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Enhanced Phosphoinositide Hydrolysis via Overexpression of Phospholipase C β1 or δ1 Inhibits Stimulus-Induced Insulin Release in Insulinoma MIN6 Cells

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To study the effects of enhanced phosphoinositide hydrolysis on insulin secretion, phosphoinositide-specific phospholipase Cβ1 (PLCβ1) or PLCδ1 was overexpressed in insulinoma MIN6 cells via adenoviral vectors. Inositol phosphate production stimulated by NaF (with AlCl3) in PLCβ1-overexpressing cells and that stimulated by KCl or glucose in both PLCβ1- and PLCδ1-overexpressing cells were greater than that in control cells. In addition, reduced phosphatidylinositol-4,5-bisphosphate levels were observed in these cells stimulated by NaF or KCl. The greater phosphoinositide hydrolysis was accompanied by 25–45% inhibition of insulin secretion. These data suggest that excessive phosphoinositide hydrolysis inhibits secretagogue-induced insulin release in MIN6 cells. © 1999 Academic Press

Key Words: **PLC; exocytosis; phosphoinositide hydrolysis; adenoviral vector; phosphatidylinositol-4,5-bisphosphate; MIN6 cell.**

The release of peptide hormones and neurotransmitters from neuronal and endocrine cells is mediated by the fusion of secretory vesicles with the plasma membrane. There has been considerable progress in identifying the protein-protein interactions involved in this exocytotic event. Both membrane and cytosolic proteins, such as synaptotagmin, synaptobrevin, and *N*-ethylmaleimide sensitive factor, have been identified as components of such interactions (1). Additional components required for regulated neuroendocrine secretion have been identified by the reconstitution of Ca^{2+}-dependent secretion in permeabilized cells. These proteins include annexin II (2), protein kinase A (3), and phosphatidylinositol-4-phosphate 5-kinases (PIP5K) (4). An important role of PIP5K-mediated phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) synthesis in regulated secretion has recently been suggested in a growing number of reports (4–7). One of the earliest evidences indicating an important role for PtdIns(4,5)P2 in regulated secretion was observation that enhanced phosphoinositide hydrolysis by exogenous phosphoinositide-specific phospholipace C (PLC) caused parallel reduction in cellular PtdIns(4,5)P2 levels and catecholamine secretion in permeabilized cells (4, 6).

Insulin secretion from the pancreatic β-cell is one of the well studied examples of regulated secretion. In intact β-cells, enhanced phosphoinositide hydrolysis was observed in response to various secretagogues, including glucose and muscarinic agonists (8, 9). This raised the possibility that enhanced phosphoinositide hydrolysis by PLC plays an important role in the potentiation of insulin secretion (8–10). This proposed role of PLC appears to contradict the observation of reduced catecholamine secretion in permeabilized chromaffin or PC12 cells to which PLC was introduced (4, 6). Phosphoinositide hydrolysis generates at least two important second messengers, inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces Ca^{2+} release from intracellular pools, raising the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), and DAG causes activation of protein kinase C (11). In permeabilized cells, however, the $[Ca^{2+}]i$ was clamped to the extracellular Ca^{2+} concentration. Addition of PLC to the permeabilized preparation affected DAG and PtdIns(4,5)P2 contents but not the $[Ca^{2+}]i$. Therefore, previous re-

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Abbreviations used: PtdIns(4,5)P2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PIP5K, phosphoatidylinositol-4-phosphate 5-kinase; InsPs, inositol phosphates; IP3, inositol-1,4,5-trisphosphate; DAG, diacylglycerol; $[Ca^{2+}]i$, intracellular free calcium concentration; CCh, carbamoylcholine.

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sults using permeabilized cells can not be directly used to infer *in vivo* roles of phosphoinositide and its hydrolysis in regulated secretion.

To study the role of phosphoinositide signaling in intact cells, putative PLC inhibitors, U73312 and neomycin, have been used (12–14). However, the specificity of these reagents has recently been questioned and their effects on regulated secretion are controversial (12–15). Therefore, in the present study, taking another approach to assess the role of phosphoinositide and its hydrolysis in regulated insulin secretion from intact cells, we have examined effects of PLCβ1 or PLCδ1 overexpression in insulin-secreting MIN6 cells.

