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PHOSPHATIDYLINOSITOL AVAILABILITY AND POLYPHOSPHOINOSITIDE SYNTHESIS IN PANCREATIC ISLET CELL MEMBRANES

LUMA J. KHALAF and SUZANNE G. LAYCHOCK*

Department of Pharmacology and Therapeutics, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, NY 14214, U.S.A.

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Abstract—Polyphosphoinositide synthesis in isolated islets of the rat was determined by the phosphorylation of endogenous phosphatidylinositol (PtdIns) by PtdIns kinase and [γ-^{32}P]ATP to form [^{32}P]-phosphatidylinositol 4-phosphate (PtdInsP) in cell homogenates. Glucose stimulation of intact islets resulted in a time- and concentration-dependent reduction in PtdInsP synthesis. Similarly, the stimulation of intact islets with carbachol (CCh), cholecystokinin (CCK-8S), or tolbutamide for 15 min reduced PtdInsP production in a concentration-dependent manner. The effects of glucose, tolbutamide and CCh were reversible. PtdInsP hydrolysis did not account for the reduction in PtdInsP recovery. The addition of exogenous PtdIns to the PtdIns kinase assay significantly increased basal PtdInsP levels. In addition, exogenous PtdIns completely reversed the inhibitory effects of glucose and increased PtdIns kinase activity in homogenates of glucose-stimulated islets to levels found in control homogenate with PtdIns. Exogenous PtdIns also increased PtdIns kinase activity in CCK-8S-treated islets, although exogenous PtdIns did not overcome the tolbutamide-induced inhibition of PtdIns kinase. The V_{max} of PtdIns kinase in homogenates of islets treated with tolbutamide was reduced significantly, although glucose did not affect the V_{max}. In addition, the K_m values for ATP and PtdIns were not altered by exposure of the islets to cell stimuli. The results suggest that the level of PtdIns in islet cell membranes is rate limiting for PtdInsP synthesis, and that tolbutamide is a noncompetitive inhibitor of PtdIns kinase.

Stimulation of the phosphoinositide cycle in pancreatic islets accompanies the activation of β cells by various insulinotropic agents [1, 2]. Membrane phosphoinositides are phospholipid substrates for phospholipase C and certain phosphomonoesterases. Phosphatidylinositol (PtdIns†) kinase, the first enzyme in the polyphosphoinositide synthesis pathway, phosphorylates PtdIns in the 4 position, generating phosphatidylinositol 4-phosphate (PtdInsP). Another enzyme, PtdInsP kinase, generates phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P) [3]. PtdInsP and PtdIns4,5P are substrates for receptor-regulated phosphoinositide phosphodiesterase (phospholipase C) [4]. The synthesis and hydrolysis of polyphosphoinositides have been demonstrated for intact isolated pancreatic islets [1, 4, 5] as well as broken cell preparations [6, 7].

Islet or insulinoma cell PtdIns kinase activity is found associated primarily with the plasma membrane, with less activity in microsomes and secretory granule membranes [6, 7]. The PtdIns kinase characteristics are similar in plasma membranes and secretory granules, with the K_m values for ATP at 120 and 110 μM, respectively [6]. PtdIns kinase in islet cells and other tissues is characterized as a Mg^{2+}-dependent enzyme, which is inhibited by Ca^{2+} [6, 8]. The present study was undertaken to investigate the effect on PtdIns metabolism of islet stimulation by several insulinotropic and phosphoinotropic agents.

* Corresponding author: S. G. Laychock, Ph.D., 102 Farber Hall, Department of Pharmacology and Therapeutics, School of Medicine, SUNY at Buffalo, Buffalo, NY 14214. Tel. (716) 831-2808; FAX (716) 831-2801.

† Abbreviations: PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol 4-phosphate; PtdIns4,5P, phosphatidylinositol 4,5-bisphosphate; CCh, carbachol; CCK-8S, cholecystokinin; EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; and BSA, bovine serum albumin.

METHODS

Materials. [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from New England Nuclear Research Products, Inc. (Boston, MA). Tolbutamide sodium salt was from the Upjohn Co. (Kalamazoo, MI). Carbachol (CCh), cholecystokinin (CCK-8S), bovine serum albumin (BSA; Type V, fatty acid free), adenosine 5′-triphosphate (ATP; disodium salt from equine muscle), trypsin inhibitor (soybean), ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), and phosphatidylinositol (bovine liver, ammonium salt) were from the Sigma Chemical Co. (St. Louis, MO). Triton X-100 was from the J. T. Baker Chemical Co. (Phillipsburg, NJ). Collagenase (Types P and D) were from Boehringer Mannheim (Indianapolis, IN). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonate (Hepes) was obtained from United States Biochemical (Cleveland, OH). PtdIns specific phospholipase C (*Bacillus thuringiensis*) was from American Radiolabeled Chemicals (St. Louis, MO). Tissue culture reagents were from GIBCO (Grand Island, NY). Other chemicals were reagent grade.

