The smooth muscle 132 kDa cyclic GMP-dependent protein kinase substrate is not myosin light chain kinase or caldesmon

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Atrial natriuretic peptide (ANP) stimulates the phosphorylation of three cyclic GMP-dependent protein kinase substrate proteins of 225, 132, and 11 kDa (P225, P132 and P11 respectively) in the particulate fraction of cultured rat aortic smooth muscle cells [Sarcevic, Brookes, Martin, Kemp & Robinson (1989) *J. Biol. Chem.* **264**, 20648–20654]. Vrolix, Raeymaekers, Wuytack, Hofmann & Casteels [(1988) *Biochem. J.* **255**, 855–863] have reported the presence of a 130 kDa cyclic GMP-dependent protein kinase substrate protein in the membrane fraction of pig aorta or stomach, and suggested that it may be myosin light chain kinase (MLCK). The aim of the present study was to determine whether P132 from rat aorta was MLCK or caldesmon. Although P132 co-migrates with purified chicken gizzard MLCK on SDS/polyacrylamide gels, it is distinct from rat aortic MLCK. Partially purified MLCK from rat aorta migrated as a 145 kDa protein on SDS/polyacrylamide gels. Immunoblotting the partially purified rat aortic MLCK with antibody to bovine tracheal MLCK identified rat aortic MLCK (145 kDa) and a corresponding 145 kDa protein in the particulate fraction of cultured rat aortic smooth muscle cells, but did not detect the 132 kDa protein. Phosphopeptide maps of purified rat aortic MLCK prepared by digestion with *Staphylococcus aureus* V8 protease were distinct from those of P132. P132 was not caldesmon, since antibodies to caldesmon cross-reacted with 136 and 76 kDa proteins in the particulate fraction of rat aortic cells, but not with P132. Furthermore, caldesmon was partially extracted from the particulate into the soluble fraction by heating at 90 °C, whereas P132 was not. These results demonstrate that the ANP-responsive cyclic GMP-dependent protein kinase substrate of 132 kDa from rat aortic smooth muscle cells is not MLCK or caldesmon.

INTRODUCTION

The relaxation of smooth muscle cells by atrial natriuretic peptide (ANP) (Fiscus *et al.*, 1985), endothelium-derived relaxing factor and nitrovasodilators such as nitroprusside is accompanied by increases in the intracellular concentration of cyclic GMP and activation of the cyclic GMP-dependent protein kinase (Rapoport *et al.*, 1983). A range of protein substrates for the cyclic GMP-dependent protein kinase have been observed in smooth muscle preparations. In particular, Parks *et al.*, (1987) reported the phosphorylation of 240, 130 and 85 kDa proteins in the particulate fraction from rabbit aortic smooth muscle, and we have observed phosphorylation of a related set of proteins from the particulate fraction of rat aortic smooth muscle cells, termed P225, P132 and P11. The P11 protein was identified as phospholamban (Sarcevic *et al.*, 1989). Phosphorylation of phospholamban *in vitro* by cyclic GMP-dependent protein kinase enhances Ca^{2+} uptake into isolated sarcoplasmic reticulum vesicles of smooth muscle (Raeymaekers *et al.*, 1988). This is consistent with a recent report that ANP reduces intracellular cytoplasmic Ca^{2+} levels in cultured smooth muscle cells as measured using fura-2 (Cornwell & Lincoln, 1989).

A number of attempts to identify the 130 kDa cyclic GMP-dependent protein kinase substrate protein in smooth muscle have been made. Furukawa & Nakamura (1987) proposed that it was the Ca^{2+} -transport ATPase, but subsequent work indicated that it was not (Vrolix *et al.*, 1988; Baltensperger *et al.*, 1988). Vrolix *et al.* (1988) suggested that the 130 kDa protein was myosin light chain kinase (MLCK) on the basis of its cross-reactivity with polyclonal antibodies to chicken gizzard MLCK and the inability of cyclic AMP-dependent protein kinase to phosphorylate this protein in the presence of calmodulin. When calmodulin is not bound to bovine tracheal MLCK, the MLCK

is phosphorylated *in vitro* by the cyclic AMP-dependent protein kinase on two sites and its catalytic activity is significantly decreased (Nishikawa *et al.*, 1984). On the other hand, phosphorylation of bovine tracheal MLCK by cyclic GMP-dependent protein kinase did not affect the catalytic activity of the enzyme. These properties are not consistent with the idea that cyclic GMP-dependent phosphorylation of MLCK is involved in smooth muscle relaxation. In the present study, we report that P132 is distinct both from MLCK and from caldesmon, a calmodulin- and actin-binding protein whose phosphorylation has recently been implicated in regulating force maintenance in vascular smooth muscle (Adam *et al.*, 1989).

