Purification and characterization of myosin light-chain kinase from the rat pancreas

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We have partially purified a protein kinase from rat pancreas that phosphorylates two light-chain subunits of pancreatic myosin, a doublet with components of 18 and 20 kDa. This protein kinase was purified approx. 1000-fold by sequential $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration, ion-exchange and affinity chromatography on calmodulin–Sephrose 4B. The resultant enzyme preparation is free of cyclic AMP-dependent protein kinase, protein kinase C and calmodulin-dependent type I or II kinase activities. The purified protein kinase is completely dependent on Ca^{2+} and calmodulin, and phosphorylates a 20 kDa light-chain subunit of intact gizzard myosin, suggesting that it belongs to a class of enzymes known as myosin light-chain kinase (MLCK). The apparent K_m values of the putative pancreatic MLCK for ATP (73 μM), gizzard myosin light chains (18 μM) and calmodulin (2 nM) are similar to those reported for MLCKs isolated from smooth muscle, platelet and other sources. The enzyme is half-maximally activated at a free Ca^{2+} concentration of 2.5 μM . A single component of the affinity-purified kinase reacts with antibodies to turkey gizzard MLCK. The apparent molecular mass of this component is 138 kDa. Immunoprecipitation of a pancreatic homogenate with these antibodies decreases calmodulin-dependent kinase activity for pancreatic myosin by over 85%. The immunoprecipitate contains a single electrophoretic band of 138 kDa. Tryptic phosphopeptide analyses of pancreatic myosin, phosphorylated by either gizzard or pancreatic MLCK, are identical. Thus the enzyme that we have purified from rat pancreas is a MLCK, as judged by (1) absolute dependence on Ca^{2+} and calmodulin, (2) high affinity for calmodulin, (3) narrow substrate specificity for the light-chain subunit of myosin, and (4) reactivity with antibodies to turkey gizzard MLCK. These studies establish the existence of a pancreatic MLCK which may be responsible for regulating myosin phosphorylation and enzyme secretion *in situ*.

INTRODUCTION

Pancreatic secretion is accompanied by changes in the extent of phosphorylation of several proteins [1,2]. It is postulated that such covalent modifications alter the physical and chemical properties of these proteins and allow them to mediate subsequent steps in secretion [3]. Myosin has recently been shown to undergo phosphorylation during agonist-stimulated enzyme secretion from the exocrine pancreas [4,5]. Supporting an effector role for myosin in secretion, Friedman *et al.* [6] have shown that microinjection of a chymotryptic fragment of myosin into adrenal chromaffin cells results in catecholamine secretion.

Phosphorylation of a light-chain subunit of myosin accompanies agonist-induced secretion from platelets [7], basophil leukaemic cells [8] and pancreatic acinar cells [4,5]. A Ca^{2+} - and calmodulin-dependent protein kinase, which phosphorylates a myosin light-chain subunit, has been isolated from several tissues, including smooth muscle [9], platelet [10], adrenal chromaffin cells [11], brain [12] and thyroid [13]. The enzyme, myosin light-

chain kinase (MLCK), is characterized by absolute dependence on Ca^{2+} and calmodulin, and a narrow substrate specificity for a light-chain subunit of myosin. Inhibition of enzyme release from the pancreas by a calmodulin antagonist suggests that a MLCK may participate in exocrine pancreatic secretion [14].

A calmodulin-dependent protein kinase has been isolated from rat pancreas [15]. The characteristics of this enzyme suggest that it is a Type II multifunctional calmodulin-dependent kinase. Unlike enzymes of the MLCK class, it has a multimeric subunit structure, broad substrate specificity and low affinity for calmodulin. Although this enzyme is capable of phosphorylating isolated light chains from gizzard myosin, its ability to phosphorylate intact myosin is unknown. Thus the presence of a protein kinase that is capable of phosphorylating myosin in the pancreas has not been demonstrated.

Therefore, in order to understand the regulation of myosin phosphorylation *in situ* and its possible role in secretion, we have partially purified and characterized MLCK from the rat pancreas. This enzyme, which is

Abbreviations used: MLCK, myosin light-chain kinase; protein kinase C, Ca^{2+} -activated, phospholipid-dependent protein kinase; $\text{MLC}_{20:18}$, pancreatic myosin light-chain doublet of 20 kDa and 18 kDa respectively; MLC_{20} , 20 kDa subunit of gizzard myosin; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis; TLE, thin-layer electrophoresis.

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absolutely dependent on Ca^{2+} and calmodulin for activation, reacts with antibodies to avian smooth-muscle MLCK, and phosphorylates a light-chain doublet of pancreatic myosin, $\text{MLC}_{20:18}$.

