Isolation of two myosin light-chain kinases from bovine carotid artery and their regulation by phosphorylation mediated by cyclic AMP-dependent protein kinase

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Myosin light-chain kinase was purified from bovine carotid artery. Approx. 90% of myosin kinase was extracted in the supernatant fraction with buffer containing EDTA during myofibril preparation. The soluble fraction yielded two distinct peaks on DEAE-Sephacel chromatography. Peak I was eluted at a conductance of 11–12 mmho and was completely dependent on Ca^{2+} –calmodulin for its activity. Peak II was eluted at a conductance of 13–14 mmho and showed approx. 15% Ca^{2+} -independent activity. The myosin kinases I and II were further purified by affinity chromatography by using calmodulin coupled to Sepharose 4B, which resulted in 960- and 650-fold purification of type I and type II kinases respectively. Myosin kinase II activity was completely Ca^{2+} -dependent after affinity chromatography on the calmodulin–Sepharose column. Myosin kinases I and II were phosphorylated by cyclic AMP-dependent protein kinase. In the presence of bound calmodulin 0.5–0.7 mol of phosphate was incorporated/mol of myosin kinases I and II. On the other hand, in the absence of bound calmodulin 1–1.4 mol of phosphate was incorporated/mol of kinases I and II. Phosphorylation in the absence of calmodulin significantly decreased the myosin kinase activity of both enzymes, and the decrease in myosin kinase activity was due to a 3–5-fold increase in the amount of calmodulin required for half-maximal stimulation of both type I and type II kinases. The regulation of myosin kinase activity by cyclic AMP-dependent phosphorylation would suggest that β -adrenergic-mediated relaxation of vascular smooth muscle may be partly due to the direct interaction of cyclic AMP at the site of contractile proteins.

Myosin light-chain kinase has been isolated from skeletal muscle (Pires & Perry, 1977), cardiac muscle (Walsh *et al.*, 1979; Wolf & Hofmann, 1980), gizzard smooth muscle (Dabrowska *et al.*, 1977; Adelstein & Klee, 1981) and other non-muscle tissues (Hathaway & Adelstein, 1979; Hathaway *et al.*, 1981). However, no attempts have been made to isolate and characterize this enzyme from vascular smooth muscle. Considerable biochemical evidence has accumulated suggesting the possibility that a reversible phosphorylation–dephosphorylation of the 20000-Da myosin light chain is involved in the regulation of smooth-muscle contraction (Small & Sobieszek, 1977; Sobieszek, 1977; Chako *et al.*, 1977; Sherry *et al.*, 1978). The alternative viewpoints are that regulation is achieved without phosphorylation of myosin and is under the control

of a system termed Leiotonin (Ebashi *et al.*, 1977; Mikawa *et al.*, 1978), and troponin control, a possibility raised most recently by Marston *et al.* (1980). In spite of these controversies, most investigators consider that myosin phosphorylation is at least part of the mechanism for the regulation of vertebrate smooth-muscle contraction. More recently, Ca^{2+} -dependent phosphorylation of 20000-Da myosin light chains has been demonstrated in intact arterial smooth muscle (Barron *et al.*, 1979) and in native actomyosin isolated from bovine aorta (DiSalvo *et al.*, 1978). A relationship between light-chain phosphorylation and the contraction cycle of pig carotid artery has been shown by Dillon *et al.* (1981) and Driska *et al.* (1981). The light-chain phosphorylation preceded tension development, suggesting that the first event of contraction is the phosphorylation of the light chain and the second event is the interaction of actin and myosin. Light-

Abbreviation used: SDS, sodium dodecyl sulphate.

chain phosphorylation has also been correlated with the shortening velocity of arterial smooth muscle. Thus Barron *et al.* (1980) have shown in KCl-contracted arterial strips that light-chain phosphorylation-dephosphorylation and rephosphorylation occurred in concert with contraction, followed by relaxation, followed by a second contraction of muscle.

