Presence of membrane-associated phosphatidate phosphohydrolase activity in cultured islets and its stimulation by glucose

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The cellular location at which exogenous phosphatidic acid is hydrolysed in cultured neonatal rat islets was examined. Phosphatidate phosphohydrolase activity could be demonstrated in both whole cell sonicates and isolated plasma membranes. In the whole cell fraction phosphatidic acid hydrolysis to diacylglycerol was stimulated 43% by the presence of Mg^{2+}. The activity present in isolated membranes was totally dependent on the presence of Mg^{2+} and was increased in plasma membranes from glucose-stimulated islets. Following exposure of islets to low glucose concentrations, raising the Ca^{2+} concentration from 150 nM to 40 μM in the presence of Mg^{2+} did not affect the formation of diacylglycerol in whole cell fractions or plasma membranes. These results indicate the presence within the islet of membrane-bound phosphatidate phosphohydrolase activity and demonstrate its activation by glucose.

Phosphatidic acid metabolism *Diacylglycerol* *Ca^{2+}* *Mg^{2+}* *(Pancreatic islet)*

1. INTRODUCTION

Several roles have been suggested for phosphatidic acid in transmembrane signalling and control of Ca^{2+} fluxes [1–3]. Phosphatidic acid is formed as an intermediate in the cycle of phosphoinositide degradation and resynthesis and it may be formed de novo from glucose or glycerol precursors [4]. Phosphatidate phosphohydrolase (EC 3.1.3.4) catalyses the dephosphorylation of phosphatidic acid to diacylglycerol, a key regulator of phospholipid-dependent, Ca^{2+}-sensitive protein kinase [5]. A number of phosphatidate phosphohydrolase activities have been described, associated with either the cytosolic or (membrane) microsomal fraction of the cell and differing in sensitivity to Mg^{2+} stimulation [6–8]. There is evidence that phosphatidate phosphohydrolase may act as a regulatory enzyme in glycerolipid biosynthesis in hepatic [9] and adipose tissue [10] but its presence and activity in the pancreatic islet have not been investigated. In the islet de novo synthesis of neutral lipid follows glucose stimulation [11–13], but the pathways involved have not been elucidated. Here, the hydrolysis of exogenous labelled phosphatidic acid was determined to investigate the activity of phosphatidate phosphohydrolase in neonatal pancreatic islets, and the effect of glucose stimulation on this activity was examined.

2. MATERIALS AND METHODS

2.1. *Glucose stimulation of neonatal islet cells and plasma membrane isolation*

Cells from pancreatic islets of neonatal rats, less than 24 h old, were grown in monolayer culture in medium RPMI 1640 for and attached to hydrated Cytodex 1 beads as described [14]. Aliquots of beads with attached cells (10^6 cells) were incubated in Krebs Ringer bicarbonate buffer containing bovine serum albumin. After 20 min incubation at a glucose concentration of 1.6 mM, glucose was added to a final concentration of 1.6 or 16.7 mM

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and the incubation continued for 15 min. The beads with attached cells were pelleted by centrifugation and either sonicated (whole cell fraction) or lysed in hypotonic buffer (10 mM Tris-HCl, pH 8.0), washed and pelleted (isolated membrane fraction) [14]. Protein was determined by the method of Bradford as described in [15].

2.2. *Hydrolysis of exogenous labelled phosphatidic acid*

Phosphatidic acid, 0.5–1.5 mM containing L-3-phosphatidic acid, 1,2-di[1-^{14}C]palmitoyl (final spec. act. 1 μCi/0.15 μmol) was added to the whole cell suspension or beads and attached membranes in a sonicated dispersion containing 2 mg fatty acid-poor albumin and EGTA, 10 μM in Tris-HCl, 100 mM, pH 7.4. Where indicated $MgCl_2$ (0.5–5.0 mM) or $CaCl_2$ (40 μM–2.5 mM) were also present in the final incubation volume of 250 μl. The incubation was continued for 30 min at 37°C. Lipids were extracted by addition of 1 ml chloroform:methanol:13 N HCl (200:100:1), followed by constant mixing at 4°C for 1 h. Two phases were generated by the addition of 1 M KCl and neutral lipids and phospholipid separated by thin-layer chromatography as in [15]. Labelled diacylglycerol and palmitate, co-chromatographing with authentic standards, were scraped from the chromatograph and added to scintillant for determination of ^{14}C content. Phosphatidate hydrolysing activity was expressed as μmol phosphatidic acid hydrolysed to diacylglycerol per 10^6 cells over 1 h.

