Identification and characterization of Ca^{2+} -phospholipid-dependent protein kinase in rat islets and hamster β -cells

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We show that extracts of rat islets of Langerhans and of cloned hamster β -cells (HIT-T15 cells) contain Ca^{2+} -phospholipid-dependent protein kinase and endogenous protein substrates for the kinase. We purified Ca^{2+} -phospholipid-dependent protein kinase from HIT-T15 β -cells and report here its physical and kinetic properties.

Changes in the intracellular concentrations of Ca^{2+} and cyclic AMP (for reviews see Hedeskov, 1980; Wollheim & Sharp, 1981), as well as alterations in phosphatidylinositol turnover (Freinkel *et al.*, 1975; Clements & Rhoten, 1976; Clements *et al.*, 1977), have been proposed to be key events in stimulus–secretion coupling in the pancreatic β -cell. The principal mediator of the action of cyclic AMP is cyclic AMP-dependent protein kinase, which has been characterized in rat islets of Langerhans (Sugden *et al.*, 1979). One important intracellular target for Ca^{2+} is calmodulin, and a Ca^{2+} -calmodulin-dependent protein kinase has been identified in rat islets (Gagliardino *et al.*, 1980; Harrison & Ashcroft, 1982) and in hamster insulinoma cells (Schubart *et al.*, 1980). Various workers have established the existence in many tissues (Takai *et al.*, 1977; Kuo *et al.*, 1980) of another protein kinase, which is sensitive to Ca^{2+} but is quite independent of calmodulin (Wrenn *et al.*, 1980). This kinase has the unique property of being dependent on phospholipid for its activity, and its sensitivity to Ca^{2+} is in addition modulated by diacylglycerol. Since diacylglycerol is liberated as a consequence of the breakdown of phosphoinositides by phospholipase C (Michell, 1975), an important role for the kinase has been suggested in stimulus–response systems showing a phosphatidylinositol effect (Nishizuka & Takai, 1981); the latter include islets of Langerhans (Clements & Rhoten, 1976; Best & Malaisse, 1983). Although the presence of a Ca^{2+} -phospholipid-dependent protein kinase in rat islets has been shown (Tanigawa *et al.*, 1982), the properties of the enzyme and the existence of endogenous protein substrates have not been reported. The aims of this present study were to confirm the presence of the enzyme in islets of Langerhans, to show that the

activity is in the β -cells, to characterize the enzyme, and to investigate endogenous protein substrates.

Experimental

Materials

Tissue-culture materials were from Gibco (Europe). DEAE-cellulose (DE52) and 31ET filter paper were from Whatman, Maidstone, Kent, U.K. [γ - ^{32}P]ATP (3 Ci/mmol; carrier-free) was from Amersham International, Amersham, Bucks., U.K. Bromophenol Blue, 2-mercaptoethanol, phosphatidylserine, diolein, lysine-rich histone H1 (type V-S), octyl-agarose and standard proteins for M_r determination were from Sigma Chemical Co., Poole, Dorset, U.K. Sephacryl S-300 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Reagents for polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories, Watford, Herts., U.K. Other reagents, of analytical grade, were from British Drug Houses, Poole, Dorset, U.K.

Preparation of tissues

Islets of Langerhans were isolated from pancreases of fed Wistar rats by a collagenase method (Coll-Garcia & Gill, 1969) and collected in bicarbonate medium (Krebs & Henseleit, 1932).

β -Cells were from a cloned cell line of simian-virus-40-transformed hamster β -cells (HIT-T15), which was originally described by Santerre *et al.* (1981) and shown to synthesize and secrete insulin. The cells were maintained in tissue culture in Ham's F-12 medium supplemented with 15% (v/v) horse serum and 2.5% (v/v) foetal-calf serum, and containing streptomycin (100 $\mu\text{g}/\text{ml}$), penicillin (100 units/ml), 0.1 μM -selenous acid and glutathione (10 $\mu\text{g}/\text{ml}$); the gas phase was humidified air/ CO_2 (19:1) and the temperature was 37°C. Cells

Abbreviation used: SDS, sodium dodecyl sulphate.

were collected after brief trypsin treatment in tissue-culture medium, and washed in bicarbonate medium containing 3 mM-glucose. Islets of β -cells were sedimented by centrifugation, resuspended in 20 mM-Tris/HCl (pH 7.4)/2 mM-EDTA/50 mM-2-mercaptoethanol and disrupted by sonication for 2×10 s (Soniprobe 7532A). The sonicated extract was centrifuged at 20 000 *g* for 20 min at 4°C, and the supernatant was retained.

