Effects of Phosphatidic Acid on Islet Cell Phosphoinositide Hydrolysis, Ca^{2+}, and Adenylate Cyclase

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Phosphatidic acid may be raised in glucose-stimulated islet cells through hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and de novo synthesis with glucose-derived trioses. The mechanism by which exogenous phosphatidic acid from egg yolk lecithin may augment insulin secretion was investigated in neonatal β-cells. In whole cells labeled with [2,8-^{3}H]-adenine, a dose-dependent increase in phosphatidic acid–stimulated adenylate cyclase activity was seen, and a small intracellular transient free-Ca^{2+} rise was seen in Fura 2AM–loaded cells. In [γ-^{32}P]ATP-labeled membranes from those β-cells, phosphatidic acid effected PIP_2 hydrolysis. These phosphatidic acid–stimulated effects were not sensitive to preincubation with Bordetella pertussis exotoxin. The findings are consistent with a stimulatory effect of exogenous phosphatidic acid on insulin release and indicate an effect at the plasma membrane. It is possible that newly synthesized phosphatidic acid may function similarly to amplify intracellular events in glucose-stimulated islet cells through both local Ca^{2+} concentration and cyclic AMP–sensitive mechanisms. The participation of newly synthesized phosphatidic acid derived from glucose could provide a link between the metabolism of glucose and insulin release. *Diabetes* 38:1187–92, 1989

In pancreatic islets, rapid metabolism of glucose is required to maintain glucose-induced insulin release (1). The complex intraislet events that accompany glucose metabolism do not indicate a direct link between a particular metabolic event and secretion of insulin. Glucose stimulates the metabolism of membrane phosphoinositides and phosphatidic acid in a Ca^{2+}-dependent manner (2–4), but unlike agonists acting directly on the cell membrane, its intraislet metabolism via glycolysis provides a route for de novo phosphatidic acid synthesis (5,6). We have shown that phosphatidic acid levels in developing islets correlate with the capacity for glucose-induced insulin release (7). Stimulation of Ca^{2+} influx accompanies exogenous phosphatidic acid stimulation of neuroblastoma (8) and hepatic (9) cells. This stimulus has been shown in human A-431 carcinoma cells to elicit an increase in intracellular Ca^{2+} subsequent to phosphoinositide hydrolysis and is presumed to follow phosphatidic acid entry into the cell and direct activation of phospholipase C (10). Additionally, in 3T3 fibroblasts, some natural but not synthetic phosphatidic acids may stimulate membrane receptors mediating adenylate cyclase inhibition in a pertussis toxin–sensitive manner (11). In this study, the effect of exogenous phosphatidic acid in augmenting intracellular Ca^{2+} and influencing cellular signaling pathways was investigated in cultured neonatal β-cells.

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RESEARCH DESIGN AND METHODS

Fura 2AM and bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate acetoxymethyl ester (BAPTA-AM) were obtained from Molecular Probes (Junction City, OR). Bordetella pertussis exotoxin was from List Biological (Campbell, CA) and [2,8-^{3}H]adenine, the D-*myo*-inositol 1,4,5-trisphosphate (^{3}H) assay system, and [γ-^{32}P]ATP were from Amersham (Amersham, UK). All other chemicals were from Sigma (St. Louis, MO).

Cultured neonatal islet cells were prepared from dispersions of 1-day-old rat pancreases under conditions previously described to give a β-cell–enriched population (12). We obtained >90% of cells with positive fluorescence after incubation with guinea pig anti-porcine insulin immunoglobulin followed by fluorescein isothiocyanate–conjugated rabbit anti-guinea pig globulin (13). Islet cells were cultured for 60 h in RPMI-1640 medium containing 10% (vol/vol) fetal bovine serum. When present, Bordetella pertussis exotoxin at a final concentration of 20 ng/ml was included for the last

24 h of culture. The sodium salt of phosphatidic acid (0.1–1000 μM) was dissolved in culture medium or Krebs-Ringer bicarbonate buffer (KRBB) after sonication or was added as homogeneous fine suspension when required at higher concentrations.