Tissue preparation and incubation. Pancreatic

islets from male Sprague–Dawley rats (250–275 g) were prepared by collagenase digestion of excised pancreas as described previously [9]. Equal numbers of islets (50–70 per sample) were incubated in polypropylene microfuge tubes in a modified Krebs Ringer bicarbonate (KRB) buffer, pH 7.4, containing (mM): NaCl (119.8); KCl (4.8); $MgSO_4$ (1.2); KH_2PO_4 (1.2); $CaCl_2$ (2.5); $NaHCO_3$ (24.6); Hepes (16); glucose (2.8); and 0.01% BSA. The islets were preincubated for 30 min at 37° under an atmosphere of O_2/CO_2 (19:1) in a shaking water bath (120 rpm). Then, the KRB buffer was replaced with fresh buffer and the incubation continued in the absence or presence of various secretogogues as specified in the text. In some experiments, islets which had been incubated with various agents for 15 min were washed three times with fresh KRB buffer within 30 min, and the incubation was continued for a total of 2 hr. Then, the islets were resuspended in ice-cold KRB buffer in the absence of BSA and Hepes, microfuged for 50 sec, and the wash supernatant discarded. In certain experiments, islets were cultured overnight under an atmosphere of 95% air/5% CO_2 in RPMI 1640 containing 0.5% penicillin, 0.5% streptomycin, and 10% fetal bovine serum. The next day, the culture medium was washed from the islets and they were incubated in KRB buffer for 1 hr, prior to stimulation with agents investigated for effects on PtdIns kinase activity. Basal PtdIns kinase activity was not different in freshly isolated islets compared to islets cultured overnight.

After washing, the islets were resuspended in a modified PtdIns kinase assay buffer (pH 7) [10] consisting of (mM): Tris–HCl (30); EGTA (0.5); $MgCl_2$ (20); and trypsin inhibitor (0.1 mg/mL). Islets were homogenized by sonication for 10 sec, using a Kontes micro-tip sonicator. An aliquot of the homogenate was saved for determination of protein [11]. The homogenate was warmed for 2 min at 30° prior to initiation of the assay with ATP. PtdIns kinase activity in the islet homogenate was assayed in a final volume of 0.11 mL Tris–HCl buffer containing 0.18 mM [γ-^{32}P]ATP (15 μCi/tube), at 30°. This concentration of ATP exceeded the K_m and was optimal for generation of PtdInsP. When exogenous PtdIns was used in the assay, the PtdIns was initially solubilized in ethanol, and then diluted 10-fold with assay buffer prior to sonication for 2 min in an ultrasonic bath. The solubilized PtdIns was added to the assay 1 min before the addition of ATP. Vehicle-treated control samples were run in parallel with PtdIns-supplemented assays, and ethanol did not exceed 1% of the final volume.

To determine the K_m and V_{max} for ATP dependence of homogenate PtdIns kinase, five concentrations of ATP were used (0.1 to 0.9 mM) in the presence of 140 μM PtdIns, with a reaction time of 15 sec. The K_m for PtdIns and the V_{max} for PtdIns kinase were determined in homogenates depleted of endogenous PtdIns by pretreatment with exogenous PtdIns-specific phospholipase C (1 U/mL) with 0.1 mM Ca^{2+} in Tris homogenization buffer lacking EGTA, for 15 min at 37°. Basal PtdInsP formation in phospholipase C-treated homogenates was reduced by 69% compared to untreated controls. PtdIns kinase activity of the homogenate was then determined in Tris assay buffer containing EGTA (1 mM), Triton X-100 (0.1%), and the absence or presence of PtdIns (0.1 to 20 μM), and ATP (0.36 mM); the reaction was stopped after 15 sec. The exogenous PtdIns concentrations were corrected for hydrolysis by phospholipase C as determined by [^{14}C]phosphatidylinositol hydrolysis. Basal values of PtdInsP formation were subtracted from values obtained in the presence of exogenous PtdIns for the determination of K_m and V_{max}.

