin digestion was incomplete, that partial proteolysis was also occurring at non-trypsin cleavage sites or a combination of these. Two-dimensional phosphopeptide mapping (see below) supported these observations. Although some phosphothreonine was detected by two-dimensional thin-layer electrophoresis of acid hydrolysates of calponin phosphorylated by CaM kinase II, we did not identify phosphothreonine in any of the phosphopeptides. We conclude from these results that calponin is phosphorylated by CaM kinase II primarily at Ser-175.

To address the question of whether calponin is phosphorylated when bound to the thin filament, as it is free in solution, synthetic thin filaments were prepared by mixing purified actin, tropomyosin and calponin, and centrifugation, as described in the Materials and methods section. The pellet (synthetic thin filaments) was gently resuspended in buffer A and analysed by SDS/PAGE (Figure 3a, lane 1). Free calponin was found in the supernatant (Figure 3a, lane 2) but not in the pellet (Figure 3a, lane 3) after ultracentrifugation. Figure 3(b) depicts the time course of PKC-catalysed phosphorylation of calponin by SDS/PAGE and autoradiography. A phosphorylation stoichiometry of 1.25 mol of P_i /mol of calponin was determined by quantification of total ^{32}P incorporation and laser scanning densitometry of the autoradiogram. The level of actin phosphorylation was determined to be < 0.1 mol of P_i /mol of actin.

An alternative approach to this issue was to examine calponin phosphorylation in a reconstituted contractile system and in washed myofibrils. Contractile filaments were prepared from purified actin, myosin, tropomyosin, calmodulin, MLCK, calponin and caldesmon, and incubated with CaM kinase II (Figure

Figure 1 Phosphoamino acid analysis of calponin phosphorylated by PKC

^{32}P Calponin phosphorylated by PKC was acid hydrolysed and the phosphoamino acids were separated and identified as described in the Materials and methods section. (a) Ninhydrin-stained electrophoretogram; (b) autoradiogram. Abbreviations: PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

Figure 2 H.p.l.c. separation of tryptic phosphopeptides of calponin phosphorylated by CaM kinase II

Trypsin digestion of phosphorylated calponin and separation of the phosphopeptides by h.p.l.c. was performed as described in the Materials and methods section. [^{32}P]Phosphopeptides were detected by Čerenkov counting of column fractions. Fractions A–J were individually purified by h.p.l.c. for N-terminal sequence analysis (Table 1). ○, ^{32}P radioactivity; ----, [acetonitrile].

Table 1 Amino acid sequence analysis of tryptic phosphopeptides of calponin phosphorylated by CaM kinase II

Peptide A				Peptide B				Peptide C				Peptide D				Peptide E			
Cycle no.	Residue	Yield (pmol)	Radio-activity (c.p.m.)	Residue	Yield (pmol)	Radio-activity (c.p.m.)		Residue	Yield (pmol)			Residue	Yield (pmol)			Residue	Yield (pmol)		
1	L	97	255	F	66	580		G	25			F	101			F	97		
2	Q	123	50	A	88	200		A	17			A	73			A	82		
3	P	73	60	S	—*	2780		S	2			S	3			S	26		
4	G	60	60	Q	55	835		Q	3			Q	14			Q	26		
5	S	18	690	Q	32	340		Q/A	3/8			Q	15			Q	26		
6	V	44	290	G	Trace	170						G	+†						
7	Q	25	125	M	Trace	115						M	2						
8	K	24	45									T	2						
Peptide F				Peptide G				Peptide H				Peptide I				Peptide J			
Cycle no.	Residue	Yield (pmol)	Radio-activity (c.p.m.)	Residue	Yield (pmol)			Residue	Yield (pmol)	Radio-activity (c.p.m.)		Residue	Yield (pmol)	Radio-activity (c.p.m.)		Residue	Yield (pmol)	Radio-activity (c.p.m.)	
1	F	456	1605	F	52	F	112	F	170	754		F	170	754		G	16	830	
2	A	470	1135	A	34	A	88	A	166	220		A	166	220		A	25	415	
3	S	—*	20450	S	—*	S	1	S	21	1794		S	21	1794		S	—*	2880	
4	Q	119	7905	Q	6	Q	34	Q	69	982		Q	69	982		Q	6	2020	
5	Q	130	3350	Q	9	Q	37	Q	78	270		Q	78	270		Q	7	310	
6	G	71	2085	G	10	G	21	G	36	148		G	36	148					
7	M	70	1485	M	Trace	M	18	M	39	112		M	39	112					
8	T	62	835	T	3	T	11	T	22	104		T	22	104					
9	A	83	755			A	19	A	25	78		A	25	78					
10	Y	51	640			Y	10	Y	20	62		Y	20	62					
11	G	38	445			G	12	G											
12	T	35	745			T	6	T											
13	R	20	530			R	Trace	R											