EXPERIMENTAL

Materials

Protein molecular-mass standards, electrophoretic-grade Tris, glycine, SDS, Bromophenol Blue, ammonium persulphate, *NNN'*-tetramethylethylenediamine, acrylamide, bisacrylamide and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad (Richmond, CA, U.S.A.). Chromatography resins Sepharose 4B, Sephacryl S-300, DEAE-Sephacel, calmodulin-Sepharose 4B, phenyl-Sepharose and Protein A-Sepharose were obtained from Pharmacia (Piscataway, NJ, U.S.A.). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from ICN Radiochemicals (Costa Mesa, CA, U.S.A.). ^{125}I -Protein A was obtained from Amersham (Chicago, IL, U.S.A.). Electrophoresis coolant for peptide mapping was obtained from Savant Instruments (Farmingdale, NY, U.S.A.). All other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.) and were reagent grade unless specified otherwise. X-Omat AR5 film and cellulose thin-layer plates (Chromagram sheets) were purchased from Kodak (Rochester, NY, U.S.A.). Nitrocellulose sheets for electroblotting were obtained from Schleicher and Schuell (Keene, NH, U.S.A.). The filtration manifold and Millipore type HA (0.45 μm pore size) filters were obtained from Millipore-Waters (Bedford, MA, U.S.A.). Fresh chicken gizzards were purchased from Alliance Poultry (Chicago, IL, U.S.A.). Protein kinase C and synapsin from bovine brain, and the catalytic subunit of cyclic AMP-dependent protein kinase from bovine heart, were generously given by Dr. Clive Palfrey of the University of Chicago. Calmodulin was purified from goat testis to electrophoretic homogeneity by the method of Gopalakrishna & Anderson [16]. Myosin light-chain 20 kDa subunit (MLC_{20}) was purified from chicken gizzards by the method of Hathaway & Haeberle [17]. MLCK was prepared from chicken gizzards by the method of Adelstein & Klee [9]. Affinity-purified antibodies to turkey gizzard MLCK were prepared as previously described [18]. Proteolytic inhibitors soya-bean trypsin inhibitor, phenylmethanesulphonyl fluoride (PMSF) and benzamidine were obtained from Sigma (St. Louis, MO, U.S.A.).

Preparation of pancreatic myosin

Fresh pancreata (11 g wet wt.) from male Sherman rats (150–200 g) were removed and homogenized with a Polytron (Brinkman Instruments, Westbury, NY, U.S.A.) in 5 vol. of extraction buffer containing 0.34 M-sucrose, 50 mM-Tris/HCl (pH 7.5), 5 mM-EGTA, 1 mM-EDTA, 5 mM-2-mercaptoethanol and 5 mM- NaN_3 . Purification steps were carried out at 4 °C, and all aqueous buffers were prepared with deionized water. The extraction buffer contained 0.1 mg of soya-bean trypsin inhibitor/ml, 1 mM-PMSF and 1 mM-benzamidine, as proteinase inhibitors. PMSF was added immediately before needed from a 2 M stock made up in dimethyl sulfoxide. The extract was centrifuged at 13000 g for 20 min, after which the supernatant was fractionated

with solid $(\text{NH}_4)_2\text{SO}_4$. The K-EDTA-stimulated ATPase peak, precipitated between 35 and 65 % $(\text{NH}_4)_2\text{SO}_4$ saturation, was resuspended in high-salt buffer, containing 0.5 M-NaCl, 50 mM-Tris/HCl (pH 7.5), 5 mM-EGTA, 1 mM-EDTA, 5 mM-2-mercaptoethanol and 5 mM- NaN_3 . This fraction was separated by gel filtration on a 2.6 cm $\times$ 90 cm Sepharose 4B column. The fractions containing myosin, identified by the K-EDTA-ATPase peak [19] and by SDS/polyacrylamide-gel electrophoresis (PAGE), were pooled and concentrated under vacuum dialysis in a buffer of 20 mM- MgCl_2 , 20 mM-NaCl and 20 mM-Mops, pH 6.4. Myosin, which precipitates under these conditions, was collected by centrifugation at 10000 g for 20 min. The precipitated protein was resuspended at 10 mg/ml in 0.5 M-NaCl/50 mM-Tris/HCl (pH 7.5)/5 mM-EGTA/1 mM-EDTA/5 mM-2-mercaptoethanol, made 50 % (v/v) in glycerol and stored at -20°C .

Assays for MLCK activity

Unless indicated otherwise, MLCK activity was assayed in 0.1 ml of 25 mM-Tris/HCl (pH 7.5) containing 0.1 mM-dithiothreitol, 10 mM- MgCl_2 , 0.2 mM- CaCl_2 (in excess over EGTA) or 2 mM-EGTA, 0.2 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2000 c.p.m./pmol) and 0.1 mg of gizzard MLC_{20} /ml at 30 °C. The reaction was initiated by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Calmodulin (0.1 μM) was included in all assays, unless indicated otherwise. One of two methods was used for the measurement of ^{32}P incorporation into gizzard MLC_{20} or pancreatic $\text{MLC}_{20:18}$. In the first method, a portion was sampled into a trichloroacetic acid/ $\text{Na}_4\text{P}_2\text{O}_7$ solution to give final concns. (w/v) of 10 % trichloroacetic acid and 2 % $\text{Na}_4\text{P}_2\text{O}_7$. The precipitated protein was trapped by filter on a Millipore filtration apparatus. After washing with 3×10 ml of 5 % trichloroacetic acid/2 % $\text{Na}_4\text{P}_2\text{O}_7$, the filter was immersed in 15 ml of Aquasol (Amersham) and counted for radioactivity in a Beckman scintillation counter. In the second method, selected samples were boiled in Laemmli [20] sample buffer and resolved by electrophoresis on a SDS/polyacrylamide (5–18 % gradient) gel. The ^{32}P -labelled gizzard MLC_{20} or pancreatic $\text{MLC}_{20:18}$ was identified from an autoradiogram and excised. The labelled bands were solubilized in Aquasol and counted for radioactivity, as for the filters. Unless indicated otherwise, ^{32}P incorporation into gizzard MLC_{20} or pancreatic $\text{MLC}_{20:18}$ is expressed in c.p.m. in the presence of Ca^{2+} and calmodulin minus c.p.m. in the presence of EGTA. The Ca^{2+} - and calmodulin-independent phosphorylation was always less than 10 % of total radioactivity. Selected column fractions from the chromatography steps were assayed with intact pancreatic myosin in place of gizzard MLC_{20} to verify MLCK activity. When pancreatic myosin was assayed as substrate, 20 μg was included and the assay contained 60 mM-NaCl. The concentrations of the other substrates assayed with myosin kinase were histone II-A, 0.20 mg/ml, and synapsin, 0.10 mg/ml.