MATERIALS AND METHODS

Materials

The materials used were as described previously (Sarcevic *et al.*, 1989) except that cyclic AMP-agarose, Ficoll-70, Tween-20, phenylmethanesulphonyl fluoride (PMSF), 3,3'-diaminobenzidine and *Staphylococcus aureus* V8 protease were from Sigma. Affigel Blue was from Bio-Rad. Nonidet P-40 and Tween-80 were from BDH. Phosphocellulose ion-exchange paper (P 81) was from Whatman. Nitrocellulose was from Schleicher and Schull. Horseradish peroxidase-conjugated swine anti-rabbit antibodies were from Dakopatts (Copenhagen, Denmark). Antibodies to MLCK from bovine trachea (raised in rabbits) were a gift from Dr. J. T. Stull, Southwestern Medical Centre, Dallas, TX, U.S.A. Purified chicken gizzard caldesmon and monoclonal antibody to chicken gizzard caldesmon were gifts from Dr. M. P. Walsh, University of Calgary, Alberta, Canada. Synthetic peptides were synthesized as the C-terminal amide forms by the Merrifield solid-phase synthesis procedure (Hodges & Merrifield, 1975).

Abbreviations used: ANP, atrial natriuretic peptide; MLCK, myosin light chain kinase; IBMX, 3-isobutyl-1-methylxanthine; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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Cell culture and preparation of smooth muscle cell particulate fraction

Isolation and culture of smooth muscle cells from the aortae of Sprague-Dawley rats and preparation of the particulate fraction from cultured rat aortic smooth muscle cells were as described previously (Sarcevic *et al.*, 1989).

Protein purification

Cyclic GMP-dependent protein kinase. Bovine lung cyclic GMP-dependent protein kinase was purified using a cyclic AMP-agarose affinity column (Lincoln, 1983a), with a slight modification. The cyclic GMP-dependent protein kinase which was eluted from the affinity column (10 ml) was further purified by f.p.l.c. using a Mono-Q column (HR 5/5, Pharmacia) equilibrated in 20 mM-potassium phosphate buffer containing 2 mM-EDTA, 1 mM-dithiothreitol (DTT) and 0.05% (v/v) Tween-80, at pH 7.0. The cyclic GMP-dependent protein kinase was eluted with a linear gradient from 50–500 mM-NaCl over 30 min at 1 ml/min. The fractions containing enzyme activity were pooled and dialysed overnight against the above buffer, and then concentrated in a dialysis bag to a final volume of approx. 1.3 ml by addition of powdered Ficoll-70 to the outside of the bag.

Chicken gizzard MLCK. This enzyme was purified as described previously (Walsh *et al.*, 1983). Calmodulin was purified from ram testes as described previously (Dedman & Kaetzel, 1983). Chicken gizzard myosin light chains were purified as described previously (Kemp *et al.*, 1982) with a slight modification. The final myosin light chain solution was centrifuged at 9000 *g* for 15 min and the supernatant was chromatographed on a Sephadex G-100 column equilibrated in 10 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl, 2 mM-MgCl₂ and 15 mM- β -mercaptoethanol. The peak fractions were recovered by precipitation with 15% (w/v) trichloroacetic acid and centrifuged at 9000 *g*. The pellet was dissolved in 1 M-Tris base and dialysed against 20 mM-imidazole (pH 7.0)/100 mM-NaCl/15 mM- β -mercaptoethanol, and then against the same buffer containing 30% (v/v) glycerol.

Rat aortic MLCK. The purification of rat aortic MLCK involved the use of affinity chromatography and two ion-exchange chromatography steps. Thirty Sprague-Dawley rats of approx. 3–4 months were killed by decapitation. The aortae were removed, stripped of fat and immediately placed in liquid N₂. The aortae were then powdered with a mortar and pestle in the presence of liquid N₂. All subsequent procedures were performed at 4 °C unless otherwise stated. The powdered aortae were homogenized in 20 ml of ice-cold extraction buffer (40 mM-Tris/HCl, pH 7.5, 80 mM-KCl, 30 mM-MgCl₂, 1 mM-EGTA, 15 mM- β -mercaptoethanol, 1 μ g of leupeptin/ml, 1 μ g of pepstatin/ml and 75 μ g of PMSF/ml) using an Ultraturax. The homogenate was centrifuged at 10000 *g* for 15 min at 4 °C, and the supernatant was filtered through Miracloth, made to 60% ammonium sulphate and stirred for 20 min. The supernatant was then diluted with extraction buffer to 40% ammonium sulphate and centrifuged at 10000 *g* for 20 min. The resulting supernatant was collected and brought to 60% ammonium sulphate and again stirred for 20 min, then centrifuged at 10000 *g* for 30 min. The pellet was resuspended in 15 ml of 25 mM-potassium phosphate buffer, pH 8.0, containing 1 mM-EGTA, 1 mM-EDTA, 15 mM- β -mercaptoethanol, 0.02% (w/v) NaN₃, 1 μ g of leupeptin/ml, 1 μ g of pepstatin/ml and 75 μ g of PMSF/ml (buffer A), and recycled three times through a 1 ml Affi-gel Blue column previously equilibrated in buffer A. The column was