MATERIALS AND METHODS

Generation of recombinant adenoviruses and culture of MIN6 cells. Bovine PLCβ1 cDNA (16) was a generous gift from Dr. Matilda Katan (CRC Center for Cell and Molecular Biology, UK). Human PLCδ1 cDNA was cloned by polymerase chain reactions using human brain Quick Clone (Clontech). Recombinant adenoviruses bearing PLCβ1 (Adex1CAbPLCβ1) and PLCδ1 (Adex1CAhPLCδ1) were generated as described previously (17). As a control, a recombinant adenovirus bearing the bacterial β-galactosidase gene (Adex1CalacZ) was used (18). MIN6 cells were cultured and infected as published (19). Recombinant adenoviruses were used at a multiplicity of infection of 30–50, which induces expression of foreign genes in nearly 100% of the cells (19). Insulin secretion was determined using static incubation methods in Hepes-balanced Krebs–Ringer bicarbonate buffer.

Western blots and assay for PLC activity. Western blots were performed as described previously (20) using monoclonal antibody to PLCβ1 or PLCδ1 (UBI, lake Placid, NY) and ECL reagents (Amersham, UK). The PLC activity *in vitro* was measured using PtdIns(4,5)P2/phosphatidylethanolamine micelles as the substrate as described by Leonis and Silbert (21).

Assay of [^{3}H]inositol phosphates production and [^{3}H]phosphoinositide. Infected MIN6 cells in 6-well plates were prelabeled with 5 μCi/ml [2-^{3}H]inositol during a 36-h incubation and stimulated with insulin secretagogues in the presence of 20 mM LiCl for 30 min. At the end of incubation, 1 ml of cold methanol:concentrated HCl (100:1) was added. The cells were scraped off and mixed with 2 ml of chloroform and centrifuged. The aqueous upper phase was neutralized and used for inositol phosphate measurements. Total inositol phosphates were separated using Amprep SAX columns (Amersham, UK) as described (22). For measurements of phosphoinositide mass, the lower phase was separated by thin layer chromatography as described previously (20) and detected fluorographically after being sprayed with ENHANCE (Du Pont-New England Nuclear). In several experiments, [^{32}P]-labeled phosphatidylinositol monophosphate and PtdIns(4,5)P2 were added to the sample to determine the position of these lipids. Spots on chromatography were scraped off and counted for [^{3}H] activity.

Statistical analysis. Results are presented as means ± SEM. The statistical significance of differences between mean values was assessed using Student's *t* test.

RESULTS

Expression of PLCβ1 and PLCδ1 in MIN6 cells via recombinant adenoviruses. Bovine PLCβ1 and human PLCδ1 cDNAs were introduced into MIN6 cells via recombinant adenovirus vectors. As shown in Fig. 1, Western blot analysis revealed 150 kDa PLCβ1 and 85 kDa PLCδ1 proteins to have abundantly expressed in MIN6 cells infected with recombinant adenoviruses Adex1CAbPLCβ1 (PLCβ1-MIN6 cells) and Adex1CAh PLCδ1 (PLCδ1-MIN6 cells), respectively. Endogenous PLCβ1 or PLCδ1 was detectable in control MIN6 cells as a 150/140 kDa doublet form or as an 85-kDa band, respectively, when immunoprecipitated MIN6 cell lysates were used for western blotting (data not shown). When PLC activity was measured *in vitro* using PtdIns(4,5)P2 as the substrate, the homogenate from PLCβ1- or PLCδ1-MIN6 cells exhibited a 15- or 13-fold increase in activity, respectively, compared to the homogenate from control MIN6 cells (lacZ-MIN6 cells) (Fig. 1).