3. RESULTS

The formation of diacylglycerol from phosphatidic acid by the whole cell fraction and by the isolated membrane fraction is shown in fig.1. Hydrolytic activity in the whole cell fraction was increased 43% in the presence of Mg^{2+}. In the isolated membrane fraction, no hydrolysing activity could be demonstrated in the absence of Mg^{2+} but this activity was clearly shown in the presence of 5 mM Mg^{2+}. Under the chosen assay conditions (1.0 mM phosphatidic acid, 5 mM $MgCl_2$, 150 nM or 40 μM free Ca^{2+}) the hydrolytic reaction was linear over a range of times and protein concentrations in isolated membranes prepared from islets pre-incubated with 1.6 mM glucose. Under these

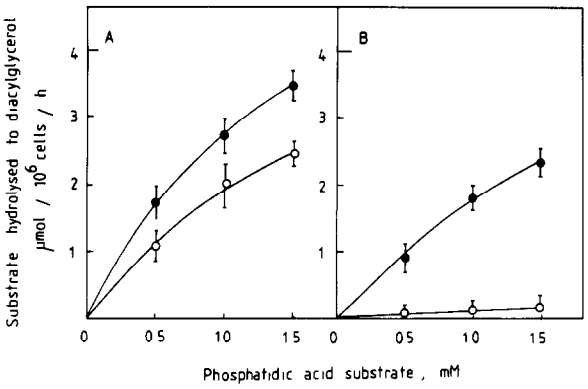

Fig.1. Effect of Mg^{2+} concentration on hydrolysis of exogenous phosphatidic acid to diacylglycerol. Phosphatidic acid, 1,2-distearoyl containing 1 μCi phosphatidic acid, 1,2-di[1-^{14}C]palmitoyl was incubated with the whole cellular fraction (A) or plasma membrane fraction (B) in the presence (●) or absence (○) of 5 mM Mg^{2+}. The results are the mean ± SE of 3 experiments (9 observations at each point).

conditions activity in the whole cell fraction rose from 2.81 ± 0.31 μmol phosphatidic acid hydrolysed to diacylglycerol/10^6 cells to 4.46 ± 0.20 μmol/10^6 cells following pre-incubation of the cells for 15 min with 16.7 mM glucose (table 1). This glucose stimulation has been shown previously to be accompanied by a 3-fold increase in release of insulin from these cells [14]. An increase in phosphatidate hydrolysing activity could be shown to be accounted for by a rise in membrane-associated activity as the formation of diacylglycerol rose from 1.80 ± 0.18 to 3.75 ± 0.35 μmol/10^6 cells in the isolated membrane fraction prepared from glucose-stimulated cells. Raising the free Ca^{2+} from 150 nM to 40 μM did not alter the phosphatidate hydrolysing activity in either the whole cell or the membrane preparation under basal glucose conditions.

As well as diacylglycerol formation from labelled phosphatidic acid, labelled palmitate was also formed in the incubations containing the whole cell fraction under basal glucose conditions. Formation of palmitate was negligible in the isolated membrane fraction. In the whole cell fraction, palmitate formation was increased significantly in homogenates prepared from islets stimulated with 16.7 mM glucose (table 1). Raising the Ca^{2+} concentration in the incubation also in-

Table 1

Effect of glucose and Ca^{2+} concentration on hydrolysis of phosphatidic acid by whole cell and isolated membrane fraction of islets

Assay conditions	Pre-incubation conditions			
	Glucose (1.6 mM)		Glucose (16.7 mM)	
	Diacylglycerol	Palmitate	Diacylglycerol	Palmitate
Whole cell fraction, 150 nM free Ca^{2+}	2.81 ± 0.31	5.62 ± 0.34	4.46 ± 0.20[b]	6.52 ± 0.36
Isolated membrane fraction, 150 nM free Ca^{2+}	1.80 ± 0.18	<0.5	3.75 ± 0.35[b]	<0.5
Whole cell fraction, 40 μM free Ca^{2+}	2.92 ± 0.35	7.84 ± 0.36[a]	0.64 ± 0.20[ab]	13.86 ± 0.42[ab]
Isolated membrane fraction, 40 μM free Ca^{2+}	2.09 ± 0.18	<0.5	1.84 ± 0.22[a]	4.21 ± 0.40

[a] Significant difference when compared to 150 nM free Ca^{2+}, $p < 0.005$
[b] Significant difference when compared to 1.6 mM glucose, $p < 0.005$ (Student's t-test)

Values (expressed as μmol phosphatidate hydrolysed to product/10^6 cells per h) are mean ± SE for 9–12 determinations at each point