Islet cells were grown to confluence on cover glass and loaded for 40 min at 37°C with 5 μM Fura 2AM in KRBB at a constant extracellular Ca^{2+} concentration. Fluorescence (F) was assessed with a Spex cation-measuring system (CM2, Edison, NJ) at 37°C in 2 ml KRBB. Intracellular Ca^{2+} levels were quantitated after addition of phosphatidic acid to cells that had been preincubated in the presence or absence of EGTA or BAPTA-AM. Free-Ca^{2+} concentration was calculated with the formula $K_d[(F-F_{min})/(F_{max}-F)]$, with F_{max} and F_{min} obtained after cell solubilization, the application of 10 μM of the nonfluorescent ionophore ionomycin with 10 mM $CaCl_2$ and 1 mM $MnCl_2$, respectively, and K_d = 224 nM (14).

Intact-cell adenylate cyclase activity was assayed by measurement of [^{3}H]cyclic AMP (cAMP) production after prelabeling of cells with [2,8-^{3}H]adenine (2 μCi/ml) for 2 h (15). After prelabeling, the medium was replaced with medium containing 0.1% bovine serum albumin with or without 3-isobutyl-1-methylxanthine (IBMX) for 20 min. In some experiments, 10 μM BAPTA-AM was included during the 20-min incubation. Phosphatidic acid from egg yolk lecithin was added and the incubation continued for another 5 min at 37°C. [^{3}H]cAMP formed over this period was measured after separation by sequential Dowex and alumina chromatography (16).

Formation of inositol 1,4,5-trisphosphate in intact cell monolayers was determined after stimulation with phosphatidic acid in KRBB with the addition of 5 mM LiCl. After 30 s, cells were disrupted with 10% trichloracetic acid (TCA) (wt/vol) followed by sonication, and TCA was extracted twice with four volumes of diethyl ether, neutralized, and lyophilized. Reconstituted extracts were assayed for inositol 1,4,5-trisphosphate with a quantitative binding protein assay in which cross-reactivity of the binding protein is <10% for inositol 1,3,4,5-tetrakisphosphate and <1% for the other inositol phosphates.

Insulin in the medium was determined by radioimmunoassay and was measured over 5 min incubation in duplicate experiments in which IBMX was omitted (12).

Membranes were prepared from cultured cell and membrane phospholipids labeled by exposure to 0.2 mM [γ-^{32}P]-ATP at a free-Ca^{2+} concentration of 150 nM. Labeled membranes were treated with phosphatidic acid for 1–5 min, the incubations terminated, and the phosphoinositides extracted and determined by thin-layer chromatography as described previously (17). Total protein was determined with Coomassie brilliant blue dye (18).

RESULTS

Phosphatidic acid consistently induced a small, rapid increase in intracellular free Ca^{2+} in Fura 2AM–loaded cells. This was apparent at 0.1 μM phosphatidic acid (means ± SE 106 ± 5 to 120 ± 6 nM, NS) and was significant at 1 μM phosphatidic acid (106 ± 5 to 138 ± 9 nM, $P < .01$) in six determinations. Ca^{2+} concentration was maintained above basal for up to 4 min after phosphatidic acid stimulation and was unaffected by pretreatment of cells with pertussis toxin (Fig. 1*B*). A further concentration dependence of peak Ca^{2+} response to phosphatidic acid was not readily demonstrated, because increasing the concentration of phosphatidic acid to 100 or 1000 μM resulted in an apparent decrease of intracellular free Ca^{2+}, possibly due to Ca^{2+} binding (data not shown). The increase in intracellular Ca^{2+} seen after 1 μM phosphatidic acid was not significantly affected by removal of extracellular Ca^{2+} with 1 mM EGTA, in Ca^{2+}-free medium. However, after removal of extracellular Ca^{2+} and concurrent exposure to BAPTA-AM, the increase in intracellular Ca^{2+} seen after addition of phosphatidic acid was prevented, and only a progressive decline in Ca^{2+} concentration was seen (Fig. 1*D*). Stimulation of these cells over 30 s with 0.1 and 1 μM phosphatidic acid resulted in an increase in inositol 1,4,5-trisphosphate formation to 142 ± 4 and 227 ± 7% of basal, respectively ($P < .05$).