Silver & DiSalvo (1979) have suggested involvement of cyclic AMP at the level of contractile proteins in vascular smooth muscle. These authors have shown that Ca^{2+} -dependent phosphorylation of myosin light chains in native actomyosin, isolated from vascular smooth muscle, is considerably inhibited in the presence of cyclic AMP and cyclic AMP-dependent protein kinase. This depression of light-chain phosphorylation was associated with an inhibition of actomyosin ATPase activity, with a concomitant phosphorylation of a 100 000-Da protein, presumed to be myosin light-chain kinase. On the other hand, Bárány & Bárány (1981) have reported no increase in the phosphorylation of any protein in the range of 100 000–140 000 Da after relaxation of pig carotid arteries under many different conditions. Although the findings of Silver & DiSalvo (1979) suggest a possible role of cyclic AMP-dependent myosin kinase phosphorylation in the modulation of actin-myosin interactions in vascular smooth muscle, no direct information is available on the cyclic AMP-dependent phosphorylation of purified myosin kinase and its effect on Ca^{2+} -calmodulin-dependent enzyme activity. In order to understand the role of cyclic AMP-dependent protein kinase in vascular smooth-muscle relaxation, it is necessary to isolate myosin kinase from this tissue and study its regulation by cyclic AMP-dependent protein kinase. In this paper, we report the isolation and purification of two types of myosin kinases separated by ion-exchange chromatography on DEAE-Sephacel from bovine carotid artery and their phosphorylation by the catalytic subunit of bovine cardiac cyclic AMP-dependent protein kinase.

Materials and methods

[γ - ^{32}P]ATP was obtained from New England Nuclear. Histone II AS, bovine serum albumin, catalytic subunit of protein kinase, protein kinase inhibitor and CNBr-activated Sepharose 4B were purchased from Sigma Chemical Co. Molecular-weight marker kits for polyacrylamide-gel electrophoresis were purchased from Pharmacia Fine Chemicals. All other chemicals were of analytical grade. Proteolysis inhibitors were obtained from the following sources: pepstatin A and leupeptin were from Vega Biochemicals; phenylmethanesulphonyl fluoride, Tos-Phe- CH_2Cl (1-chloro-4-phenyl-3-L-tosylamido-

butan-2-one; 'TPCK') and soya-bean trypsin inhibitor were from Sigma.

Purification of proteins

Bovine brain calmodulin was isolated and purified by a modification of the procedure described by Teo *et al.* (1973) for the purification of bovine cardiac calmodulin, as described in detail by Sharma & Wang (1979).

Mixed myosin light chains used as substrate for myosin kinase were prepared from fresh bovine myocardium by the method of Blumenthal & Stull (1980). The resulting mixed light-chain fraction was free of detectable calmodulin, as judged by the fractions' inability to activate calmodulin-deficient brain phosphodiesterase.

Preparation of calmodulin-Sepharose

Calmodulin was coupled to CNBr-activated Sepharose 4B (Sigma) by the method of Klee & Krinks (1978). The resin was equilibrated with 40 mM-Tris/HCl (pH 7.6)/10 mM-2-mercaptoethanol/0.05 M-NaCl/0.5 mM- CaCl_2 and packed into a column 2.8 cm $\times$ 16 cm. The Sepharose contained approx. 1.5 mg of calmodulin/ml of packed resin. When not in use, the resin was stored in the presence of 0.05 mM- CaCl_2 and 0.02% NaN_3 at 4°C.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed on 7.5%-acrylamide gels as described by Weber & Osborn (1969).

Enzyme assay

Enzyme activity was determined by the rate of ^{32}P incorporation into bovine cardiac light chains. The assay medium (final volume 100 μl) contained 20 mM-Tris/HCl, pH 7.6, 20 mM- MgCl_2 , 2 mM-dithiothreitol, 0.2 mM-[γ - ^{32}P]ATP (100–200 c.p.m./pmol), and either 0.2 mM- CaCl_2 and 1 μM -calmodulin (unless otherwise mentioned) or 2 mM-EGTA. Reaction was initiated by addition of enzyme and incubated at 30°C for various periods of time. Reaction was terminated by pipetting 50 μl of the incubation mixture on to Whatman no. 3MM filter-paper discs (1.5 cm square), which were then washed as described by Corbin & Reimann (1975). For enzyme assays of fractions obtained during purification, 70 mM-NaF was included to inhibit phosphatase activity. The initial velocity of the enzyme reaction was determined in the presence or absence of Ca^{2+} for each fraction during purification by incubating 750 μl of reaction mixture at 30°C and withdrawing 50 μl samples at 1 min intervals to minimize possible interference by phosphatase activity.

Phosphorylation by catalytic subunit of protein kinase

Myosin light-chain kinase (150–200 µg/ml) was incubated at 30°C for 20 min in 40 mM-Tris/HCl, pH 7.6, containing 1 mM-dithiothreitol, 10 mM-MgCl₂, 0.2 mM-[γ-³²P]ATP, 20 µg of catalytic subunit of bovine cardiac cyclic AMP-dependent protein kinase/ml and EGTA or Ca²⁺, with or without calmodulin as noted in the text. To a portion (80 µl) of reaction mixture was added 2 ml of ice-cold 10% (w/v) trichloroacetic acid, and the mixture was filtered through Millipore filters (45 µm pore size) and counted for radioactivity in a liquid-scintillation spectrometer, in toluene-based scintillant. A second portion was diluted 4-fold with chilled 40 mM-Tris/HCl buffer, pH 7.6, and assayed immediately for myosin light-chain kinase activity in the presence of protein kinase inhibitor (Sigma) (20 µg/ml) with various concentrations of calmodulin (1 nM–1 µM).