To determine PtdInsP hydrolysis during the PtdIns kinase assay, [^{32}P]PtdInsP formation in islet homogenates was determined after 1 min, and then in an aliquot of the reaction mixture [^{32}P]ATP incorporation was quenched either by 100-fold dilution by volume with reaction buffer lacking radiolabeled ATP or a 10-fold excess of unlabeled ATP. Both methods allow for continued PtdInsP synthesis although the ^{32}P incorporation was negligible, and hydrolysis of [^{32}P]PtdInsP was quantitated. Quenched samples were allowed to react for an additional 4 min, after which the reaction was terminated as described below. The difference in PtdInsP levels recovered 4 min following the dilution compared with PtdInsP levels at 1 min served as an estimate of the rate of PtdInsP hydrolysis in the homogenates.

The reaction was terminated by placing a 25-μL aliquot of the reaction mixture in 1 mL of cold chloroform/methanol (1:2, v/v). The samples were extracted overnight at 4°, and then 0.03 mL HCl (2N), and 0.4 mL each of 2 M KCl and chloroform were added to achieve phase separation. The organic phase was removed and pooled with the organic phase resulting when the aqueous phase was re-extracted with 0.7 mL chloroform. The organic phase was dried under a stream of N_2. Thin-layer chromatography of the extracted lipids was carried out as described previously [1] for the isolation of phosphoinositides. Lipid zones were identified by autoradiography, and then scraped into scintillation vials and counted by liquid scintillation spectrometry.

Statistical analysis. PtdInsP formation is expressed as picomoles PtdInsP produced per milligram of protein, or as a percent of control. Values are the means ± SEM. Differences between treatments were determined using a one-way or two-way analysis of variance, or Student's *t*-test.

RESULTS

Effect of glucose on [^{32}P]PtdInsP formation. PtdInsP synthesis in homogenates of isolated pancreatic islets is defined as the picomoles of PtdInsP formed by PtdIns kinase utilizing endogenous PtdIns as substrate. PtdInsP formation from endogenous PtdIns was time dependent for at least 90 sec (Fig. 1). The incubation of intact islets with the primary physiological insulin secretogogue glucose affected PtdIns kinase activity in islet homogenates in a time- and concentration-dependent manner. PtdInsP synthesis in homogenates of islets that had been incubated with a maximum insulinotropic concentration of glucose for 15 min was significantly lower than in unstimulated controls

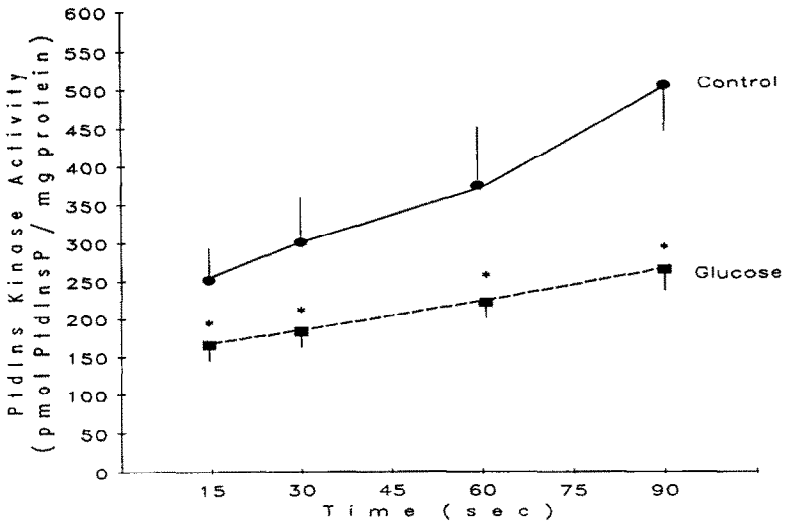

Fig. 1. PtdInsP synthesis in homogenates of isolated rat islets. Islets were incubated with 2.8 mM glucose (control) or 17 mM glucose for 15 min, and then they were homogenized for determination of PtdInsP synthesis. Values, expressed as PtdIns kinase activity levels, are the means ± SEM for seven experimental determinations. Key: *statistically significant ($P < 0.01$) compared to control values, as determined by two-way analysis of variance.

