Purification, characterization and substrate specificity of calmodulin-dependent myosin light-chain kinase from bovine brain

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A substrate-specific calmodulin-dependent myosin light-chain kinase (MLCK) was purified 45000-fold to near homogeneity from bovine brain in 12 % yield. Bovine brain MLCK phosphorylates a serine residue in the isolated turkey gizzard myosin light chain (MLC), with a specific activity of 1.8 $\mu\text{mol/min}$ per mg of enzyme. The regulatory MLC present in intact gizzard myosin is also phosphorylated by the enzyme. The M_r -19000 rabbit skeletal-muscle MLC is a substrate; however, the rate of its phosphorylation is at best 30 % of that obtained with turkey gizzard MLC. Phosphorylation of all other protein substrates tested is less than 1 % of that observed with gizzard MLC as substrate. SDS/polyacrylamide-gel electrophoresis of purified MLCK reveals the presence of a major protein band with an apparent M_r of 152000, which is capable of binding ^{125}I -calmodulin in a Ca^{2+} -dependent manner. Phosphorylation of MLCK by the catalytic subunit of cyclic-AMP-dependent protein kinase results in the incorporation of phosphate into the M_r -152000 protein band and a marked decrease in the affinity of MLCK for calmodulin. The presence of Ca^{2+} and calmodulin inhibits the phosphorylation of the enzyme. Bovine brain MLCK appears similar to MLCKs isolated from platelets and various forms of muscle.

INTRODUCTION

Calmodulin-dependent myosin light-chain kinases (MLCKs) are substrate-specific, tightly regulated, enzymes proposed to render actomyosin-based motility systems in smooth muscle and non-muscle cells subject to regulation by changes in intracellular Ca^{2+} concentrations (for reviews, see Walsh & Hartshorne, 1982; Kamm & Stull, 1985). In smooth muscle, Ca^{2+} regulates force generation through phosphorylation of the regulatory light chain of myosin by a calmodulin-dependent MLCK (Sherry *et al.*, 1978; Adelstein & Klee, 1981). Phosphorylation of MLCs by the activated enzyme promotes a conformational change that allows stimulation by actin of the myosin ATPase (Lebowitz & Cooke, 1979). Hydrolysis of ATP provides the energy necessary for tension development and motility. The skeletal- and smooth-muscle forms of MLCK are well characterized, and the amino acid sequence of portions of the molecules have been determined (Takio *et al.*, 1985; Lukas *et al.*, 1986). A mechanism similar to that of muscle actomyosin has been postulated for the control of force generation in non-muscle cells (Adelstein & Conti, 1975; Bourguignon *et al.*, 1982); however, MLCKs from these sources are less well characterized.

There is ample evidence for the existence of an actomyosin-based motility system in neuronal tissues. Actin and myosin have been observed immunocytochemically in neuronal and glial cells as well as in synaptic regions in brain (Kuczmarski & Rosenbaum, 1979b; Drenkhahn & Kaiser, 1983). The structural components of the brain actomyosin system have been isolated (Kuczmarski & Rosenbaum, 1979a; Malik *et al.*, 1983a), and a substrate-specific MLCK activity has been detected in astrocytes (Scordilis *et al.*, 1977). Calmodulin-

dependent protein kinases capable of phosphorylating purified smooth-muscle MLC have been isolated from brain. Dabrowska & Hartshorne (1978) and Hathaway *et al.* (1981) have reported partial purifications of a MLCK from bovine brain, but did not study its substrate specificity. More recently, multifunctional calmodulin-dependent protein kinases capable of phosphorylating MLC have been isolated from rat brain (Fukunaga *et al.*, 1982; Bennett *et al.*, 1983; Goldenring *et al.*, 1983; Yamauchi & Fujisawa, 1983; Kuret & Shulman, 1984). These brain kinase activities have subsequently been found to be members of a class of enzymes that is now widely termed calmodulin-dependent protein kinase II. We wished to determine whether brain contains a specific MLCK as well as protein kinase II. Here we report the purification of a calmodulin-dependent MLCK from bovine brain with a narrow substrate specificity similar to the enzymes isolated from various forms of muscle.

EXPERIMENTAL

Materials

Protein M_r standards and Affi-Gel 15 were purchased from Bio-Rad, Richmond, CA, U.S.A. Casein–Affi-gel and calmodulin–Affi-Gel were prepared according to the manufacturer's instructions. DEAE-cellulose (Whatman DE52), leupeptin, calf thymus mixed histone (II AS), histone I-enriched fraction (VI-S), bovine type I haemoglobin, sweet potato (*Ipomoea batatas*) β -amylase type 1B, the catalytic subunit of bovine heart cyclic-AMP-dependent protein kinase, and rabbit skeletal-muscle MLCs were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ultrogel AcA 34 was purchased from LKB, Rockville, MD, U.S.A. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased

Abbreviations used: DFP, di-isopropyl fluorophosphate; DTT, dithiothreitol; MAP, microtubule-associated protein; MLC, myosin light chain; MLCK, myosin light-chain kinase; Tween 40, polyoxyethylene sorbitan monopalmitate.

