The Role of Calcium in Phospholipid Turnover following Glucose Stimulation in Neonatal Rat Cultured Islets*

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Phospholipid turnover was studied in cultured neonatal rat pancreatic islets. In islets prelabeled with [32P]Pi, 15-min stimulation with glucose (16.7 mM) caused increased labeling of phosphatidic acid (93%) and phosphatidylinositol (94%) and decreased labeling of the polyphosphoinositides (20%). Omission of calcium ion during the period of glucose stimulation did not modify the changes in inositol phospholipids. In islets equilibrated with [32P]Pi, in the presence and absence of stimulatory glucose concentrations (11.1 and 1.7 mM, respectively), chelation of calcium by ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′'-tetraacetic acid prevented the increase in phosphatidic acid and phosphatidylinositol labeling. However, the decrease in polyphosphoinositide labeling was inhibited by the chelator only in islets labeled in the absence of stimulatory glucose concentrations, the decrease persisting in islets labeled in the presence of glucose. This suggests that a specific pool of polyphosphoinositides is labeled in the presence of agonist and decreases in response to acute glucose stimulation irrespective of availability of external calcium.

In the absence of calcium, the addition of [γ-32P]ATP to a membrane preparation of cultured islets yielded three lipid phosphorylation products (phosphatidic acid, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate). In broken cell preparations, [32P]Pi-labeled phosphatidylinositol was also detected. The extent of all these phosphorylations was decreased by the presence of free calcium ion (40 μM).

These data indicate that polyphosphoinositide turnover takes place after glucose stimulation independent of extracellular calcium and support the possibility that this may play a primary role in altering cell calcium availability.

Extensive investigations have established that glucose-induced insulin release requires an increase of calcium ions (Ca++) within the pancreatic β cell (reviewed in Refs. 1 and 2) and that intracellular and extracellular sources may contribute to raised free cytosolic Ca++ on stimulation (3-5). While the inter-relationship of islet calcium sources is complex, it is possible that both intra- and extracellular Ca++ availability may be affected by glucose-induced changes in islet phospholipids and accompanying changes in the plasma membrane microenvironment. The precedent is seen in a number of tissues (reviewed in Refs. 6-8) in which hormones which exert their effects through mobilization of Ca++ show coincident changes in phosphatidylinositol metabolism. In these diverse systems, a role for inositol phospholipids in the maintenance and disposition of cellular calcium seems likely. Furthermore, the polyphosphoinositides phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate formed in the plasma membrane from phosphatidylinositol by the action of specific kinases have been shown to be rapidly degraded by a number of calcium-mobilizing stimuli (9-11). Similarly, insulin secretagogues glucose (12-15), leucine, and arginine (16) stimulate the metabolism of various β cell phospholipids with enhanced P1 metabolism and catabolism of its polyphosphorylated derivatives shown in response to glucose. The calcium dependency of agonist-induced breakdown of P1, located in most membrane systems of the cell, and the polyphosphoinositides, located primarily in the plasma membrane (6), has been studied in many systems (3). In most of these, interpretation of calcium dependence remains equivocal. A requirement for calcium has been inferred in the adult islet as no phosphoinositol turnover was seen in the absence of Ca++ and the presence of EGTA (13,14). In studies employing chelators, it is necessary to consider the capacity of EGTA to deplete intramembrane stores from which Ca++ may be released by agonists (17). We have demonstrated that glucose stimulation of neonatal islet cells affects a plasma membrane complement of Ca++ ionophoretic lipids (18), which may be an indication of a capacity for glucose to alter intramembrane calcium.

The following study was undertaken to determine the changes in neonatal islet inositol phospholipids following glucose stimulation and to establish whether these changes are a primary response independent of Ca++ or whether they require the presence of Ca++, which could indicate a response secondary to calcium entry.

EXPERIMENTAL PROCEDURES

Materials—[32P]Pi and [γ-32P]ATP were obtained from The Radiochemical Centre, Amersham, England. RPMI 1640 medium and HEPES were from Flow Laboratories, Inc. All phospholipids were from Sigma. Precatted silica gel plates were from Merck. All other chemicals and solvents were from BDH Chemicals Ltd. (AnalaR grade).