Protein kinase assay

The standard assay mixture (50 μ l) contained 1 μ mol of Tris/HCl (pH 7.4), 0.25 μ mol of magnesium acetate, 10 μ g of histone H1, 0.25 nmol of [γ - ^{32} P]ATP, 1 nmol of ATP, 2.5 nmol of CaCl_2 , 0.8 μ g of phosphatidylserine and 0.06 μ g of diolein. When Ca^{2+} was omitted, the reaction mixture also contained 10 nmol of EGTA. For study of the dependence of reaction rate on Ca^{2+} concentration, the reaction mixture contained 10 mM-EGTA and concentrations of CaCl_2 calculated (Severson *et al.*, 1974) to give the desired free Ca^{2+} concentration. The reaction was initiated by the addition of 12 μ l of enzyme. The extent of incorporation of ^{32} P from [γ - ^{32} P]ATP into histone H1 was measured by the method of Corbin & Reimann (1974), modified by the addition of 0.25% sodium tungstate to the trichloroacetic acid solution.

For the study of the phosphorylation of endogenous islet and β -cell proteins, histone H1 was omitted from the reaction mixture. ^{32} P-labelled proteins were resolved by electrophoresis in SDS/12%-polyacrylamide gels and quantified by autoradiography and densitometry as previously described (Gagliardino *et al.*, 1980). Proteins used for M_r calibration were phosphorylase *b* (98 000), albumin (68 000), ovalbumin (43 000), trypsinogen (24 000) and lysozyme (14 300).

Purification of Ca^{2+} -phospholipid-dependent protein kinase from hamster β -cells (HIT-T15)

This was done by the method of Schatzman *et al.* (1983), with omission of the final Affigel step. Purified enzyme was stored in 30% (v/v) glycerol at -80°C.

Samples of purified Ca^{2+} -phospholipid-dependent protein kinase were run on SDS/16%-polyacrylamide gels and stained with a silver stain as described by Morrissey (1981). Protein was determined by the method of Bradford (1976), with albumin as standard.

Sucrose-density-gradient centrifugation

Sedimentation coefficients were determined by the method of Martin & Ames (1961) by using a linear sucrose gradient (13 ml; 5–20%, w/v) formed in 5 mM-Tris/HCl/1 mM-EDTA, pH 7.5. Standards

used were bovine haemoglobin (4.6S), rabbit muscle phosphorylase *b* (8.2S) and bovine liver catalase (11.2S).

Gel filtration

Stokes radii were determined on a column (1.5 cm $\times$ 25 cm) of Sephacryl S-300 equilibrated with 20 mM-Tris/HCl/2 mM-EDTA/50 mM-2-mercaptoethanol/10% glycerol, pH 7.4. The column was standardized with the following proteins of known Stokes radius: chymotrypsinogen (2.1 nm), bovine serum albumin (3.55 nm) and pyruvate kinase (5.5 nm). Inclusion and exclusion volumes were determined with potassium dichromate and Blue Dextran respectively. Data were plotted by the method of Laurent & Killander (1964); see also Siegel & Monty (1966). For determination of M_r by gel filtration, the M_r values used for protein standards were pyruvate kinase (273 000), albumin (68 000) and chymotrypsinogen (25 000).

Results and discussion

Ca^{2+} -phospholipid-dependent protein kinase in extracts of islets and HIT-T15 β -cells

A 20 000 *g* supernatant fraction from extracts of rat islets catalysed the incorporation of ^{32} P from [γ - ^{32} P]ATP into exogenous histone H1 in the presence of Ca^{2+} and phospholipid (Fig. 1). The

Fig. 1. Effects of Ca^{2+} and phospholipid on protein phosphorylation in islet extracts

The time course of incorporation of ^{32} P from [γ - ^{32} P]ATP into histone H1 catalysed by a 20 000 *g* supernatant from islet extract was measured at 30°C in the presence of: ●, 50 μ M- Ca^{2+} , diolein (1.2 μ g/ml) and phosphatidylserine (16 μ g/ml); □, 10 mM-EGTA, diolein (1.2 μ g/ml) and phosphatidylserine (16 μ g/ml); ○, 50 μ M- Ca^{2+} alone. Data are mean values of triplicate determinations.

reaction was linear for 2 min. Incorporation was markedly decreased by omission of Ca²⁺ (and addition of EGTA) or in the absence of added phospholipid. Essentially similar results (not shown) were obtained with a crude extract of HIT-

T15 β -cells. Stimulation of phosphorylation in the presence of phospholipid could be achieved with 1 μ M-Ca²⁺, and maximal phosphorylation was obtained with 50 μ M-Ca²⁺.