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from ICN Radiochemicals, Costa Mesa, CA, U.S.A. Centricon micro-concentrators were purchased from Amicon Corp., Danvers, MA, U.S.A. Synthetic peptide kinase substrates were purchased from Peninsula Laboratories, Belmont, CA, U.S.A. Bovine brains were obtained from the Bermelon Abattoir, Newark, NJ, U.S.A., and turkey gizzards were from Larrison's Turkey Farm, Chester, NJ, U.S.A. Rat brain calmodulin-dependent protein kinase II, bovine brain synapsin I and Protein A-purified rabbit polyclonal antibody to rat brain kinase II were generously given by Dr. Mary Kennedy of the California Institute of Technology, Pasadena, CA, U.S.A. Glycogen synthase was generously given by Dr. Thomas Soderling of Vanderbilt University and the Howard Hughes Institute, Nashville, TN, U.S.A. Turkey gizzard myosin, devoid of MLCK activity, was generously given by Dr. Bershan Sharma of Schering-Plough, Bloomfield, NJ, U.S.A. Calmodulin was prepared from bovine brain as described by Wolff & Sved (1985) and iodinated with 1 mCi of Na¹²⁵I/mg of protein by using lactoperoxidase and H₂O₂ as described by Carlin *et al.* (1980). Calmodulin-dependent bovine brain phosphodiesterase was prepared by the method of Brostrom & Wolff (1976).

Preparation of substrate proteins

Pig brain tubulin was prepared by the method of Williams & Lee (1982). Pig brain microtubule-associated protein (MAP) 2 was purified from pig brain microtubule proteins as described by Sloboda & Rosenbaum (1982) and boiled for 3 min to destroy endogenous kinase activity. Histone 1 was prepared from histone 1-enriched fraction (V-S), and histone 2b was prepared from histone 2a-enriched fraction (VII-S) as described by Wolff *et al.* (1981). Casein was solubilized and dephosphorylated by heating a 10 mg/ml solution in 0.1 M-Na₂CO₃, pH 11.3, to 55 °C for 30 min and re-adjusting the pH to 7.5. Myelin basic protein was prepared by the method of Oshiro & Eylar (1970). Rabbit skeletal-muscle MLCs were prepared by a modification of the method of Perrie & Perry (1970) in which MLCs were purified on hydroxyapatite. Phosphorylase *b* was prepared from rabbit skeletal muscle by the procedure of Fischer & Krebs (1958). The *M_r*-20000 light chain of turkey gizzard myosin was prepared by a modification of the method of Grand & Perry (1983), in which MLCs were prepared from a urea extract of gizzard by 50–80% ethanol precipitation, followed by gradient elution from hydroxyapatite and chromatography on DEAE-cellulose to remove calmodulin.

Gel electrophoresis and ¹²⁵I-calmodulin gel overlay

Polyacrylamide-gel electrophoresis in the presence of SDS was performed on 7.5–15% acrylamide gradient gels by the procedure of Laemmli (1970). Calmodulin-binding proteins were detected after SDS/polyacrylamide-gel electrophoresis by binding of ¹²⁵I-calmodulin as described by Carlin *et al.* (1980) with the modifications described by Bartelt *et al.* (1986).

Endogenous phosphorylation of bovine brain proteins

Bovine brains were homogenized in 2 vol. of 100 mM-Pipes (pH 6.9)/10 mM-EDTA/10 mM-EGTA containing 0.2 mM-DFP and leupeptin (1 µg/ml). The homogenate was centrifuged for 10 min at 10000 *g*, and the resulting

postmitochondrial supernatant was centrifuged at 100000 *g* for 1 h. The microsomal pellet derived from 35 g of tissue was resuspended in 5 ml of homogenization buffer with a Dounce homogenizer. Samples of the postmitochondrial and postmicrosomal supernatants (15 µl) and the microsomal suspension (10 µl) were incubated for 1 min at 30 °C in 25 mM-Tris (pH 7.5)/30 mM-Pipes (pH 6.9)/10 mM-MgCl₂/3 mM-EDTA/3.5 mM-EGTA/100 µM-[γ-³²P]ATP (1060 c.p.m./pmol) in the absence and the presence of 5 mM-CaCl₂ in a final volume of 50 µl. The reactions were terminated by the addition of 50 µl of Laemmli sample buffer containing 100 mM-DTT and heating to 90 °C. Samples were subjected to SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue, destained, dried and placed on X-ray film. Bands of labelled protein were excised and counted for radioactivity in liquid scintillant in a liquid-scintillation counter.

Assay of calmodulin-dependent MLCK

[³²P]Phosphate incorporation into MLC catalysed by MLCK was measured at 30 °C in a final volume of 50 µl under conditions described in the legends to the appropriate Figures and Tables. When assaying column fractions, reactions were terminated and samples processed by the procedure of Reiman *et al.* (1971). All other incubations were terminated and samples processed as described above for assays of endogenous phosphorylation. Other proteins were tested as substrates for MLCK, at concentrations of 10–25 µg/50 µl assay. Protein concentrations were measured by the method of Lowry *et al.* (1951). Optimal phosphorylation of myosin was measured and compared with MLC phosphorylation under conditions described by Walsh *et al.* (1983) and detailed in the legend to Table 3. Phosphate-acceptor peptides were incubated with MLCK under conditions described in the legend to Table 4. Reactions were terminated by acidification with acetic acid, and peptides were adsorbed to phosphocellulose paper by the method of Glass *et al.* (1978).

Peptide hydrolysis and separation of phosphoamino acids

Samples of ³²P-MLC were electroeluted from polyacrylamide gels. Eluates were desalted by repeated ultrafiltration and dilution by using a Centricon ultrafiltration device. Samples of phosphorylated MLC were hydrolysed under vacuum either in constant-boiling HCl at 110 °C for 2 h or in 5 M-KOH at 140 °C for 35 min by the method of Martensen & Levine (1983). Acid hydrolysates were freeze-dried and redissolved in water, whereas alkaline hydrolysates were neutralized and salts precipitated by the addition of an equal volume of 5 M-HClO₄. After centrifugation at 10000 *g* for 20 min, the pH of the supernatant was adjusted to 6 with acetic acid. Samples of either hydrolysate to which 100 µg each of phosphoserine, phosphothreonine and phosphotyrosine had been added were electrophoresed and chromatographed on cellulose thin-layer sheets as described in the legend to Fig. 2. Amino acid standards were detected by ninhydrin staining, and labelled phosphoamino acids by autoradiography. The identity of phosphoamino acids was confirmed and their recovery through hydrolysis was quantified by reverse-phase h.p.l.c. of their o-phthalaldehyde derivatives.