Over 5 min of stimulation, adenylate cyclase activity, expressed as a percentage of basal [^{3}H]cAMP production, increased significantly (half-maximal stimulation at 1 μM phosphatidic acid; Fig. 2). Inclusion of pertussis toxin for the final 24 h of culture raised basal adenylate cyclase significantly (167 ± 7% of control in absence of toxin, $P < .001$). However, stimulation by 1 μM phosphatidic acid was not affected by pretreatment with pertussis toxin but was significantly reduced in the presence of 50 μM polymyxin B and 50 μM calmidazolium (compound R24571) to 69 ± 4 and 46 ± 2%, respectively, of control stimulation at the 5th

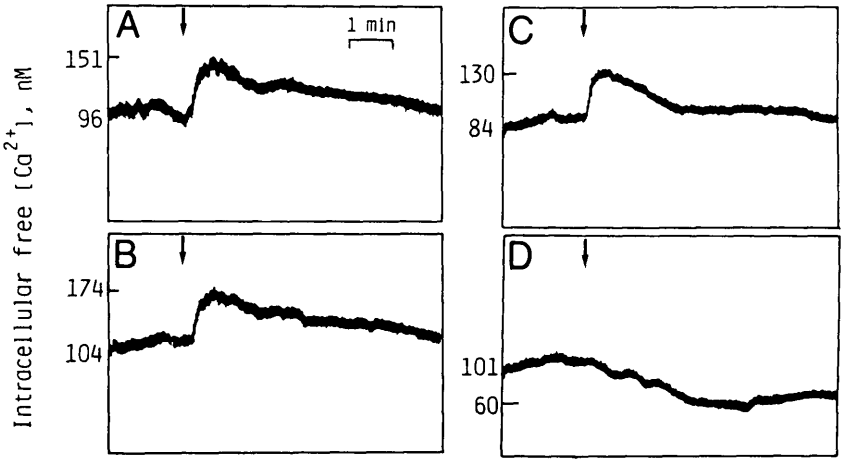

FIG. 1. Effect of phosphatidic acid on islet cell intracellular free Ca^{2+}. Phosphatidic acid (1 μM) was added (*arrows*) to curvettes containing Fura 2AM–loaded islet cells attached to cover glass after preincubation of representative cell monolayer in Krebs-Ringer bicarbonate buffer. *A*: control. *B*: preincubated with 20 ng/ml pertussis toxin. *C*: preincubated and stimulated in presence of 1 mM EGTA. *D*: bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate acetoxymethyl ester–loaded cells preincubated and stimulated in Ca^{2+}-free medium.