Results

Purification of bovine carotid-artery myosin light-chain kinase

Homogenization. Bovine carotid arteries were obtained from the packing plant at Dubuque, IA. Arterial smooth muscle was freed from fat and loose connective tissue and kept in ice during the process of cleaning. After cleaning, the tissue was stored at –70°C until used. Frozen tissue (500 g) was thawed at 4°C in 3–4 vol. of chilled homogenizing buffer, containing 40 mM-Tris/HCl, pH 7.6, 10 mM-2-mercaptoethanol, 4 mM-EDTA, 2 mM-EGTA, 1 mM-MgCl₂, 75 mg of phenylmethanesulphonyl fluoride/litre, 100 mg of streptomycin sulphate/litre, 1 mg of leupeptin/litre, 1 mg of pepstatin A/litre, 10 mg of Tos-Phe-CH₂Cl/litre and 10 mg of soya-bean trypsin inhibitor/litre. After thawing, the arteries were cut into small pieces by scissors and homogenized in a commercial Waring blender at the top speed for 3 × 30 s pulses with 1 min intervals in between. The pH of the extract was adjusted to 7.6 by addition of 0.5 M-NaOH. The homogenate was centrifuged at 13 000 g for 30 min. The supernatant was filtered through glass wool. The myofibrillar pellet was re-extracted with 3 vol. of homogenizing buffer and the two supernatants were combined. The myofibrillar pellet was further extracted for 1 h with homogenizing buffer containing 60 mM-KCl and 25 mM-MgCl₂ as described for extraction of myosin light-chain kinase from chicken gizzard myofibrils by Adelstein & Klee (1981). This step resulted in the extraction of myosin light-chain kinase activity, which was approx. 10% of the activity present in the homogenate supernatant. These data suggest that most of the myosin light-chain kinase in vascular smooth muscle is in the soluble fraction. For further purification only the soluble enzyme was used.

Batchwise ion-exchange chromatography on DEAE-cellulose

DEAE-cellulose (500 g wet wt.) from Sigma, equilibrated with 40 mM-Tris/HCl (pH 7.6)/4 mM-EDTA / 2 mM-EGTA / 10 mM-2-mercaptoethanol / 0.1 mM-phenylmethanesulphonyl fluoride, was added slowly to the supernatant and mixed at very low speed for 60 min. The mixture was filtered in a Buchner funnel and washed with 8 litres of equilibration buffer containing 0.05 M-NaCl. The myosin light-chain kinase was eluted from DEAE-cellulose by incubating the resin for 60 min in 1 litre of equilibration buffer containing 0.3 M-NaCl. This mixture was filtered in a Buchner funnel and the resin was washed again with 2 × 500 ml of the buffer containing 0.3 M-NaCl. The eluate and two washings were combined and solid (NH₄)₂SO₄ was added slowly up to 60% saturation. The mixture was stirred gently for 60 min and centrifuged at 13 000 g for 30 min. The pellet was suspended by a hand-powered glass homogenizer in 400 ml of buffer containing 40 mM-Tris/HCl, pH 7.6, 4 mM-EDTA, 2 mM-EGTA, 10 mM-2-mercaptoethanol, 0.1 mM-phenylmethanesulphonyl fluoride and 0.05 M-NaCl, and dialysed against the same buffer for 16 h with two buffer changes of 8 litres each.