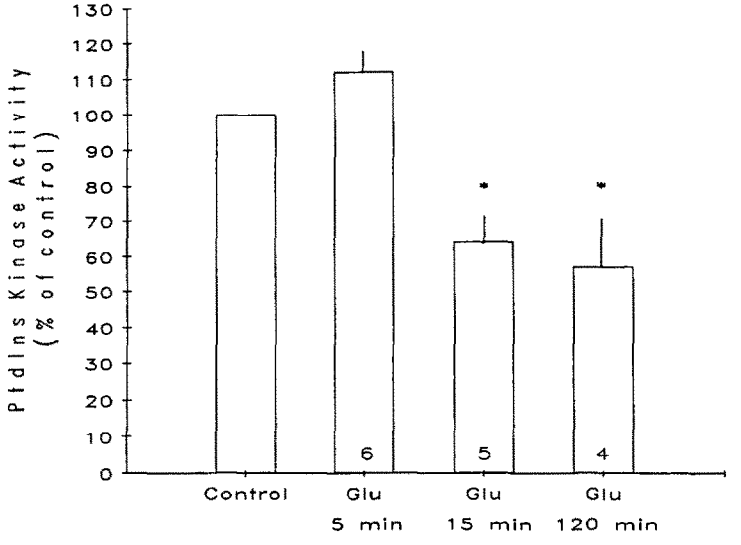

Fig. 2. Time-dependent effects of glucose on PtdInsP synthesis. Islets were incubated in KRB buffer containing 2.8 mM glucose (control) or 17 mM glucose, for the times indicated. Then, the islets were homogenized for determination of PtdInsP synthesis. Values are the means ± SEM for PtdInsP formation expressed as percent of control values after 60 sec, for the number of determinations indicated at the base of each bar. Control value: 270 ± 50 pmol PtdInsP/mg protein. Key: *$P < 0.05$ compared to control values as determined by one-way analysis of variance.

(Fig. 1). The reduced capacity to synthesize PtdInsP following exposure of intact islets to glucose was time related. PtdInsP synthesis was 112% of control in homogenates from islets incubated with glucose for 5 min, whereas stimulation for 15 min and 120 min reduced PtdInsP synthesis by more than 30% (Fig. 2). The reduction in PtdInsP synthesis following islet glucose exposure was concentration dependent. A concentration of glucose (8.5 mM) which is half-maximum for insulin release did not affect PtdInsP synthesis significantly, in contrast to the significant reduction by a maximally insulinotropic concentration of glucose (17 mM) (Fig. 3).

The change in PtdInsP formation in glucose-

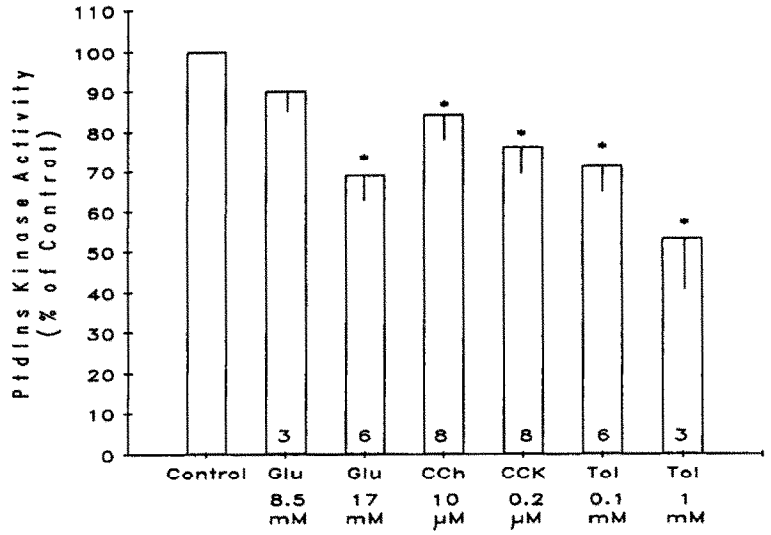

Fig. 3. Effects of insulinotropic agents on PtdInsP synthesis. Islets were incubated in KRB buffer containing 2.8 mM glucose (control) in the absence or presence of CCh, CCK-8S (CCK), tolbutamide (Tol), or increasing concentrations of glucose (Glu), as indicated. Values are the means ± SEM for PtdInsP formation expressed as percent of control values after 60 sec. The number of experimental determinations is shown at the base of each bar. Control values: 172 ± 14 pmol PtdInsP/mg protein.
Key: *P < 0.05 compared to control values as determined by one-way analysis of variance.

Table 1. Kinetic analysis of islet PtdIns kinase

		Islet PtdIns kinase	
Group	Treatment	K_m ATP (μM)	V_{max} (pmol PtdInsP/sec/mg protein)
A	Control	67 ± 21	7.7 ± 1.3
	Glucose	92 ± 8	6.6 ± 0.4
B	Control	62 ± 4	7.4 ± 0.7
	CCK-8S	44 ± 5	6.2 ± 0.6
C	Control	55 ± 2	8.8 ± 0.4
	Tolbutamide	42 ± 1	5.9 ± 0.1*

Islets were incubated for 15 min in KRB buffer in the absence (control) or presence of glucose (17 mM), CCK-8S (0.2 μM), or tolbutamide (1 mM). The islets were washed and homogenized, and the activity of PtdIns kinase was determined from the formation of PtdInsP during 15 sec. Values, derived from Lineweaver–Burk analysis of data using linear regression, are the means ± SEM from three independent paired determinations per group.
* P < 0.01 compared to Group C control values, as determined by Student's *t*-test (paired).

treated islet homogenates did not appear to be due to a change in enzyme utilization of the substrate ATP since the apparent K_m for ATP and the V_{max} of PtdIns kinase between control and glucose-treated islet homogenates were similar (Table 1).