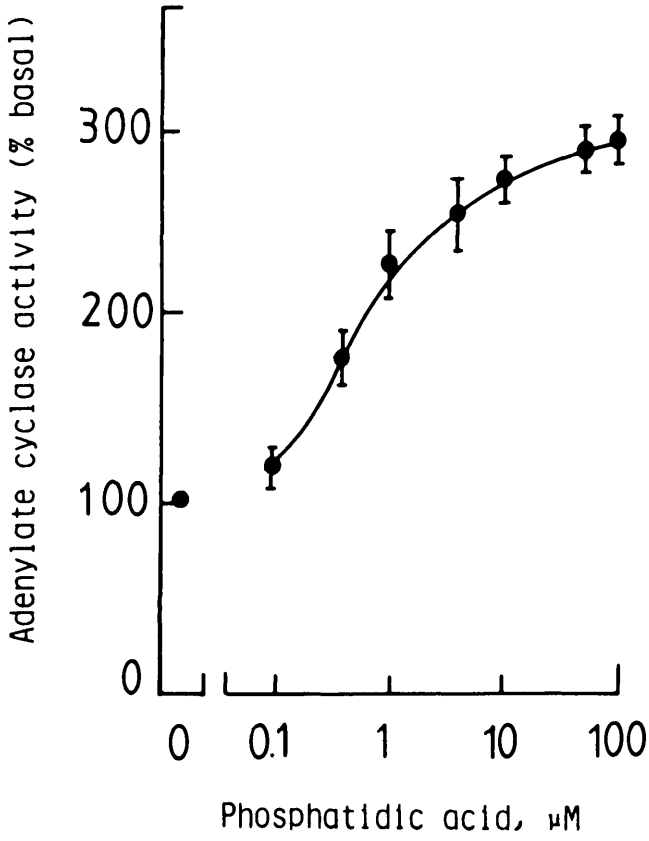

FIG. 2. Stimulatory effect of increasing concentrations of phosphatidic acid on islet cell adenylate cyclase activity. Adenylate cyclase activity is expressed relative to control islet cells under basal incubation conditions with 763 ± 13 dpm [^{3}H]cyclic AMP formed/100 μg islet cell protein at 100% activity.

min (Fig. 3; Table 1). No increase in adenylate cyclase activity was seen in control or pertussis toxin–treated cells that had been preincubated with BAPTA-AM. Over this period, a concentration-dependent stimulation of insulin release in response to phosphatidic acid was seen, with half-maximal stimulation occurring at 1 μM phosphatidic acid (Fig. 4). Pretreatment with pertussis toxin raised the basal level of insulin release by a nonsignificant amount, but a significant increase was seen after addition of 1 μM phosphatidic acid. Phosphatidic acid–stimulated insulin release was significantly reduced in the presence of polymyxin B and calmidazolium (Table 2). Stimulation of ^{32}P-labeled islet cell membranes with 0.1–1000 μM phosphatidic acid resulted in significant reduction of ^{32}P-labeled phosphatidylinositol 4,5-bisphosphate (PIP_2). The prompt hydrolysis occurring over the 1st min of stimulation at 1 μM phosphatidic acid was not affected by preexposure of the membrane preparation to activated pertussis toxin (Fig. 5) or pretreatment of cells with pertussis toxin before membrane preparation (not shown).

DISCUSSION

Until recently, phosphatidic acid had not been implicated in the mechanism of receptor-operated Ca^{2+} mobilization. This study indicates that such membrane stimulation is possible in a secretory cell and, together with observations in Rat-1 fibroblasts, mouse embryonal mesodermal cells, A-431 carcinoma cells (10), and 3T3 fibroblasts (11), indicates that this may be a widespread phosphatidic acid–promoted event. As with these other cells, in islets, transient intracellular free Ca^{2+} can be proposed to result from PIP_2 hydrolysis, with the formation of Ca^{2+} mobilizing inositol 1,4,5-trisphosphate. In this study, phosphatidic acid–stimulated formation of inositol 1,4,5-trisphosphate in intact cells is demonstrated together with the hydrolysis of PIP_2 in isolated membranes at a low free-Ca^{2+} concentration (150 nM). Additionally, it has been shown that in these isolated membrane preparations, in which inositol 1,4,5-trisphosphate–induced release of ^{45}Ca is present (19), a concentration-response effect for phosphatidic acid–induced PIP_2 hydrolysis and ^{45}Ca release can be demonstrated. For both responses, phosphatidic acid is not effective at <0.1 μM, with half-maximal stimulation of PIP_2 hydrolysis and ^{45}Ca release at 1 and 0.5 μM phosphatidic acid, respectively (20). In the intact islet cell, PIP_2 hydrolysis and Ca^{2+} release may precede the Ca^{2+} influx shown to accompany phosphatidic acid stimulation in many cell types. It is not possible to determine from these studies whether phosphatidic acid acts on a receptor at the cell surface or, by entering the cell, stimulates phospholipase C directly, an alternative suggested by Moolenaar et al. (10).