washed to baseline and the MLCK was eluted with 20 ml of buffer A containing 2 M-NaCl, and 1 ml fractions were collected and assayed for MLCK activity. The active fractions were pooled and dialysed overnight against 40 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EGTA, 1 mM-DTT, 1 μ g of leupeptin/ml, 1 μ g of pepstatin/ml and 75 μ g of PMSF/ml (buffer B). The sample was applied to a Mono-S column (HR 5/5, Pharmacia) equilibrated in buffer B, and the MLCK was eluted with a linear NaCl gradient to 500 mM over 60 min at 1 ml/min. The active fractions were collected and dialysed overnight against 30 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EGTA, 0.5 mM-DTT, 1 μ g of leupeptin/ml, 1 μ g of pepstatin/ml and 75 μ g of PMSF/ml (buffer C). The sample was applied on a Mono-Q column (HR 5/5) equilibrated in buffer C and the MLCK was eluted with a linear NaCl gradient to 500 mM over 60 min at 1 ml/min. The active fractions were pooled and glycerol (10%, v/v) and Tween-80 (0.1%, v/v) were added. The sample was then concentrated to a final volume of 50 μ l using the Centricon filter system (Pharmacia).

MLCK assay. During all stages of the purification, MLCK activity was assayed as described previously (Kemp *et al.*, 1983) in a reaction mixture of 40 μ l containing 40 mM-Hepes, pH 7.0, 5 mM-magnesium acetate, 0.25 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (500 c.p.m./pmol), 0.55 mM-CaCl₂, 1 mg of BSA/ml, 0.1% (w/v) Tween-80 and mixed myosin light chains containing 33 μ M of the phosphorylatable 20 kDa form in the presence of either 1 mM-EGTA or 50 μ g of calmodulin/ml. Incubations were carried out for 20 min at 30 °C and then 35 μ l portions were applied to P 81 phosphocellulose ion-exchange paper squares (2 cm $\times$ 2 cm), which were washed with three changes of 75 mM-phosphoric acid then once in ethanol, and were dried and counted for radioactivity in Beckman Ready-Solv NA scintillation cocktail. For phosphorylation of peptides by rat aortic MLCK, the composition of the assay buffer was as described above and incubations were carried out for 10 min at 37 °C. When the peptide analogue of myosin light chains, [Ala^{14,15}]MLC-(11–23)-peptide (sequence KKRAARATSNVFA), was used as substrate (Kemp & Pearson, 1985), the reaction was terminated by placing 35 μ l of the reaction mixture on to P 81 paper, then washing and counting the P 81 paper for radioactivity as described above. When the peptide analogue of glycogen synthase, [Ala^{9,10}]GS-(1–10)-peptide (sequence PLSRTLVA), was used as substrate (Pearson *et al.*, 1985), the phosphopeptide was separated from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by the anion-exchange procedure in 30% (v/v) acetic acid (Pearson *et al.*, 1985).

Protein phosphorylation and PAGE

Phosphorylation of smooth muscle cell particulate fractions was performed as described previously (Sarcevic *et al.*, 1989). The standard phosphorylation reaction mixture for smooth muscle cell particulate fractions contained 50 mM-Tris/HCl buffer, pH 7.6, 15 mM-MgCl₂, 1 mM-EGTA, 500 μ M-3-isobutyl-1-methylxanthine (IBMX), 20 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (12.5 Ci/mmol), 1 μ M-cyclic GMP (where indicated) and 30–50 μ g of protein in a final volume of 60 μ l. The reaction was initiated by the addition of ATP and incubated at 0 °C for 1 min. Phosphorylation of purified MLCK or caldesmon with purified cyclic GMP-dependent protein kinase was performed in a reaction mixture containing 50 mM-Tris/HCl, pH 7.6, 15 mM-MgCl₂, 1 mM-EGTA and 50 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1 Ci/mmol) in a final volume of 60 μ l. The reaction was initiated by the addition of ATP and was carried out for 5 min at 37 °C. For phosphopeptide maps, purified rat aortic MLCK was phosphorylated in the above buffer containing 20 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (8.3 Ci/mmol) for 30 min at 37 °C. All phosphorylation reactions were terminated by the addition of

30 μ l of 187 mM-Tris/HCl buffer, pH 6.8, containing 30 % (w/v) glycerol, 6 % (w/v) SDS and 15 % (v/v) β -mercaptoethanol and freezing on dry ice pellets. The samples were then boiled for 2 min and 80 μ l portions were subjected to PAGE according to the method of Laemmli (1970), using a linear 7.5–20 % polyacrylamide gradient gel. After electrophoresis the gels were either fixed with 20 % trichloroacetic acid and stained with Coomassie Brilliant Blue, or silver-stained according to the method of Wray *et al.* (1981), dried under vacuum and exposed to Kodak X-AR film for autoradiography.