* No residue detected.

† Detected but not quantified.

Table 2 Summary of phosphopeptide sequencing results

Peptide	Radioactivity (% of total)	N-terminal sequence	Phosphorylation site
A	5.3	LQPGSVQK	Ser-71
B	4.0	FASQQGM	Ser-175
C	15.9	GASQQ/A	Ser-215 or Ser-254
D	7.2	FASQQGMT	Ser-175
E	9.8	FASQQ	Ser-175
F	17.8	FASQQGMTAYGTR	Ser-175
G	5.2	FASQQGMT	Ser-175
H	10.6	FASQQGMTAYGTR	Ser-175
I	19.4	FASQQGMTAY	Ser-175
J	4.8	GASQQ	Ser-254

Figure 3 Phosphorylation of calponin in synthetic thin filaments

Synthetic thin filaments prepared from purified actin, tropomyosin and calponin as described in the Materials and methods section were incubated with PKC under standard phosphorylation reaction conditions. Duplicate samples (50 μ l each) of the reaction mixture were withdrawn at the indicated times for quantification of [32 P]phosphate incorporation (Walsh et al., 1983) and SDS/PAGE and autoradiography. (a) Coomassie Blue-stained gel of synthetic thin filaments (lane 1) and purified calponin (CaP) centrifuged as for the preparation of synthetic thin filaments: supernatant (lane 2), pellet (lane 3). Tm γ and Tm β , tropomyosin γ and β . (b) Time course of phosphorylation by PKC of calponin (CaP) in synthetic thin filaments. Similar results were obtained with CaM kinase II.

4b). Phosphorylation of calponin and caldesmon (by CaM kinase II) and of myosin LC₂₀ (by MLCK) was observed (Figure 4a) as well as a low level of actin phosphorylation. Phosphorylation of calponin resulted in its dissociation from the thin filaments as shown by its appearance in the supernatant after centrifugation at 50000 *g* (Figure 4c, lane 2). The small amount of phosphorylated calponin recovered in the pellet (Figure 4c, lane 3) is probably the result of trapping. Similar results were obtained with PKC in place of CaM kinase II (results not shown).

Two-dimensional phosphopeptide mapping was used to show that the same sites in calponin are accessible for phosphorylation whether calponin is free in solution or bound to the thin filaments in a reconstituted contractile system (Figure 5). This was true whether the kinase used was PKC (Figures 5a–5c) or CaM kinase II (Figures 5d–5f).

Figure 4 CaM kinase II-catalysed phosphorylation of calponin in a reconstituted contractile system

Contractile filaments were reconstituted from purified actin, myosin, tropomyosin, calmodulin, MLCK and calponin as described in the Materials and methods section. Caldesmon (0.2 mg/ml) containing endogenous CaM kinase II was added to the resuspended filaments and phosphorylation begun by addition of [γ - 32 P]ATP (2 mM; 65 c.p.m./pmol). After incubation at 30 °C for 60 min, a sample (50 μ l) of the reaction mixture was subjected to SDS/PAGE and autoradiography. (a) Autoradiogram; (b) Coomassie Blue-stained gel. The remainder of the reaction mixture was centrifuged at 50000 *g* for 30 min at 4 °C. (c) The separated supernatant (lane 2) and resuspended pellet (lane 3) (50 μ l each) and a sample of the total reaction mixture (lane 1) were subjected to SDS/PAGE and autoradiography. The region of the autoradiogram showing calponin and LC₂₀ is shown. Abbreviations: CaD, caldesmon; CaP, calponin; LC₂₀, LC₁₇, the 20 kDa and 17 kDa light chains of myosin respectively.