The lack of effect of pertussis toxin indicates that, as with other islet cell PIP_2 hydrolytic stimuli (17,21), any phos-

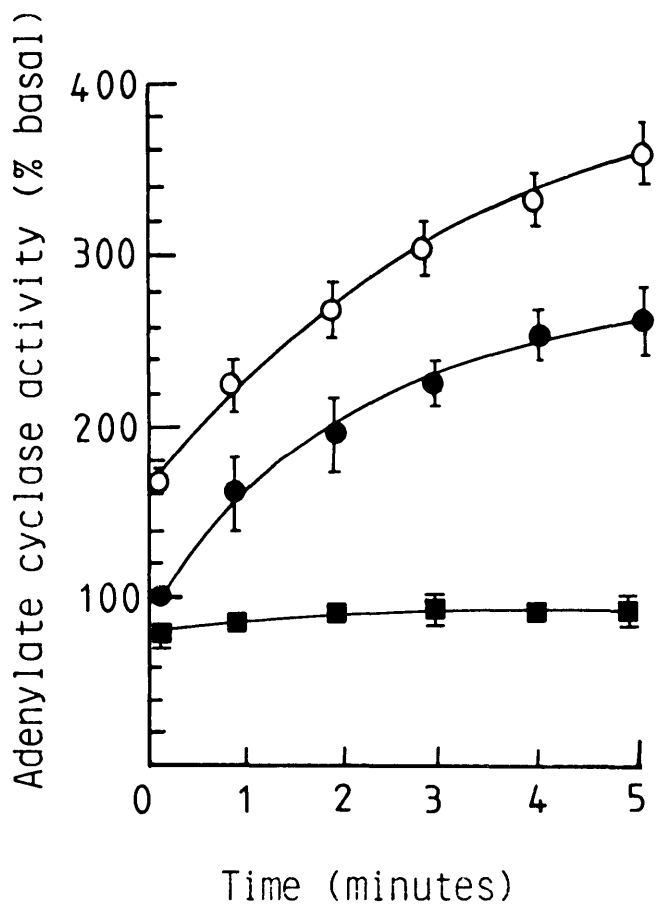

FIG. 3. Effect of phosphatidic acid on adenylate cyclase activity. Adenylate cyclase activity expressed relative to control islet cells under basal incubation conditions at 100% activity. Values shown for addition of 1 μM phosphatidic acid to control (●), pertussis toxin–pretreated (○), or bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate acetoxymethyl ester–preloaded (■) islet cells. Values are means ± SE for 6–8 determinations at each point.

TABLE 1
Effects of inhibitors on phosphatidic acid–stimulated adenylate cyclase activity

Addition	Activity (% of basal)
None	100
Phosphatidic acid (1 μM)	236 ± 11
Phosphatidic acid (1 μM) plus polymyxin B (50 μM)	165 ± 10*
Phosphatidic acid (1 μM) plus calmidazolium (50 μM)	109 ± 5†

Values are means ± SE for 6 triplicate determinations.
*$P < .005$, †$P < .001$, vs. phosphatidic acid treatment.

TABLE 2
Effect of inhibitors on insulin release (ng · 5 min^{-1} · 100 μg^{-1} protein) in response to phosphatidic acid

Addition	Control	Pertussis toxin pretreatment
None	0.41 ± 0.05	0.52 ± 0.04
Phosphatidic acid (1 μM)	0.92 ± 0.09*	1.61 ± 0.21*†
Phosphatidic acid (1 μM) plus polymyxin B (50 μM)	0.62 ± 0.09‡	1.06 ± 0.19†
Phosphatidic acid (1 μM) plus calmidazolium (50 μM)	0.52 ± 0.07§	0.87 ± 0.14†‡

Values are means ± SE for 6 triplicate determinations.
*$P < .001$ vs. absence of phosphatidic acid.
†$P < .05$ vs. control treatment.
‡$P < .05$, §$P < .01$, vs. phosphatidic acid treatment.

pholipase C modified subsequent to phosphatidic acid stimulation is not regulated by a pertussis toxin–sensitive mechanism. Whatever the mechanism, because de novo phosphatidic acid synthesis dependent on glucose metabolism has been demonstrated in islets (5,6), the role of phosphatidic acid may be a physiologically relevant link between glucose metabolism and insulin release.

