Journal of Cellular Biochemistry 32:143–150 (1986)

Enzymes of Phospholipid Metabolism in Rat Pancreatic Islets: Subcellular Distribution and the Effect of Glucose and Calcium

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The effect of glucose and calcium on the activities of the phosphatidylinositol cycle enzymes, CDP-diglyceride inositol transferase, diacylglycerokinase, and lysophosphatidylcholine 2-acyltransferase in rat pancreatic islets was studied. Calcium inhibited the activity of CDP-diglyceride inositol transferase but had no effect on lysophosphatidylcholine 2-acyltransferase and diacylglycerokinase activites. Upon preincubation of islets in a concentration of glucose known to stimulate insulin release, the activity of lysophosphatidylcholine 2-acyltransferase, but not that of diacylglycerokinase or the CDP-diglyceride inositol transferase, was stimulated. Subcellular fractionation of pancreatic islets showed that secretory granule membranes were enriched in CDP-diglyceride inositol transferase, whereas lysophosphatidylcholine 2-acyltransferase activity was highest in the microsomal membranes. The activation of 2-acyltransferase by incubating islets in insulinotropic glucose, and the calcium sensitivity of CDP-diglyceride inositol transferase, suggest that these enzymes may have roles in regulation of insulin secretion.

Key words: lysophosphatidylcholine 2-acyltransferase stimulation, phosphatidylinositol cycle, calcium inhibition

Due to the possibility that phospholipids may be involved in the regulation of calcium-mediated secretory phenomena, considerable research effort has been directed to studying the role of phospholipids in glucose-induced insulin secretion in intact pancreatic islets. So far, the primary focus of research in this area has been to demonstrate increased phospholipid turnover when islets are incubated in the presence of insulinotropic concentrations of glucose or other insulin secretagogues.

Studies of the "phospholipid effect" in various excitable tissues have implicated the PtIns cycle [1–4] as an integral part of the stimulus-secretion coupling. This has

Abbreviations used: DAG, diacylglycerol; EGTA, ethylene glycol bis (P-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Ins, inositol; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; MSH, mannitol-sucrose-Hepes buffer; PA, phosphatidic acid; PC, phosphatidylcholine; PtIns, phosphatidylinositol.

Received February 20, 1986; revised and accepted June 18, 1986.

prompted investigations of phospholipid breakdown in pancreatic islets [5–10]. Breakdown of PtIns putatively indicates involvement of the PtIns cycle, but except for phospholipase C [11], enzymes of the PtIns cycle (Fig. 1) have not been studied in pancreatic islets in respect to glucose metabolism or calcium effects. We were interested in studying diacylglycerokinase and CDP-diglyceride inositol transferase, two PtIns cycle enzymes, because the former may affect calcium ionophoresis by regulating the concentration of PA, which has been found to have ionophoretic properties, and the latter may control the amount of PtIns available as a substrate for phospholipase C. We were also interested in lysophosphatidylcholine 2-acyltransferase because of the ability of insulinotropic glucose to enhance arachidonate incorporation into islet phospholipids [5–8]. Recently available data show that pancreatic islets contain at least two pools of phosphatidylinositol and phosphatidylcholine. The turnover of only one of these pools appears to be responsive to glucose and this turnover depends on an adequate extracellular concentration of calcium [8]. The current report describes the subcellular distribution of these enzymes and the effect of glucose metabolism and of calcium and calcium modifiers on their activities.

MATERIALS AND METHODS

Isolation of Islets

Pancreatic islets were isolated from well-fed 200–250 g Sprague-Dawley rats by digesting minced pancreata with collagenase. The islets were then separated from the bulk of pancreas fragments by use of a discontinuous Ficoll gradient [12,13], picked manually, and distributed into test tubes with the aid of a microscope.