Immunoblot analysis

Phosphoproteins separated on polyacrylamide gels were electrophoretically transferred on to nitrocellulose using a Bio-Rad transblot apparatus. Before electrophoresis the nitrocellulose was moistened in transfer buffer [25 mM-Tris/HCl, pH 8.25, 19 mM-glycine, 20 % (v/v) methanol and 0.1 % (w/v) SDS]. The transfer was conducted for 2 h at 60 V. After transfer, the nitrocellulose sheet was incubated in blocking buffer [50 mM-Tris/HCl, pH 8.0, 80 mM-NaCl, 2 mM-CaCl₂, 5 % (w/v) dry skim milk powder and 0.2 % (v/v) Nonidet P-40] for 1 h with gentle agitation. The nitrocellulose was then incubated in fresh blocking buffer containing rabbit polyclonal antibodies raised against bovine tracheal MLCK at a dilution of 1:1500 or monoclonal antibodies raised against chicken gizzard caldesmon at a dilution of 1:10000 for 1 h with gentle agitation. The nitrocellulose sheet was washed extensively with phosphate-buffered saline containing 0.1 % (v/v) Tween-20, and then incubated in blocking buffer containing horseradish peroxidase-conjugated swine anti-rabbit antibodies (1.3 μ g/ml, final concentration) for another 1 h. The nitrocellulose sheet was rewashed extensively in phosphate-buffered saline containing 0.1 % (v/v) Tween-20 and the peroxidase reaction was performed using a freshly prepared solution of 0.15 mM-3,3'-diaminobenzidine/0.02 % H₂O₂, and then thoroughly washed in water. The nitrocellulose sheet was then carefully dried with tissue paper and exposed to Kodak X-AR film.

Phosphopeptide maps

Phosphopeptide maps of the proteins of interest were performed as described by Cleveland *et al.* (1977), with modifications according to Dunkley *et al.* (1986).

RESULTS

ANP stimulates phosphorylation of a 132 kDa protein (P132) in the particulate fraction of cultured rat aortic smooth muscle cells via the cyclic GMP-dependent protein kinase (Sarcevic *et al.*, 1989). To determine if this phosphoprotein is MLCK, we partially purified and characterized MLCK from rat aorta. During all stages of the purification, MLCK activity was assayed in the absence or presence of calmodulin, to show that the enzyme was dependent on calmodulin for activity. The major peak of calmodulin-dependent MLCK activity eluted from the cation-exchange column (Mono-S) at approx. 180 mM-NaCl, with a minor peak at 115 mM-NaCl (Fig. 1a). Elution of MLCK activity from the anion-exchange column (Mono-Q) yielded a single peak of calmodulin-dependent activity at 320 mM-NaCl (Fig. 1b). Analysis of the substrate specificity of the partially purified MLCK shows that it was dependent on calmodulin for activity and phosphorylated myosin light chains (MLC) and a synthetic analogue peptide substrate, corresponding to [Ala^{14,15}]MLC-(11–23)-peptide (Kemp & Pearson, 1985) (Table 1). The velocity obtained with the synthetic peptide was 13-fold lower than with the parent protein, which is the same as the difference observed using chicken gizzard MLCK (Kemp &

Fig. 1. Purification of rat aortic MLCK using ion-exchange chromatography

(a) The active fractions eluted from the Affi-gel Blue column were combined, dialysed in Mono-S buffer and loaded on to a Mono-S column. The MLCK was eluted with Mono-S buffer using a 0–500 mM linear NaCl gradient over 60 min at 1 ml/min. Each 1 ml fraction was assayed for MLCK activity as described in the Materials and methods section in the presence (Δ) or absence (□) of calcium/calmodulin. (b) The active fractions of MLCK eluted from the Mono-S column (fractions 8–28) were combined, dialysed in Mono-Q buffer and loaded on to a Mono-Q column. The MLCK was eluted with a 0–500 mM linear NaCl gradient over 60 min at 1 ml/min. Each 1 ml fraction was assayed for MLCK activity as described in the Materials and methods section in the presence (Δ) or absence (□) of calcium/calmodulin.

