# Phosphatidylinositol 3-Kinase Suppresses Glucose-Stimulated Insulin Secretion by Affecting Post-Cytosolic [$Ca^{2+}$] Elevation Signals

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**The role of phosphatidylinositol (PI) 3-kinase in the regulation of pancreatic β-cell function was investigated. PI 3-kinase activity in p85α regulatory subunit–deficient (*p85α*$^{-/-}$) islets was decreased to ~20% of that in wild-type controls. Insulin content and mass of rough endoplasmic reticula were decreased in β-cells from *p85α*$^{-/-}$ mice with increased insulin sensitivity. However, *p85α*$^{-/-}$ β-cells exhibited a marked increase in the insulin secretory response to higher concentrations of glucose. When PI 3-kinase in wild-type islets was suppressed by wortmannin or LY294002, the secretion was also substantially potentiated. Wortmannin's potentiating effect was not due to augmentation in glucose metabolism or cytosolic [$Ca^{2+}$] elevation. Results of *p85α*$^{-/-}$ islets and wortmannin-treated wild-type islets stimulated with diazoxide and KCl showed that inhibition of PI 3-kinase activity exerted its effect on secretion, at least in part, distal to a cytosolic [$Ca^{2+}$] elevation. These results suggest that PI 3-kinase activity normally plays a crucial role in the suppression of glucose-stimulated insulin secretion. *Diabetes* 51: 87–97, 2002**

Defects in insulin secretion from pancreatic β-cells and insulin resistance in the target tissues are two major elements that cause type 2 diabetes (1,2). However, diabetes develops only when adequate secretion of the hormone, in proportion to insulin resistance, fails to occur (3–6). This suggests that preservation of glucose-stimulated insulin secretion is crucial for maintaining glucose homeostasis in the presence of insulin resistance. To trigger insulin secretion, glucose metabolism and subsequent mitochondrial ATP generation are required (7,8). An increase in the cellular ATP-to-ADP ratio closes ATP-sensitive $K^+$ ($K_{ATP}$) channels, which depolarizes the plasma membrane and evokes $Ca^{2+}$ influx into the cytosol. This finally induces exocytosis of insulin from secretory vesicles (9,10). Moreover, increased glucose metabolism augments insulin secretion under conditions in which cytosolic [$Ca^{2+}$] is fixed at higher levels, where glucose metabolism amplifies efficacy of $Ca^{2+}$ on exocytosis of insulin granules (11–13). The mediators of this amplifying pathway may include ATP (14,15), guanosine triphosphate (16), long-chain acyl-CoAs (17), and glutamate (18).

Phosphatidylinositol (PI) 3-kinase is a key element in transducing various actions of insulin on downstream effectors (19,20) and has been implicated in controlling vesicle trafficking and secretory function in many cell types (21–27). Pancreatic β-ells also contain PI 3-kinase activity (28–30). Its activity is important for stimulating the transcription of the preproinsulin gene and liver-type pyruvate kinase gene (31,32). Insulin is hypothesized to enhance its own gene transcription in an autocrine manner through insulin receptor and PI 3-kinase pathway. However, the role of this enzyme in β-cell secretory function remains largely unknown. Involvement of PI 3-kinase in glucose-stimulated insulin secretion has been studied by some investigators using the pharmacological PI 3-kinase inhibitor wortmannin. Thus, 100 nmol/l wortmannin had no effect on insulin secretion induced by basal concentration of glucose in HIT-T15 cells (33), and 50–100 nmol/l wortmannin potentiated glucose-stimulated insulin secretion at higher glucose ranges in MIN6 cells (29); the same concentrations of wortmannin seemed to show no effect on secretion induced by glucose plus carbamyl choline in rat islets or β-TC3 cells (30). Very recently, two articles reported wortmannin's potentiating effect on glucose-stimulated insulin secretion at 8–16.7 mmol/l glucose in rat islets (34,35). However, the mechanism of the potentiation has been elusive and a few experiments only suggested that inositol phosphate accumulation or protein kinase C activity in islets was not correlated with the phenomenon (35).