Subcellular Fractionation

Subcellular fractionation was carried out at 4°C according to published procedures [14,15]. Approximately 1,200 islets were washed three times in 230 mM mannitol, 70 mM sucrose, and 5 mM Hepes, pH 7.4, and pelleted in a 1.5-ml Eppendorf microtube. The pellet was then homogenized by using a Pellet Pestle (Kontes, Vineland, New Jersey) and resuspended in 600 μl of the same buffer solution. A portion (120 μl) of the homogenate was removed for the measurement of

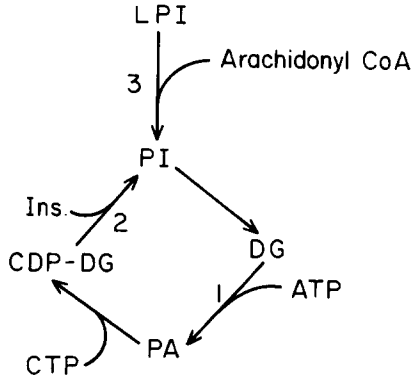

Fig. 1. PtIns cycle enzymes: 1, diacylglycerokinase; 2, CDP diglyceride inositol transferase; 3, lysophosphatidylcholine 2-acyltransferase.

enzyme activities and the rest was centrifuged at 600g for 5 min to obtain a pellet containing plasma membranes and nuclei. The resulting supernatant fractions were sequentially centrifuged at 5,500g for 10 min, 25,000g for 20 min, and 105,000g for 60 min to obtain fractions enriched in mitochondria, secretory granules, and microsomes, respectively. The final supernatant fraction was the cytosol. Each of the pellets was washed once with the buffer used for homogenization and resuspended in 200 μl of the same buffer for enzyme and protein measurements.

Enzyme Assays

Diacylglycerokinase. The activity of diacylglycerokinase was assayed by a slight modification of the procedure described by Kanoh [16]. The reaction was initiated by the addition of 20 μl of islet homogenate to 110 μl of the reaction mixture to obtain final concentrations of Tris chloride buffer, pH 7.4, 100 mM; NaF, 20 mM; deoxycholate, 1 mM; dithiothreitol, 0.5 mM; $MgCl_2$, 10 mM; diacylglycerol, 1 mM; and [^{32}P]ATP, 1.6 mM (specific activity 6.25 mCi/mmol). The mixture was incubated at 37°C for specified times and the reaction was terminated by adding 50 μl of 12 N HCl. One and one-half milliliters of water and 1 ml of n-butanol were added and the contents were mixed vigorously. After the phases separated, the top (butanol) phase was transferred to another tube and washed once with 1 ml of water saturated with butanol. The butanol was evaporated under a stream of nitrogen and then the residue was dissolved in 100 μl of chloroform containing 20 μg of PA. After thin-layer chromatography (TLC) in chloroform/methanol/ammonia/water (70/30/2/3), the spot corresponding to PA was scraped into 10 ml of Aquasol, and the radioactivity incorporated into PA was determined by liquid scintillation spectrometry.

Lysophosphatidylcholine 2-acyltransferase. The activity of lysophosphatidylcholine 2-acyltransferase was measured as described by Rubin [17]. The reaction was initiated by adding 20 μl islet homogenate to 130 μl of the reaction mixture containing the following agents to give final concentrations of Tris chloride buffer, pH 8.3, 30 mM; ATP, 6 mM; EGTA, 1 mM; lysophosphatidylcholine, 1 mM; and [^{14}C]arachidonate, 50 μM (51.6 mCi/mmol). The reactants were incubated at 37°C for 10 min. Three milliliters of ice-cold chloroform methanol (1:2 v/v) was added and the lipid was extracted by the procedure of Bligh-Dyer [18] and dissolved in 100 μl chloroform. Twenty micrograms of PC was added to the extract and the reaction product (PC) was separated by TLC in tetrahydrofuran:acetone:methanol:water (50:20:40:8, v/v/v/v). The PC spot was scraped into 10 ml of Aquasol and the radioactivity was determined by liquid scintillation spectrometry.

