CALCIUM-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE IN RAT PANCREAS ISLETS OF LANGERHANS

Its possible role in glucose-induced insulin release

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1. Introduction

Both Ca^{2+} and cyclic AMP play roles as intracellular messengers which regulate the release of insulin from β-cells of Langerhans' islets (reviews [1,2]). The principal mediator of cyclic AMP has been identified as cyclic AMP-dependent protein kinase (protein kinase A) [3] and this species of protein kinase has been found in islets [4]. In contrast, possible factors mediating the regulatory properties of Ca^{2+} are far less understood. Calmodulin has been identified in rat pancreatic islets [5], and a calmodulin-dependent protein kinase has also been found in hamster insulinoma cells [6]. Another line of evidence has shown that PI turns over rapidly during the activation of islets by high concentrations of glucose [7–9]. This PI turnover has been proposed to be intimately related to glucose-induced insulin release [7–9], but the definitive mechanism involved has not yet been clarified. In [10,11], a new species of protein kinase (protein kinase C) was described which absolutely requires Ca^{2+} and phospholipid. Protein kinase C is usually present as an inactive form, but is activated by diacylglycerol which may be transiently produced from PI during its receptor-linked turnover [12,13]. In [14–16], protein kinase C was shown to play an indispensable role in the thrombin-induced release of serotonin in human platelets. To facilitate our understanding of the insulin release mechanism, these studies were done to examine and characterize protein kinase C in rat pancreas islets of Langerhans.

Abbreviations: PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid

2. Materials and methods

Male Sprague-Dawley rats (150–250 g body wt) were used. Pancreatic islets were isolated as in [17]. *Clostridium perfringens* phospholipase C was obtained from Sigma. Chlorpromazine hydrochloride and dibucaine hydrochloride were kindly donated by Shionogi Research Laboratory and Teikoku Chemical Industry, respectively. PS was a generous gift from Drs T. Fujii and A. Tamura, Kyoto College of Pharmacy. Synthetic diolein was purchased from Nakarai Chemicals. [γ-^{32}P]ATP, calf thymus H1 histone, various lipids, and other chemicals employed for the present studies were prepared as specified in [10–16]. All reagents were taken up in water which was prepared by a double distillation apparatus followed by passing through a Chelex 100 column to remove Ca^{2+} as in [13].

2.1. *Determination of insulin release*

The islets were preincubated for 45 min at 37°C in a Krebs Ringer bicarbonate buffer supplemented with 0.25% bovine serum albumin and 3.75 mM glucose under the gas phase of 95% O_2 and 5% CO_2. Incubation was performed in the presence of test substances for 30 min. Insulin concentration in the medium was measured by the polyethylene glycol radioimmunoassay [18].

2.3. *Assay for PI turnover*

PI turnover was assayed by measuring the incorpo-

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Table 1
Enhancement of ^{32}P-incorporation into PI and insulin release by a high concentration of glucose

	^{32}P-Incorporation into			Insulin release (μunit . islet^{-1} . h^{-1})
	PI (%)	PC (%)	PE (%)	
3.75 mM glucose	30.2 ± 0.9	30.0 ± 4.5	36.0 ± 1.6	14.4 ± 4.8
16.7 mM glucose	49.6 ± 3.4	26.0 ± 4.5	24.4 ± 7.8	293.1 ± 46.1

The numbers of ^{32}P-incorporation into each phospholipid are expressed as percent of total radioactivity that was incorporated into the phospholipid fraction as 100. The results are means ± SEM for triplicate experiments

ration of ^{32}P into PI as in [19]. The islets were preincubated with 100 μCi of $^{32}P_i$ for 90 min under the above conditions except that KH_2PO_4 was omitted from the buffer. The islets were than incubated with test substances for 60 min. Phospholipids were extracted from the islets as in [7], and PI was separated from other phospholipids by silica gel thin-layer chromatography as in [10]. The area corresponding to PI was scraped into a vial and the radioactivity was determined.