SDS/PAGE

PAGE was performed in 5–18 %-gradient slab gels with the buffer system described by Laemmli [20]. Gels were stained with Coomassie Blue, or with a silver stain [21], if greater sensitivity was required. After staining, the gels were destained and dried down under vacuum.

Protein immunoblotting

Samples were subjected to SDS/PAGE as described above. After electrophoresis, the gels were transferred to nitrocellulose sheets at 15 mA for 15 h in a buffer of 20 % (v/v) methanol/25 mM-Tris/192 mM-glycine (pH 8.3). The method of Towbin *et al.* [22] was used for subsequent immunoblotting. After incubation with the primary antibody, the blots were incubated for 2 h with ^{125}I -Protein A (0.1 $\mu\text{Ci/ml}$) and an autoradiogram was prepared.

Protein determination

Protein concentrations were determined by the methods of Lowry *et al.* [23] or Bradford [24], with bovine serum albumin as the standard.

Phosphopeptide mapping and phosphoamino acid analysis

Two-dimensional cellulose thin-layer maps of tryptic peptides of phosphorylated pancreatic myosin were prepared by the method of Axelrod [25], as modified by Alper *et al.* [26]. Pancreatic myosin (45 μg), in 25 mM-Tris/HCl (pH 7.5) containing 0.1 mM-dithiothreitol, 0.2 mM- Ca^{2+} in excess of EGTA, 0.1 μM -calmodulin and 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2000 c.p.m./pmol), was incubated in the presence of gizzard MLCK (1.5 $\mu\text{g/ml}$) or affinity-purified pancreatic MLCK (2 $\mu\text{g/ml}$) for 30 min at 30 °C. After the reaction was stopped by boiling in SDS, myosin was resolved by SDS/PAGE and an autoradiogram was prepared. The ^{32}P -labelled pancreatic $\text{MLC}_{20:18}$ was identified from the autoradiogram and excised. The bands were digested with trypsin (50 $\mu\text{g/ml}$) in 0.05 M- NH_4HCO_3 for 12 h at 37 °C. The supernatant from the gel digest was collected and the gel was washed with 500 μl of 0.05 M- NH_4HCO_3 . The digest and wash were combined and freeze-dried. The freeze-dried protein was solubilized in TLE buffer (acetic acid/pyridine/water, 10:1:100, by vol.), pH 3.5, and re-freeze-dried. The samples were dissolved a second time in a small volume of TLE buffer and spotted on a t.l.c. plate with Phenol Red added as a dye marker. Separation in the first dimension was by electrophoresis at 400 V for 2 h in TLE buffer, pH 3.5. Separation in the second dimension was by ascending liquid t.l.c. in buffer containing butan-1-ol/pyridine/acetic acid/water (13:10:2:8, by vol.). After chromatography, the plate was dried and exposed to an X-ray film.

The method of Bitte & Kabat [27], as modified by Alper *et al.* [26], was used for phosphoamino acid-analyses of the ^{32}P -labelled $\text{MLC}_{20:18}$. The conditions for myosin phosphorylation were identical with those described previously for the phosphopeptide analyses. After trypsin digestion and freeze-drying, samples were hydrolysed in 6 M-HCl at 110 °C under vacuum for 2 h. After hydrolysis and re-freeze-drying, samples were dissolved in acetic acid/formic acid/water (9:3:48, by vol.), pH 1.9. Phosphoamino acid standards (Sigma) of phosphoserine and phosphothreonine were added to the sample, and Phenol Red was included as a dye marker. The samples and standards were separated by electrophoresis in the acetic acid/formic acid/water buffer at 500 V for 2 h. The plate was dried and the standards were identified by spraying the plate with 0.1 % ninhydrin in acetone. An autoradiogram of the plate was prepared and migration

of the ^{32}P -labelled amino acids was compared with migration of the standards.

Calculation of free Ca^{2+} concentrations

Free Ca^{2+} , MgATP and CaATP concentrations were calculated by using the computer program of Fabiato & Fabiato [28]. The CaEGTA buffers were prepared as described by Ashley & Moisesescu [29]. The free Ca^{2+} concentrations were confirmed by using an independently standardized Fura II Ca^{2+} -indicator fluorescence assay [30].