CDP-diglyceride inositol transferase. The activity of CDP-diglyceride inositol transferase was measured according to the method described by Egawa et al [19] with some modifications. The activity was measured in a reaction mixture containing 30 mM glycylglycine buffer, pH 8.6, 20 mM $MgCl_2$, 100 μM CDP-diglyceride, 0.2% Triton X-100, 0.6 mM myo-[2^{3}H]inositol (19 mCi/mmol). The reaction was initiated by adding 20 μl of islet homogenate prepared by sonication to 80 μl of the reaction mixture. The reaction was stopped by adding of 3 ml of ice-cold chloroform-methanol (1:2). One milliliter of chloroform and 1.6 ml 0.01 M HCl were added and phase separation was carried out at 4°C. The top phase was removed and the bottom phase was washed three times with 2 ml of a synthetic top phase (chloroform/methanol/0.01 M HCl, 3/48/47 v/v/v). The radioactivity remaining in

the bottom phase (> 95% PI as judged by TLC) was determined by liquid scintillation spectrometry.

Effect of glucose on enzyme activities in intact islets. Islets (200–400/test tube, in triplicate) were incubated for 30 min at 37°C in 100 μl of Krebs Ringer solution, pH 7.4, containing either 3.3 mM or 16.7 mM glucose. At the end of the incubation period, the islets were washed twice in cold MSH buffer and homogenized in the same buffer in a final volume of 200 μl. The activities of the various enzymes were then measured as described above.

Calcium concentrations. When the effect of calcium on enzyme activities was studied, calcium concentrations in the range of 0–20 μM were obtained by use of calcium-EGTA buffers [20].

RESULTS

Assay conditions were selected such that product formation was linear. On the basis of preliminary studies the rate of product formation (PA, PC, or PI) was found to be linear under the following conditions: 25–35 μg protein/assay for each assay and incubation times of 4 min for the diacylglycerokinase assay, 10 min for the 2-acyltransferase assay, and 15 min for CDP-diglyceride inositol transferase assay.

Effect of Glucose

The activities of diacylglycerokinase and CDP-diglyceride inositol transferase were unaffected by preincubating the islets in the presence of either 3.3 or 16.7 mM glucose. However, incubating the islets with 16.7 mM glucose resulted in a 66% stimulation of the lysophosphatidylcholine 2-acyltransferase activity. The rate of acylation (nmoles arachidonate recovered in PC/min/mg protein) after incubating islets with 16.7 mM glucose was 5.13 ± 0.06 (n=3) compared to 3.21 ± 0.04 (n=3) after incubation with 3.3 mM glucose ($P < .005$).

Effect of Calcium

The effect of calcium on the three enzyme reactions was examined. The activity of diacylglycerokinase was not affected by the addition of calcium or EGTA (nmol PA/min/mg protein: control, 3.29 ± 0.02 (n=8); 100 μM calcium, 3.18 ± 0.01 (n=3); 10 mM EGTA, 3.33 ± 0.01 (n=3)). Similarly, the addition of calcium had no effect on lysophosphatidylcholine 2-acyltransferase activity. (The rate of arachidonate incorporation into PC remained constant at about 3.25 ± 0.03 nmol/min/mg protein in the presence of 0–500 μM calcium.) However, CDP-diglyceride inositol transferase was inhibited 80% by 25 μM calcium (Fig. 2). The Dixon plot of inositol incorporation vs calcium concentration (inset Fig. 2) revealed two slopes corresponding to inhibition constants ($K_{0.5}$) of 5 μM and 90 μM. When 1 mM $MnCl_2$ was added to the reaction mixture containing 20 mM Mg^{++}, the enzyme activity was stimulated fourfold (Table I). Furthermore, 1 mM $MnCl_2$ prevented the inhibition of enzyme activity by calcium (Table I).