Sucrose-density-gradient ultracentrifugation

In a modification of the procedure described by Payne *et al.* (1983), samples containing MLCK, 25 μg of ^{125}I -calmodulin (1.85 S), 500 μg of haemoglobin (4.09 S), 100 μg of calmodulin-dependent bovine brain phosphodiesterase (6.85 S) and 500 μg of catalase (11.4 S) were centrifuged at 286 000 g for 15 h through linear gradients of 5–20% (w/v) sucrose containing 25 mM-Tris/HCl, pH 7.5, 1 mM-MgCl₂, 0.5 mM-DTT, 0.2 mM-DFP and leupeptin (1 $\mu\text{g}/\text{ml}$). Fractions were assayed for calmodulin-dependent MLCK and for calmodulin-dependent phosphodiesterase by the method of Wolff *et al.* (1981).

Phosphorylation of MLCK by cyclic-AMP-dependent protein kinase

MLCK was incubated with the catalytic subunit of cyclic-AMP-dependent protein kinase under conditions described in the legend to Fig. 4. Reactions containing labelled ATP were stopped by the addition of an equal volume of Laemmli sample buffer containing 100 mM-DTT and electrophoresed as described above. MLCK protein bands were excised from the gel and counted for radioactivity. The protein content of bands of MLCK was estimated by densitometric scanning of the Coomassie-Blue-stained gel and comparing the absorbance measured with that for known amounts of bovine serum albumin electrophoresed on the same gel. Other phosphorylation reactions were terminated by dilution (1:200) with 0.1 mg of albumin/ml at 0 °C. Samples of dilutions were assayed for MLCK activity as described above in the presence of Ca²⁺ and various concentrations of calmodulin (4–600 nM).

RESULTS

Purification of bovine brain MLCK

During a study of endogenous protein phosphorylation in bovine brain, we observed the Ca²⁺-dependent phosphorylation of a M_r -20 000 protein present in a postmitochondrial supernatant, but absent from a postmicrosomal supernatant. The M_r -20 000 endogenous substrate protein co-migrated with added turkey gizzard MLC, which was phosphorylated in a Ca²⁺-dependent manner by the brain postmicrosomal supernatant (results not shown). Quantification of Ca²⁺-dependent MLC phosphorylation present in the various fractions showed that, of the MLCK activity present in the postmitochondrial supernatant, 69% was recovered in the postmicrosomal supernatant and only 0.6% in the microsomal membrane pellet, suggesting that, unlike smooth muscle (Walsh *et al.*, 1982) and like both cardiac muscle (Wolf & Hofmann, 1980) and platelets (Hathaway & Adelstein, 1979), most of the MLCK in bovine brain is in the soluble fraction.

Bovine brains (2.7 kg) were trimmed of the brain stem, corpus callosum and large blood vessels, diced, and washed with 100 mM-Pipes/10 mM-EDTA/10 mM-EGTA/0.2 mM-DFP/leupeptin (2 $\mu\text{g}/\text{ml}$), pH 6.8 (buffer A). The washed tissue was transported in buffer A on ice. Subsequent manipulations were performed at 4 °C. The diced tissue was homogenized in 2 vol. of buffer A in a Waring blender for 3 $\times$ 30 s. The homogenate was filtered through cheesecloth and centrifuged at 15 000 g for 45 min. The resulting supernatant was made 1 mM in cyclic

AMP to dissociate the cyclic-AMP-dependent protein kinase holoenzyme (Payne *et al.*, 1983), so that the catalytic subunit of this kinase would be tightly bound to phosphocellulose. Phosphocellulose (1000 ml) pre-equilibrated with 30 mM-Pipes/0.2 mM-DFP/leupeptin (1 $\mu\text{g}/\text{ml}$), pH 6.8 (buffer B), was added to the supernatant, and the resulting slurry was stirred for 1 h. The resin was collected by filtration, suspended in 500 ml of buffer B and poured into a column 5.0 cm in diameter. Calcineurin, a calmodulin-dependent phosphatase, and calmodulin were not retained by the resin (results not shown). After no additional protein could be eluted with buffer B, MLCK was eluted with buffer B containing 0.15 M-NaCl. Fractions containing MLCK activity were combined and made 60% saturated with (NH₄)₂SO₄. Precipitated protein was collected by centrifugation (30 min, 18 000 g), dissolved in a minimal volume of 25 mM-Tris/50 mM-NaCl/1 mM-MgCl₂/1 mM-EGTA/0.2 mM-DFP/leupeptin (1 $\mu\text{g}/\text{ml}$), pH 8.0 (buffer C), and dialysed against 4 litres of this buffer.

DEAE-cellulose chromatography

After clarification by centrifugation at 30 000 g for 30 min, the dialysed enzyme was applied to a column (2.5 m $\times$ 25 cm) of DEAE-cellulose equilibrated in buffer C. After no further protein was eluted with buffer C, the column was washed with buffer C 75 mM in NaCl. Calmodulin-dependent MLCK was then eluted with buffer C containing 125 mM-NaCl.