When histone was omitted from the reaction mixture, phosphorylation of several endogenous proteins could be enhanced by Ca²⁺ plus diolein and phosphatidylserine. Resolution and detection of the phosphorylated proteins by SDS/polyacrylamide-gel electrophoresis and autoradiography (Fig. 2) showed that the main endogenous substrates for Ca²⁺-phospholipid-dependent protein kinase in islet extracts had M_r values of 11 000, 15 000, 20 000, 35 000 and 38 000. Bands of similar M_r were also found for extracts of HIT-T15 β -cells.

Purification of Ca²⁺-phospholipid-dependent protein kinase from HIT-T15 β -cells

The procedure of Schatzman *et al.* (1983) was scaled down to accommodate the small amount of starting material; the final Affigel step was omitted because its low yield was found to give insufficient activity for further study. The results of a typical purification of Ca²⁺-phospholipid-dependent protein kinase from HIT-T15 β -cells are given in Table 1. Purification to 250-fold yielded an enzyme with specific activity of 37.4 nmol of phosphate incorporated/min per mg of protein at 30°C. Activity was stable for several weeks when stored in glycerol. SDS/polyacrylamide-gel electrophoresis of the purified enzyme followed by silver staining of the gel showed one major protein band of M_r 81 300 $\pm$ 4000 ($n = 3$).

Properties of Ca²⁺-phospholipid-dependent protein kinase from HIT-T15 β -cells

The physical properties of the enzyme are summarized in Table 2. From the sedimentation coefficient of 4.7S and the Stokes radius of 4.4 nm, the M_r of the native enzyme was calculated to be 85 200. Since the M_r under denaturing conditions was 81 300, the enzyme is likely to be monomeric; a similar conclusion has been reached for the heart

Fig. 2. Phosphorylation of endogenous protein substrates by islet Ca²⁺-phospholipid-dependent protein kinase

A 20 000g supernatant of islet extract was incubated for 5 min with the following additions to the basic incubation buffer: (a) 50 μ M-Ca²⁺, diolein (1.2 μ g/ml) and phosphatidylserine (16 μ g/ml); (b), 10 mM-EGTA, diolein (1.2 μ g/ml) and phosphatidylserine (16 μ g/ml); (c), 50 μ M-Ca²⁺ alone. Phosphorylated proteins were separated by electrophoresis in a 12%-polyacrylamide gel in the presence of SDS and detected by autoradiography. The Figure shows densitometer scans of the autoradiogram, with M_r values indicated for certain peaks.

Table 1. Purification of Ca²⁺-phospholipid-dependent protein kinase from HIT-T15 β -cells
Details of the purification procedures and enzyme assay are given in the Experimental section.

Step	Protein (mg)	Specific activity (pmol/min per mg)	Stimulation (fold)		Purification (fold)
			Ca ²⁺ †	PL†	
Crude‡	6.4	150	1.3	1.2	—
DEAE-cellulose DE52	0.13	3918	1.8	2.1	26
Octyl-agarose	0.008	14 208	4.0	2.5	95
Sephacryl S-300	0.002	37 400	3.0	6.0	250

* Stimulation by 50 μ M-Ca²⁺.

† Stimulation by phosphatidylserine (16 μ g/ml) plus diolein (1.2 μ g/ml).

‡ 20 000g fraction from extract of cells.

Table 2. *Properties of Ca^{2+} -phospholipid-dependent protein kinase purified from HIT-T15 β -cells*

Results are given as means $\pm$ S.E.M. for the numbers of determinations in parentheses.