The effects of glucose on PtdInsP synthesis were reversible. When islets that had been treated for 15 min with glucose (17 mM) were washed and incubated in KRB buffer containing 2.8 mM glucose for 2 hr, PtdInsP synthesis was 98 ± 14% of control (N = 9).

Effects of CCh, CCK-8S and tolbutamide on [^{32}P] *PtdInsP formation.* CCh, CCK-8S and tolbutamide stimulate phosphoinositide hydrolysis and inositol phosphate production, and secretion in pancreatic islets [1,12]. The incubation of isolated islets with CCh for 15 min resulted in a modest 15% reduction in PtdInsP synthesis by homogenates (Fig. 3). A shorter period of CCh stimulation (1 min or 5 min) did not affect PtdInsP formation (data not shown). Similarly, CCK-8S, at a concentration which evokes insulin release and inositol release from phosphoinositides [12], suppressed PtdInsP synthesis by 25% in homogenates of islets exposed to the

hormone for 15 min (Fig. 3). When islets were treated with the sulfonylurea tolbutamide, PtdInsP synthesis in islet homogenates was reduced by 40% (Fig. 3). In addition, tolbutamide effects on PtdInsP synthesis were concentration related (Fig. 3). Glucose (17 mM)- and tolbutamide (1 mM)-treated islet PtdInsP syntheses were reduced to similar levels (Fig. 3). The V_{max} of PtdIns kinase for ATP was reduced significantly by treatment of the islets with tolbutamide, but not CCK-8S (Table 1). The apparent K_m values for ATP of PtdIns kinase of islets exposed to CCK-8S or tolbutamide were not changed compared to control values (Table 1).

The tolbutamide and CCh responses were reversible. The treated islets were washed and incubated in KRB buffer for up to 2 hr. Although 1 hr was not sufficient to completely reverse the drug effects, a 2-hr washout period restored PtdInsP synthesis activity to 92 ± 14% ($N = 6$; $P > 0.05$) and 114 ± 20% ($N = 5$; $P > 0.05$) of control, respectively, for tolbutamide (0.1 mM)- and CCh (10 μM)-treated islets.

Effect of exogenous PtdIns on PtdIns kinase activity. Intact islets were incubated in the absence (control) or presence of glucose (17 mM) for 15 min, and homogenized for assay of PtdIns kinase activity in the presence or absence of exogenous PtdIns. Exogenous PtdIns substrate added to the PtdIns kinase assay enhanced the production of PtdInsP. A concentration response to 70, 140 and 280 μM PtdIns demonstrated that 140 μM PtdIns supported maximal PtdInsP formation, in agreement with the findings of others [7]. Exogenous PtdIns significantly ($P < 0.05$) increased PtdInsP synthesis in homogenates of control and glucose-treated islets (Fig. 4). Exogenous PtdIns not only completely reversed the inhibitory effects of glucose on PtdInsP synthesis, but increased PtdInsP formation to the levels found in control membrane preparations supplemented with exogenous PtdIns (Fig. 4). The K_m for PtdIns and the V_{max} of PtdIns kinase in control and glucose-treated islets were similar (Table 2). However, treatment of the homogenates with Triton X-100 increased the V_{max} of PtdIns kinase by approximately 2-fold (compare Tables 1 and 2).

Exogenous PtdIns also affected PtdIns kinase activity in membranes of islets pretreated with CCK-8S. CCK-8S-treated islet homogenate PtdInsP synthesis did not differ significantly (259 ± 39 pmol PtdInsP/min/mg protein) from basal values (264 ± 26 pmol PtdInsP/min/mg protein) in the presence of 140 μM exogenous PtdIns. In contrast, tolbutamide-treated islet homogenate PtdInsP synthesis was suppressed ($P < 0.05$) even in the presence of 140 μM exogenous PtdIns (158 ± 32 pmol PtdInsP/min/mg protein), when compared to basal values obtained with the addition of PtdIns (see above). The V_{max} values for PtdIns kinase in tolbutamide-treated and control samples were significantly different (Table 2). The K_m values for PtdIns of PtdIns kinase in tolbutamide-treated and control islet homogenates were similar.