The mechanisms by which increased insulin release is stimulated by phosphatidic acid may be complex. Glucose-induced insulin secretion is mediated by submicromolar increases in intracellular Ca^{2+} and enhanced by any elevation in cellular cAMP levels (22). Addition of exogenous phosphatidic acid stimulates adenylate cyclase in islet cells. This is in contrast to the findings of Murayama and Ui (11), in which phosphatidic acid decreased the cAMP level and inhibited membrane adenylate cyclase activity in a pertussis toxin–sensitive manner in 3T3 fibroblasts. This inhibition was

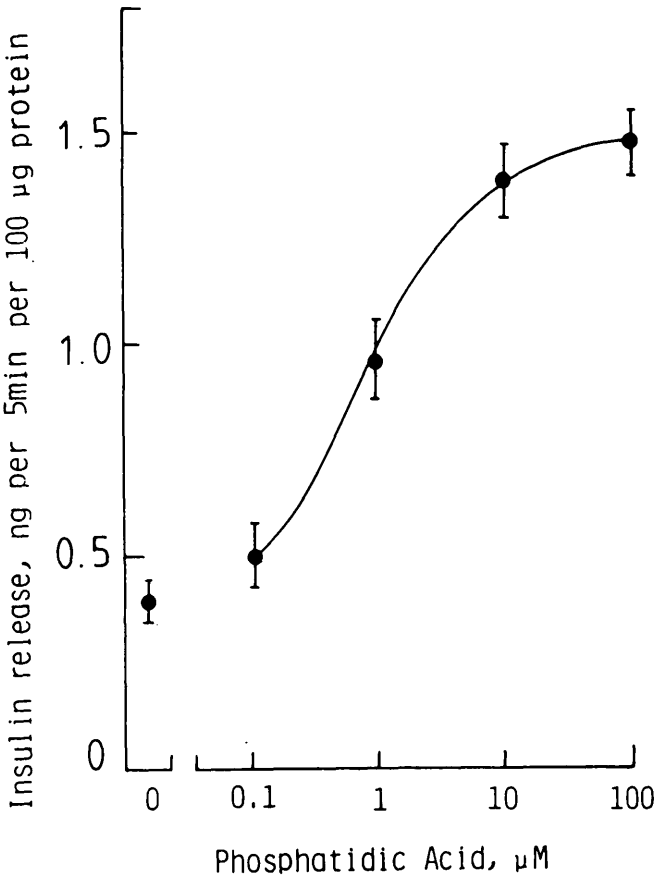

FIG. 4. Effect of phosphatidic acid on insulin release from islet cells. Dose-dependent increase in insulin release over 5-min stimulation with phosphatidic acid is shown as means ± SE of 6–8 determinations.