Calponin phosphorylation was also observed when washed myofibrils were treated with PKC (Figure 6). Similar results were obtained with CaM kinase II.

We extended these studies on phosphorylation of chicken gizzard calponin to toad stomach smooth muscle for the following reasons: (i) a great deal is known about the mechanism of contraction in this tissue at the single-cell level; (ii) we have previously isolated calponin from this tissue and, using specific antibodies, have demonstrated its localization on the thin filament (Walsh et al., 1993); and (iii) this is a suitable tissue in which to carry out physiological (tension) measurements which can be correlated with biochemical parameters because of the availability of relatively large amounts of tissue. Figure 7 shows that calponin is a major substrate of PKC in an extract of toad stomach smooth muscle containing thin filaments. The calponin band was positively identified by immunoblotting (results not shown). Calponin in the extract was dephosphorylated by turkey gizzard SMP-I (Figure 7c, lane 1) or chicken gizzard calponin phosphatase (Figure 7c, lane 2), type 2A protein phosphatases. These observations were confirmed by two-dimensional gel electrophoresis (Figure 8). The stained gel in Figure 8(a) of toad stomach extract incubated with [γ - 32 P]ATP in the absence of added kinase or phosphatase reveals four spots of calponin. The corresponding autoradiogram (Figure 8b) shows very slight phosphorylation correlating with the most acidic calponin spot

Figure 5 Calponin is phosphorylated at the same sites in the isolated state and in the reconstituted contractile system

Calponin (free in solution or bound to thin filaments in the reconstituted contractile filament system, prepared as described in the Materials and methods section) was phosphorylated by incubation with PKC or CaM kinase II for 60 min under standard conditions. After SDS/PAGE, calponin bands (identified by brief autoradiography) were cut out of the gel, extracted overnight with 10% (v/v) acetic acid/1% (w/v) SDS at 37 °C, dialysed against four changes (2 litres each) of 50 mM NH_4HCO_3 , lyophilized and digested with trypsin. Phosphopeptides were separated by thin-layer electrophoresis (t.l.e.)/t.l.c. (Colburn et al., 1988) and identified by autoradiography. (a) Purified calponin phosphorylated by PKC; (b) calponin in the reconstituted system phosphorylated by PKC; (c) a mixture of tryptic digests from (a) and (b); (d) purified calponin phosphorylated by CaM kinase II; (e) calponin in the reconstituted system phosphorylated by CaM kinase II; (f) a mixture of tryptic digests from (d) and (e).

and strong phosphorylation of myosin LC_{20} by endogenous MLCK. Addition of PKC resulted in a marked increase in calponin phosphorylation in three spots, suggesting that all isoforms become phosphorylated (Figure 8c). Coomassie Blue staining revealed two additional spots of lower pI which were not observed in the absence of added kinase (results not shown). Subsequent treatment with EDTA, to prevent further kinase activity, and turkey gizzard SMP-I resulted in almost complete dephosphorylation of calponin (Figure 8d). Dephosphorylation also resulted in the disappearance of the more acidic stained calponin spots observed in the presence of kinase (results not shown). The observed dephosphorylation of myosin LC_{20} is probably due to the action of endogenous myosin-associated phosphatase, as SMP-I does not dephosphorylate intact myosin (Pato, 1985) and the endogenous myosin phosphatase is bound to myosin (Sellers and Pato, 1984; Alessi et al., 1992).

Figure 9(a) confirms the expression of at least three isoforms of calponin in the toad stomach. The pattern of calponin spots was unaffected by treatment with a high concentration of SMP-I (Figure 9b), indicating that the more acidic of the three spots

are not phosphorylated forms of calponin but probably represent distinct isoforms.