Pearson, 1985). On the other hand, a synthetic analogue peptide corresponding to the N-terminal portion of glycogen synthase, [Ala^{9,10}]glycogen synthase-(1–10)-peptide, which is a known substrate of several protein kinases, including the multifunctional calmodulin-dependent protein kinase (Pearson *et al.*, 1985), was relatively poorly phosphorylated (Table 1). These results distinguish the kinase activity from other calmodulin-dependent protein kinases such as the multifunctional calmodulin-dependent protein kinase that phosphorylates [Ala^{9,10}]glycogen synthase-(1–10) at a 5-fold faster rate over [Ala^{14,15}]MLC-(11–23). PAGE and silver staining of partially purified rat aortic MLCK yielded several bands, with a major band migrating at 145 kDa (Fig. 2a). Immunoblot analysis with a polyclonal antiserum raised against bovine tracheal MLCK was used to determine if the 145 kDa protein was MLCK. This antiserum cross-reacted with purified chicken gizzard 132 kDa MLCK and with the 145 kDa protein (Fig. 3). Since MLCK is a known substrate of cyclic GMP-dependent protein kinase (Nishikawa *et al.*, 1984), we sought to determine if the 145 kDa protein is phosphorylated by this

Table 1. Substrate specificity of purified rat aortic MLCK

MLCK activity was assayed in a volume of 40 μ l containing 40 mM-Hepes, pH 7.0, 5 mM-magnesium acetate, 0.25 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (500 c.p.m./pmol) and 0.55 mM- CaCl_2 , with the substrate as indicated in the Table, in the presence of either 1 mM-EGTA or 50 μ g of calmodulin/ml. Myosin light chains were purified from chicken gizzards as described in the Materials and methods section and the concentration of the phosphorylatable 20 kDa form in the assay was 33 μ M. The sequence of the myosin light chain (MLC) peptide analogue, corresponding to residues 11–23 of MLC with alanine residues at positions 14 and 15, is KKRAARATSNVFA (Kemp & Pearson, 1985). The sequence of the glycogen synthase (GS) peptide analogue, corresponding to residues 1–10 of glycogen synthase with alanine residues at positions 9 and 10, is PLSRTLVA (Pearson *et al.*, 1985). The assay was performed for 10 min at 30 °C and terminated as described in the Materials and methods section, and the activity is expressed as pmol of phosphate transferred from ATP to the substrate per min.

Substrate	Protein kinase activity (pmol of P_i /min)	
	EGTA	calmodulin
Myosin light chains (33 μ M)	0	38.4
MLC peptide (150 μ M)	0	2.87
GS peptide (150 μ M)	0.27	0.67

kinase. Incubation of the partially purified MLCK preparation with the cyclic GMP-dependent protein kinase resulted in the phosphorylation of only the 145 kDa protein and autophosphorylation of the 80 kDa cyclic GMP-dependent protein kinase (Figs. 2b and 4). Furthermore, incubation of the partially purified MLCK preparation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ also resulted in the phosphorylation of only the 145 kDa protein, and addition of calcium/calmodulin inhibited 145 kDa-protein phosphorylation, consistent with this protein binding calcium/calmodulin and thereby inhibiting its autophosphorylation (Fig. 4). On the basis of its cross-reactivity with antibody to MLCK, phosphorylation by cyclic GMP-dependent protein kinase and inhibition of phosphorylation by addition of calcium/calmodulin, the 145 kDa protein is identified as MLCK.

These results indicate that rat MLCK and P132 are distinct proteins. It is unlikely that P132 is a proteolysed form of the 145 kDa MLCK, since phosphopeptide maps generated by partial digestion with *S. aureus* V8 protease following P132 phosphorylation by cyclic GMP-dependent protein kinase were different to those obtained with MLCK. The most prominent and characteristic P132 phosphopeptide was of 33 kDa, and this is not present in rat aortic MLCK phosphorylated by cyclic GMP-dependent protein kinase (Fig. 5). Furthermore, a polyclonal antiserum raised against bovine tracheal MLCK cross-reacted with both the purified chicken gizzard 132 kDa MLCK and the partially purified 145 kDa rat aortic MLCK, as well as with a 145 kDa protein in the rat aortic smooth muscle cell particulate fractions, but not with P132 in these fractions (Fig. 3). These results indicate that P132 is not a proteolytic fragment of the native MLCK.