Evaluation of PtdInsP hydrolysis. The suppressed levels of PtdInsP formed in membranes from stimulated islets may not only be due to reduced synthesis of PtdInsP but also to the enhanced hydrolysis of PtdInsP. To test the latter possibility, homogenates of unstimulated islets, and glucose-, tolbutamide- or CCh-treated islets were allowed to generate PtdInsP in the usual manner for 60 sec. Then, an aliquot of the reaction mixture was either diluted 100-fold with assay buffer or a 10-fold excess of unlabeled ATP. Then, the incubation was continued for an additional 4 min. The dilution of the [^{32}P]ATP by either the volume or mass method quenched the formation of radiolabeled PtdInsP, and after 4 min PtdInsP control levels were modestly reduced from the levels quantitated at 60 sec (Table 3). In homogenates of glucose-stimulated islets, PtdInsP hydrolysis was only 2.3%/min, whereas control hydrolysis was 3.5%/min (Table 3). Similarly, PtdInsP hydrolysis in tolbutamide- and CCh-treated islet homogenates was slightly lower, 3.3% and 4.3%/min, respectively, than control values (Table 3). The percentage changes in PtdInsP formation in treated islet homogenates were not significantly different from those for control islets. Moreover, the percentage reductions in PtdInsP levels due to hydrolysis were not sufficient to account for the reduced levels of PtdInsP observed during 60-sec incubations of homogenates from glucose-, tolbutamide-, or CCh-treated islets, compared to control PtdInsP levels.

DISCUSSION

The results of this study demonstrate that insulinotropic agents affect PtdIns kinase activity and PtdInsP formation in isolated pancreatic islets. PtdInsP synthesis in islet homogenates was linear for 1–2 min with endogenous PtdIns as substrate, which is similar to the kinetics for PtdIns kinase activity reported by others [6, 7]. In addition, the presence of exogenous PtdIns substrate increased PtdInsP formation in a concentration-dependent manner. In agreement with others [7], 140 μM PtdIns was optimal for islet PtdIns kinase activity. The PtdInsP formation and K_m for ATP of PtdIns kinase in this study are similar to those reported for insulinoma cell PtdIns kinase [6]. In contrast, other investigators have reported that PtdInsP formation in the absence of exogenous PtdIns is less than 1% of the enzyme activity in the presence of substrate [7]. However, the former investigation utilized a low specific activity and molar concentration of ATP similar to the K_m for ATP of PtdIns kinase, making it difficult to detect PtdInsP formation in the presence of endogenous PtdIns. The method of homogenization may also account for differences in enzyme activity.

Glucose stimulates the hydrolysis of PtdIns4,5P in islet membranes by the sequential depolarization of the β cell, increased Ca^{2+} influx, and stimulated phospholipase C activity [1,13]. In contrast to enhanced polyphosphoinositide hydrolysis, membranes from glucose-stimulated islets exhibited a reduced capacity to synthesize PtdInsP. Basal or submaximal insulinotropic levels of glucose did not affect PtdInsP synthesis during a short-term incubation. The inhibitory effects of glucose on PtdInsP synthesis were reversed by incubation of the islets for 2 hr under basal conditions. A similar

