Ca^{2+}–CALMODULIN DEPENDENT MYOSIN LIGHT-CHAIN PHOSPHORYLATING ACTIVITY IN INSULIN-SECRETING TISSUES

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1. Introduction

Calmodulin, a calcium-binding protein of wide tissue and species distribution, appears to be important in Ca^{2+}-mediated intracellular processes (review [1,2]). The Ca^{2+}–calmodulin complex has been demonstrated to modulate several enzymatic activities including cyclic nucleotide phosphodiesterase, adenyl cyclase, phosphorylase kinase, myosin light-chain kinase (MLCK) and Ca^{2+}-ATPase.

The stimulation of insulin secretion in vitro by most secretagogues requires extracellular Ca^{2+} [3,4] and is associated with changes in Ca^{2+} handling by the β-cell [5,6]. The key regulatory component in this process may be an increase in the intracellular free $[Ca^{2+}]$ (review [7]) which would promote Ca^{2+}/calmodulin interaction. Insulin secreting tissues have a high content of calmodulin [8,9] and insulin secretion is inhibited by calmodulin antagonists [10–12].

The Ca^{2+}–calmodulin-mediated phosphorylation of myosin light-chains (MLC) prepared from non-muscle cells leads to aggregation of myosin molecules into short bipolar filaments and activation of the actomyosin ATPase [13]. In smooth muscle cells also, activation of the actomyosin ATPase accompanies phosphorylation [14]. In skeletal muscle changes in MLC phosphorylation are also evident, however, Ca^{2+} sensitivity of the actomyosin ATPase seems to be conferred principally by the troponin/tropomyosin complex [15]. Actin and myosin have been detected in islets of Langerhans and contractile processes have been implicated in the mechanism whereby insulin secretory granules move within the cell and fuse with the plasma membrane during exocytosis [16–18]. Such processes may be influenced by a Ca^{2+}–calmodulin-dependent MLCK.

This paper demonstrates MLCK activity in rat pancreatic islets and in a rat transplantable islet cell tumour. The partially purified tumour enzyme was activated by calmodulin at physiological $[Ca^{2+}]$. The tumour used in these studies releases insulin by a mechanism indistinguishable from islet tissue [11], and calmodulin isolated from the tumour is identical to that purified from bovine or rat brain as assessed on a broad range of physical and biochemical criteria [9].

Abbreviations: MLCK, myosin light-chain kinase; MLC, myosin light-chains; MES, 2-(*N*-morpholino)ethane-sulphonic acid; DTT, dithiothreitol; EGTA, ethyleneglycol bis (β-aminoethylether) *N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulphate; PMSF, phenylmethylsulphonylfluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid

2. Methods

2.1. *MLCK assays*

The rat islet cell tumour was propagated by subcutaneous implantation in an inbred strain of rats [9,19]. Pancreatic islets were isolated by collagenase digestion [20] from untreated animals of the same strain.

MLCK activity was usually determined electrophoretically. Samples (2–50 μg protein) were incubated for 1–20 min at 30°C in 50 μl 50 mM MES (pH 6.9) containing 1 mM $MgCl_2$, 0.1 mM DTT, 5.1 mM EGTA and 60 μM [γ-^{32}P]ATP (4×10^6 dpm; prepared as in [21] from carrier free $^{32}P_i$; Amersham Intl., Bucks.). Other additions included 5 μM chicken gizzard MLC (prepared as in [22]), 1.2 μM bovine brain calmodulin (prepared as in [9]) and 5 mM $CaCl_2$ which resulted in free Ca^{2+} estimated at 15 μM [23]. The reaction

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was terminated by addition of 30 μl 200 mM Tris–HCl (pH 6.8) containing 20% (w/v) sucrose, 2% (w/v) SDS, 10 mM DTT, 0.02% bromophenol blue and heating to 100°C for 5 min. Samples (50 μl) were electrophoresed on polyacrylamide slab gels polymerised from 17.5% (w/v) acrylamide and 0.073% *N,N'*-methylene–bisacrylamide using the buffer system in [24]. After staining with Kenacid blue R, radioactivity incorporated into MLC was determined either by autoradiography or by removal of the MLC band and determination of its radioactivity by Cerenkov counting.