RESULTS

Rat pancreatic myosin

We purified myosin from rat pancreas in order to study its phosphorylation *in vitro*. The extraction and purification steps were carried out in the presence of several proteinase inhibitors, as the pancreas is a rich source of these enzymes. After Sepharose 4B gel filtration and precipitation in the presence of Mg^{2+} at low ionic strength and acid pH, pancreatic myosin is over 95 % pure by electrophoretic criteria. Pancreatic myosin has a multimeric subunit structure, consisting of a large subunit of 200 kDa and three smaller subunits of 20, 18 and 15 kDa (Fig. 1). Densitometry of the Coomassie-stained

Fig. 1. Coomassie Blue stain and autoradiogram of SDS/polyacrylamide gradient gel of gizzard and pancreatic myosins (10 μg)

Gizzard (GIZZ.) and pancreatic (PAN.) myosins were phosphorylated with gizzard MLCK as described in the Materials and methods section. Pancreatic myosin is composed of a heavy-chain subunit (MHC) of 200 kDa and three light-chain subunits of 20, 18 and 15 kDa. The light-chain doublet of 20 and 18 kDa, $\text{MLC}_{20:18}$, is phosphorylated by gizzard MLCK in the presence of Ca^{2+} and calmodulin.

gel of pancreatic myosin gives the proportions 20:1:1:2 (by wt.) respectively for these subunits. Approx. 2.5 mg of purified myosin was obtained per g of pancreatic homogenate protein, reflecting a 22% recovery, based on K-EDTA-ATPase activity.

Initially, we examined the ability of gizzard MLCK to phosphorylate pancreatic myosin. We found that gizzard MLCK, in the presence of Ca^{2+} and calmodulin, phosphorylated two light-chain components of pancreatic myosin, one of 20 kDa and the other of 18 kDa. Both components of this myosin light-chain doublet ($\text{MLC}_{20:18}$) were phosphorylated equally (Fig. 1). To test for the presence of an endogenous pancreatic MLCK, we investigated the ability of pancreatic homogenate to phosphorylate purified pancreatic myosin. A Ca^{2+} - and calmodulin-dependent increase in ^{32}P labelling of pancreatic $\text{MLC}_{20:18}$ was demonstrated (results not shown).

Purification of MLCK from rat pancreas

Kinase extraction. Purification steps were carried out at 4 °C, and all aqueous buffers were prepared with deionized water. Fresh pancreata (40 g wet wt.) from male Sherman rats (150–250 g) were homogenized with a Polytron in 5 vol. of extraction buffer, containing 0.34 M-sucrose, 50 mM-Tris/HCl (pH 7.5), 5 mM-EGTA, 1 mM-EDTA, 15 mM-2-mercaptoethanol and 5 mM- NaN_3 . The extraction buffer was made 0.1 mg/ml in soya-bean trypsin inhibitor, 10 mM in benzamidine and 1 mM in PMSF to inhibit proteinase activity. The extract was centrifuged at 50 000 *g* for 30 min. The supernatant was recovered and centrifuged at 100 000 *g* for 60 min. After this ultracentrifugation, the supernatant was filtered through glass wool to remove lipid and fractionated by adding solid $(\text{NH}_4)_2\text{SO}_4$. The protein precipitated between 35 and 65% satd. of $(\text{NH}_4)_2\text{SO}_4$ was collected by centrifugation at 27 000 *g* for 20 min. The precipitate was resuspended by using a glass Dounce homogenizer in a small volume of buffer containing 0.5 M-NaCl, 50 mM-Tris/HCl (pH 7.5), 5 mM-EGTA, 1 mM-EDTA, 15 mM-2-mercaptoethanol and 5 mM- NaN_3 . After homogenization, soya-bean trypsin inhibitor (0.1 mg/ml) and PMSF (1 mM final concn.) were added. The sample was dialysed against the same buffer without inhibitors for 2 h and centrifuged for 60 min at 100 000 *g*.

Column-chromatography steps. The supernatant from this ultracentrifugation step (15 ml, 24 mg/ml) was applied to a column (2.6 cm $\times$ 80 cm) of Sephacryl S-300. The column was equilibrated and developed in 0.5 M-NaCl/50 mM-Tris/HCl (pH 7.5)/5 mM-EGTA/1 mM-EDTA/15 mM-2-mercaptoethanol/5 mM- NaN_3 . The flow rate was adjusted to 35 ml/h, and 5.2 ml fractions were collected. A single peak of MLCK activity was eluted from the column with $K_{av} = 0.24$. This peak was pooled and dialysed for 12 h against 4 litres of 20 mM-Tris/HCl (pH 7.8)/1 mM-EGTA/5 mM-2-mercaptoethanol/1 mM-PMSF.

After dialysis, the pooled Sephacryl S-300 peak was separated by ion-exchange chromatography. A 32 ml portion of the pancreatic MLCK peak (0.75 mg/ml) was applied at 20 ml/h to a column (1.6 cm $\times$ 7 cm) of DEAE-Sephacel, which was equilibrated with 20 mM-Tris/HCl (pH 7.8)/1 mM-EGTA/5 mM-2-mercaptoethanol. The kinase was eluted with a 200 ml linear NaCl gradient (20–600 mM-NaCl) made up in the column

equilibration buffer; 2.5 ml fractions were collected. The kinase was eluted as a single peak at approx. 0.26 M-NaCl. The fractions containing MLCK activity were pooled and dialysed for 12 h against 1 litre of 20 mM-Tris/HCl (pH 7.8)/50 mM-NaCl/1 mM-EGTA/10 mM-2-mercaptoethanol.