DEAE-Sephacel ion-exchange chromatography

The dialysed sample was centrifuged at 40 000 g for 20 min, and the clear supernatant was applied to a column (3.8 cm × 40 cm) of DEAE-Sephacel (Pharmacia) previously equilibrated with 40 mM-Tris / HCl (pH 7.6 / 4 mM-EDTA / 2 mM-EGTA / 10 mM-2-mercaptoethanol / 0.1 mM-phenylmethanesulphonyl fluoride/0.05 M-NaCl. The column was washed with 1 litre of equilibration buffer and eluted with a 2-litre linear gradient of NaCl (50–400 mM). Myosin kinase activity was eluted in two peaks, at conductances of 10–11 and 13–14 mmho (Fig. 1). Enzyme from both peaks required Ca²⁺ and calmodulin for maximal activity. Peak-I enzyme was found to be totally Ca²⁺-dependent, whereas peak-II enzyme when assayed in the presence of EGTA exhibited approx. 15% of the activity measured in the presence of Ca²⁺ and calmodulin (Fig. 1). The relative distribution of Ca²⁺-dependent myosin kinase activity between peaks I and II was 56% and 44% respectively. On the other hand, distribution on the basis of total enzyme activity (Ca²⁺-dependent plus independent) was 45% and 55% for peaks I and II respectively. Both peaks were pooled separately and dialysed overnight against two changes (6 litres each) of buffer containing 40 mM-Tris/HCl, pH 7.6, 4 mM-EDTA, 10 mM-2-mercaptoethanol, 0.2 mM-EGTA and 0.1 mM-phenylmethanesulphonyl fluoride.

Fig. 1. DEAE-Sephacel ion-exchange chromatography of dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction

The column ($3.8 \text{ cm} \times 40 \text{ cm}$) was equilibrated with 40 mM-Tris/HCl buffer ($\text{pH } 7.6$)/ 4 mM-EDTA / 2 mM-EGTA / $10 \text{ mM-2-mercaptoethanol}$ / $0.1 \text{ mM-phenylmethanesulphonyl fluoride}$ / 50 mM-NaCl , and to this was applied 500 ml of myosin kinase (6.5 mg/ml). The enzyme was eluted at a flow rate of 55 ml/h with a 2-litre linear NaCl gradient ($50\text{--}400 \text{ mM-NaCl}$) made up in the equilibration buffer. Portions ($20 \mu\text{l}$) of the 8 ml fractions were assayed for myosin light-chain kinase activity in the presence of 2 mM-EGTA ($\bigcirc$) or 0.5 mM-Ca^{2+} and $1 \mu\text{M-calmodulin}$ ($\bullet$) as described in the Materials and methods section. Δ , A_{280} (protein); $\square$, conductivity (mmho).

Table 1. Purification of bovine carotid-artery smooth-muscle myosin light-chain kinase

For details see the Results section. One unit is defined as the amount of enzyme that catalyses the incorporation of 1 nmol of ^{32}P into cardiac mixed light chains/min under the standard conditions described in the Materials and methods section.

Purification step	Total volume (ml)	Protein concn. (mg/ml)	Total protein (mg)	Activity/mg* (units/mg)	Total activity* (units)	Yield (%)	Purification (-fold)
Homogenate supernatant	3130	2.65	8295	0.13	1070	100	—
DEAE-cellulose batch 0.3 M-NaCl eluate	2200	1.78	3931	0.23	904	84	1.8
$(\text{NH}_4)_2\text{SO}_4$, $0\text{--}60\%$ satn.	500	6.50	3250	0.25	806	75	1.9
DEAE-Sephacel							
Type I	150	2.11	317	0.84	266	25	6.5
Type II	114	3.16	360	0.63	227	21 (46)	4.9
Calmodulin affinity chromatography							
Type I	9	0.17	1.50	124.0	186	17	961.0
Type II	9	0.25	2.25	84.0	189	18 (35)	651.0

* Activity presented here refers only to the Ca^{2+} -calmodulin-sensitive enzyme activity, which is obtained by subtracting the enzyme activity obtained with EGTA from the total activity obtained with Ca^{2+} and calmodulin.

Affinity chromatography on calmodulin-Sephacel

The dialysed peaks I and II obtained from the DEAE-Sephacel column were loaded separately on to a column of brain calmodulin coupled to Sepharose 4B previously equilibrated with 40 mM-Tris/HCl ($\text{pH } 7.6$)/ 4 mM-EDTA / $10 \text{ mM-2-mercaptoethanol}$ / 3 mM-MgCl_2 / 50 mM-NaCl . Just before chromatography, enough Ca^{2+} was added to the pooled peak fractions to give a final concentration of 0.5 mM-Ca^{2+} . The column was first washed with 120 ml of equilibration buffer, followed by 120 ml of equilibration buffer containing 0.2 M-NaCl . Finally the kinase was eluted with equilibration

buffer containing 0.2 M-NaCl in which Ca^{2+} was replaced by 7.5 mM-EGTA (Fig. 2). Fractions containing enzyme activity were pooled and dialysed against 40 mM-Tris/HCl ($\text{pH } 7.6$)/ 4 mM-EDTA / 2 mM-EGTA / $10 \text{ mM-2-mercaptoethanol}$ overnight, with two changes of 4 litres of buffer. A summary of the purification is shown in Table 1. Myosin kinases I and II were purified 960- and 650-fold respectively, with a yield of 17% for type I and 18% for type II.