Caldesmon is another major smooth muscle cell phosphoprotein with an apparent size of 130–140 kDa, whereas the non-muscle form is approx. 75–77 kDa (Bryan *et al.*, 1989). It was of interest to determine the relationship between P132 and caldesmon. Monoclonal antisera to chicken gizzard caldesmon cross-reacted with a protein of 136 kDa and also with a band at 76 kDa in the particulate fraction of cultured rat aortic smooth muscle cells, suggesting that these cells synthesize both forms of

Fig. 2. Migration of partially purified rat aortic MLCK on SDS/PAGE and comparison of phosphorylation of partially purified rat aortic MLCK, chicken gizzard MLCK and P132 by cyclic GMP-dependent protein kinase

(a) Rat aortic MLCK (rMLCK) was purified as described in the Materials and methods section, electrophoresed on a 7.5–20% polyacrylamide gel and silver-stained. (b) Autoradiograph representing phosphorylation of P132 and MLCK. Cultured aortic smooth muscle cell particulate fractions were phosphorylated for 1 min at 0 °C as described in the Materials and methods section in the absence (lane 1) or presence (lane 2) of 1 μ M-cyclic GMP. Lanes 3–6 represent ^{32}P -labelled proteins phosphorylated by 1.5 μ g of purified cyclic GMP-dependent protein kinase: 1.5 μ g of cyclic GMP-dependent protein kinase autophosphorylation (lane 3), 1.5 μ g of cyclic GMP-dependent protein kinase plus purified rat aortic MLCK (lane 4), 1.5 μ g of cyclic GMP-dependent protein kinase plus 1 μ g of purified chicken gizzard caldesmon (lane 5), or 1.5 μ g of cyclic GMP-dependent protein kinase plus 1 μ g of purified chicken gizzard MLCK (lane 6). The positions of standard molecular mass marker proteins are indicated on the right in kDa.

Fig. 3. Immunoblot analysis of MLCK

(a) Immunoblotting with antibodies to bovine tracheal MLCK of proteins separated on a 7.5–20% polyacrylamide gel. Lanes 1 and 2 contain rat aortic smooth muscle cell particulate fractions which were phosphorylated for 1 min at 0 °C under standard reaction conditions in the absence (lane 1) or presence (lane 2) of 1 μ M-cyclic GMP and then immunoblotted. Lane 3 contains 1 μ g of purified chicken gizzard MLCK and lane 4 contains purified rat aortic MLCK. The positions of the rat aortic MLCK and P132 are indicated with arrows at 145 kDa and 132 kDa respectively. (b) The position of P132 was confirmed by autoradiography of the nitrocellulose and is indicated with an arrow at 132 kDa.

caldesmon (Fig. 6). Heating the smooth muscle cell particulate fraction for 5 min at 90 °C, a procedure which has previously been reported to result in the extraction of particulate caldesmon into the soluble fraction (Bretscher, 1984), resulted in the appearance of bands at 136 kDa and 76 kDa in the particulate as

Fig. 4. Autophosphorylation of rat aortic MLCK

Purified rat aortic MLCK was phosphorylated for 5 min at 37 °C as described in the Materials and methods section in the absence (lane 1) or presence (lane 2) of CaCl_2 (200 μM) plus calmodulin (25 $\mu\text{g}/\text{ml}$). Lane 3 represents phosphorylation of rat aortic MLCK by 1.5 μg of cyclic GMP-dependent protein kinase. The position of rat aortic MLCK and cyclic GMP-dependent protein kinase are indicated on the right with arrows as 145 and 80 kDa respectively. The positions of standard molecular mass marker proteins are indicated on the left in kDa.

Fig. 5. Phosphopeptide map analysis of P132 and rat aortic MLCK phosphorylated by the cyclic GMP-dependent protein kinase

Smooth muscle cell particulate fractions were phosphorylated for 1 min at 0 °C in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (12.5 Ci/mmol) in the absence or presence of 1 μM cyclic GMP and 1 μg of cyclic GMP-dependent protein kinase. Purified rat aortic MLCK was phosphorylated with purified cyclic GMP-dependent protein kinase (1 μg) and 1 μM -cyclic GMP in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (8.3 Ci/mmol) for 30 min at 37 °C. The proteins were then separated on SDS/polyacrylamide gels and the positions of P132 and phosphorylated rat aortic MLCK were determined by autoradiography. These bands were cut out, digested with *S. aureus* V8 protease and run on the second gel to generate a phosphopeptide map. The autoradiograph of the second gel is shown with phosphopeptides derived from P132 phosphorylated in the absence (lane 1) and presence of cyclic GMP-dependent protein kinase and cyclic GMP (lane 2) or rat aortic MLCK phosphorylated by cyclic GMP-dependent protein kinase (lane 3). The major phosphopeptides in P132 and rat aortic MLCK phosphorylated by cyclic GMP-dependent protein kinase are indicated on the right. The positions of standard molecular mass marker proteins are indicated on the left.