The DEAE-Sephacel pooled peak was adjusted to 0.2 mM free Ca^{2+} and 3 mM free Mg^{2+} , immediately before affinity chromatography, for which 19 ml (0.19 mg/ml) of the pooled pancreatic MLCK peak was applied to a column of calmodulin-Sephacrose 4B. The column (bed volume 1.6 ml) was previously equilibrated with 40 mM-Tris/HCl (pH 7.2)/50 mM-NaCl/0.2 mM- CaCl_2 /3 mM- MgCl_2 /10 mM-2-mercaptoethanol. The column was washed with 3 vol. of column equilibration buffer, and then with 3 vol. of equilibration buffer made 0.2 M in NaCl. The Ca^{2+} /calmodulin-dependent kinase activity was eluted as a prominent peak when 2 mM-EGTA was added to the column buffer. The pooled kinase peak was made 1% in bovine serum albumin, divided into 20 μl batches and stored at -70°C . Under these conditions, the MLCK activity was stable for more than 2 months.

The final enzyme purification represented a 1000-fold increase in MLCK specific activity compared with that in the initial extract. The purification steps are summarized in Table 1. The recovery of pancreatic MLCK was 6% of the activity present in the 35–65%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction.

Characterization of pancreatic MLCK

The kinase fraction was tested for contaminating protein kinase C activity by examining its ability to phosphorylate histone II-A in the presence of 0.5 mM- Ca^{2+} and 10 μg of phosphatidylserine. Similarly, we examined the phosphorylation of synapsin in the presence of 20 μM -8-bromo cyclic AMP or 0.5 mM- Ca^{2+} and 0.1 μM -calmodulin to test for the presence of cyclic AMP-dependent protein kinase or the multifunctional calmodulin-dependent Type I or II protein kinase respectively. In each case the affinity-purified kinase caused no increase in phosphorylation compared with incubation of the substrates with buffer alone. Histone II-A or synapsin was phosphorylated stoichiometrically under the same conditions with purified protein kinase C or the catalytic subunit of cyclic AMP-dependent kinase respectively (results not shown).

The kinetic parameters of pancreatic MLCK were studied by using enzyme purified through the DEAE-Sephacel step, as there was insufficient affinity-purified enzyme available to perform these experiments. The specific activity of this fraction was approx. 60 nmol/min per mg of kinase. The dependence of pancreatic MLCK activity on gizzard MLC_{20} , calmodulin, ATP or Ca^{2+} was measured under linear conditions. Rates of the reactions are expressed in relative units, V_R , normalized to 100% for maximal velocity. To examine the calmodulin activation of pancreatic MLCK, we tested the phosphorylation of gizzard MLC_{20} by pancreatic MLCK in the presence of increasing concentrations of calmodulin (Fig. 2a). The K_m for calmodulin was 2.0 nM, as estimated from the concentration required for half-maximal activation of the enzyme. A free Ca^{2+} concentration of 2.5 μM was found to cause 50% activation of pancreatic MLCK (Fig. 2b). The dependence of pancreatic MLCK activity on gizzard

Table 1. Purification of MLCK from rat pancreas

Activity of the kinase was determined in the presence of Ca^{2+} and calmodulin. Specific activity refers to nmol of phosphate transferred to gizzard MLC_{20} /min per mg.

Purification step	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)
1. Extract	2220	1021	0.46	— *
2. 35–65 % -satd. $-(\text{NH}_4)_2\text{SO}_4$	356	819	2.3	100
3. Sephacryl S-300	24	360	15.0	44
4. DEAE-Sephacel	3.6	220	61.0	27
5. Calmodulin-Sephacel	0.1	47	470.0	6

* The presence of other kinases in the extract, which are capable of phosphorylating isolated gizzard MLC_{20} , make accurate estimation of pancreatic MLCK recovery from the extract unreliable.

Fig. 2. Analyses of the kinetic parameters of pancreatic MLCK

DEAE-Sephacel-purified pancreatic MLCK was assayed in triplicate at 30 °C under linear conditions with saturating concentrations of the non-varied cofactors/substrates. Maximum MLCK activity is normalized to 100 %. (a) Calmodulin activation. Pancreatic MLCK was assayed in 0.2 mM- CaCl_2 with the indicated concentrations of calmodulin. The reaction (100 μl) contained 25 mM-Tris/HCl (pH 7.5), 10 mM- MgCl_2 , 0.02 mM-EGTA, 0.2 mM free Ca^{2+} , 0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (250 c.p.m./ μmol) and 50 μM gizzard MLC_{20} . Pancreatic MLCK concentration was 50 $\mu\text{g}/\text{ml}$. The reaction was terminated by boiling in SDS, and ^{32}P incorporation was measured as described in the text. (b) Ca^{2+} activation. Pancreatic MLCK was assayed under the same conditions as in (a), except that calmodulin was 0.1 μM in all fractions and free Ca^{2+} was adjusted to the indicated concentrations by using Ca^{2+} -EGTA buffers as outlined in the text. (c) Lineweaver-Burk analysis of the dependence of pancreatic MLCK activity on gizzard MLC_{20} . Pancreatic MLCK was assayed under the conditions in (a), except that all reactions contained 0.1 μM -calmodulin and the indicated concentrations of gizzard MLC_{20} . The total protein in each assay was adjusted to 200 μg by addition of bovine serum albumin. Points corresponding to concentrations of light chain ranging from 2.5 μM to 100 μM were used to determine the slope and intercept of the line. The line was fitted by the method of least squares. (d) Lineweaver-Burk analysis of the dependence of pancreatic MLCK activity on ATP. Pancreatic MLCK was assayed under the conditions in (a), except that all reactions contained 0.1 μM -calmodulin and the indicated concentrations of ATP. The specific radioactivity of ATP was held constant at 250 c.p.m./ μmol . Points corresponding to concentrations of ATP ranging from 25 μM to 500 μM were used to determine the slope and intercept of the line. The line was fitted by the method of least squares.