Casein-Affi-Gel chromatography

To remove Ca²⁺-activated proteinases suspected of being present in the preparation, MLCK was subjected to chromatography on casein-Affi-Gel (Malik *et al.*, 1983b). The eluate from the DEAE-cellulose column containing MLCK was adjusted to 4 mM-CaCl₂ and 2 mM-DTT and applied to a 1.5 cm $\times$ 7 cm column of casein-Affi-Gel equilibrated in 50 mM-Tris/HCl (pH 7.5)/100 mM-NaCl/1 mM-MgCl₂/3 mM-CaCl₂/2 mM-CaCl₂/2 mM-DTT/0.2 mM-DFP/leupeptin (5 $\mu\text{g}/\text{ml}$). Protein not bound to the column was immediately subjected to affinity chromatography on calmodulin-Affi-Gel.

Affinity chromatography of MLCK on calmodulin-Affi-Gel

A column (1.5 cm $\times$ 10 cm) of calmodulin-Affi-Gel was equilibrated with 25 mM-Tris/HCl (pH 7.5)/100 mM-NaCl/1 mM-MgCl₂/0.5 mM-CaCl₂/0.2 mM-DFP/leupeptin (1 $\mu\text{g}/\text{ml}$) (buffer D). The effluent from the casein-Affi-Gel column was applied to calmodulin-Affi-Gel. The column was washed with buffer D, followed by buffer D containing 5% (w/v) glycerol and 0.05% Tween 40, until no further protein was eluted. Calmodulin-dependent MLCK was eluted with 25 mM-Tris/HCl (pH 7.5)/100 mM-NaCl/2 mM-EGTA/1 mM-MgCl₂/0.2 mM-DFP/leupeptin (1 $\mu\text{g}/\text{ml}$)/5% glycerol/0.05% Tween 40 (buffer E). Glycerol and Tween 40 were added to the buffer to stabilize the enzyme and diminish its adsorption to glass and plastic surfaces (Pearson *et al.*, 1982).

Gel filtration of MLCK on AcA 34

A column (1.0 cm $\times$ 55 cm) of Ultrogel AcA 34 was equilibrated in buffer E and calibrated by individually gel-filtering calmodulin (M_r 33 400; Stokes radius 2.9 nm),

Table 1. Purification of bovine brain MLCK

Starting material was 2.7 kg of bovine brain. Protein was determined by the method of Lowry *et al.* (1951).

	Protein (mg)	Calmodulin- dependent MLCK ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Yield (%)	Purification (fold)
15000 g supernatant	51978	2.085	0.00004	(100)	1.00
Phosphocellulose 0.15 M- NaCl eluate	396	0.804	0.0020	38.6	51
DEAE-cellulose 0.15 M- NaCl eluate	106	0.715	0.0067	34.3	168
Calmodulin-Affi-Gel EGTA eluate	0.74	0.358	0.484	17.2	12082
Ultrogel AcA-34	0.140	0.256	1.834	12.4	45843

bovine serum albumin (M_r 67000; 3.55 nm), bovine brain calmodulin-dependent phosphodiesterase (M_r 116000; 4.42 nm) and sweet-potato amylase (M_r 152000; 4.15 nm) as protein standards. MLCK eluted from the calmodulin-Affi-Gel column was concentrated approx. 15-fold by ultrafiltration with a Centricon 10 micro-concentrator and was gel-filtered on the calibrated AcA 34 column. MLCK activity was eluted as a single symmetrical peak containing 72 % of the activity applied to the column. From the elution position of MLCK, the Stokes radius is estimated to be 6.6 nm. MLCK was stored at -80°C .

A quantitative summary of the purification of bovine brain MLCK is presented in Table 1. The procedure developed for the isolation of MLCK was designed so that the enzyme was purified and concentrated at each step, since MLCK is not stable to freeze-drying. Introduction of the casein-Affi-Gel chromatographic step in the purification greatly increased the relative proportion of M_r -152000 material present in MLCK preparations after calmodulin-Affi-Gel chromatography. Samples of bovine brain MLCK after chromatography on calmodulin-Affi-Gel and gel filtration were subjected to SDS/polyacrylamide-gel electrophoresis as shown in Fig. 1. Integration of the peaks in a densitometric scan of purified MLCK after SDS/polyacrylamide-gel electrophoresis (results not shown) indicated that the M_r -152000 protein band accounted for 70 % of the Coomassie Blue staining of the sample, with no other protein band accounting for as much as 10 % of the total staining. Comparison of the Coomassie-Blue-staining patterns of MLCK samples before and after gel filtration show that only the M_r -152000 band is enriched during this step of purification, in which over 70 % of MLCK activity was recovered. A single calmodulin-binding protein of M_r 152000 was detected in the sample by the ^{125}I -calmodulin gel-overlay technique (Fig. 1b). Binding of calmodulin was Ca^{2+} -dependent.