Subcellular Distribution

The subcellular distribution of lysophosphatidylcholine 2-acyltransferase was studied by differential centrifugation (Table II). The enzyme activity was detected in all fractions including cytosol, which accounted for 44% of the total activity. How-

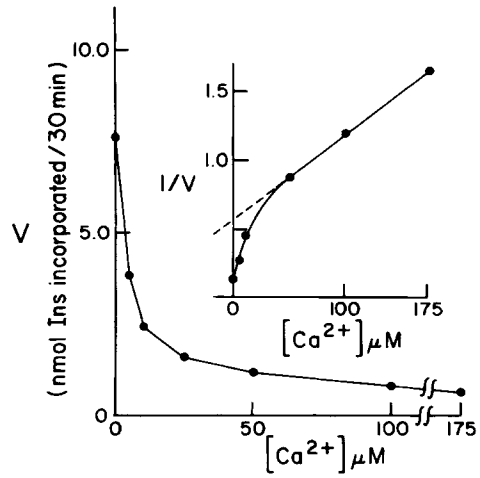

Fig. 2. Effect of calcium on the activity of CDP-diglyceride inositol transferase. Points are the average of three determinations. Inset: a Dixon plot of the data.

TABLE I. Effect of Calcium on the Activity of CDP-Diglyceride Inositol Transferase in the Presence or Absence of Manganese*

Additions	Relative enzyme activity
$MgCl_2$	100
$MgCl_2$ + $CaCl_2$	15 ± 3
$MgCl_2$ + EGTA	426 ± 20
$MgCl_2$ + $MnCl_2$	725 ± 35
$MnCl_2$	12 ± 3
$MgCl_2$ + $MnCl_2$ + $CaCl_2$	620 ± 28
$MgCl_2$ + $MnCl_2$ + EGTA	760 ± 31

*Enzyme activity was measured in the presence of either 1 mM $MnCl_2$ or 20 mM $MgCl_2$ or both, as well as in the presence of 200 μm $CaCl_2$ or 1 mM EGTA. Results are the mean ± SD of triplicate observations.

ever, the specific activity of lysophosphatidylcholine 2-acyltransferase was maximum in the microsomal fraction followed by the secretory granule and mitochondrial fractions which each had about half the specific activity of microsomes. The rest of the total activity was equally distributed among the nuclear-plasma membrane-cell debris, mitochondrial, and secretory-granule-rich fractions. The microsomal fraction had the highest specific activity, even though it only accounted for 10% of the total activity.

The subcellular distribution of CDP-diglyceride inositol transferase was studied by the same fractionation procedure which was used for lysophosphatidylcholine 2-acyltransferase. The activity in the various fractions was assayed in the presence of 1 mM EGTA to eliminate any possible inhibition due to endogenous calcium (Table II). The activity in the secretory granule fraction accounted for almost half of the total activity and the specific activity of the enzyme in this fraction was approximately

TABLE II. Subcellular Distribution of Lysophosphatidylcholine 2-Acyltransferase and CDP-Diglyceride Inositol Transferase in Rat Pancreatic Islets*

	2-acyltransferase		Inositol transferase	
Fraction	Specific activity (units/mg protein)	Percent distribution	Specific activity (units/mg protein)	Percent distribution
Islet homogenate	3.39	100	6.2	100
Nuclei and plasma membrane	2.16	14.2	8.0	28
Mitochondria	5.43	14.2	5.4	10.6
Secretory granule	5.75	16.8	32.3	46.8
Microsomes	10.85	10.1	5.8	10.8
Cytosol	4.80	44.5	Absent	0.0

*The various subcellular fractions were prepared as described in Methods. The activity of the inositol transferase was determined in the presence of 1 mM EGTA to chelate any endogenous calcium which would be inhibitory. One unit of enzyme activity equals the incorporation of arachidonate or myoinositol at the rate of 1 nmol/min.

fivefold higher than in the other fractions. The nuclear plus the plasma membrane fraction accounted for 30% of the total activity and the mitochondrial and microsomal fractions each accounted for 10%.