MLC_{20} and ATP both followed Michaelis-Menten kinetics. The double-reciprocal plots indicate that the K_m for gizzard MLC_{20} was 18 μM (Fig. 2c) and that the K_m for ATP was 73 μM (Fig. 2d). Table 2 summarizes the concentrations of gizzard MLC_{20} , calmodulin, ATP and free Ca^{2+} for half-maximal activation of pancreatic

MLCK. The K_m values measured with this preparation were similar to those found in preliminary experiments with the affinity-purified kinase.

The antigenic similarity of pancreatic and smooth-muscle MLCKs was tested by studying the ability of affinity-purified antibodies to turkey gizzard MLCK to

Table 2. Kinetic characterization of rat pancreatic MLCK

Phosphorylation assays contained approx. 50 μ g of DEAE-Sephacel-purified pancreatic MLCK/ml in 25 mM-Tris/HCl (pH 7.5). Incubations were carried out under linear conditions at 30 °C. Concentrations of non-varied components were saturating, with 50 μ M gizzard MLC_{20} , 0.5 mM-ATP, 10 mM- Mg^{2+} , 0.1 μ M-calmodulin and 0.2 mM- Ca^{2+} in excess of EGTA. For determination of K_m and $V_{\max}$, gizzard [MLC_{20}] was varied from 2.5 to 100 μ M. [Calmodulin] was varied from 0.01 to 100 nM, [ATP] from 25 to 500 μ M, and free [Ca^{2+}] from 0.01 to 10 μ M.

	K_m (μ M)	$V_{\max}$ (μ mol/min per mg)
Chicken gizzard MLC_{20}	18	0.470
Calmodulin	0.002	—
ATP	73	—
Ca^{2+}	2.5	—

react with pancreatic MLCK. As shown in Fig. 3(a), the antibodies react with the kinase eluted from the calmodulin-Sepharose 4B column, and a co-migrating component in the pancreatic homogenate. By electrophoretic determination of molecular mass [31], the kinase is of 138 kDa in SDS under reducing conditions. Turkey gizzard MLCK migrates slightly faster, with an apparent molecular mass of 130 kDa. We ensured that the component reacting with gizzard anti-MLCK antibodies is indeed a MLCK, by immunoprecipitating pancreatic MLCK from a pancreatic homogenate. The immunoprecipitate was collected with Protein A-Sepharose and the residual kinase activity in the supernatant was quantified by using intact pancreatic myosin as substrate. Antibodies to the gizzard kinase were found to immunoprecipitate more than 85% of MLCK activity. The immunoprecipitate was solubilized and resolved on an SDS/polyacrylamide gel. Silver staining of the gel revealed a single band of 138 kDa (Fig. 3b).

Finally, to compare the phosphorylation sites, pancreatic myosin was phosphorylated by either gizzard or pancreatic MLCK in the presence of [γ - ^{32}P]ATP. The peptide maps of the ^{32}P -labelled $\text{MLC}_{20:18}$ were identical (Fig. 4). Two major ^{32}P -labelled tryptic peptides were identified. Each may represent one component of the phosphorylated $\text{MLC}_{20:18}$ doublet. Both gizzard and pancreatic MLCK phosphorylated predominantly serine residues of $\text{MLC}_{20:18}$. However, pancreatic MLCK also phosphorylated a small amount of threonine (Fig. 5).

DISCUSSION

The recent demonstration that myosin is phosphorylated during stimulated pancreatic enzyme secretion suggests that a myosin-specific kinase may be involved in exocrine secretion. We therefore sought to isolate from pancreas a kinase capable of regulating myosin phosphorylation *in vitro*. Our initial experiments demonstrated a Ca^{2+} - and calmodulin-stimulated protein kinase activity in the homogenate with specificity for pancreatic myosin. Interestingly, two light-chain subunits

Fig. 3. Immunoreactivity of pancreatic MLCK

(a) Immunoblot analyses of turkey gizzard MLCK (G), affinity-purified pancreatic MLCK (P) and pancreatic homogenate (PH). Turkey gizzard MLCK (0.01 μ g), calmodulin-Sepharose-purified pancreatic MLCK (0.1 μ g) and pancreatic homogenate (20 μ g) were resolved by electrophoresis on a SDS/polyacrylamide (5–18% gradient) gel. The proteins were transferred to nitrocellulose sheets by electroblotting for 12 h at 15 mA as described in the text. The nitrocellulose blots were incubated for 2 h with antibody to MLCK (1:1000 dilution), followed by an incubation for 2 h with ^{125}I -Protein A. An autoradiogram was then prepared. (b) Immunoprecipitation of pancreatic MLCK from pancreatic homogenate. A 5 mg sample of pancreatic homogenate (100000 g extract supernatant) was incubated with anti-(gizzard MLCK) antibodies at a dilution of 1:1000. After a 2 h incubation at 4 °C, Protein A-Sepharose (50 μ l) was added and the incubation continued for 30 min. The Protein A-Sepharose was collected by centrifugation (12500 g for 5 min) and the precipitate recovered. Silver staining of the SDS/polyacrylamide gel of the immunoprecipitate reveals a single band with an apparent molecular mass of 138 kDa (arrowhead). The bands of approx. 55 kDa are antibody heavy chains. Samples in (a) and (b) were resolved on separate gels. Electrophoretic mobilities of molecular-mass standards for each are shown to the right.

of myosin were phosphorylated, a doublet consisting of a 20 kDa and an 18 kDa component ($\text{MLC}_{20:18}$).