M_r of bovine brain MLCK

The sedimentation coefficient of MLCK was determined by sucrose-density-gradient ultracentrifugation to be 4.65 S. By using this value in the equation $M_r = 6\pi\eta N a^3 / (1 - \nu\rho)$, where N = Avogadro's number, $\eta_{20,w} = 0.01002 \text{ P}$ (0.001 Pa·s) (viscosity of 100 mM-KCl at 20°C), $\nu = 0.725 \text{ ml/g}$ (literature value for the average

partial specific volume of proteins), a = the Stokes radius in $\text{nm} \times 10^{-1}$, and $\rho_{20,w} = 1.0012 \text{ g/ml}$ (density of 100 mM-KCl), the M_r of MLCK was calculated to be 127000. From this value, a value of 1.97 was calculated for the frictional coefficient of MLCK by using the equation:

$$f/f_0 = a(4\pi N/3\eta M_r)^{1/3}$$

Phosphorylation of isolated turkey gizzard MLC by MLCK

Phosphorylation of isolated gizzard MLC and the light chain of intact gizzard myosin by MLCK required the presence of both Ca^{2+} and calmodulin. When 5.8 ng of MLCK was incubated with myosin or MLC for 2 min in the presence of Ca^{2+} and calmodulin, under the conditions described in Table 2, 9.2 pmol of phosphate was incorporated into MLC and 27.8 pmol of phosphate was incorporated into myosin. No phosphate incorporation was observed in the absence of either Ca^{2+} or calmodulin. The maximal specific activity determined for MLCK after gel filtration with isolated MLC as substrate is $1.83 \mu\text{mol}$ of phosphate transferred/min per mg of protein. Assuming the degree of purity obtained for MLCK in the current study is 70 %, the minimal V_{max} of homogeneous MLCK toward gizzard MLC is $2.6 \mu\text{mol}/\text{min}$ per mg.

Phosphorylation of isolated MLC by MLCK is dependent on concentrations of ATP, Mg^{2+} , MLC and calmodulin. The rates of MLC phosphorylation as a function of MLC, ATP and calmodulin concentrations followed Michaelis-Menten kinetics, and the concentrations at half-maximal activity are given in Table 2. The apparent K_m for Mg^{2+} is 2.0 mM. The rate of phosphorylation was maximal at 10 mM- MgCl_2 and decreased with further added MgCl_2 . The inhibition of kinase activity at high Mg^{2+} concentrations may be due to the competition of Mg^{2+} with Ca^{2+} for binding to calmodulin (Wolff *et al.*, 1977).

In order to determine the amino acids in MLC phosphorylated by MLCK, partial acid hydrolysates of ^{32}P -MLC were separated by two-dimensional thin-layer electrophoresis/chromatography. As shown in Fig. 2, ^{32}P -labelled material co-migrating with both phosphoserine and phosphotyrosine was present in the partial acid hydrolysate. However, two-dimensional separation of an alkaline hydrolysate which contained unlabelled

Fig. 1. Purification of bovine brain MLCK

(a) Samples of MLCK after chromatography on calmodulin-Affi-Gel and gel filtration were subjected to SDS/polyacrylamide-gel electrophoresis, and the gel was stained with Coomassie Blue. Lane 1, EGTA eluate of calmodulin-Affi-Gel; lane 2, MLCK after gel filtration on Ultrogel Aca 34. (b) Autoradiograms resulting from ^{125}I -calmodulin binding to MLCK after SDS/polyacrylamide-gel electrophoresis. Gels were incubated in the presence of CaCl_2 (lane 1) and EGTA (lane 2). Arrowheads mark the positions of protein standards of M_r values given at the left of the Figure.

phosphotyrosine revealed that no ^{32}P -labelled material co-migrated with phosphotyrosine, although 80 % of the unlabelled phosphotyrosine was recovered after alkaline hydrolysis. Therefore, the labelled material present in the partial acid hydrolysate and co-migrating with phosphotyrosine is not phosphotyrosine. Cooper *et al.* (1983) have reported the presence in partial acid protein hydrolysates of material with chromatographic behaviour similar to that of phosphotyrosine which, when subjected to further acid hydrolysis, yields phosphoserine. Confirmation and quantification of phosphoserine and phosphotyrosine in the hydrolysates were accomplished by h.p.l.c. separation and comparison with known amounts of phospho-amino acid standards. No

Table 2. Kinetic characterization of bovine brain MLCK

Except where indicated, phosphorylation assays contained 400 ng of MLCK/ml in 25 mM-Pipes, pH 6.8, and were incubated for 15 min at 30 °C. Concentrations of components were held at optimal values while calmodulin, ATP and Mg^{2+} concentrations were varied. For measurement of kinetic parameters, the Mg^{2+} concentration was varied from 0.4 to 30 mM, ATP from 5 to 300 μM , calmodulin from 0.5 to 500 nM and turkey gizzard MLC from 2 to 125 μM .

	K_m (μM)	$V_{\max}$ ($\mu\text{mol/min}$ per mg)
Turkey gizzard MLC	20	1.83
Calmodulin	0.002	—
ATP	20	—
Mg^{2+}	2000	—
Turkey gizzard myosin*	≥ 9.5	≥ 2.51
Kemptamide†	110	0.078

* Phosphorylation assays containing 400 ng of MLCK/ml in 25 mM-Tris (pH 7.5), 60 mM-KCl, 10 mM- MgCl_2 , 500 μM -[γ - ^{32}P]ATP (0.07 $\mu\text{Ci/pmol}$), 1 mM- CaCl_2 and 10 μg of calmodulin/ml were incubated for 2 min at 30 °C. Myosin concentrations were varied from 0.13 to 3.8 mg/ml (0.5–16 μM).
† The concentration of Kemptamide was varied from 10 μM to 2.5 mM. Peptides were isolated from reaction mixtures by adsorption to phosphocellulose paper (Glass *et al.*, 1978).