Finally, calponin phosphorylation was investigated in intact toad stomach smooth-muscle strips metabolically labelled with $^{32}\text{P}_i$. Toad stomach is a phasic smooth muscle which undergoes spontaneous contractions in amphibian Ringer's solution. Contractions could be induced in a more controlled manner by addition of carbachol to the bathing medium. Near the peak of a carbachol-induced contraction, the muscle strip was quick-frozen and tissue proteins were extracted and separated by two-dimensional gel electrophoresis. The stained gel (Figure 10a) shows four spots at the molecular mass of calponin and their identity was confirmed by immunoblotting (Figure 10b). Figure 10(c) shows an autoradiogram of the stained gel in Figure 10(a) which indicates that the two most acidic calponin spots were phosphorylated. The carbachol-induced increase in calponin phosphorylation is shown in Figure 11. A low level of calponin phosphorylation is apparent in muscle maintained at resting tension (Figure 11a). This is markedly increased in response to carbachol in parallel with the expected increase in

Figure 6 PKC-catalysed phosphorylation of calponin in washed myofibrils

Chicken gizzard washed myofibrils (2 mg of protein/ml), prepared as described by Sobieszek and Bremel (1975), were incubated with PKC under standard conditions (see the Materials and methods section). Samples (50 μ l) of the reaction mixture were withdrawn at the indicated times for SDS/PAGE and autoradiography and duplicate samples (50 μ l) for quantification of protein-bound [32 P]phosphate as previously described (Walsh et al., 1983). The stoichiometry of calponin phosphorylation was determined by laser densitometric scanning of the autoradiograph. The inset shows, by autoradiography, the time-dependent phosphorylation of calponin.

Figure 7 Phosphorylation and dephosphorylation of calponin (CaP) in a toad stomach extract: one-dimensional gel analysis

Toad stomach homogenate supernatant (containing thin filaments) was incubated at 30 $^{\circ}$ C in the presence of [γ - 32 P]ATP with no added kinase or phosphatase (a), with PKC (1 μ g/ml) (b), or with PKC for 60 min followed by EDTA (30 mM) and either turkey gizzard SMP-I (c, lane 1) or chicken gizzard calponin phosphatase (c, lane 2). Phosphorylation reaction conditions were: 17 mM imidazole/HCl (pH 7), 0.1 M KCl, 0.3 mM dithiothreitol, 5 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.1 mg of phosphatidylserine/ml, 2 μ g of dioleoin/ml and 1 mM [γ - 32 P]ATP. Reactions were started with ATP and samples withdrawn at the indicated times for SDS/PAGE and autoradiography.

Figure 8 Phosphorylation and dephosphorylation of calponin (CaP) in a toad stomach extract: two-dimensional gel analysis

Samples were prepared as described in the legend to Figure 7 and analysed by two-dimensional gel electrophoresis (NEPHGE/SDS/PAGE). (a) Coomassie Blue-stained gel (control conditions); (b)–(d) autoradiographs of the region of gels corresponding to the boxed region in (a). (b) Control conditions (30 min incubation no added kinase or phosphatase); (c) 30 min incubation with PKC; (d) 30 min incubation with PKC followed by 30 min incubation with EDTA (30 mM) and SMP-I. Reaction mixtures were quick-frozen in liquid N_2 , lyophilized and dissolved in NEPHGE sample buffer before two-dimensional gel electrophoresis and autoradiography.

Figure 9 Identification of at least three isoforms of toad stomach calponin

Toad stomach extract was incubated at 30 $^{\circ}$ C for 30 min in the absence (a) or presence (b) of SMP-I (7 μ g/ml), quick-frozen, lyophilized and dissolved in NEPHGE sample buffer. Two-dimensional gel electrophoresis (NEPHGE/SDS/PAGE) was carried out as described in the Materials and methods section. The large protein spot in (b) is BSA used as a stabilizing protein in the SMP-I solution.