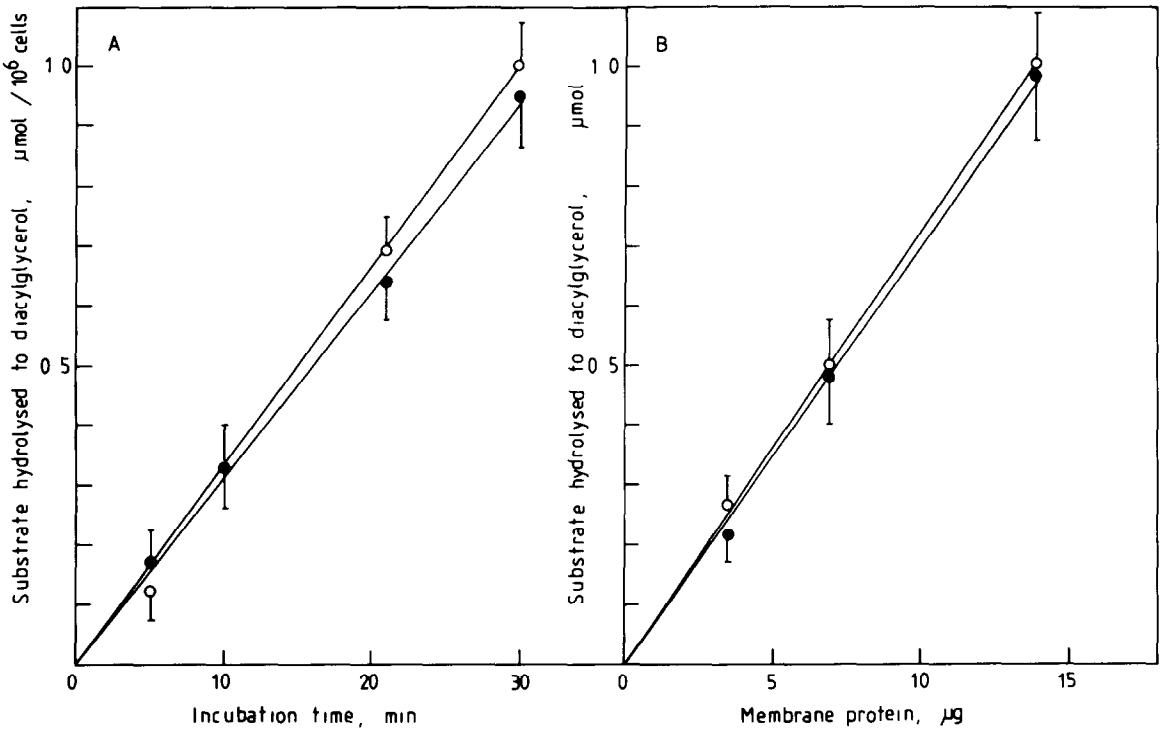

Fig.2. Hydrolysis of phosphatidic acid by isolated membrane fractions of islet cells. The time course of hydrolysis of phosphatidic acid (1.0 mM) in the presence of Mg^{2+} (5 mM) following addition of 14 μg islet membrane protein, equivalent to 10^6 isolated cells is shown in panel A. The hydrolysis over 30 min for a range of membrane protein concentrations is shown in panel B. The reactions were performed in the presence of 150 nM (○) or 40 μM (●) free Ca^{2+}. Results are expressed as mean ± SE of 3 experiments (9 observations at each point).

creased the formation of palmitate by the whole cell and membrane preparations from glucose-stimulated islets.

4. DISCUSSION

These studies demonstrate the presence in the islet cell of a phosphatidate phosphohydrolase, capable of hydrolysing an aqueous exogenous substrate. The islet phosphatidate phosphohydrolase activity exists in different cellular compartments. Increased activity of the membrane-associated fraction could be demonstrated following glucose stimulation. This may be responsible for the increased diacyl- and triacylglycerol synthesis from glycerol following stimulation of adult islets with glucose and ketoisocaproate [12] and de novo synthesis of diacylglycerol from glucose in adult [10] and neonatal islets [16].

Here, increased phosphatidate phosphohydrolase activity was found concomitant with raised cellular fatty acid levels. This latter observation confirms a previous finding of fatty acid release from islet-derived phosphatidic acid following glucose stimulation [17]. The utilization of phosphatidic acid double-labelled with palmitic acid prevented the channeling of products of phospholipase A_2 or diacylglycerol lipase action to active eicosanoid products, seen in the earlier study. It is not possible to determine the contribution of these 2 fatty acid mobilizing enzymes to raised palmitate levels.

The finding that palmitate is increased in glucose-stimulated preparations in the presence of raised calcium levels concomitant with a decrease in diacylglycerol suggests strongly that diacylglycerol lipase is active under these conditions.

Butterwith et al. [8] have proposed that hepatic triacylglycerol synthesis is enhanced by activation of phosphatidate phosphohydrolase subsequent to fatty acid-facilitated transfer of the enzyme to a membrane-associated cellular compartment, containing membranes of the endoplasmic reticulum. The isolated membrane fraction used here is essentially characteristic of a plasma membrane fraction using established marker enzymes [14]. However, it should be noted that there is a consistent increase in the recovery of glucose-6-phosphatase associated with glucose stimulation (130% of basal glucose preparation) indicating some plasma membrane association of endoplasmic reticulum components following glucose stimulation.

In the present experiments, phosphatidate has been presented in micellar form as an exogenous substrate. It is not possible to determine the source of the endogenous phosphatidic acid to which the phosphohydrolase activity is directed. This may be phosphatidic acid synthesized de novo or that generated by the action of diacylglyceride kinase [18]. Our previous demonstration of de novo diacylglycerol formation coincident with phosphatidic acid synthesis from glucose [16] suggests strongly that the action can be directed against phosphatidic acid synthesized de novo.

In conclusion, the demonstration of islet phosphatidate phosphohydrolase activity provides a possible mechanism by which phosphatidate formed directly from glucose can be converted to diacylglycerol, a regulator of phospholipid- and calcium-dependent protein phosphorylation. The relative importance of this pathway compared with phosphoinositide hydrolysis in the formation of diacylglycerol in the islet remains to be determined.

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