Fig. 2. Separation of phosphoamino acids in hydrolysates of turkey gizzard MLC phosphorylated by MLCK

Samples of ^{32}P -MLC were hydrolysed under vacuum either in constant-boiling HCl at 110 °C for 2 h (a) or in 5 M-KOH at 140 °C for 35 min (b). Hydrolysates were treated as described in the Experimental section, combined with 100 μg each of phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY), and separated on cellulose thin-layer sheets in the vertical dimension by electrophoresis in butanol/pyridine/acetic acid/water (2:1:1:36, by vol.) for 90 min at 450 V and in the horizontal dimension by chromatography in pyridine/butanol/acetic acid/water (95:61:19:75, by vol.). The positions of amino acid standards detected by ninhydrin staining are outlined in continuous lines. [^{32}P]Phosphate (Pi) and phosphoamino acids were detected by autoradiography. The arrowhead points to the position of the origin (O). Unlabelled phosphotyrosine was recovered in 80 % yield through alkaline hydrolysis.

Table 3. Calmodulin-dependent phosphorylation of protein substrates by bovine brain MLCK

Phosphorylation by MLCK (16.5 ng) was measured in the presence of 25 mM-Pipes, pH 6.8, 10 mM-MgCl₂, 100 μ M-[γ -³²P]ATP (0.07 μ Ci/pmol), approx. 300 μ g of substrate/ml, and either 0.5 mM-EGTA or 1 mM-CaCl₂ and 10 μ g of calmodulin (CaM)/ml. Incubation was for 20 min at 30 °C.

Substrate	MLCK activity (pmol/min)			CaM-dependent	(%)
	+ Ca ²⁺ + CaM	– Ca ²⁺ – CaM			
Turkey gizzard MLC	29.0	0.090		28.90	(100)
Rabbit skeletal muscle MLC*	7.7	0.014		7.69	26.6
Rabbit skeletal muscle MLC†	0.366	0.025		0.341	1.18
Synapsin	0.125	0.036		0.089	0.31
MBP	0.066	0.025		0.041	0.14
Histone 1	0.020	0.014		0.006	0.02
Glycogen synthase	0.648	0.650		0	0
Tubulin	0.012	0.013		0	0
MAP2	0.002	0.005		0	0
Casein	0.011	0.017		0	0
Histone 2b	0.024	0.027		0	0
Phosphorylase b	0.011	0.028		0	0

* Prepared by the method of Perrie & Perry (1970).

† Commercially available preparation.

Table 4. Calmodulin-dependent phosphorylation of peptide substrates by bovine brain MLCK

Phosphorylation was measured in the presence of 25 mM-Pipes, pH 6.8, 10 mM-MgCl₂, 100 μ M-[γ -³²P]ATP (0.07 μ Ci/pmol), 30 ng of enzyme and either 0.5 mM-EGTA or 1 mM-CaCl₂ and 10 μ g of calmodulin (CaM)/ml. After incubation for 15 min at 30 °C, peptides were adsorbed to phosphocellulose paper by the procedure of Glass *et al.* (1978) and counted for radioactivity. Peptide-sequences are in the one letter code.

Substrate	Concn. (μ M)	Activity (pmol/min)			(%)
		+ Ca ²⁺ + CaM	– Ca ²⁺ – CaM	CaM-dependent	
Turkey gizzard MLC	50	35.3	0.21	35.1	(100)
K-K-R-P-Q-R-A-T-S-N-V-F-S-NH* (Kemptide)	132	5.9	0.78	5.1	14.5
L-R-R-A-S-L-G-OH† (Kemptide)	150	0.8	1.22	0.0	0
R-R-L-I-E-D-A-E-Y-A-A-R-G-OH‡	197	0.0	0.0	0.0	0

* A synthetic peptide containing the phosphorylation site of chicken gizzard MLC.

† A synthetic peptide containing the phosphorylation site from pig liver pyruvate kinase phosphorylated by cyclic AMP-dependent protein kinase.

‡ A synthetic peptide containing the site of tyrosine phosphorylation of pp60^{src}.

phosphothreonine was detected in any of the hydrolysates, indicating that in MLC only serine is phosphorylated by MLCK.

Phosphorylation of MLC in native gizzard myosin by MLCK

Only the regulatory light chain of myosin was phosphorylated by MLCK (results not shown). The maximal specific activity measured for MLCK against MLC in native myosin was 2.51 μ mol/min per mg of protein (Table 2). Since the rate of myosin phosphorylation was increasing with increasing myosin concentration at the highest myosin concentration tested

(16 μ M-myosin; M_r = 240 000/MLC), the data for V_{max} and K_m given in Table 2 are minimal values. Assays of MLCK at higher concentrations of myosin were not attempted, owing to the ATPase activity of myosin. Assuming that the degree of purity obtained for MLCK in the current study is 70 %, the V_{max} of homogeneous MLCK toward gizzard myosin is minimally 3.6 μ mol/min per mg.

Substrate specificity of MLCK

Proteins known to be substrates for both calmodulin-dependent and calmodulin-independent kinases were tested as substrates for MLCK. Rates of phosphorylation

Fig. 3. Comparison of protein substrate specificity of bovine brain MLCK with that of rat brain protein kinase II

Phosphorylation was measured at 30 °C for 15 min in 25 mM-Pipes (pH 6.8)/10 mM-MgCl₂/100 µM-[γ-³²P]ATP/0.5 mM-EGTA in the absence (–) or presence (+) of 1 mM-CaCl₂ and 10 µg of calmodulin (CaM)/ml and subjected to SDS/polyacrylamide-gel electrophoresis. Panel (a), Coomassie Blue (CB)-stained gel. Panels (b) and (c), autoradiograms (AR) of dried gels: (b), substrates phosphorylated by bovine brain MLCK; (c), substrates phosphorylated by rat brain protein kinase II (CaM-PKII). Lane 1, turkey gizzard MLC (450 µg/ml); lane 2, rabbit skeletal-muscle MLCs (500 µg/ml); lane 3, bovine brain synapsin (200 µg/ml). Arrowheads mark the positions of protein standards of M_r values indicated at the left of the Figure.

of these proteins by MLCK are presented in Table 3. Other than turkey gizzard MLC, the only substrate to be phosphorylated by MLCK to any significant extent (above 1% of gizzard MLC) is the M_r -19000 rabbit skeletal-muscle MLC. The maximal rate of phosphorylation of this, when measured at molar concentrations of phosphorylatable MLC equal to that for turkey gizzard MLC, is 30% of that for turkey gizzard MLC. The apparent K_m of MLCK for the M_r -19000 rabbit skeletal-muscle MLC is 21 µM (results not shown), which is equal to the K_m for turkey gizzard MLC.