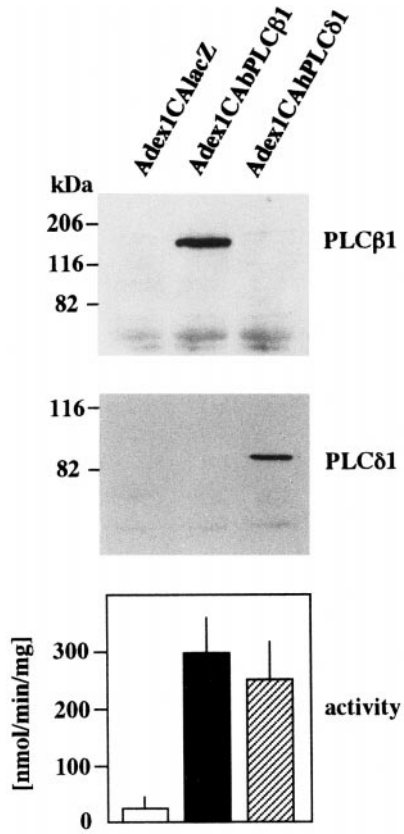

FIG. 1. Expression of PLCβ1 and PLCδ1 in MIN6 cells via recombinant adenoviruses. Lysates (10 μg/lane) of MIN6 cells infected with Adex1CAlacZ, Adex1CAbPLCβ1, or Adex1CAhPLCδ1 were subjected to SDS–polyacrylamide gel electrophoresis (7.5%) and then probed with monoclonal anti-PLCβ1 antibody (upper), or anti-PLCδ1 antibody (middle). Homogenates of infected MIN6 cells were assayed for PLC activity using PtdIns(4,5)P2/phosphatidylethanolamine micelles as the substrate (lower). Assays were terminated after 5 min incubation by the addition of chloroform:methanol:concentrated HCl (100:100:0.6). Following centrifugation at 2000*g* for 3 min, an aliquot of the resulting aqueous phase was counted. Data are means ± SEM of three independent experiments.

Phosphoinositide hydrolysis in PLCβ1- and PLCδ1-MIN6 cells. To examine the effects of PLCβ1 or PLCδ1 overexpression on phosphoinositide hydrolysis,

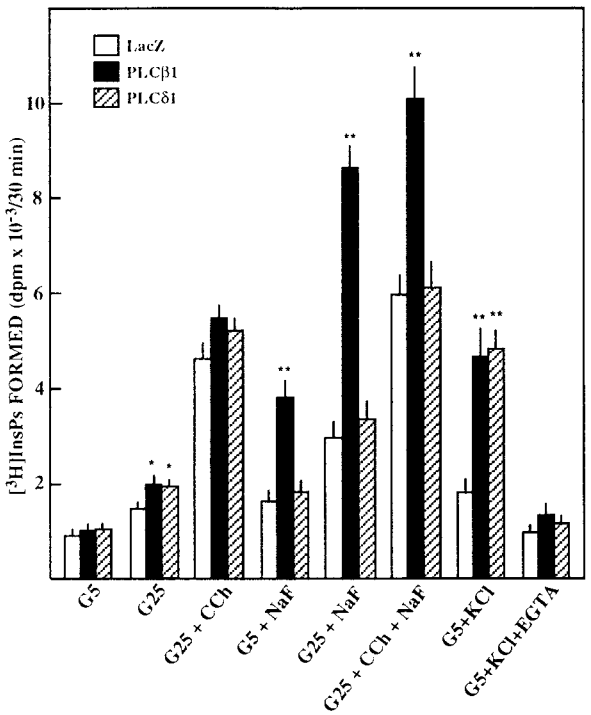

FIG. 2. Effects of PLCβ1 or PLCδ1 overexpression on secretagogues-stimulated total inositol phosphates production in MIN6 cells. [^{3}H]Inositol-labeled lacZ-, PLCβ1-, or PLCδ1-MIN6 cells were stimulated for 30 min at 37°C with 5 mM or 25 mM glucose (G5 or G25), 0.5 mM CCh in the presence of 25 mM glucose, 10 mM NaF plus 10 μM AlCl3 in the presence of 5 or 25 mM glucose, 0.5 mM CCh plus 10 mM NaF plus 10 μM AlCl3 in the presence of 25 mM glucose, or 40 mM KCl in the presence of 5 mM glucose with or without 5 mM EGTA. InsPs production was measured as described under Materials and Methods. Values are means ± SEM of four to seven independent experiments. *Differences from control cells stimulated with the same stimuli at $p < 0.05$ and **differences at $p < 0.01$.

the production of inositol phosphates (InsPs) was examined in PLCβ1- or PLCδ1-MIN6 cells stimulated with several insulin secretagogues (Fig. 2). Basal InsPs production at 5 mM glucose was not significantly different among PLCβ1-, PLCδ1- and lacZ-MIN6 cells. As was the case with murine islets (10), control MIN6 cells respond to 25 mM glucose stimulation with only minimal increments in InsPs production (approximately 1.5-fold). Both PLCβ1- and PLCδ1-MIN6 cells exhibited an approximately 2-fold increase in InsPs production in response to 25 mM glucose, which was significantly greater than that in lacZ-MIN6 cells (Fig. 2).