To investigate the role of PI 3-kinase in β-cell function, we attempted to analyze islets from class I PI 3-kinase p85α regulatory subunit–deficient (*p85α*$^{-/-}$) mice, a β-cell model with genetically decreased PI 3-kinase activity. The mice showed increased systemic insulin sensitivity attributable to upregulation of alternative PI 3-kinase regulatory subunits (36). PI 3-kinase activity in *p85α*$^{-/-}$ islets was

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ER, endoplasmic reticulum; IRS-1, insulin receptor substrate-1; $K_{ATP}$, ATP-sensitive $K^+$ channel; KRB, Krebs-Ringer bicarbonate; MAP, mitogen-activated protein; PDE, phosphodiesterase; PI, phosphatidylinositol.

decreased to ~20% of that in wild-type controls. We observed insulin content or rough endoplasmic reticulum (ER) mass to be decreased in $p85\alpha^{-/-}$ β-cells. Nevertheless, glucose-stimulated insulin release from β-cells was significantly increased at higher glucose concentrations. Wortmannin and another structurally distinct PI 3-kinase inhibitor, LY294002, also exhibited marked potentiation of glucose-stimulated insulin secretion at higher glucose concentrations, as was consistent with previous reports (29,34,35). Our data indicated that inhibition of PI 3-kinase activity did not interfere with the secretion-triggering pathway, including the increase in glucose oxidation, ATP content, or cytosolic [$Ca^{2+}$] in response to glucose, but it did exert its potentiating effect distal to cytosolic [$Ca^{2+}$] elevation, presumably through pathways that amplified efficacy of $Ca^{2+}$ in the presence of glucose metabolism. These results suggest a crucial role of PI 3-kinase in the suppression of β-cell secretory function in a normal state.

## RESEARCH DESIGN AND METHODS

**Materials.** Insulin radioimmunoassay kit, enhanced chemiluminescence protein detection kit, and D-[U-$^{14}$C]glucose were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden); anti-p85α$^{PAN}$ was from Upstate Biotechnology (Lake Placid, NY); collagenase, wortmannin, actinomycin D, LY294002, rapamycin, PD98059, diazoxide, and ATP bioluminescent assay kit were from Sigma (St. Louis, MO); and nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany).

**Animals.** $p85\alpha^{-/-}$ mice were maintained by a crossing of $p85\alpha^{+/-}$ mice on the original C57BL6/CBA hybrid background (36). The mice were housed on 12-h light/dark cycle with standard diet and water available ad libitum.

**Preparation of islets.** Islets were isolated by collagenase digestion and manual picking from the pancreata of $p85\alpha^{-/-}$ male mice and their wild-type male littermate mice. These islets were used for experiments immediately after isolation. The number of islet cells was microscopically counted after dispersing 10 islets per tube into single cells with trypsinization and pipetting through microtips.

**Electron microscopic examination of β-cells.** For electron microscopic examination (37), islets were immersed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4). They were then subjected to double-fixation with 2.5% glutaraldehyde and 1% osmic acid, embedded in Epon 812, and cut into ultrathin sections. The sections were doubly stained with uranyl acetate and lead acetate and examined under a Hitachi HU-11B electron microscope. The number of insulin-containing dense core granules was counted in a 12.5-μm$^2$ rectangular area, except for the nuclear region, using ×4,000 photographs. The counting was performed on 150 β-cells from at least three mice in each group. For measurement of rough ER mass, their areas were traced and calculated on 150 β-cells using ×4,000 photographs.

**Insulin content.** For insulin content assay (38), islet homogenates were solubilized in 74% ethanol and 1.4% HCl solution at 4°C overnight, and insulin liberated into the solution was measured by radioimmunoassay.

**Immunoblotting and immunoprecipitation.** Islets were homogenized in ice-cold buffer A (25 mmol/l Tris-HCl [pH 7.4], 10 mmol/l sodium orthovanadate, 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l EGTA, and 1 mmol/l phenylmethylsulfonyl fluoride). For immunoblotting, the samples were boiled in Laemmli's sample buffer for 5 min, separated on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes, followed by detection with an ECL kit using anti-p85α$^{PAN}$ antibody (36). For immunoprecipitation, islet homogenates were incubated in buffer A with anti-p85α$^{PAN}$ antibody and protein G agarose for 2 h at 4°C. After the immunoprecipitates were washed three times with buffer A and three times with PI-3 kinase buffer (25 mmol/l Tris-HCl [pH 7.4], 100 mmol/l sodium chloride, 0.5 mmol/l EGTA), they were immunoblotted with anti-p85α mouse monoclonal antibody (Upstate Biotechnology).

**PI 3-kinase assay.** After the islets had been incubated at 37°C for 60 min in the presence of the indicated concentrations of glucose, islet homogenates were immunoprecipitated as described above and used for a PI phosphorylation reaction (36). The reaction products were applied to thin-layer chromatography and quantified with an image analyzer (BAS 2000; Fuji Film, Tokyo, Japan), as described (39).