DEAE-cellulose chromatography

Chromatography on the affinity column resulted in a large enrichment of enzyme activity; however, it

Fig. 2. Affinity chromatography of myosin light-chain kinases I and II on calmodulin-Sepharose

Myosin kinases I (150 ml, 2.11 mg/ml) (a) and II (114 ml, 3.16 mg/ml) (b) of the pooled peaks from DEAE-Sephacel chromatography were separately applied to a 60 ml column of calmodulin-Sepharose 4B equilibrated with 40 mM-Tris/HCl (pH 7.6)/50 mM-NaCl/0.5 mM-CaCl₂/3 mM-MgCl₂/10 mM-2-mercaptoethanol. The column was successively washed with 2 vol. of equilibrating buffer, followed by 2 vol. of the same buffer containing 200 mM-NaCl. Myosin light-chain kinase was eluted with 7.5 mM-EGTA in the buffer containing 200 mM-NaCl. Fractions (10 μ l) of myosin kinase were assayed by using cardiac mixed myosin light chains in the presence of 1 μ M-calmodulin and 0.5 mM-Ca²⁺ (●) or 2 mM-EGTA (O) as described in the Materials and methods section. Δ , A_{260} (protein).

chromatography (Wolf & Hofmann, 1980). Data presented in Fig. 3 show the elution profiles of myosin kinases I and II on a DEAE-cellulose column; most of the protein peak corresponded to the enzyme activity. A slight contamination of peak I by peak II, which could have remained after DEAE-Sephacel chromatography, was separated by this step (Fig. 3). This was further confirmed by SDS/polyacrylamide-gel electrophoresis.

Second calmodulin-Sepharose affinity chromatography

In order to remove non-specific contamination, which could have remained after the first affinity chromatography, pooled active kinase fractions of peaks I and II from the DEAE-cellulose step were applied to the affinity column as described above. Active fractions eluted with 40 mM-Tris/HCl (pH 7.6)/10 mM-2-mercaptoethanol/200 mM-NaCl/7.5 mM-EGTA were pooled, and dialysed against 40 mM-Tris/HCl (pH 7.4)/1 mM-EGTA/2 mM-EDTA/1 mM-dithiothreitol. Enzyme was concentrated to 0.25 mg of protein/ml and stored at -70°C until used. No attempts were made to calculate the purification (fold) achieved by DEAE-cellulose column chromatography and the second affinity chromatography. However, SDS/polyacrylamide-gel electrophoresis revealed removal of minor contaminations from both types I and II of myosin kinase by these additional steps.

Molecular weights of myosin kinases I and II

Myosin kinases I and II obtained after the second affinity column were subjected to SDS/polyacrylamide-gel electrophoresis on 7.5%-acrylamide gels. For type I enzyme a single strongly staining band and two very faintly staining bands were observed. However, with the type II enzyme, two bands of equal intensity and two very minor bands were observed (Fig. 4). Storage of either enzyme at 4°C for up to 3 weeks in EGTA or Ca²⁺ buffer did not alter the SDS/polyacrylamide-gel pattern, suggesting no proteolytic degradation of enzymes during this period. The molecular weight of type I was 66 000, whereas type II showed two bands, with molecular weights of 79 000 and 67 000 (Fig. 4). At present it is not clear which of these two bands is associated with the myosin kinase activity. These two proteins could not be separated by ion-exchange chromatography on DEAE-cellulose (Fig. 3). Further work is needed to separate these two moieties and characterize their properties.

Phosphorylation of myosin kinases I and II by cyclic AMP-dependent protein kinase

Conti & Adelstein (1981) have shown that gizzard smooth-muscle myosin kinase is phosphorylated by cyclic AMP-dependent protein kinase.

had been shown previously (Klee & Krinks, 1978) that the calmodulin affinity column binds several other calcium-binding proteins besides myosin kinase, which could be removed by ion-exchange

Fig. 3. Separation of myosin light-chain kinase from other calmodulin-binding proteins on a DEAE-cellulose column. The pooled and dialysed fractions of myosin kinases I (a) and II (b) from calmodulin affinity chromatography were applied separately to a DEAE-cellulose column (1 cm $\times$ 16 cm) previously equilibrated with 40 mM-Tris/HCl (pH 7.6)/4 mM-EDTA/2 mM-EGTA/1 mM-MgCl₂/10 mM-2-mercaptoethanol/50 mM-NaCl. The enzyme was eluted at a flow rate of 20 ml/h with a 250 ml linear NaCl gradient (50–400 mM) made up in the equilibrium buffer. Portions (10 μ l) of the 2 ml fractions were assayed for the kinase activity in the presence of 0.5 mM-Ca²⁺ and 1 μ M-calmodulin (●) or 2 mM-EGTA (○) as described in the Materials and methods section. Δ , A_{280} (protein); $\square$, conductivity (mmho).