Because PLCβ1 is known to be coupled to heterotrimeric G proteins activated by muscarinic acetylcholine receptors in several tissues (23), carbamoylcholine (CCh)-stimulated InsPs production was then studied in these cells. InsPs production was increased approximately 3-fold by 25 mM glucose plus 0.5 mM CCh compared to 25 mM glucose alone in control cells. Although InsPs production by CCh was found to be slightly greater in PLCβ1-MIN6 cells than in lacZ-MIN6 cells, the difference was not significant (Fig. 2). We also tested 0.1 and 1.0 mM CCh and found no significant differences in InsPs production among three cell preparations (data not sown). These data suggest that PLCβ1 is not coupled to muscarinic acetylcholine receptors in MIN6 cells. Alternatively, PLCβ1 activity may not be rate-limiting for the muscarinic receptor-PLCβ1 system and that overexpression of muscarinic receptors is needed to increase the CCh response in MIN6 cells. We also failed to find differences in InsPs production in response to cholecystokinin octapeptide among these cells (data not shown). Therefore, in order to activate heterotrimeric G proteins directly in intact cells, NaF was used with AlCl3 (Fig. 2). When stimulated with 10 mM NaF (with 10 μM AlCl3) in the presence of 5 or 25 mM glucose, marked enhancement in InsPs production, approximately 4-fold, was observed in PLCβ1-MIN6 cells, with only 1.5- to 2-fold increase in PLCδ1- or lacZ-MIN6 cells.

The effects on phosphoinositide hydrolysis of increasing the $[Ca^{2+}]i$ were also studied by stimulating cells with 40 mM KCl. A high K^+ stimulation leads to plasma membrane depolarization, opening of voltage-dependent calcium channels and thereby to an increase in the $[Ca^{2+}]i$. As shown in Fig. 2, 40 mM KCl in the presence of 5 mM glucose caused an approximately 2.5-fold higher InsPs production in both PLCβ1- and PLCδ1-MIN6 cells than in lacZ-MIN6 cells. These increases in InsPs production were almost completely abolished by the addition of 5 mM EGTA, suggesting an essential role for Ca^{2+} influx for both PLCβ1- and PLCδ1-mediated phosphoinositide hydrolysis. An essential role of Ca^{2+} has previously been reported for PLCβ1 (24) and PLCδ1 (25).

Effect on PtdIns(4,5)P2 levels of PLCβ1 or PLCδ1 overexpression. Effects of glucose, CCh, NaF, and KCl on phosphoinositides levels were then examined in PLCβ1- and PLCδ1-MIN6 cells. There were no significant differences in phosphatidylinositol or phosphatidylinositol-4-phosphate levels among lacZ-, PLCβ1-, or PLCδ1-MIN6 cells stimulated with these secretagogues (data not shown). We also failed to detect differences in basal PtdIns(4,5)P2 levels and PtdIns(4,5)P2 levels in response to 25 mM glucose or 25 mM glucose plus 0.5 mM CCh in these three cell preparations (Fig. 3). NaF caused a reduction in PtdIns(4,5)P2 levels in all three preparations and it was most pronounced in PLCβ1-MIN6 cells compared to those in lacZ- or PLCδ1-MIN6 cells. In addition, PtdIns(4,5)P2 levels at 40 mM KCl were found to be lower by approximately 30% in both PLCβ1- and PLCδ1-MIN6 cells than in lacZ-MIN6 cells (Fig. 3).

Effect on insulin secretion of PLCβ1 or PLCδ1 overexpression. Having established the functional expression of PLCβ1 and PLCδ1, the effects of enhanced

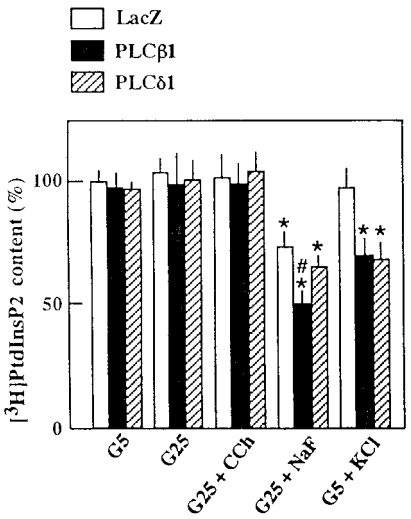

FIG. 3. PtdIns(4,5)P2 levels in PLCβ1- or PLCδ1-MIN6 cells. Cells were stimulated as described in legend for Fig. 2. PtdIns(4,5)P2 levels were expressed as percentage of that in control MIN6 cells exposed to 5 mM glucose. Values are means ± SEM of four to six independent experiments. *Differences from lacZ-MIN6 cells stimulated with 25 mM glucose at $p < 0.05$. #Difference from lacZ-MIN6 cells stimulated with 25 mM glucose plus 10 mM NaF at $p < 0.05$.