The kinetic constants of the partially purified MLCK were determined as in [25,26]. Samples (0.3 μg protein) were incubated for 20 min in 100 μl of Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$, 0.1 mM DTT, 50 mM KCl, 5 mM EGTA or 0.1 mM $CaCl_2$, 0.025–0.3 mM [γ-^{32}P]ATP (2×10^6 dpm), 8–85 μM MLC and 1.2 μM calmodulin. Each reaction was terminated by addition of trichloroacetic acid, the phosphorylated proteins recovered by filtration (type HA 0.45 μm, Millipore Corp.) and the radioactivity determined by Cerenkov counting.

2.2. *Partial purification of islet cell tumour MLCK*

Fresh tumour was homogenised in 10 mM MES (pH 6.9) containing 0.27 M sucrose, 1 mM EGTA and 1 mM PMSF. The supernatant obtained after centrifugation at 40 000 × *g* for 60 min was passed through a 1 × 5 cm column of DEAE–Sephadex equilibrated with 0.4 M NH_4HCO_3 (pH 7.9) containing 1 mM EGTA, and eluted by 4 bed vol. of this buffer. This was done to remove endogenous calmodulin which remained bound to the column. The eluate, which contained 90% of the initial protein was adjusted to 2 mM $CaCl_2$ and chromatographed on a 2 × 25 cm affinity column prepared by coupling 20 mg bovine brain calmodulin to 80 ml Sepharose 4B by the CNBr method [27].

3. Results and discussion

3.1. *MLCK activity in tissue extracts*

Little incorporation of radioactivity into peptides with M_r-values equivalent to MLC was observed in crude extracts of the islet cell tumour (fig.1A) in the presence of EGTA alone, Ca^{2+} alone or with Ca^{2+} combined with calmodulin (tracks 1–4). Added MLC were markedly phosphorylated under these conditions

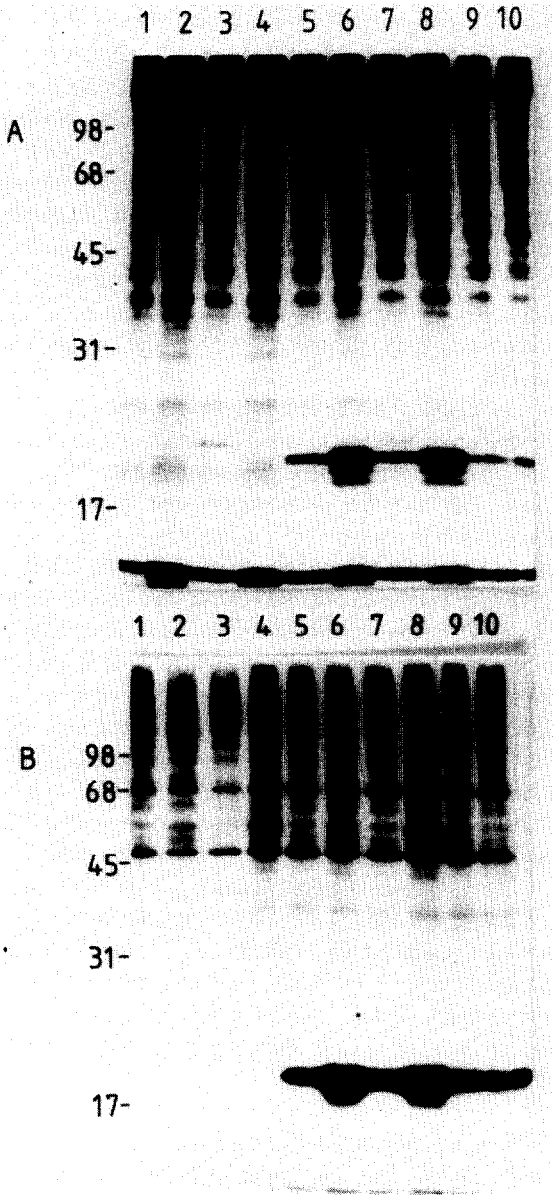

Fig.1. Autoradiogram showing phosphorylation of MLC by (A) an islet cell tumour and (B) pancreatic islets. Tissues (5–10 mg/ml) were disrupted in 50 mM MES (pH 6.9) by sonication for 10 s at 4°C (medium setting MSE ultrasonic disintegrator Mk2, MSE Crawley, Sussex). Extracts were incubated with [γ-^{32}P]ATP for 1 min, the phosphorylated proteins separated by polyacrylamide gel electrophoresis and the gels stained, dried and autoradiographed. Tracks 2,4,6,8,10 have 5 mM Ca^{2+} added in addition to 5.1 mM EGTA. Other additions were: (3,4) calmodulin; (5,6) MLC; (7,8) calmodulin and MLC; (9,10) calmodulin, MLC and trifluoperazine (100 μM). M_r standards used were: phosphorylase (98 000); bovine serum albumin (68 000); ovalbumin (45 000); carbonic anhydrase (31 000); myoglobin (17 000).