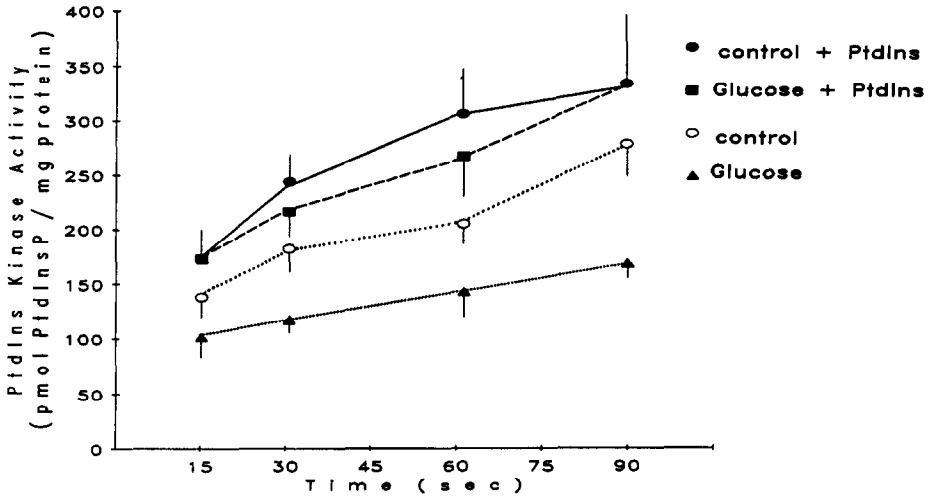

Fig. 4. Effect of exogenous PtdIns on PtdInsP synthesis. Islets were incubated in KRB buffer containing 2.8 mM glucose (control) or 17 mM glucose, for 15 min. Then, the islet homogenates were assayed for PtdIns kinase activity in the absence or presence (±PtdIns) of 140 μM PtdIns for up to 90 sec. Values are the means ± SEM for three to five experimental determinations. Two-way analysis of variance of the data showed that control(+PtdIns) values were higher ($P < 0.05$) than control values; values for glucose-treated islet homogenates were lower than control values ($P < 0.01$); values for control(+PtdIns) samples were not different from glucose(+PtdIns) values.

Table 2. PtdIns kinetic analysis of PtdIns kinase

		Islet PtdIns kinase	
Group	Treatment	K_m PtdIns (μM)	V_{max} (pmol PtdInsP/sec/mg protein)
A	Control	6.2 ± 0.7	17.7 ± 1.1
	Glucose	6.0 ± 1.5	15.7 ± 3.1
B	Control	5.9 ± 1.4	16.3 ± 3.9
	Tolbutamide	3.9 ± 0.8	9.3 ± 2.8*

Islets were incubated in KRB buffer in the absence (control) or presence of glucose (17 mM) or tolbutamide (1 mM) for 15 min. The islets were then homogenized, endogenous PtdIns was depleted, and the activity of the PtdIns kinase was determined in the presence of ATP (360 μM) and Triton X-100 (0.1%). Values derived from Lineweaver–Burk analyses are the means ± SEM for four independent determinations per group.

* $P < 0.05$ compared to Group B control, as determined by Student's *t*-test (paired).

period of recovery was effective for glucose-stimulated islets exhibiting blunted insulin secretory and inositol phosphate responses [14].

The effects of glucose in this study are at variance with an earlier report [7] that glucose stimulation of isolated islets did not affect PtdIns kinase activity. However, those studies utilized exogenous PtdIns to quantitate PtdInsP formation. The present study demonstrates that the addition of exogenous substrate to the PtdIns kinase assay overcomes the inhibitory effect of glucose stimulation. Since the K_m and V_{max} values for ATP and PtdIns of PtdIns kinase were not altered by islet exposure to glucose, the data support the conclusion that PtdIns depletion in islet membranes can account for suppressed PtdIns kinase activity. It has been proposed that a limited pool of endogenous PtdIns is available to PtdIns kinase [15], and it is possible that glucose and other secretogogues which promote polyphosphoinositide hydrolysis through the stimulation of phospholipase C deplete a pool of PtdIns during polyphosphoinositide synthesis. Evidence has been reported that in the absence of extracellular Ca^{2+} and suppressed phospholipase C activity, glucose stimulates PtdIns conversion to polyphosphoinositides [16].

The hypothesis that glucose stimulation of islets reduces PtdIns phosphorylation is supported by the findings of Zawalich *et al.* [17] which demonstrate that the incorporation of [^{3}H]inositol into polyphosphoinositides of intact islets is reduced following

Table 3. PtdInsP hydrolysis in islet homogenates

	PtdInsP hydrolysis (%/min)	(N)
Group A		
Control	3.5 ± 1.3	6
Glucose	2.3 ± 1.5	3
Group B		
Control	5.8 ± 1.5	3
CCh	4.3 ± 3.0	3
Tolbutamide	3.3 ± 2.3	4

Islets were incubated for 15 min with 2.8 mM glucose (control) or 17 mM glucose, tolbutamide (1 mM), or CCh (10 μM), and then homogenized for determination of PtdIns kinase activity. After 60 sec, an aliquot of the PtdIns kinase reaction mixture was extracted for quantitation of PtdInsP levels, while an equal aliquot was either diluted 100-fold with reaction mixture lacking the [^{32}P]ATP (Group A), or the mass of [^{32}P]ATP was diluted 10-fold by addition of 1.8 mM ATP (Group B). Diluted samples were incubated for an additional 4 min, with subsequent extraction and quantitation of PtdInsP. The hydrolysis of PtdInsP was determined from the difference between the PtdInsP levels at 60 sec and 5 min. Values are the means ± SEM for the number of determinations (N).