Fig. 6. Immunoblot analysis of heat-treated smooth muscle cell particulate fractions

Smooth muscle cell particulate fractions were heated for 5 min at 90 °C, chilled on ice and centrifuged at 20000 g for 30 min. The heat-treated soluble and particulate fractions were then dissolved in 90 μl of SDS buffer and subjected to SDS/PAGE on a 7.5–15% acrylamide gradient. The proteins were then transferred to nitrocellulose and immunoblotted with antibodies to chicken gizzard caldesmon. Lane 1 contains smooth muscle cell particulate fractions not subjected to heat treatment, and lane 2 contains the particulate fraction and lane 3 the soluble fraction after heat treatment. The positions of the 136 and 76 kDa bands are indicated on the right. The positions of standard molecular mass marker proteins are indicated on the left.

Fig. 7. Autoradiogram of heat-treated smooth muscle cell particulate fractions

Smooth muscle cell particulate fractions were phosphorylated for 1 min at 37 °C in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The samples were either frozen before electrophoresis or heated for 5 min at 90 °C, chilled on ice and centrifuged at 20000 g for 30 min. The heat-treated soluble and particulate fractions were then subjected to SDS/PAGE on a 7.5–15% acrylamide gradient. (a) Smooth muscle cell particulate fractions phosphorylated with no heat treatment in the presence of 1.5 μg of purified cyclic GMP-dependent protein kinase and 1 μM -cyclic GMP (lane 1) or 1 μM -cyclic GMP alone (lane 2) (lane 3, control). (b) Particulate (lanes 1–3) or soluble (lanes 4–6) fractions after heating at 90 °C for 5 min prephosphorylated in the presence of 1.5 μg of purified cGK plus 1 μM cyclic GMP (lanes 1 and 4) or 1 μM -cyclic GMP alone (lanes 2 and 5). Lanes 3 and 6 are controls. The position of P132 is indicated. The position of the 116 kDa standard molecular mass protein is shown on the left.

well as the soluble fractions upon Western blotting (Fig. 6). This behaviour is distinct from that of P132, which was not extracted into the soluble fraction after heating at 90 °C for 5 min (Fig. 7). Furthermore, the monoclonal antisera to chicken gizzard caldesmon cross-reacted with purified chicken gizzard caldesmon of 141 kDa and with a protein of 136 kDa in the particulate fraction of cultured rat aortic smooth muscle cells, but not with P132 (Fig. 8). Finally, purified chicken gizzard caldesmon is not phosphorylated by cyclic GMP-dependent protein kinase under conditions where rat aortic and chicken gizzard MLCK are phosphorylated, indicating that caldesmon is apparently not a substrate of cyclic GMP-dependent protein kinase (Fig. 2b). Based on these criteria, P132 is not caldesmon.

Fig. 8. Immunoblot analysis of caldesmon

(a) Immunoblotting with antibodies to chicken gizzard caldesmon of proteins separated on a 7.5–20% polyacrylamide gel. Lane 1 contains 3 μ g of purified chicken gizzard caldesmon, which is indicated with an arrow (141 kDa). Lanes 2 and 3 contain rat aortic smooth muscle cell particulate fractions which were phosphorylated under standard reaction conditions in the absence or presence respectively of 1 μ M-cyclic GMP and then immunoblotted. The positions of the rat aortic caldesmon and P132 are indicated (136 kDa and 132 kDa respectively). (b) The position of P132 in lane 2 was confirmed by autoradiography of the nitrocellulose.

DISCUSSION

In contrast with smooth muscle contraction, the biochemical mechanism of smooth muscle relaxation is not well characterized. In general, however, lowering of the cytoplasmic concentration of Ca^{2+} is invariably associated with smooth muscle relaxation (Lincoln, 1983b). We have previously shown that ANP-stimulated phosphorylation of three proteins of 225, 132 and 11 kDa in the particulate fraction of cultured smooth muscle cells is mediated by cyclic GMP-dependent protein kinase (Sarcevic *et al.*, 1989). Furthermore, the 11 kDa protein was identified as phospholamban, suggesting that one of the ways in which ANP may mediate relaxation in smooth muscle is by decreasing the cytoplasmic concentration of Ca^{2+} via phosphorylation of phospholamban. Observations that phosphorylation of MLCK *in vitro* by cyclic AMP-dependent protein kinase decreases MLCK activity by increasing the concentration of calmodulin required for half-maximal activation of the enzyme led to the concept that phosphorylation of MLCK may be important in smooth muscle relaxation (Adelstein *et al.*, 1978; Conti & Adelstein, 1981). However, the physiological significance of MLCK phosphorylation by the cyclic AMP-dependent protein kinase has been questioned (Kamm & Stull, 1989). MLCK has also been shown to be a substrate of cyclic GMP-dependent protein kinase (Nishikawa *et al.*, 1984), and Vrolix *et al.* (1988) have suggested that a 130 kDa smooth muscle cyclic GMP-dependent protein kinase substrate may be MLCK. These observations raised the possibility that smooth muscle relaxation may in part be due to inhibition of MLCK activity via phosphorylation by cyclic GMP-dependent protein kinase. However, cyclic GMP-dependent protein kinase phosphorylation of bovine tracheal MLCK *in vitro* is not accompanied by changes in enzyme activity (Nishikawa *et al.*, 1984).