Purification of a myosin-specific kinase from pancreas is made difficult by the limited quantities of the enzyme present and by the high proteolytic activity of this tissue. In the case of smooth muscle, MLCK is greatly enriched before the chromatography steps by preparation of myofibrils. However, for pancreatic MLCK, the first chromatography step, gel filtration, begins with a much cruder cell fraction, containing many proteinases. In both muscle and platelets, proteinase activity has led to discordant findings regarding the molecular mass and

Fig. 4. Autoradiograms of two-dimensional tryptic peptide maps of the ^{32}P -labelled pancreatic $\text{MLC}_{20:18}$

Pancreatic myosin ($45\text{ }\mu\text{g}$) was incubated under the conditions described in the text in the presence of (a) gizzard MLCK ($1.5\text{ }\mu\text{g/ml}$) or (b) affinity-purified pancreatic MLCK ($2\text{ }\mu\text{g/ml}$) for 30 min at $30\text{ }^{\circ}\text{C}$. The reaction was stopped by boiling in SDS. The ^{32}P -labelled pancreatic $\text{MLC}_{20:18}$ was separated by SDS/PAGE, identified from the autoradiogram and excised. Subsequent tryptic digestion and analyses were carried out as described in the text. Origins are indicated by the open circles. Electrophoresis was in the horizontal dimension with the anode at the right. T.l.c. was in the vertical dimension.

Ca^{2+} /calmodulin regulation for MLCKs [10,32]. The presence of single co-migrating electrophoretic bands in pancreatic homogenate and in the calmodulin-Sepharose-purified kinase, that react with antibodies to gizzard MLCK, indicate the effectiveness of our proteinase inhibition. The absence of significant Ca^{2+} -independent MLCK activity during the preparation also suggests effective inhibition of proteolysis.

MLCK has been purified from several tissues, including smooth muscle [9], platelets [10], adrenal chromaffin cells [11], brain [12] and thyroid [13]. However, only in the first two cases was myosin, the putative substrate for MLCK, purified from the same tissue and confirmed to be a specific substrate. Importantly, we have demonstrated that the enzyme which we have isolated is capable of phosphorylating light-chain subunits of intact myosin from rat pancreas. Moreover, it has several characteristics consistent with its assignment as a MLCK, including: (1) absolute dependence on Ca^{2+} and calmodulin, (2) high affinity for calmodulin, (3) narrow substrate specificity for myosin light-chain subunits and (4) reactivity with antibodies to turkey gizzard MLCK.

Pancreatic and gizzard MLCK are comparable in their affinity for calmodulin (K_m values of 2.0 nM and 1.0 nM respectively) [9]. Compared with gizzard MLCK, pancreatic MLCK has slightly higher K_m values for ATP ($73\text{ }\mu\text{M}$ versus $50\text{ }\mu\text{M}$) and for isolated gizzard MLC_{20} ($18\text{ }\mu\text{M}$ versus $5\text{ }\mu\text{M}$). With respect to these kinetic parameters, pancreatic MLCK more closely resembles MLCK purified from human platelets than that purified from gizzard [10]. Although the specific activity of

the calmodulin-Sepharose-purified pancreatic MLCK represents a 1000-fold purification over the starting homogenate, V_{max} for the isolated gizzard MLC_{20} is 6-fold lower than that of platelet MLCK (0.5 versus $3\text{ }\mu\text{mol/min per mg}$ of kinase) [10]. This, in part, reflects incompleteness of the purification, but isolated gizzard MLC_{20} may also be a better substrate for platelet MLCK than for pancreatic MLCK. Like gizzard MLCK, pancreatic MLCK exhibits Michaelis-Menten kinetics toward gizzard MLC_{20} . In contrast, Serventi & Coffee [11] reported positive co-operativity of adrenal chromaffin-cell MLCK towards phosphorylatable skeletal-muscle myosin light chains. Brain MLCK also displayed non-hyperbolic kinetics toward gizzard myosin light chains [33]. Whether these kinetic differences reflect functional differences remains to be seen.

Pancreatic MLCK is absolutely dependent on Ca^{2+} for activation. The concentration of free Ca^{2+} required for 50% activation is higher than that reported for adrenal chromaffin-cell MLCK ($2.5\text{ }\mu\text{M}$ versus $0.17\text{ }\mu\text{M}$) [11]. In contrast, thyroid MLCK requires much higher Ca^{2+} concentrations for activation ($1000\text{ }\mu\text{M}$) than does pancreatic MLCK [13]. Interestingly, a transient increase in cytosolic free Ca^{2+} occurs in pancreatic acinar cells after cholecystokinin-octapeptide-stimulated secretion, and the reported peak stimulated free Ca^{2+} concentration is sufficient to activate pancreatic MLCK [34].