(track 5). The activity, then, in the presence of Ca^{2+} was 2–5-fold greater than that with EGTA alone (track 6). Addition of calmodulin (track 8) did not further enhance the Ca^{2+}-dependent phosphorylation of MLC, however, there was presumably sufficient endogenous calmodulin in the tumour extract for full expression of activity. Consistent with this interpretation, the Ca^{2+}-enhanced phosphorylation was abolished by the calmodulin antagonist trifluoperazine (track 10). Homogenates of islets of Langerhans, subjected to the same protocol, gave identical results (fig.1B).

It was important to establish whether the MLCK activity observed was a property of the insulin-containing cell or derived from associated tissue. The tumour used in these studies is principally composed of cells with a typical β-cell morphology. Unlike islets it does not appear to possess glucagon-, pancreatic polypeptide- or somatostatin-containing cells as determined by sensitive immunoassay procedures (Professor L. H. Rees, Drs T. E. Adrian and S. R. Bloom, personal communication). In the adrenal medulla localisation studies have shown that much of the myosin and hence possibly MLCK activity is associated with muscle cells of the vascular bed [28]. The Ca^{2+}–calmodulin-dependent MLCK activity determined in the vessel entering the tumour (1.4 ± 0.4 nmol . min^{-1} . mg^{-1} ($n = 4$)) was not significantly greater than that of the whole tumour (1.2 ± 0.1). The uterus, which like the vasculature is a rich source of smooth muscle, had an activity of 3.9 ± 0.2 nmol . min^{-1} . mg^{-1} in the present assay. It would seem therefore that the β-cell contains a substantial proportion of the MLCK activity observed.

3.2. *Partially purified MLCK activity*

The tumour was used for the partial purification of the Ca^{2+}–calmodulin-dependent MLCK activity; a summary of the procedure and the resultant yield of enzyme activity is shown in table 1. The calmodulin-affinity column used in the final step of the purification (fig.2) resolved 2 major forms of MLCK activity. The presence of 2 forms has been observed in other tissues [30]. The first of these which did not interact with the affinity support, did not depend on Ca^{2+} or calmodulin for activity. The second form which was Ca^{2+}–calmodulin-dependent was further characterised.

Phosphorylation of added MLC in the absence of Ca^{2+} occurred at a rate equivalent to 4% of the maximum achieved with added Ca^{2+} and calmodulin. Ca^{2+}

Table 1
Summary of the purification of islet cell tumour MLCK

		Ca^{2+}–calmodulin-dependent MLCK act.	
	Protein (mg)	Total act. (nmol/min)	Spec. act. (nmol . min^{-1} . mg^{-1})
Initial homogenate	768	1175	1.5
Supernatant: 40 000 × *g* for 60 min	234	662	2.8
DEAE–Sephadex	197	660	3.4
Calmodulin–Sepharose 4B	3.2	199	62.3

MLCK activity was determined electrophoretically at each stage in the purification as described except ATP and MLC were increased to 0.2 mM and 55 μM, respectively. Values were corrected for activity observed in the presence of EGTA