Figure 10 Phosphorylation of calponin in intact toad stomach smooth-muscle strips

Toad stomach smooth-muscle strips metabolically labelled with $^{32}\text{P}_i$ were induced to contract phasically with carbachol ($1\ \mu\text{M}$). Near the peak of force development the tissue was quick-frozen in acetone/10% trichloroacetic acid on solid CO_2 . Frozen tissue was pulverized after removal of trichloroacetic acid and extracted with NEPHGE sample buffer before two-dimensional gel electrophoresis. Duplicate gels were stained with Coomassie Blue (a) and immunoblotted using affinity-purified anti-calponin (b). The gel in (a) was autoradiographed (c). The arrowhead (Δ) indicates the location of the calponin spots.

Figure 11 Calponin phosphorylation is enhanced in response to carbachol treatment

Calponin ($\blacktriangleright$) and myosin LC_{20} ($\blacktriangleright$) phosphorylation was analysed by two-dimensional gel electrophoresis in toad stomach smooth-muscle strips under resting tension and at the peak of carbachol-induced tension development. Samples corresponding to $50\ \mu\text{g}$ of tissue were applied to each gel. For autoradiography, the two gels were exposed simultaneously to the same X-ray film. (a) Autoradiogram of resting muscle; (b) autoradiogram of contracted muscle. Coomassie Blue-stained gels corresponding to (a) and (b) are shown in (c) and (d) respectively.

myosin LC_{20} phosphorylation (Figure 11b). It is also apparent that other unidentified proteins increase in phosphorylation in response to carbachol. Inspection of the corresponding

Coomassie Blue-stained gels (Figures 11c and 11d) confirms that the same amount of protein was applied to each gel.

DISCUSSION

Calponin is an attractive candidate for a thin-filament-linked mechanism of regulation of smooth-muscle contraction for the following reasons: (i) the protein is localized on the thin filaments of cultured (Gimona et al., 1991; Takeuchi et al., 1991) and freshly isolated smooth-muscle cells (Winder et al., 1992a; Walsh et al., 1993), consistent with high-affinity binding to actin *in vitro* (Winder et al., 1991) and co-purification with native thin filaments (Ngai et al., 1987; Nishida et al., 1990; Marston, 1991); (ii) calponin is present at the same molar concentration as tropomyosin, i.e. 1 calponin:7 actin monomers (Takahashi et al., 1986); (iii) purified calponin inhibits the actin-activated MgATPase activity of smooth-muscle myosin without affecting myosin phosphorylation (Abe et al., 1990; Winder and Walsh, 1990a). Maximal (approx. 80%) inhibition of actin-activated myosin MgATPase activity was observed at a molar ratio of 1 calponin:1 tropomyosin:3 actin monomers (Winder and Walsh, 1990a; Winder et al., 1992b) which coincides with the maximal binding stoichiometry *in vitro* of 1 calponin:3 actin monomers (Winder et al., 1991). Modest inhibition (approx. 20%) was observed at 1 calponin:1 tropomyosin:7 actin monomers, the physiological ratios of these proteins. This suggests that the inhibitory effect of calponin *in vivo* is rather modest. However, the inhibitory potency of calponin may be less in the *in vitro* reconstituted system because of, for example, the use of denaturing conditions during the purification of calponin (e.g. Takahashi et al., 1986; Winder and Walsh, 1990a). Alternatively, or additionally, calponin may be restricted to the contractile actin-containing domain (Small et al., 1986) and/or confined to specific regions of actin filaments which overlap with myosin during a normal contractile cycle, so that a higher local stoichiometry of calponin/actin may occur *in vivo*.