(a) Physical properties

Parameter	Value
Sedimentation coefficient (S)	4.70 ± 0.16 (3)
Stokes radius (nm)	4.40 ± 0.01 (4)*
M_r	$120\,500 \pm 2400$ (4)*
	$81\,300 \pm 4000$ (3)†
	$85\,200$ ‡
Frictional ratio (f/f_0)	1.53§

(b) Kinetic properties

Substrate or effector	Concn. for half-maximum rate†
ATP	10 ± 1 μM (3)
Histone H1	0.5 ± 0.05 μM (3)
Ca^{2+}	3.9 ± 0.23 μM (7)
Phosphatidylserine	18 ± 2 $\mu\text{g/ml}$ (4)
Diolein	2.5 ± 0.2 $\mu\text{g/ml}$ (7)

* By gel filtration on Sephacryl S-300.

† By SDS/polyacrylamide-gel electrophoresis.

‡ From $s_{20,w}$ and Stokes radius.

§ From Stokes radius and calculated M_r .

|| For conditions under which these values were obtained, see the text.

and spleen enzymes (Schatzman *et al.*, 1983; Wise *et al.*, 1982). From the Stokes radius and the calculated M_r , the frictional ratio was estimated to be 1.53, suggesting that the enzyme is asymmetric. This conclusion is reinforced by the higher estimate of M_r obtained from gel filtration, as previously noted for the heart enzyme (Wise *et al.*, 1982).

Kinetic properties of the enzyme are also summarized in Table 2. In contrast with results with crude homogenates, the rate of phosphorylation of histone H1 by the purified enzyme was linear for at least 30 min, presumably because of removal of phosphatase and/or ATPases during purification. When assayed in the presence of $50 \mu\text{M}$ - Ca^{2+} , phosphatidylserine ($16 \mu\text{g/ml}$) and diolein ($1.2 \mu\text{g/ml}$), the dependence of reaction rate on concentrations of ATP or of histone H1 gave K_m values for these substrates of 10 and $0.5 \mu\text{M}$ respectively. Similar values have been reported for the spleen and heart enzymes (Wise *et al.*, 1982; Schatzman *et al.*, 1983). In the presence of phosphatidylserine ($16 \mu\text{g/ml}$) and diolein ($1.2 \mu\text{g/ml}$) the enzyme was markedly stimulated by $1 \mu\text{M}$ - Ca^{2+} ; maximal stimulation was achieved with $50 \mu\text{M}$ - Ca^{2+} and half-maximal with $3.9 \mu\text{M}$ - Ca^{2+} . In the presence of diolein ($1.2 \mu\text{g/ml}$) and $1 \mu\text{M}$ - Ca^{2+} , enzyme activity was increased by phosphatidylserine over the range 4 – $32 \mu\text{g/ml}$, with half-maximal effect at $18 \mu\text{g/ml}$. Similarly, diolein (0.3 –

$3.5 \mu\text{g/ml}$), with $1 \mu\text{M}$ - Ca^{2+} and phosphatidylserine ($16 \mu\text{g/ml}$), increased the rate of reaction, with half-maximal effect at $2.5 \mu\text{g/ml}$. It is necessary to specify the conditions under which these values were obtained, because of interactions between the various enzyme effectors, e.g. sensitivity to diolein was decreased at higher Ca^{2+} concentrations. Detailed characterization of these kinetic interactions was not possible with the limited amount of purified enzyme available. Under the conditions used here, the sensitivity to lipid effectors was similar to that reported for the brain enzyme (Kaibuchi *et al.*, 1981).

General conclusions

We have shown that β -cells contain substantial amounts of Ca^{2+} -phospholipid-dependent protein kinase and have defined physical and kinetic properties of the enzyme. In addition we have shown the presence of several endogenous β -cell substrates for the enzyme. This is to our knowledge the first characterization of this enzyme in an endocrine tissue.

Two lines of evidence implicate Ca^{2+} -phospholipid-dependent protein kinase in insulin secretion. Incubation of rat islets with phospholipase C enhanced turnover of phosphatidylinositol and stimulated insulin release (Tanigawa *et al.*, 1982). The data were interpreted in terms of increased formation of diacylglycerol from phosphatidylinositol leading to stimulation of Ca^{2+} -phospholipid-dependent protein kinase. Secondly, it has been shown that tumour-promoting phorbol esters, which rather specifically activate Ca^{2+} -phospholipid-dependent protein kinase (Castagna *et al.*, 1982), elicit marked stimulation of insulin release (Deleers *et al.*, 1981; Malaisse *et al.*, 1983). Confirmation of such a role for the kinase requires elucidation of the nature of its endogenous β -cell substrates and demonstration of changes in their phosphorylation state on physiological stimulation of insulin secretion.

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