ase and that sites phosphorylated are either two (in the absence of bound calmodulin) or one (in the presence of bound calmodulin). Further, phosphorylation of myosin kinase only in the absence of bound calmodulin results in 2–3-fold decrease in

enzyme activity at lower concentration of calmodulin during assay. To test whether similar regulation of myosin kinase by cyclic AMP-dependent protein kinase exists in vascular smooth muscle, attempts were made to phosphorylate type I and II

Fig. 4. Determination of subunit molecular weight of myosin light-chain kinases (MLCK) I and II

Myosin kinases I and II (approx. 4–5 μg) and various molecular-weight marker proteins (S) (6–14 μg each) were subjected to electrophoresis on 7.5%-acrylamide gels (120 mm $\times$ 5 mm) in the presence of 0.1% SDS as described in the Materials and methods section. Gels were stained by Coomassie Brilliant Blue R250 by diffusion staining for 24 h, and then destained. The marker proteins used were: 1, phosphorylase *b*; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase; 5, soya-bean trypsin inhibitor; 6, lactalbumin. Mobility is relative to that of Bromophenol Blue.

kinases with the catalytic subunit of cyclic AMP-dependent protein kinase as described in the Materials and methods section. The phosphorylation of myosin kinases I and II in the absence of catalytic subunit of cyclic AMP-dependent protein kinase was negligible in the presence of either EGTA or Ca^{2+} plus calmodulin. However, addition of catalytic subunit of protein kinase (20 $\mu\text{g}/\text{ml}$) in the presence of Ca^{2+} and calmodulin resulted in incorporation of approx. 0.6 mol and 0.5 mol of phosphate per mol of type I and type II kinase respectively. On the other hand, in the presence of EGTA, the phosphate incorporation was 1.2 mol and 1 mol per mol of enzyme for myosin kinases I and II respectively. The molecular weights of type I and type II myosin kinases for these calculations were taken as 66 000 and 79 000 respectively. These data suggest that the rate of phosphate incorporation in the presence of EGTA was approximately twice that in the presence of bound calmodulin. These observations are qualitatively similar to those reported for gizzard smooth muscle (Conti & Adelstein, 1981).

Effect of myosin light-chain kinase phosphorylation on enzyme activity and affinity for calmodulin

Myosin kinase types I and II, either phos-

phorylated by catalytic subunit of cyclic AMP-dependent protein kinase in the presence of EGTA or unphosphorylated (control), were assayed for enzyme activity at concentrations of 70 nM-type I and 100 nM-type II over a range of calmodulin concentrations of 1 nM–1 μM . Data presented in Fig. 5 show that phosphorylation of both myosin kinases I and II resulted in a significant decrease in the affinity of kinase for calmodulin. When these data were analysed by double-reciprocal plots, the calmodulin concentration required for half-maximal stimulation of isoenzyme I phosphorylated and unphosphorylated was 55.5 nM and 10.5 nM, respectively. The values for myosin kinase II phosphorylated and unphosphorylated were 36.2 nM and 14.6 nM, respectively. These data suggest a 3–5-fold increase in the calmodulin concentration required for half-maximal stimulation of phosphorylated myosin light-chain kinase activity. In contrast with the differences in the affinity of the phosphorylated and unphosphorylated forms of myosin kinases I and II for calmodulin, phosphorylation did not affect the maximal rate of kinase activity for kinases I and II. However, at low calmodulin concentration (10–25 nM), activity of phosphorylated myosin kinases I and II was considerably decreased (Fig. 5). The rate at which myosin kinases I and II phosphorylated

Fig. 5. Effect of phosphorylation on the affinity of myosin kinases I and II for calmodulin

Myosin kinases I (a) and II (b) were phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase as described in the Materials and methods section. The kinase activity of phosphorylated enzyme (O) and non-phosphorylated control (●) was immediately assayed in the presence of different concentrations of calmodulin and a fixed Ca^{2+} concentration (0.5 mM), by using myocardial mixed light chains free of calmodulin as substrate. Arrows indicate the quantity of calmodulin required for half-maximal stimulation of myosin kinase(s).

bovine cardiac mixed light chains was 0.124 and 0.084 $\mu\text{mol/min per mg}$ of protein respectively. Myosin kinases I and II did not phosphorylate histone II AS.