Maintenance and Labeling of Neonatal Islets with [32P]Pi, in Culture—Cultured neonatal islets were prepared from dispersions of 1-day-old rat pancreas as described by Hellerstrom et al. (19). After culture for 40 h in RPMI 1640 medium, [32P]Pi (30 μCi/400 islets) was added and the culture was continued for up to 24 h. Islets were

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Glucose-induced Phospholipid Turnover

RESULTS

The time course of incorporation of $^{32}$P into islet phospholipids is shown in Fig. 1. Following 24-h labeling, steady state was achieved for PC, PE, PI, PA, PI-4-P, and PI-4,5-P$_2$. Acute exposure to high glucose concentration stimulated insulin release ($0.87 \pm 0.40$ ng/10 islets/15 min, 1.7 mM glucose versus $3.42 \pm 0.47$, 16.7 mM glucose, $p < 0.001$). The phospholipid changes seen following glucose stimulation at this time are shown in Table I. There was increased labeling of PC, PS, PA, and PI and a decrease in the $^{32}$P content of the combined fraction containing polyphosphoinositides. Over the 15-min stimulation, PC, PS, and PI labeling occurs gradually, while the increased labeling of PA and the decrease in polyphosphoinositides occur more rapidly (within the first 5 min of stimulation). These phosphoinositide changes in islets equilibrated with $^{32}$P, in the presence of stimulatory (11.1 mM) or nonstimulatory (1.7 mM) glucose prior to the acute stimulation are further shown in Table II. A difference in the basal labeling of the phosphoinositides is seen. In those islets equilibrated in the presence of glucose (11.1 mM), the ratio of PI-4-P to PI-4,5-P$_2$ is 1.35 to 1, while in islets equilibrated in the presence of glucose (1.7 mM), this ratio is reversed, being 1 to 1.35 ($p < 0.05$). A further difference in the basal labeling is seen as decreased PI labeling and increased PA labeling of glucose-equilibrated islets. In both these equilibration conditions, however, acute glucose stimulation increased PI and PA labeling and decreased the labeling of PI-4-P and PI-4,5-P$_2$ as seen for islets equilibrated with glucose for 24 h.

Omission of Ca$^{2+}$ during acute glucose stimulation had no effect on the enhancement of $^{32}$P incorporation into PI and the decrease in the polyphosphoinositides was still apparent. When in addition to the omission of Ca$^{2+}$ during acute glucose stimulation had no effect on the enhancement of $^{32}$P incorporation into PI and the decrease in the polyphosphoinositides was still apparent. When in addition to the omission of Ca$^{2+}$ during acute glucose stimulation had no effect on the enhancement of $^{32}$P incorporation into PI and the decrease in the polyphosphoinositides was still apparent.
Glucose-induced Phospholipid Turnover

Effect of glucose on \([^{32}P]P\), labeling of phospholipids in neonatal rat islets prelabeled to steady state

Islets were incubated in RPMI 1640 medium buffered with HEPES and containing the salt concentration of bicarbonate-buffered Krebs solution following 24-h prelabeling with \([^{32}P]P\) in RPMI 1640 medium. \([^{32}P]P\) content of PS, PA, PI, and the polyphosphoinositides was determined from acidic chloroform:methanol-extracted samples applied to solid phase columns of neomycin coupled to glycoxyphosphatidyleoline and PE were determined following further thin layer chromatographic separation. Values shown are mean ± S.E. (n = six to eight observations). Statistically significant differences between control (1.7 mM glucose) and glucose stimulation are indicated by an asterisk (p < 0.05) and a double asterisk (p < 0.001).

### Table I

| Phospholipid | 1.7 mM glucose | 16.7 mM glucose |
|--------------|----------------|----------------|
|              | 15 min | 5 min | 10 min | 15 min |
| PC           |         |       |        |        |
| PE           |         |       |        |        |
| PS           |         |       |        |        |
| PA           |         |       |        |        |
| PI           |         |       |        |        |
| PI-4-P + PI-4,5-P₂ |   |       |        |        |

| Phospholipid | 1.7 mM glucose | 16.7 mM glucose |
|--------------|----------------|----------------|
|              | 15 min | 5 min | 10 min | 15 min |
| PC           |         |       |        |        |
| PE           |         |       |        |        |
| PS           |         |       |        |        |
| PA           |         |       |        |        |
| PI           |         |       |        |        |
| PI-4-P + PI-4,5-P₂ |   |       |        |        |

Effect of calcium removal on \([^{32}P]P\), labeling of inositol phospholipids and phosphatidic acid in response to acute glucose stimulation following prelabeling in the presence of stimulatory and nonstimulatory glucose concentrations

Islets were incubated in RPMI 1640 medium modified to bicarbonate-buffered Krebs solution containing glucose (1.7 or 16.7 mM) for 15 min. Calcium was omitted in the presence and absence of EGTA (5 mM). Inositol phospholipids were determined following thin layer chromatography. Values shown are mean ± S.E. (n = five to eight observations). For each prelabeling condition, the statistical significance of the difference from control (1.7 mM glucose) is indicated by an asterisk (p < 0.05), a double asterisk (p < 0.01), and a triple asterisk (p < 0.005).