Phosphorylation of synthetic phosphoacceptor peptides by MLCK

Three synthetic peptides containing sequences homologous to the phosphorylation sites of gizzard MLC (Kemptamide), pyruvate kinase (Kemptide) and the transforming gene product of Rous sarcoma virus pp60^{src} were tested as substrates for MLCK. These three peptides are substrates for MLCKs, cyclic-AMP-dependent protein kinase and tyrosine kinases respectively. As shown in Table 4, Kemptamide was the only peptide to be phosphorylated in a Ca²⁺-calmodulin-dependent manner. The K_m for Kemptamide is 5-fold greater and the V_{max} 20-fold less than the values obtained for MLC as substrate (Table 2).

Comparison of MLCK with protein kinase II

Bovine brain MLCK was compared directly with rat brain calmodulin-dependent protein kinase II, for substrate specificity by using gizzard and skeletal-muscle MLCs and synapsin, and for inhibition by a Protein A-

purified antibody raised against and inhibitory to rat brain protein kinase II. As shown in Fig. 3, the preferred substrate for both enzymes was gizzard MLC under the conditions employed. However, the proportions of rate of phosphorylation by MLCK of gizzard MLC:skeletal muscle MLC:synapsin were 100:16.2:0.31, whereas that by rat brain protein kinase II was 100:1.63:17.0. The inclusion of inhibitory antibody to protein kinase II sufficient to inhibit 95% of the activity of protein kinase II towards gizzard MLC did not affect the rate of phosphorylation of gizzard MLC by bovine brain MLCK (results not shown).

Phosphorylation of MLCK by cyclic-AMP-dependent protein kinase

Incubation of purified MLCK with the catalytic subunit of cyclic-AMP-dependent protein kinase resulted in the incorporation of phosphate into MLCK (Fig. 4). Incorporation of ³²P into the M_r -152000 protein was time- and enzyme-concentration-dependent. There was no significant autophosphorylation of MLCK under the conditions employed (Fig. 4, lane 2). Phosphorylation of MLCK in the presence of Ca²⁺ was inhibited by calmodulin. In the experiment illustrated in Fig. 4, after 30 min, 0.38 mol of phosphate was incorporated into the M_r -152000 MLCK in the absence of calmodulin (lane 3), whereas in the presence of calmodulin (lane 4) phosphate incorporation into MLCK was limited to 0.11 mol/mol. The kinetic consequences of MLCK phosphorylation on the affinity of the enzyme for calmodulin were examined in a series of experiments in which MLCK was phosphorylated in the absence and presence of Ca²⁺ and

Fig. 4. Effect of calmodulin on the phosphorylation of MLCK by cyclic-AMP-dependent protein kinase

MLCK (500 ng) was incubated for 30 min at 30 °C in 25 mM-Pipes (pH 6.8)/10 mM-MgCl₂/1 mM-[γ -³²P]ATP/1.5 mM-DTT/0.5 mM-EGTA, in the absence (lanes 2 and 3) and the presence (lane 4) of 1.5 mM-CaCl₂ and 15 μ g of calmodulin/ml, in the absence (lane 2) and the presence (lanes 3 and 4) of 25 ng of catalytic subunit of cyclic-AMP-dependent protein kinase in a final volume of 30 μ l. Reaction mixtures were subjected to SDS/polyacrylamide-gel electrophoresis. Lane 1, Coomassie-Blue (CB)-stained gel; lanes 2–4, autoradiogram (AR) of dried gel. Arrowheads mark the positions of protein standards of M_r values given at the left of the Figure.

calmodulin. Phosphorylation of MLCK by the cyclic-AMP-dependent protein kinase in the presence of EGTA to the extent of 0.90 mol of phosphate/mol of MLCK resulted in an increase in the K_m of MLCK for calmodulin from 3 to 15 nM (results not shown). In the presence of Ca²⁺ and calmodulin, 0.50 mol of phosphate/mol of MLCK was incorporated, with no change in the K_m for calmodulin.

DISCUSSION

The presence of a calmodulin-dependent protein kinase in bovine brain, capable of phosphorylating the

light chains of smooth-muscle myosin, was first reported by Dabrowska & Hartshorne (1978) and later by Hathaway *et al.* (1981). Dabrowska & Hartshorne (1978) partially purified MLCK from both human platelets and bovine brain and observed that both kinases had monomeric M_r values of 105 000 as estimated by SDS/polyacrylamide-gel electrophoresis and gel filtration. Hathaway *et al.* (1981) partially purified MLCK from bovine brain and correlated kinase activity with a protein of apparent M_r 130 000 as estimated by SDS/polyacrylamide-gel electrophoresis. They reported a sedimentation coefficient which is similar to that observed for our preparation; however, the K_m of MLCK for ATP reported (80 μ M) is 4 times as high as that observed in the present study. The specific activity of MLCK for gizzard MLC reported was approximately one-third that observed in the current study. Since neither of the previous reports on bovine brain MLCK describe the substrate specificity of the enzyme, it is not possible to compare the preparations by using this parameter.