DISCUSSION

It was previously reported that stimulatory glucose enhances the incorporation of arachidonate into islet phospholipids [5–8] and it was suggested that the incorporation of arachidonate is increased secondary to the increased turnover of phospholipid as a result of the stimulation of phospholipase A_2 by glucose. Indeed, Laychock [21] showed that incubating islets with stimulatory glucose prior to assaying for phospholipase A_2 activity results in an increased enzyme activity that persists after islets are homogenized. In the current report it is shown that incubating intact islets with stimulatory glucose leads to a stimulation of lysophosphatidylcholine 2-acyltransferase activity which also is retained after homogenization (Table II). Thus, it appears likely that the simultaneous stimulation of these two enzymes could increase the turnover of fatty acid moieties at the C-2 position of the glycerol backbone of phospholipids by a deacylation/reacylation mechanism. This may serve to increase the unsaturation of phospholipids by substituting some of the saturated fatty acids with arachidonate. The increased unsaturation of phospholipids has been shown by Lucy to favor membrane fusion [22]. Thus, stimulation of lysophosphatidylcholine 2-acyltransferase in the presence of high glucose could serve to promote exocytosis by, for example, facilitating the fusion of the insulin secretory granule with the plasma membrane or it could have other local effects on the islet plasma and/or intracellular membranes. Alternatively, stimulation of 2-acyltransferase may also serve to replenish the phospholipase A_2-induced loss of arachidonate from phospholipids. The mechanism by which glucose stimulates the activity of the acyltransferase is not clear at present. It is unlikely that this effect is calcium related because neither added calcium nor EGTA affected the assayable activity of this enzyme. Laychock [23] has recently suggested that acylation of endogenous acceptor molecules may be modulated by hydroxyeicosatetraenoic acids.

The assayable activity of diacylglycerokinase was unaffected by adding calcium to the enzyme reaction mixture or by preincubating islets with stimulatory glucose prior to assaying for enzyme activity. However, the other PtIns cycle enzyme, CDP-diglyceride inositol tranferase, although not affected by preincubation with stimulatory glucose, was strongly inhibited by calcium. The incubation period of 30 min with stimulatory glucose was chosen because it can take that long for the rate of glucose-induced insulin release by isolated islets to reach the maximal plateau. Thus, it is not possible to rule out any stimulatory or inhibitory effects that were transient and/or occurred prior to 30 min. Mn^{2+} antagonized the inhibition of inositol transferase by calcium (Table I). This property of the islet enzyme is similar to that of CDP-diglyceride inositol transferase from rat aorta and vas deferens [18]. A Dixon plot of calcium vs the CDP-diglyceride inositol transferase activity showed two slopes corresponding to $K_{0.5}$'s of 5 μM and 90 μM. This suggests the presence of two inositol transferase enzymes or two sites on one enzyme in islets with different sensitivities to inhibition by calcium.

The widely observed ability of stimulatory glucose to increase the incorporation of [^{32}P]Pi or myo[2-^{3}H]inositol into islet PtIns in intact islets [8,24,25] is consistent with an activation of CDP-diglyceride inositol transferase. In the current study, most of the activity of this enzyme was found in the secretory-granule enriched fraction. This fraction has also been reported to show the greatest stimulation of myoinositol incorporation in the presence of high glucose [24]. Since calcium inhibits the activity of CDP-diglyceride inositol transferase, it is tempting to speculate that increased myoinositol incorporation into secretory granule membranes may result from a glucose-stimulated efflux of calcium from secretory granules. Hahn et al, [26] have presented evidence for an efflux of calcium from the secretory granule fraction to the cytosol upon stimulation of pancreatic islets.

In regard to possible physiological roles for lysophosphatidylcholine 2-acyltransferase and CDP-diglyceride inositol transferase, it is likely that lysophosphatidylcholine 2-acyltransferase may be involved in altering membrane fatty acid composition, thus favoring secretion. CDP-diglyceride inositol transferase, on the other hand, may be involved in replenishing the PtIns which undergoes hydrolysis upon agonist-induced phospholipase C activation. This replenishment of PTIns may prepare islets for the next round of stimulation.

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