2.3. *Assay for protein kinases C and A*

Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into calf thymus H1 histone in the presence of Ca^{2+}, PS and diolein. The complete reaction mixture (0.25 ml) contained 5 μmol Tris–HCl at pH 7.5, 1.25 μmol magnesium acetate, 50 μg H1 histone, 2.5 nmol [γ-^{32}P]ATP (5–10 × 10^5 cpm/nmol), 0.25 μmol $CaCl_2$, 2 μg PS and 0.2 μg diolein. Incubation was carried out for 5 min at 30°C. The reaction was stopped by the addition of 25% trichloroacetic acid and the acid-precipitable radioactivity was determined as in [10]. Protein kinase A was similarly assayed except that 250 pmol cyclic AMP was added instead of Ca^{2+}, PS and diolein.

2.4. *Determinations*

The radioactivity of ^{32}P-samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, model 3320, with Bray's solution [20]. Sedimentation coefficient and M_r-values were estimated as in [21]. Protein was determined as in [22] with bovine serum albumin as a standard.

3. Results and discussion

When islets were incubated with high concentrations of glucose (16.7 mM), ^{32}P-incorporation into PI was specifically increased, and the relative ratios of various phospholipid labelling are shown in table 1. ^{32}P-Incorporation into other phospholipids such as PC and PE was not increased under these conditions. This result agrees with that in [7]. Such a relative enhancement of ^{32}P-incorporation into PI was also observed when the islets were incubated with bacterial phospholipase C instead of high concentrations of glucose as shown in table 2. Presumably, bacterial phospholipase C reacted with various membrane phospholipids and produced diacylglycerol, but the latter compound was converted more predominantly to PI by way of its resynthetic pathway. Bacterial phospholipase C

Table 2
Enhancement of ^{32}P-incorporation into PI and insulin release by bacterial phospholipase C

	^{32}P-Incorporation into			Insulin release (μunit . islet^{-1} . h^{-1})
	PI (%)	PC (%)	PE (%)	
3.75 mM glucose	30.1 ± 0.6	30.1 ± 3.3	31.2 ± 1.7	13.3 ± 2.8
3.75 mM glucose + 0.4 μg/ml phospholipase C	47.5 ± 4.2	26.0 ± 4.5	24.6 ± 4.2	236.7 ± 25.5

The numbers of ^{32}P-incorporation into each phospholipid are expressed as % of total radioactivity that was incorporated into the phospholipid fraction as 100. The results are means ± SEM for triplicate experiments

also induced insulin release as shown in table 2. This insulin release, however, was not simply due to the disruption of islet cells, since other subcellular constituents did not measurably leak into the medium. These results imply that diacylglycerol formation may be intimately related to the regulation of insulin release.

We next examined whether diacylglycerol-activated protein kinase C was present in islets of Langerhans. Islets were subjected to sonication in a buffer given in fig.1, and the sonicate was centrifuged for 60 min at 100 000 × g. The supernatant was then analyzed by sucrose density gradient ultracentrifugation under the conditions specified. When each fraction was assayed for protein kinase in the presence of 1 μM cyclic AMP, a small peak appeared in fractions 10–13. This small peak bound cyclic AMP and was identified as protein kinase A. Next, all fractions were assayed for protein kinase in the presence of 1 mM $CaCl_2$, 2 μg PS and 0.2 μg diolein. A large single symmetrical peak appeared in fractions 13–15. This peak, that was not detected in the absence of these 3 activators, was identified as protein kinase C. The active fractions were collected and directly employed for subsequent studies. This enzyme showed an S-value of ~5.1, which corresponded to 77 000 M_r.