Although chicken gizzard MLCK was phosphorylated by cyclic GMP-dependent protein kinase and co-migrated with P132 on SDS/polyacrylamide gels, partially purified rat aortic MLCK, which was also phosphorylated by cyclic GMP-dependent protein kinase, was significantly larger (145 kDa), in agreement with earlier studies which show that the mammalian MLCK is larger than avian MLCK (Yamazaki *et al.*, 1987). Phosphopeptide maps using *S. aureus* V8 protease were performed on purified rat aortic MLCK and P132. The phosphopeptide maps from rat aortic MLCK and P132 were distinct, suggesting, but not proving, that these two proteins are unrelated. Moreover, antibodies to bovine tracheal MLCK cross-reacted with purified

chicken gizzard and rat aortic MLCK and with the 145 kDa MLCK in the particulate fraction, but not with P132 in the smooth muscle cell particulate fraction. This result confirms that P132 is not MLCK. P132 is unlikely to be a proteolytic breakdown product of MLCK generated during preparation of the smooth muscle cell particulate fractions, since phosphopeptide maps did not reveal any similarity with rat aortic MLCK, nor did immunoblots detect any 132 kDa forms of rat aortic MLCK.

A cyclic GMP-dependent protein kinase substrate of 130 kDa has been reported in aortic smooth muscle from rabbit (Parks *et al.*, 1987) and also in other smooth muscles such as guinea pig uterus (Casnellie & Greengard, 1974), rabbit intestine (Wallach *et al.*, 1978) and bovine tracheal smooth muscle (Hogaboom *et al.*, 1982), and it is likely that they are related to P132. Our finding that P132 is not MLCK is in apparent contrast with the suggestion that the 130 kDa protein from pig aorta or stomach, which was phosphorylated by cyclic GMP-dependent protein kinase, may be MLCK on the basis of cross-reactivity with antibodies to chicken gizzard MLCK and of the inability of cyclic AMP-dependent protein kinase to phosphorylate this protein in the presence of Ca^{2+} /calmodulin (Vrolix *et al.*, 1988).

Caldesmon is a major smooth muscle protein that inhibits actomyosin ATPase activity, and phosphorylation of this protein is generally correlated with muscle contraction (Ngai & Walsh, 1987; Adam *et al.*, 1989; Kamm & Stull, 1989). The apparent size of caldesmon from smooth muscle tissues analysed by SDS/PAGE varies between duck gizzard (140 kDa) (Vorotnikov *et al.*, 1988), chicken gizzard (141 kDa) (Ngai & Walsh, 1985), porcine carotid arteries (145 kDa) (Adam *et al.*, 1989) and bovine carotid artery (148 kDa) (Yamazaki *et al.*, 1987), whereas the non-muscle form is 75–77 kDa (Bryan *et al.*, 1989). In view of the similar size of smooth muscle caldesmon to P132, we investigated whether caldesmon is phosphorylated by the cyclic GMP-dependent protein kinase. Purified chicken gizzard caldesmon was not phosphorylated by cyclic GMP-dependent protein kinase, suggesting that the rat aortic form of this protein is not a substrate of this kinase. Antibodies to chicken gizzard caldesmon cross-reacted with proteins of 136 kDa and 76 kDa in the particulate fraction of cultured rat aortic smooth muscle cells, and with purified chicken gizzard caldesmon of 141 kDa, but not with P132. The cross-reactivity of the antibody to a 76 kDa band in the smooth muscle cell particulate fraction suggests that smooth muscle cells in culture synthesize the lower molecular mass non-muscle form as well as the larger muscle form of caldesmon. Furthermore, heating of the smooth muscle cell particulate fractions at 90 °C for 5 min, a procedure previously reported to extract particulate caldesmon into the soluble fraction, resulted in the partial extraction of the 136 and 76 kDa proteins but not of P132 into the soluble fraction. Therefore, based on these criteria, P132 is not caldesmon.

In conclusion, we have demonstrated that a 132 kDa phosphoprotein in the particulate fraction of smooth muscle cells, whose phosphorylation is stimulated by ANP and mediated by cyclic GMP-dependent protein kinase, is neither MLCK nor caldesmon. P132 may therefore be a novel protein whose phosphorylation may contribute to the mechanism of smooth muscle relaxation.

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