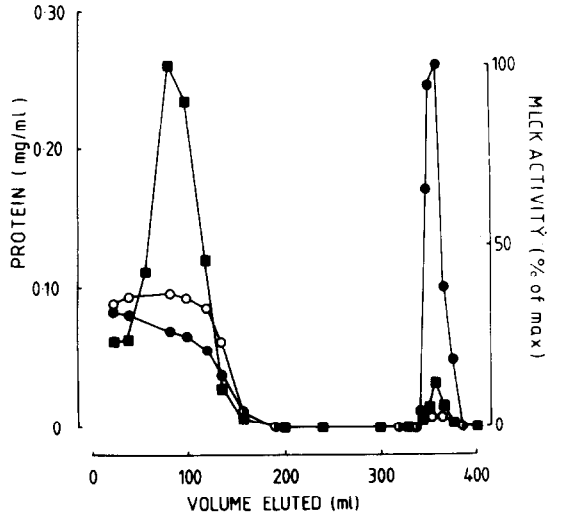

Fig.2. Calmodulin-affinity chromatography of MLCK. The calmodulin-depleted material from the ion-exchange column (~20 mg) was applied to a calmodulin–Sepharose 4B column equilibrated with 20 mM Hepes (pH 6.9) containing 0.3 M NaCl and 1 mM $CaCl_2$. The eluate was recirculated 4 times, the column washed with 4 vol. buffer then with the same buffer containing 1 mM EGTA. The eluate was stored at 4°C with 1 mM PMSF, 0.1 mg/ml lima bean trypsin inhibitor, 0.1 mM *p*-tosyl-L-arginine methyl ester hydrochloride and 0.1 mM L-1-tosylamide-2-phenylethyl-chloromethyl ketone. MLCK was assayed electrophoretically. Protein (■——■) was measured as in [29]; Ca^{2+}–calmodulin-dependent MLCK (●——●) and Ca^{2+}–calmodulin-independent MLCK (○——○) were both expressed as a % of the maximum observed in the presence of Ca^{2+} and calmodulin.

Table 2
The effect of Ca^{2+}, calmodulin and trifluoperazine on MLCK activity

Addition	Ca^{2+}	^{32}P incorp. into MLC (area of peak)
None	–	<0.1
	+	<0.1
MLC	–	0.7
	+	9.3
MLC + calmodulin	–	1.1
	+	18.1
MLC, calmodulin + trifluoperazine	–	1.2
	+	1.2

MLCK activity was determined electrophoretically and the relative radioactivity incorporated into MLC determined by densitometry of the autoradiograph

alone caused 50% of the maximal phosphorylation, but as in crude extracts this was abolished by trifluoperazine. It was demonstrated by direct assay as in [9] that in spite of the precautions taken there was still sufficient calmodulin in the final eluate to account for this phenomenon.

The apparent K_m-values for ATP and the M_r 20 000 light-chain of chicken gizzard myosin were 70 μM and 44 μM, respectively. These values are similar to those observed for MLCK isolated from chicken gizzard [31], human platelets [32] and bovine brain [33].

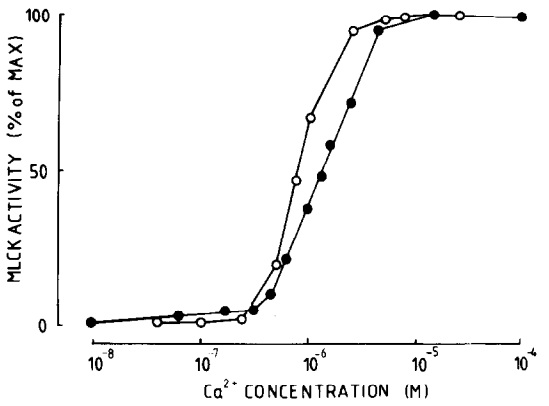

Fig.3. The effect of $[Ca^{2+}]$ on MLCK phosphorylation. MLCK activity (●——●) was determined electrophoretically in the presence of varying $[Ca^{2+}]$. The % conversion of MLCK to the calmodulin · MLCK complex (○——○) was calculated from the intrinsic K_a for MLCK · calmodulin [15], the K_a-values for Ca binding to calmodulin in the presence of 1 mM $MgCl_2$ [34] and assuming that only calmodulin bearing 4 Ca atoms/mol interacted with the enzyme. Calmodulin was maintained at 1.2 μM. [MLCK] was estimated at 2.5 nM on the basis of the specific activity of the purified brain enzyme [33]. The calculated activity was not affected significantly by variation of [enzyme] from 0.1 nM–0.1 μM.

Under the standard assay conditions the partially purified MLCK was half-maximally activated by Ca^{2+} at 1.3 μM. The Ca^{2+}-dependency showed positive cooperativity with a Hill coefficient of ~2. Such findings were concordant with those reported for the skeletal muscle enzyme [15]. The cooperativity may be accounted for by the requirement of the binding of 3 or 4 Ca atoms to calmodulin before activation can occur. Calculation of the concentration of the Ca_4^{2+} · calmodulin · MLCK complex from the reported K_a-values for Ca binding to calmodulin [34] and the intrinsic K_a for MLCK · calmodulin [15] with the assumption that only Ca_4^{2+} · calmodulin binds to MLCK and activates it, gave a Ca^{2+} concentration to activity relationship consistent with the observed findings. Under conditions prevailing in vivo in the β-cell where the calmodulin concentration may be 30–50 μM [8], MLCK would be expected to be half-maximally activated at approximately the Ca^{2+} concentration presently observed on the basis of such calculations.

The Ca^{2+}-sensitivity of MLCK activity thus falls within the concentration range of cytosolic free Ca^{2+} believed to occur during activation of insulin secretion. As such it is compatible with a physiological role for this enzyme in the secretory response.

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