glucose stimulation. Moreover, although *myo*-inositol is limiting in isolated islets and contributes to reduced PtdIns synthesis [18], glucose stimulation increases the free inositol levels of islet cells [19], suggesting that the incorporation of *myo*-inositol into PtdIns is suppressed. The mechanism for the reduced PtdIns levels and PtdIns kinase activity in stimulated islet cells is not known. However, Ca^{2+} inhibits islet CDP-diglyceride inositol transferase, the rate-limiting step in the resynthesis of PtdIns [20, 21]. An increase in cell Ca^{2+} levels upon stimulation with insulinotropic agents may inhibit the transferase enzyme and impede PtdIns synthesis. Since CDP-diglyceride levels increase in stimulated islets [18], and glucose stimulation of insulin secretion and phosphoinositide hydrolysis is not accompanied by an increase in CDP-diglyceride inositol transferase activity [20], it appears that PtdIns synthesis does not parallel the rapid rate of phosphoinositide hydrolysis in islet cell membranes.

Similar to the effects of glucose, incubation of intact islets with other agents including CCh, CCK-8S and tolbutamide also reduced PtdInsP synthesis in cell homogenates. Muscarinic receptor agonists stimulate PtdIns4,5P hydrolysis [22], and it is possible that PtdIns reserves used to synthesize polyphosphoinositides are not repleted rapidly following phosphoinositide cycle stimulation. Similar to glucose, continued stimulation with CCK-8S impairs the formation of islet inositol phosphates [23], suggesting that there is a desensitization of the phosphoinositide cycle.

In contrast to the muscarinic receptor agonists, tolbutamide receptor binding inactivates the ATP-sensitive K^+ channel of β cells, thereby depolarizing the cell and opening voltage-dependent Ca^{2+} channels [13]. Tolbutamide also increases Ca^{2+}-dependent phospholipase C and phospholipase A_2 activities at the drug concentrations used in this study [1, 24, 25]. The results of experiments in which exogenous PtdIns was added to the PtdIns kinase assay of islet homogenate derived from tolbutamide-treated cells also indicate that additional substrate does not overcome the drug effects. The reduced V_{max}, but unchanged K_m for ATP or PtdIns, observed in tolbutamide-treated islets suggests noncompetitive inhibition of PtdIns kinase. Thus, although tolbutamide stimulates phospholipase C in islets, this agent suppresses the rate of resynthesis of PtdInsP and PtdIns4,5P, and inositol phosphate accumulation and insulin secretion from islets [1, 25].

The hydrolysis of PtdInsP either by phospholipase C or phosphatases does not contribute significantly to the reduction in PtdInsP levels observed in homogenates of glucose-, tolbutamide-, or CCh-treated samples. When [^{32}P]ATP was quenched by the mass or volume method, PtdInsP hydrolysis in homogenates of treated islets was not higher than the rate of hydrolysis in control samples. Rather, the rate of hydrolysis of PtdInsP was slightly lower for treated islet samples compared to control. These observations are in agreement with results reported for the islet where phospholipase C is Ca^{2+} regulated [1], and the β cell tumor [6] where PtdInsP phosphatase activity required calcium for activation, and secretory granule membranes did not hydrolyze PtdInsP. However, other investigators have reported that muscarinic agonist addition to islet cell membranes induces the hydrolysis of PtdInsP and PtdIns4,5P by phospholipase C in the presence of a low concentration of calcium [4]. In the present studies, where receptor agonists were washed from the cells prior to homogenization and the calcium-chelating agent EGTA was present, phosphatase activity was minimized.

In conclusion, insulinotropic agents which stimulate the phosphoinositide pathway reduce the formation of PtdInsP in islets. Only tolbutamide among the agents tested affected PtdIns kinase as a noncompetitive inhibitor. For the other agents, the reduction in PtdIns kinase activity in homogenates appears to be regulated by the availability of PtdIns substrate. A specific pool of PtdIns may exist in islet membranes, the hydrolysis and synthesis of which are responsive to insulinotropic agents [26]. Moreover, glucose and tolbutamide stimulations induce a rapid fall in PtdInsP and PtdIns4,5P levels in islets, which is followed by a sustained increase in the synthesis of these phosphoinositides [1], suggesting that the synthesis of PtdInsP by PtdIns kinase may be rate limiting in the generation of polyphosphoinositides. The suppressed formation of PtdInsP and subsequent PtdIns4,5P synthesis may desensitize the phosphoinositide pathway, thus limiting inositol trisphosphate production, the elevation of intracellular Ca^{2+}, and insulin secretion. An endogenous inhibitor of PtdIns kinase generated during cell stimulation, such as adenine or adenosine compounds, may suppress PtdInsP formation. However, adenosine analogues typically inhibit PtdIns kinase in the presence or absence of exogenous PtdIns [27].

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