Energetically, it is clearly unfavourable to maintain a con-

stitutively active mechanism causing such pronounced inhibition of cross-bridge cycling, suggesting that this function may be regulated in some way. Potential regulatory mechanisms which have been considered involve: (i) interaction of Ca^{2+} /calmodulin with calponin (Takahashi et al., 1986); (ii) direct binding of Ca^{2+} to calponin (Takahashi et al., 1987; Winder and Walsh, 1990a); (iii) GTP binding to calponin (Takahashi and Nadal-Ginard, 1991); and (iv) phosphorylation-dephosphorylation of calponin, catalysed by PKC or CaM kinase II (Winder and Walsh, 1990a) and a type 2A phosphatase (Winder et al., 1992b). The fact that very high (unphysiological) amounts of Ca^{2+} /calmodulin are required to dissociate calponin from actin or actin/tropomyosin and alleviate inhibition of the actomyosin ATPase (S. J. Winder and M. P. Walsh, unpublished work) suggests that this mechanism may not be physiologically relevant. Similarly, direct Ca^{2+} or GTP binding to calponin are unlikely to regulate its inhibition of the actomyosin ATPase. The affinity of calponin for Ca^{2+} is low (K_d 7 μM) (Takahashi et al., 1987); in fact, calponin may not bind Ca^{2+} at all (Wills et al., 1993). We have been unable to detect any binding of GTP[S] to calponin or to observe any effect of GTP on calponin-mediated inhibition of the actomyosin ATPase (S. J. Winder and M. P. Walsh, unpublished work). On the other hand, stoichiometric phosphorylation of calponin by PKC or CaM kinase II results in loss of calponin-mediated inhibition of actin binding and actomyosin ATPase inhibition (Winder and Walsh, 1990a). We report here that the major site of calponin phosphorylation by CaM kinase II and PKC is Ser-175. Phosphorylation at this site disrupts the calponin-actin interaction, consistent with the observations that a 21 kDa fragment (residues 61–237 of calponin), generated by cleavage with 2-nitro-5-thiocyanobenzoic acid, inhibits the actomyosin MgATPase, and the inhibition can be released by phosphorylation (Winder and Walsh, 1990b). Furthermore, Ser-175 is within the difference peptide (residues 145–182) deduced by Mezgueldi et al. (1992) to be the minimum actin-binding fragment of calponin. Phosphorylation of this region would be expected to disrupt actin binding. PKC phosphorylation of threonine residues in calponin has also been demonstrated (Nakamura et al., 1993). Thr-184 was identified as the major phosphorylation site resulting in reduced affinity of calponin for actin. Although at odds with our identification of Ser-175 as the major PKC site, we did observe some phosphorylation of Thr-184 by PKC. Furthermore, phosphorylation of Ser-175 by PKC is indicated by the sequence data of Nakamura et al. (1993), which show a marked drop in yield at cycle 3 of the tryptic phosphopeptide [calponin (173–185)] or the corresponding synthetic peptide, although phosphoserine was not detected by one-dimensional electrophoresis. Identification of the sites phosphorylated *in vivo* will help to resolve this difference.

The effects of phosphorylation are reversed after dephosphorylation of calponin by the type 2A phosphatase (Winder et al., 1992b). On the basis of these results we have proposed a mechanism to explain the physiological role of calponin in regulation of the contractile state of smooth muscle and its regulation by phosphorylation-dephosphorylation (Walsh, 1991; Winder et al., 1991). This mechanism is based largely on *in vitro* binding properties and effects on actomyosin ATPase activity of isolated calponin. We have observed phosphorylation of calponin in intact canine tracheal smooth-muscle strips stimulated to contract with carbachol (Pohl et al., 1991). Similarly, Rokolya and Moreland (1993) observed calponin phosphorylation in swine carotid media in response to endothelin-1. However, Bárány et al. (1991) and Gimona et al. (1992) did not observe calponin phosphorylation in pig carotid artery or chicken gizzard respectively. To address the issue of the physio-

logical relevance of calponin phosphorylation, we have examined calponin phosphorylation by PKC and CaM kinase II when the protein is associated with thin filaments as it is *in situ*. We have also directly investigated calponin phosphorylation in intact toad stomach smooth-muscle fibres in response to contractile stimuli. The following main conclusions are drawn: (i) the major site of phosphorylation by PKC or CaM kinase II is Ser-175 of calponin and this site is also phosphorylated when calponin is associated with actin in synthetic thin filaments, a reconstituted contractile system, washed myofibrils and tissue extracts; (ii) calponin is phosphorylated in intact toad stomach smooth-muscle strips and calponin phosphorylation increases in response to carbachol. These results are therefore consistent with regulation of calponin function by phosphorylation-dephosphorylation.

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