phosphoinositide hydrolysis on insulin secretion were examined (Fig. 4). Although the basal insulin secretion at 5 mM glucose did not differ among PLCβ1-, PLCδ1- and lacZ-MIN6 cells, stimulation of insulin secretion in response to 25 mM glucose was smaller in PLCβ1- and PLCδ1-MIN6 cells (approximately 2.5-fold in both cases) than in lacZ-MIN6 cells (4-fold). NaF evoked insulin secretion in the presence of 5 or 25 mM glucose in all three cell preparations (Fig. 4). However, the magnitude of the stimulation of insulin secretion by NaF was the smallest in PLCβ1-MIN6 cells, which exhibited the greatest InsPs production to the stimulus among the three cell preparations (Fig. 2). In addition, with a combined stimulus of CCh and NaF, insulin secretion was greater than that evoked by either CCh or NaF alone in PLCδ1- and lacZ-MIN6 cells, whereas the combined stimulus failed to further increase insulin secretion in PLCβ1-MIN6 cells. It was also found that 40 mM KCl-induced insulin secretion was inhibited by approximately 35% by overexpression of either PLCβ1 or PLCδ1 (Fig. 4).

The reduced insulin secretion observed in PLCβ1- and PLCδ1-MIN6 cells in this study was considered unlikely to be due to non-specific or cytotoxic effects of PLCβ1 or PLCδ1 overexpression, because CCh-stimulated insulin secretion (Fig. 4) and the cellular insulin content (data not shown) did not differ among lacZ-, PLCβ1- and PLCδ1-MIN6 cells.

DISCUSSION

Here we reported that overexpression of PLCβ1 or PLCδ1 resulted in inhibition of insulin secretion induced by several secretagogues in MIN6 cells. As was expected, PLCs overexpression caused increased production of inositol phosphates. DAG, which is generated stoichiometrically with InsPs, was presumably increased in PLCβ1- or δ1-overexpressing MIN6 cells, although it was not demonstrated directly in this study. Decreased levels of PtdIns(4,5)P2 was also found in these cells in response to either NaF or KCl. It is possible that all or some of these factors mediated inhibitory effects of PLCs overexpression on stimulus-induced insulin release.

Among several inositol phosphate species, IP_3 is established to play an important role in cellular functions. IP_3 is known to elevate the $[Ca^{2+}]i$. Although we did not examine the $[Ca^{2+}]i$ in PLCβ1- or PLCδ1-MIN6 cells, it should be noted that overexpression of PLCγ in NIH3T3 cells did not increase the $[Ca^{2+}]i$ despite a marked increase in IP_3 production (26). Because an acute increase in the $[Ca^{2+}]i$ has been established to induce insulin secretion (27), a negative effect of the IP_3 induced $[Ca^{2+}]i$ increase, if any, on insulin secretion was unlikely. However, negative effects of IP_3 independent from Ca^{2+} mobilizing action can not be excluded. Inositol-1,3,4,5- tetrakisphosphate (IP4),

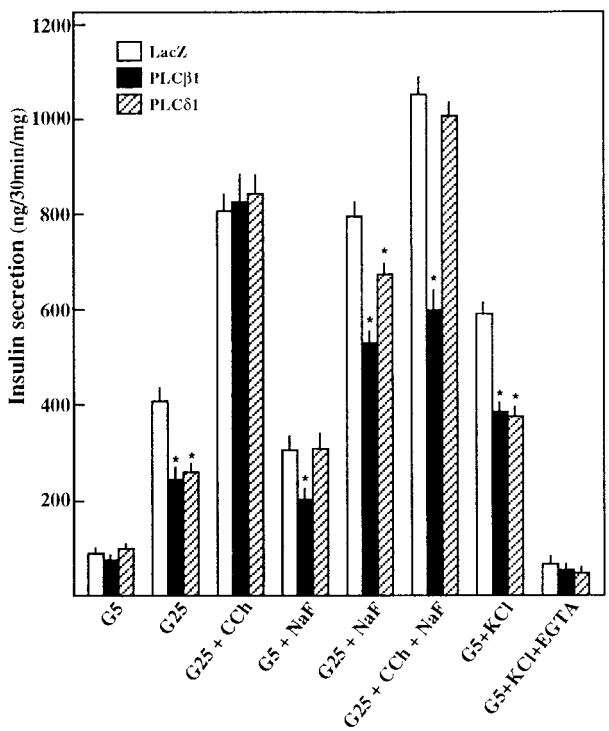

FIG. 4. Effects of PLCβ1 or PLCδ1 overexpression on insulin secretion. Control, PLCβ1- and PLCδ1-MIN6 cells were seeded onto 24-well multiwell plates at a density of 2×10^5 cells per well. Two days later, insulin secretion was measured after 30-min incubations as published previously (19) in the presence of various agents described in the legend to Fig. 2. Values are means ± SEM of 3 to 10 independent experiments. *Differences from control cells stimulated with the same stimuli at $p < 0.05$.