### Table II

| Incubation condition | 1.7 mM glucose | 16.7 mM glucose |
|----------------------|----------------|----------------|
|                      | 15 min | 5 min | 10 min | 15 min |
| PC                   |         |       |        |        |
| PE                   |         |       |        |        |
| PS                   |         |       |        |        |
| PA                   |         |       |        |        |
| PI                   |         |       |        |        |
| PI-4-P + PI-4,5-P₂   |   |       |        |        |

| Incubation condition | 1.7 mM glucose | 16.7 mM glucose |
|----------------------|----------------|----------------|
|                      | 15 min | 5 min | 10 min | 15 min |
| PC                   |         |       |        |        |
| PE                   |         |       |        |        |
| PS                   |         |       |        |        |
| PA                   |         |       |        |        |
| PI                   |         |       |        |        |
| PI-4-P + PI-4,5-P₂   |   |       |        |        |

DISCUSSION

This study has shown that glucose induces a sequence of events in cultured neonatal rat islets which indicates phosphatidylidyinositol phosphodiesterase cleavage to form diacylglycerol, its phosphorylation to form phosphatidic acid, and the resynthesis of PI through cytidylyphosphoinositides. This confirms previous findings in mature islets. The time course studies also support the finding of Laychock (14) that polyphosphoinositide hydrolysis is an early event in glucose-induced insulin secretion.

The role of Ca²⁺ in PI and polyphosphoinositide turnover in different tissues has been controversial. In adult rat islets, it has been reported that PI and polyphosphoinositide turnover measured as \([^{32}P]P\) labeling (14) or inositol phosphate release (15) is markedly inhibited by the removal of Ca²⁺ with the addition of EGTA to the extracellular medium. It was therefore inferred that this phosphoinositide turnover is dependent on an influx of calcium. Using similar conditions of labeling with \([^{32}P]P\), as employed by Laychock (14), similar results were obtained in the present study. However, when phosphoinositide pools were labeled in the presence of stimulatory concentrations of glucose, quite different findings resulted. Even in the absence of extracellular Ca²⁺, and the presence of a high concentration of EGTA, polyphosphoinositide loss was induced by acute exposure to glucose. This suggests that when labeling is carried out in the presence of agonist (stimulatory glucose concentrations), a specific pool of phosphoinositides, inaccessible to EGTA and possibly at an inner membrane leaflet site, is labeled. This may be analogous to the situation in other tissues where agonist labeling reveals a specific pool of PI which is hormone-responsive (29–31) and Ca²⁺-independent (31). In the islet, PI turnover itself remains dependent on extracellular Ca²⁺ even with agonist labeling, but polyphosphoinositide breakdown does not.

The role of Ca²⁺ in regulating phosphoinositide turnover was investigated further by looking at the incorporation of...
brane preparations, with resynthesis of PI demonstrable only in homogenates. The net formation of the polyphosphoinositides and of PA was shown in membrane preparations to be inhibited by Ca\(^{2+}\). This may reflect the sensitivity to Ca\(^{2+}\) of islet phosphodiesterases and phospholipases as described for liver (32), brain (33), lymphocytes (34), smooth muscle (35), white pancreas (36), and platelets (37). However, Ca\(^{2+}\) inhibition of the kinases involved may also contribute as micromolar diacylglycerol kinase of rat liver is inhibited by elevated Ca\(^{2+}\) (38).

The findings described in the present report carry the following implications. As the polyphosphoinositides are chelators of both Ca\(^{2+}\) and Mg\(^{2+}\) (39), a change in their amount relative to other phospholipids located at internal cell membranes may change the amount of Ca\(^{2+}\) bound to the plasma membrane. However, it must be remembered that using the techniques currently employed to measure phosphoinositide turnover, it is not possible to establish whether the pool size is sufficient to effect changes in membrane Ca\(^{2+}\) availability and/or disposition. The finding of an absolute dependence on extracellular Ca\(^{2+}\) of PA and PI turnover but not of polyphosphoinositide breakdown may indicate that the latter glucose-induced phospholipid turnover may be an initial event following glucose stimulation, which precedes Ca\(^{2+}\) entry into the islet. The sensitivity of polyphosphoinositide reaccumulation to Ca\(^{2+}\) demonstrated in the membrane preparations using \([\gamma-32P]ATP\) would indicate that while intracellular Ca\(^{2+}\) levels remain high, polyphosphoinositide reaccumulation is prevented. In support of the sequence described are the ultrastructural studies of the mature pancreatic islet which show an accumulation of calcium closely associated with the plasma membrane which is depleted following glucose stimulation (40).

The current study emphasizes the complexity of the membrane-associated inositol phospholipid pools and the importance of agonist labeling in revealing a specific, glucose-responsive, and extracellular Ca\(^{2+}\)-independent pool of polyphosphoinositides in the pancreatic islet. In thrombin-stimulated platelets, a loss of PI-4,5-P\(_2\) has been shown to precede Ca\(^{2+}\) mobilization, phospholipase activation, the formation of arachidonate metabolites, and alteration in polymerization of cytoskeletal elements (41). By analogy, the breakdown of polyphosphoinositides in the agonist-labeled pool in the neonatal islet may be an initiating step integral to glucose-induced insulin release.

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