Discussion

Two myosin light-chain kinases which specifically phosphorylated cardiac myosin light chains have been isolated from bovine carotid arteries.

Studies on the localization of myosin kinase revealed that more than 90% of the enzyme was extracted in the supernatant fraction with buffer containing EDTA during myofibril preparation. Further extraction of washed myofibrils with 25 mM-MgCl₂ and 60 mM-KCl in Tris buffer by the procedure of Adelstein & Klee (1981) yielded less than 10% of the total enzyme activity. Similar results on myosin kinase localization have been reported for bovine myocardium (Walsh *et al.*, 1979), human platelets (Hathaway & Adelstein, 1979) and rabbit skeletal muscle (Pires & Perry, 1977). On the other hand, myosin kinase in chicken (Dabrowska *et al.*, 1977) and turkey (Adelstein & Klee, 1981) gizzard smooth muscle was found to be associated with myofibrils, which could not be extracted with EDTA buffers.

The soluble fraction of bovine carotid artery on DEAE-Sephacel chromatography yielded two distinct peaks. Peak I was eluted at a conductance of 11–12 mmho and was completely dependent on Ca^{2+} and calmodulin for its activity. Peak II was eluted at a conductance of 13–14 mmho and showed approx. 15% Ca^{2+} -independent activity. However, further purification of isoenzyme II on a calmodulin-Sepharose 4B affinity column resulted in isolation of the enzyme which was completely dependent on Ca^{2+} and calmodulin for its activity. Walsh & Guilleux (1981) have isolated two myosin light-chain kinases from bovine heart by chromatography on DEAE-Sepharose CL6B. On the other hand, previous studies have shown the presence of a single myosin kinase in gizzard smooth muscle (Dabrowska *et al.*, 1977; Adelstein & Klee, 1981), skeletal muscle (Pires & Perry, 1977), platelets (Hathaway & Adelstein, 1979), brain (Hathaway *et al.*, 1981) and myocardium (Walsh *et al.*, 1979; Wolf & Hofmann, 1980).

Myosin kinases I and II were purified 960- and 650-fold respectively from the carotid arteries, with yields of 3.0 and 4.5 mg/kg of tissue respectively. The molecular weight of myosin kinase I was 66 000 as determined by its mobility on SDS/polyacrylamide-gel electrophoresis. Myosin kinase II, on the other hand, gave two equal-density bands with molecular weights of 79 000 and 67 000. The molecular weights of kinases I and II observed in the present studies are comparable with the values reported for bovine myocardium (85 000; Walsh *et al.*, 1979) and skeletal muscle (77 000; Pires & Perry, 1977) and human platelets (83 000; Daniel & Adelstein, 1976), but contrast with values for human platelets (105 000; Hathaway & Adelstein, 1979), chicken gizzard (105 000; Dabrowska *et al.*, 1977), turkey gizzard (130 000; Adelstein & Klee, 1981), bovine brain (130 000; Hathaway *et al.*, 1981), bovine myocardium (150 000; Walsh & Guilleux, 1981) and skeletal muscle (155 000; Walsh &

Guilleux, 1981). The molecular weights of myosin kinases observed in our investigation are generally lower than the reported values, therefore the possibility of a proteolytic degradation of native high-molecular-weight enzyme cannot be ruled out. However, precautions were taken to minimize proteolytic degradation by using a large number of proteolytic inhibitors during homogenization and also during subsequent steps of purification. One piece of evidence against a proteolytic degradation comes from the observations that in three different preparations the relative distribution and conductance at which myosin kinases I and II were eluted from the DEAE-Sephacel column were identical. It is unlikely that the proteolytic degradation would cleave higher-molecular-weight enzyme each time at the same site. Ca^{2+} -dependent (mol.wt. 105 000) and Ca^{2+} -independent (mol.wt. 83 000) myosin kinases have been isolated from human platelets (Daniel & Adelstein, 1976; Hathaway *et al.*, 1979). Hathaway *et al.* (1979) have provided a possible explanation for the lower molecular weight and Ca^{2+} -independent myosin kinase activity of 83 000-Da protein on the basis of limited proteolysis of native enzyme. Direct studies on the effect of limited proteolysis by treatment with α -chymotrypsin or trypsin of smooth-muscle and skeletal-muscle myosin light-chain kinases resulted in myosin kinase activation with a concomitant loss of Ca^{2+} -calmodulin-sensitivity (Hartshorne *et al.*, 1977; Tanaka *et al.*, 1980). However, the lower-molecular-weight myosin kinases I and II reported in the present paper show an absolute requirement for Ca^{2+} and calmodulin for their activity. Therefore it is likely that either these enzymes are not proteolytic products or that proteolysis has not removed the Ca^{2+} -calmodulin-binding sites of these enzymes. Moreover, the storage of purified enzyme at 4°C for up to 3 weeks in EGTA or Ca^{2+} buffer did not alter the mobility of myosin kinases I or II on SDS/polyacrylamide gels.