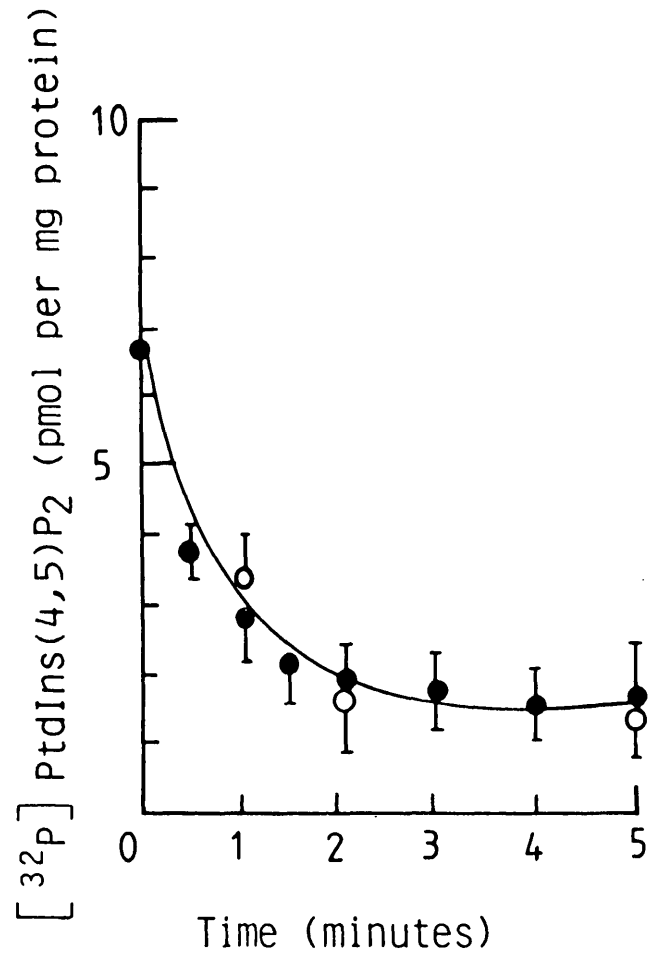

FIG. 5. Effect of phosphatidic acid on [γ-^{32}P]-labeled phosphoinositide (PtdIns(4,5)P_2) content of islet cell membranes. Time course of phosphoinositide loss from islet cell membranes prelabeled with [γ-^{32}P]ATP is shown for control (●) and pertussis toxin–pretreated (○) islet cell membranes. Values are means ± SE of 6–8 determinations at each point.

believed to follow coupling of the putative receptor for phosphatidic acid to the guanine nucleotide inhibitory regulatory (Gi) protein of adenylate cyclase. Although the α-subunit of the Gi protein is readily demonstrable in islet cells identical to those of our study, after pertussis toxin–induced ADP ribosylation (21) and its participation in regulating basal adenylate cyclase activity, indicated by increased basal activity in pertussis toxin–preincubated cells, coupling between the Gi protein and adenylate cyclase after phosphatidic acid is not effective; if receptor coupled, phosphatidic acid stimulation of adenylate cyclase in islet cells may be through the stimulatory regulatory (Gs) protein alone. Stimulation of protein kinase C is an alternative possible mechanism for the stimulation of adenylate cyclase. The demonstration of phosphatidate phosphohydrolase activity in islet cells enables the potential for metabolism of phosphatidic acid to diacylglycerol to be considered (23). Activation of protein kinase C could be responsible for phosphorylation and reduction in activity of the α-subunit of Gi (24), or alternatively, phosphorylation may alter interaction of the catalytic subunit of adenylate cyclase contributing to increased adenylate cyclase activity and cAMP levels (25).

Although stimulation by glucose of endogenous protein kinase C activity in neonatal islets has been demonstrated (26), numerous studies suggest that activation of protein kinase C may not be an essential step in initiating stimulus-secretion coupling. In mature islets, glucose-induced insulin release may still be elicited in conditions of protein kinase C depletion (27), and it has not yet been demonstrated that the diacylglycerol derived by de novo synthesis from glucose, predominantly a dipalmitoyl species (6), is responsible for the activation of protein kinase C within the islet. Alternatively, through elevation of cellular Ca^{2+}, Ca^{2+}-calmodulin activation of adenylate cyclase may occur (28,29). There is considerable evidence for the contribution of Ca^{2+}-activated calmodulin to insulin release (30). The dipalmitoyl species of phosphatidic acid has been shown to be capable of eliciting transient intracellular Ca^{2+} accompanying phosphoinositide hydrolysis. The removal of this transient Ca^{2+} by pretreatment of islet cells with BAPTA-AM prevents adenylate cyclase activation, strong evidence that exogenous phosphatidic acid may act on insulin release through intracellular Ca^{2+}-dependent mechanisms. Significant inhibition of phosphatidic acid–stimulated insulin release and adenylate cyclase activity is seen with an inhibitor of protein kinase C, polymyxin B, which has been shown to inhibit equally the effect of phorbol ester and glucose on insulin release from intact mature islets (31) but is seen also after calmidazolium treatment, inhibiting Ca^{2+}-calmodulin activation, suggesting that both transduction systems may be involved. This finding has implications for glucose-stimulated islet cells in which newly synthesized phosphatidic acid produced by the metabolism of glucose may initiate or amplify phosphoinositide hydrolysis and may serve as an intracellular messenger in the intact islet.

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