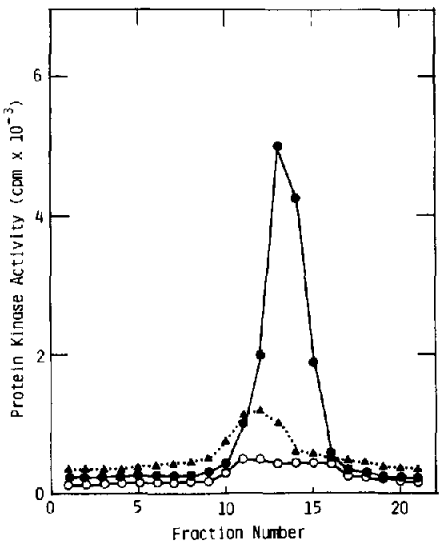

Fig.1. Analysis of protein kinases in pancreatic islets on sucrose density gradient ultracentrifugation. About 300 islets were disrupted by sonication with a Kontes sonifier, model K881440, for 30 s at 0°C in 0.3 ml 10 mM potassium phosphate buffer, at pH 7.0, containing 2 mM EDTA, 3 mM EGTA, and 10 mM 2-mercaptoethanol. The sonicate was centrifuged for 60 min at 100 000 × g. The supernatant (0.25 ml, 100 μg protein) was layered over a 4.8 ml linear gradient of 5–20% sucrose established in 20 mM Tris–HCl, at pH 7.5, containing 0.1 mM EDTA, 0.1 mM EGTA and 10 mM 2-mercaptoethanol, and was centrifuged at 4°C for 14 h at 39 000 rev./min in the SW 50 rotor of a Beckman ultracentrifuge, model L5-50. After centrifugation, fractions of 0.2 ml each were collected by punching a hole in the bottom of the tube. A 50 μl aliquot of each fraction was employed for protein kinase A and C assays under standard conditions: (●——●) with Ca^{2+}, PS and diolein; (▲- - -▲) with cyclic AMP; (○——○) with 0.5 mM EGTA instead of Ca^{2+}, PS, diolein or cyclic AMP.

Protein kinase C obtained in this way showed maximum enzymatic activity in the simultaneous presence of Ca^{2+}, PS and unsaturated diacylglycerol, diolein in this experiment, as shown in table 3. When either one of these activators was omitted, the enzymatic activity was dramatically decreased. The metal requirement for protein kinase C activation was very specific for Ca^{2+}, and other divalent cations such as Mg^{2+}, Mn^{2+}, Sr^{2+}, Ni^{2+} and Co^{2+} were not effective. PS could not be replaced by other phospholipids such as PI, PE, PC and PA. Diarachidonin and dilinolein were as effective as diolein, but diacylglycerols possessing 2 saturated fatty acids such as distearin and dipalmitin were far less effective. Monoacylglycerol, triacylglycerol, free fatty acid and cholesterol were totally inactive in this capacity. Calmodulin showed no effect over a wide range of concentrations. These properties of the islet protein kinase C were very similar to those of the enzyme originally found in rat brain soluble fraction [10–13].

In the last set of experiments, the effects of some phospholipid-interacting drugs such as chlorpromazine and dibucaine on islets protein kinase C were examined. Table 4 shows that these drugs inhibited the islet protein kinase as well as glucose-induced insu-

Table 3
Activation of islet protein kinase C by Ca^{2+}, PS, and diacylglycerol

Assay system	Protein kinase act. (cpm)
Complete system	12 870 ± 640
−enzyme	0
−Ca^{2+} + 0.5 mM EGTA	1980 ± 95
−PS	510 ± 60
−Diolein	2060 ± 110

A 20 μl aliquot of active fractions in fig.1 was employed for protein kinase assay. For the complete system 2 × 10^{-5} M $CaCl_2$, 2 μg PS, and 0.2 μg diolein were employed. The results are means ± SEM for triplicate experiments

Table 4
Inhibition by chlorpromazine and dibucaine of protein kinase C and glucose-induced insulin release

	Protein kinase C act. (cpm)	Insulin release (μunit . islet^{-1} . 0.5 h^{-1})
None	3730 ± 160	142.4 ± 20.2
Chlorpromazine		
(0.05 mM)	1870 ± 80	73.7 ± 9.2
(0.10 mM)	1240 ± 90	31.7 ± 2.2
Dibucaine		
(0.25 mM)	1610 ± 85	99.7 ± 6.8
(0.50 mM)	1370 ± 65	82.6 ± 5.7

The results are means ± SEM for triplicate experiments

lin release in parallel. Chlorpromazine was more effective than dibucaine.

The above results seem to suggest that PI turnover is coupled to the activation of protein kinase C in glucose-stimulated islets as described for thrombin-stimulated platelets [14–16], since unsaturated diacylglycerol markedly activates this protein kinase in the presence of PS and Ca^{2+}. It may be noted that some phospholipid-interacting drugs such as chlorpromazine and dibucaine inhibit both activation of protein kinase C and insulin release. Although it is generally accepted that these drugs interact with calmodulin and thereby inhibit calmodulin-dependent cellular processes [23], protein kinase C may be another target of these drugs within the cell. The precise role of this protein kinase in regulating islet cell functions may be clarified by further investigations.

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