should also be considered because it has reportedly been increased after PLC activation in insulinoma cells (28). IP4 has been shown to inhibit neuroexocytosis when injected into the squid giant synapse, possibly via binding to C2B domain of synaptotagmin isoforms (29). Further studies are needed to elucidate roles of increased inositol phosphates in insulin secreting cells.

On the other hand, the possible increase in DAG levels might cause greater activation of protein kinase C isoforms in PLCβ1- or PLCδ1-MIN6 cells. Role of PKC isoforms in regulated insulin secretion, however, was not clearly understood (30, 31). In addition, DAG also regulates several proteins, such as UNC-13 (32) and RasGRP (33), which might affect regulated secretion. We also found increased production of phosphatidic acid in PLCβ1- or PLCδ1-MIN6 cells, which was possibly generated by phosphorylation of DAG by DAG kinases (data not shown). Phosphatidic acid is known as a potent modulator of cellular functions. Therefore, increased DAG levels may affect diverse cellular processes, and thus much more efforts are needed to conclude effects of enhanced DAG levels on insulin secretion.

Decreased levels of PtdIns(4,5)P2 were observed with inhibition of insulin secretion in PLCβ1- or PLCδ1- MIN6 cells stimulated with NaF or KCl. This observation was in accordance with the current notion that PtdIns(4,5)P2 itself may play an important role in regulated secretion (7). However, regarding glucose-stimulated insulin secretion, its inhibition by overexpression of PLCβ1 or PLCδ1 appeared to be independent of changes in PtdIns(4,5)P2 levels; no apparent differences in PtdIns(4,5)P2 levels were observed among three preparations at 25 mM glucose. However, our failure of detecting the difference might be due to small phosphoinositide pools involved in glucose-stimulated insulin secretion, as suggested by small InsPs production (Fig. 2). It is thus still possible that the partial inhibition of insulin secretion by PLCs overexpression at 25 mM glucose resulted from reduction in this small PtdIns(4,5)P2 pools, which could not detected by the total cellular phosphoinositide measurement. In this context, PtdIns(4,5)P2 synthesis appears to be important, not only for supplying ample amount for generation of second messengers, IP3 and DAG, but also for the functions of this lipid molecule itself. Glucose was reported, more than 10 years ago, to stimulate phosphoinositide synthesis in pancreatic β-cells (34). CCh and other PLC activating agonists have also been demonstrated to stimulate PtdIns(4,5)P2 synthesis (35–37). Ability of these agents as insulin secretagogues might be ascribed to their action on synthesis as well as to that on hydrolysis of PtdIns(4,5)P2. They may activate one or several isoforms of recently cloned PIP5Ks (20, 38).

Although excessive PtdIns(4,5)P2 hydrolysis by overexpressed PLCs appears to inhibit insulin secretion, one should be cautious to interpret the current data regarding to roles played by PLC in insulin secretion. An inappropriately large increase or unbalanced increase in one enzyme has sometimes caused negative effects on insulin secretion (39, 40). In addition, because our measurement of insulin secretion was made under conditions of static incubation, it is possible that important roles of phosphoinositide hydrolysis were missed or masked in some respects. In this regard, it should be noted that murine islets expressed considerably reduced levels of PLCβ1 and PLCδ1 compared to rat islets. This has been considered to be one of major causes of reduced second phase insulin secretion in response to glucose from the murine islet (10). However, inhibition of glucose-stimulated insulin secretion by overexpression of PLCβ1 or PLCδ1 in MIN6 cells suggest that underexpression of these isoforms is not the primary cause of the defect in murine islets.

In summary, the present data suggest that excessive phosphoinositide hydrolysis inhibits insulin secretion. Manipulation of phosphoinositide signaling in intact insulin secreting cells would provide important means of analyzing mechanism by which phosphoinositide and its metabolism function in insulin secretion.

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