Pancreatic MLCK has antigenic similarities with the turkey gizzard kinase, as demonstrated by reactivity with affinity-purified antibodies to gizzard MLCK. Conserved antigenic sequences have also been noted among turkey gizzard, bovine tracheal and human platelet MLCKs

Fig. 5. Phosphoamino acid analyses of the ^{32}P -labelled pancreatic $\text{MLC}_{20:18}$

Pancreatic myosin was phosphorylated as described in the text, with pancreatic MLCK (P) or gizzard MLCK (G). After isolation by SDS/PAGE and tryptic digestion, the $\text{MLC}_{20:18}$ peptide fragments were hydrolysed in 6 M-HCl at 110 °C. Subsequent analysis was carried out as described in the text. Abbreviations: P-Thr, phosphothreonine; P-Ser, phosphoserine.

[35]. The apparent molecular mass of pancreatic MLCK is 138 kDa, as determined by PAGE in the presence of SDS and reducing agents. This is similar to previously reported values for MLCKs purified from other tissues. Published values of molecular mass for MLCK include 130 kDa from turkey gizzard [9], 150 kDa from bovine adrenal chromaffin cells [11], 152 kDa from bovine brain [12] and 105 kDa from human platelets [10]. Data from the immunoprecipitation experiment support a monomeric structure for pancreatic MLCK, as has been reported for other MLCKs [9–12]. However, without measurement of the molecular mass in a non-denaturing system, this conclusion is tentative.

MLCK is known to play an important role in smooth muscle and many non-muscle cells. Phosphorylation of myosin purified from these cells increases the actin-stimulated myosin MgATPase activity [36,37]. Myosin phosphorylation by MLCK is associated with stimulus-response coupling and cytoskeletal changes in both muscle and non-muscle cells. These include smooth-muscle contraction [38], platelet shape change and secretion [7,39,40], lymphocyte capping [41] and

enterocyte brush-border contraction [42]. Studies in platelets and adrenal chromaffin cells implicate myosin, the substrate for MLCK, in secretion [6,7]. In addition, we and others have shown that a myosin light-chain subunit is phosphorylated during stimulated enzyme secretion from pancreatic acini [4,5]. Burnham *et al.* noted that maximal cholecystokinin-octapeptide (CCK-8) stimulation of acinar-cell secretion from mice is accompanied by ^{32}P labelling of myosin heavy chain and a 25–40% increase in ^{32}P labelling of a 20 kDa myosin light-chain subunit compared with ^{32}P -labelled MLC_{20} from unstimulated acini. Higher concentrations of CCK-8, which cause submaximal stimulation and profound alterations in acinar-cell morphology [43], were associated with even greater light-chain labelling [5]. Moreover, observations that agents which are capable of blocking light-chain phosphorylation inhibit pancreatic secretion are consistent with a role for regulated myosin phosphorylation in secretion [14].

Watanabe *et al.* [44] have also shown that myosin, which is purified from ^{32}P -labelled pancreatic acinar carcinoma cells, is labelled on both the heavy chain and one of the light-chain subunits. The absence of a phosphorylated light-chain doublet in their studies may reflect differences between myosin phosphorylation *in situ* and *in vitro*, or differences between myosin from transformed and non-transformed acinar cells. Alternatively, the electrophoretic system employed by Watanabe *et al.* [44] may have been unable to resolve a closely spaced doublet. Burnham *et al.* [5] noted the phosphorylation of an unidentified protein of 21 kDa which was immunoprecipitated with myosin antibodies. As this 21 kDa protein is present in their purified myosin preparation, we suspect it is also a myosin light-chain subunit.

For myosin purified from pancreatic acinar carcinoma cells, actin stimulates pancreatic myosin MgATPase activity [44]. However, for the ATPase experiments, the extent of myosin phosphorylation was not quantified. In preliminary studies, we have found that MLCK phosphorylation of pancreatic $\text{MLC}_{20:18}$ increases actin-stimulated pancreatic myosin MgATPase activity. In addition to light-chain phosphorylation, myosin heavy-chain phosphorylation also occurs *in situ* [5,44]. However, the effect of this phosphorylation on pancreatic myosin function, including ATPase activity or filament formation, is at present unknown.

Additional insights into the function of pancreatic MLCK are provided by ultrastructural studies of the subcellular localization of myosin, the specific substrate for this kinase. Bendayan [45] has localized pancreatic myosin to the sub-apical cytoskeleton and limiting membranes of immature and mature secretory granules. Drenckhahn & Mannherz [46] have emphasized the sub-apical distribution of myosin, interposed between the zymogen granules and the luminal (secretory) membrane. Both the periductular apposition of myosin, and its phosphorylation during hormonal stimulation, support a role for regulated myosin phosphorylation in exocrine pancreatic secretion.

Thus previous biochemical and ultrastructural studies have suggested the involvement of myosin in pancreatic exocrine secretion. Moreover, there are substantial data from physiological studies that myosin is regulated by phosphorylation of a light-chain subunit during secretion. However, the protein kinase responsible for

catalysing this phosphorylation has not previously been identified and characterized. In this study, we demonstrate the presence of a protein kinase that phosphorylates MLC_{20:18} and has many of the biochemical and immunological properties of MLCK purified from smooth muscle and many non-muscle cells. These studies could be extended to understand how the regulation of pancreatic MLCK affects exocrine secretion by the pancreas.

This work was supported in part by a Biomedical Research Support Grant from the National Institutes of Health (BRSG S07RR05476) to M.B. and a grant from the National Science Foundation (BNS 8700881) to P.deL.

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Received 1 August 1988/31 October 1988; accepted 16 November 1988