The M_r of 152 000 for bovine brain MLCK as estimated by SDS/polyacrylamide-gel electrophoresis is virtually identical with that reported for the enzyme from bovine stomach muscle (Walsh *et al.*, 1982). MLCKs isolated from other types of bovine smooth muscle have been reported to range in M_r from 67 000 and 79 000 from bovine carotid artery (Bhalla *et al.*, 1982) to 142 000 for MLCK isolated from bovine aorta (Vallet *et al.*, 1981). Walsh & Hartshorne (1982) have suggested that the wide variability in M_r of MLCKs may be an artifact, the lower- M_r forms being active proteolytic fragments of native MLCKs of M_r > 100 000. The difference in M_r values observed for bovine brain MLCK here and in previous reports could be explained in this manner. The similarities between the brain enzyme and that isolated from smooth muscle invite the speculation that the MLCK described here is derived solely from the smooth muscle of the brain vasculature. Several lines of evidence appear to be in opposition to this premise. Care was taken to trim the major blood vessels from the tissue before homogenization. Although the amount of MLCK found in brain is small as compared with smooth- and skeletal-muscle sources, it is comparable with the amount of MLCK isolated from bovine cardiac muscle (Wolf & Hofmann, 1980). The vast majority of MLCK activity in bovine brain was found in the soluble fraction, whereas MLCK from smooth-muscle sources is prepared from pellet fractions (Walsh *et al.*, 1982; Bhalla *et al.*, 1982). Resolution of this issue awaits the localization, perhaps by immunocytochemistry, of MLCK to cells of neural origin.

Of the substrates tested, bovine brain MLCK was found to phosphorylate appreciably only MLCs from turkey gizzard and rabbit skeletal muscle, and Kempamide, a synthetic peptide containing the phosphorylation site (residues 11–23) of chicken gizzard MLC. The specific activity of bovine brain MLCK towards gizzard myosin is comparable with that reported for the turkey gizzard enzyme (Walsh *et al.*, 1983), but is one-third the value obtained with bovine stomach muscle MLCK (Walsh *et al.*, 1982). With Kempamide as substrate, bovine brain MLCK exhibited a K_m 5 times higher and a V_{max} 30 times lower than that reported for turkey gizzard MLCK (Kemp *et al.*, 1983). These differences in kinetic parameters between the gizzard and brain enzymes may reflect the use of substrates derived from hetero-

logous species and types of tissues in the assay of bovine brain MLCK.

Ikebe *et al.* (1986) have reported that threonine-18 as well as serine-19 of turkey gizzard MLC are phosphorylated when phosphate incorporation exceeds 1 mol/mol. Upon incubation of the same substrate with bovine brain MLCK, only serine was phosphorylated when 0.6 mol of phosphate was incorporated/mol of MLC. These data, when considered in the light of the ability of MLCK to phosphorylate Kemptamide, suggest that serine-19 of turkey gizzard MLC is phosphorylated by MLCK.

Like MLCKs from smooth and skeletal muscle, bovine brain MLCK is phosphorylated by the catalytic subunit of cyclic-AMP-dependent protein kinase. Its phosphorylation is inhibited by the presence of Ca^{2+} -calmodulin. Conti & Adelstein (1981) have shown that, in the absence of Ca^{2+} -calmodulin, two sites on gizzard MLCK are phosphorylated, resulting in an increase in the K_m for calmodulin. When calmodulin is bound to MLCK, only one site is phosphorylated and there is no change in the affinity of MLCK for calmodulin. The site phosphorylated in a Ca^{2+} -calmodulin-dependent manner has been shown to be located immediately adjacent to the calmodulin-binding domain of MLCK (Lukas *et al.*, 1986). Phosphorylation of cardiac and skeletal-muscle MLCKs in the absence of calmodulin results in the incorporation of 1 mol of phosphate/mol of enzyme, with no changes in the calmodulin-activation properties (Kamm & Stull, 1985). The highest phosphate incorporation into bovine brain MLCK (0.90 mol/mol) was achieved in the presence of calmodulin and EGTA, and was accompanied by an increase in the K_m for calmodulin from 3 to 15 nM. Phosphorylation of MLCK occurred in the presence of Ca^{2+} in the absence of calmodulin, with no resulting change in the K_m for calmodulin.

Multifunctional calmodulin-dependent kinases, denoted calmodulin-dependent protein kinase II and capable of phosphorylating gizzard MLCs, have been isolated from rat brain by several laboratories (Fukunaga *et al.*, 1982; Bennett *et al.*, 1983; Goldenring *et al.*, 1983; Yamauchi & Fujisawa, 1983; Kuret & Schulman, 1984). Comparison of the activity of bovine brain MLCK and protein kinase II towards turkey gizzard and rabbit skeletal-muscle MLCs and bovine brain synapsin, and the failure of an antibody inhibitory to protein kinase II to inhibit bovine brain MLCK, indicated that the enzyme of narrow substrate specificity described here is not the bovine brain form of protein kinase II.

Rat brain protein kinase II has been shown to phosphorylate both the heavy chain and light chain of brain myosin, resulting in an increase in the actin-activated MgATPase activity of myosin (Tanaka *et al.*, 1986). A M_r -120000 calmodulin-dependent MLCK isolated from the tissue phosphorylated only the light chain of brain myosin, with an equivalent increase in the actin-activated MgATPase activity. The question of whether actin-myosin interactions in brain are regulated by MLCK or protein kinase II or both remains to be answered.

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