Conti & Adelstein (1981) have shown that turkey gizzard smooth-muscle enzyme is phosphorylated by cyclic AMP-dependent protein kinase. In their studies, when calmodulin was not bound to myosin kinase, protein kinase incorporated phosphate into two sites in myosin kinase with a concomitant decrease in the rate of myosin kinase activity. The decrease in the myosin kinase activity was due to a 10–20-fold decrease in the affinity of calmodulin for myosin kinase. On the other hand, when calmodulin was bound to myosin kinase, cyclic AMP-dependent protein kinase incorporated phosphate into a single site in myosin kinase with no effect on myosin kinase activity. We decided to investigate if this mechanism of regulation of myosin light-chain kinase by cyclic AMP-dependent protein kinase also exists in vascular smooth muscle. Our results show

that when myosin kinases I and II were phosphorylated by catalytic subunit of protein kinase in the presence of calmodulin, approx. 0.5–0.7 mol of phosphate was incorporated/mol of myosin kinase. However, when phosphorylation of myosin kinases I and II was performed in the absence of calmodulin, the amount of phosphate incorporated was 1.0–1.4 mol/mol of myosin kinase. These data are qualitatively similar to those for gizzard smooth muscle, but the net amount of phosphate incorporated is slightly lower. Similar phosphorylation of cardiac myosin light-chain kinase by protein kinase has been demonstrated by Walsh & Guilleux (1981). However, in earlier studies protein kinase-dependent phosphorylation was not observed for cardiac myosin light-chain kinase (Walsh *et al.*, 1979). The possible explanation for the lack of phosphorylation was attributed to the fact that this enzyme could be a proteolytic product from which the phosphate acceptor site has been removed (Walsh *et al.*, 1979). Wolf & Hoffman (1980) have reported phosphorylation of cardiac myosin kinase, but this phosphorylation is not dependent on protein kinase and does not have any effect on enzyme activity. Since we observed phosphorylation of myosin kinases I and II of vascular smooth muscle, and it was qualitatively comparable with that of gizzard smooth-muscle kinase, these observations would suggest that at least the phosphate-acceptor site and calmodulin-binding sites of myosin kinases I and II are not cleaved by proteinases during purification. Additional support for our contention that myosin kinases I and II are not proteolytic products of the holoenzyme comes from our studies on the enzyme activity and calmodulin affinity of phosphorylated and unphosphorylated myosin kinases. Our data show that phosphorylation of myosin kinases results in 4–5-fold increase in the concentration of calmodulin required for 50% activation of myosin kinase activity. Further, the activity of myosin kinases I and II at very low calmodulin concentration was considerably decreased. Similar results have been reported for gizzard smooth-muscle enzyme (mol.wt. 130 000) and cardiac enzyme (mol.wt. 150 000). Radioautographic analysis of these isoenzymes revealed that ^{32}P incorporation corresponded to the main band of isoenzyme I. For isoenzyme II, most of the ^{32}P incorporation corresponded to the 79 000-Da band; however, significant ^{32}P incorporation was also present in the 67 000-Da band. SDS/polyacrylamide-gel electrophoresis performed at weekly intervals for 3 weeks did not reveal any difference in the pattern of mobility or the intensity of the two bands of isoenzyme II. These data would suggest that the two bands are not the proteolytic product of isoenzyme II after it has been purified. At present, it is not clear whether the enzyme activity is associated with either band I or

band II or both of these bands. Further work is needed to isolate and characterize the isoenzymes by using fresh tissue to avoid the possibility of proteolytic degradation during the process of freezing and thawing.

The results presented in this paper on the decrease of myosin kinase activity by phosphorylation of myosin kinase with catalytic subunit of cyclic AMP-dependent protein kinase provide direct support for the observation by Kerrick *et al.* (1980), using chemically skinned smooth-muscle cells, and Silver & DiSalvo (1979), using crude actomyosin preparations of bovine aorta, that cyclic AMP and cyclic AMP-dependent protein kinase decrease the myosin light-chain kinase activity by modulating its affinity for calmodulin and Ca^{2+} .

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