**Insulin secretion from islets.** Insulin release from pancreatic islets was measured in static incubation or perifusion incubation with Krebs-Ringer bicarbonate (KRB) buffer composed of 129 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l $KH_2PO_4$, 2.5 mmol/l $CaCl_2$, 5 mmol/l $NaHCO_3$, 0.2% bovine serum albumin, and 10 mmol/l HEPES (pH 7.4) (40). In static incubation, batches of 10 freshly isolated islets were preincubated at 37°C for 30 min in KRB buffer containing 2.8 mmol/l glucose. The preincubation solutions were replaced with KRB buffer containing test agents, and batches of islets were incubated at 37°C for 60 min. Insulin released into these supernatants was measured by radioimmunoassay. In perifusion incubation, 30 freshly isolated islets were suspended in 500 μl of Bio-Gel G-10 beads in each perifusion chamber and perifused with KRB buffer at 37°C at a flow rate of 0.6 ml/min. Islets were perifused for 30 min in the presence of 2.8 mmol/l glucose before higher glucose stimulation. Perifusate fractions were periodically collected, and insulin in these samples was measured by radioimmunoassay. When wortmannin was added to incubation media, it was also included in preincubation media if not otherwise stated.

**Glucose oxidation.** Glucose oxidation in islets was measured by generation of $^{14}CO_2$ from D-[U-$^{14}$C]glucose (41). Batches of 10 islets were incubated at 37°C for 90 min in KRB buffer containing the isotope. When wortmannin was added to incubation media, it was also included in preincubation for 30 min. The $^{14}CO_2$ generated was made volatile by adding HCl, captured by NaOH, and measured with liquid scintillation counting.

**ATP content.** For determining ATP contents in whole islets (40), batches of 10 islets were incubated at 37°C for 60 min in KRB buffer. When wortmannin was added to incubation media, it was also included in preincubation for 20 min. The incubation was stopped by the addition of ice-cold $HClO_4$, and islets were homogenized by sonication. The homogenates were neutralized by addition of NaOH. ATP contents in the supernatants were measured with ATP bioluminescent assay kit.

**Cytosolic [$Ca^{2+}$].** Islets were loaded with 15 μmol/l fura-2/acetoxymethylester at 37°C for 60 min in Sol AII buffer (150 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 2 mmol/l $CaCl_2$, and 10 mmol/l HEPES [pH 7.4]) (40). An islet on a glass coverslip was placed on the stage of an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan) equipped with a charged-coupled device camera (Photometrics). The islet was viewed under a water immersion objective lens (LUMPlanFL ×60, Olympus) with alternate excitation wavelengths of 355 nm and 380 nm. The images of 256 × 256 pixels (170 × 170 μm) were obtained with an exposure time of 100 ms (42). The image analysis was carried out using an IPLab program (Signal Analysis Corporation).

**Statistical analysis.** Statistical analysis was performed using Student's *t* test for unpaired comparisons or analysis of variance with Tukey's post hoc comparison. Values are presented as means ± SE.

## RESULTS

**Reduced insulin content in $p85\alpha^{-/-}$ β-cells.** At 8 weeks of age, body weights of $p85\alpha^{-/-}$ male mice were 22.4 ± 0.5 g, which were similar to those of wild-type control mice (22.2 ± 0.3 g). Pancreas weights were also similar in wild-type (0.22 ± 0.02 g) and $p85\alpha^{-/-}$ mice (0.25 ± 0.02 g). We next measured the insulin contents of isolated islets. The $p85\alpha^{-/-}$ islets that we used for the present studies were comparable in size (~150 μm diameter) to those of wild-type islets and contained a number of cells (1,601 ± 104 cells/islet) similar to that of wild-type islets (1,549 ± 76 cells/islet). The insulin content of $p85\alpha^{-/-}$ islets was 33.7 ± 1.8 ng/islet, which was significantly decreased ($P < 0.01$) as compared with that of wild-type islets (53.1 ± 1.4 ng/islet). When insulin content was expressed per cell, the content in individual $p85\alpha^{-/-}$ cells was 21.4 ± 1.4 pg/cell and was decreased by 39% ($P < 0.01$) as compared with that of wild-type cells (34.9 ± 2.1 pg/cell).

**Decreased β-granule number and rough ER area on electron microscopic examination of $p85\alpha^{-/-}$ β-cells.** To investigate further the cause of decreased insulin content in $p85\alpha^{-/-}$ β-cells, we examined these cells with an electron microscope (Fig. 1*B*) (37). The number of insulin-containing dense-core granules (2.8 ± 0.3/μm$^2$) was decreased ($P < 0.05$) as compared with wild-type β-cells (4.3 ± 0.4/μm$^2$) (Fig. 1*A*) across ultrathin sections. In addition, as compared with wild-type β-cells (2.39 ± 0.21% of whole cell area), $p85\alpha^{-/-}$ β-cells possessed markedly reduced rough ER (0.53 ± 0.11% of whole cell

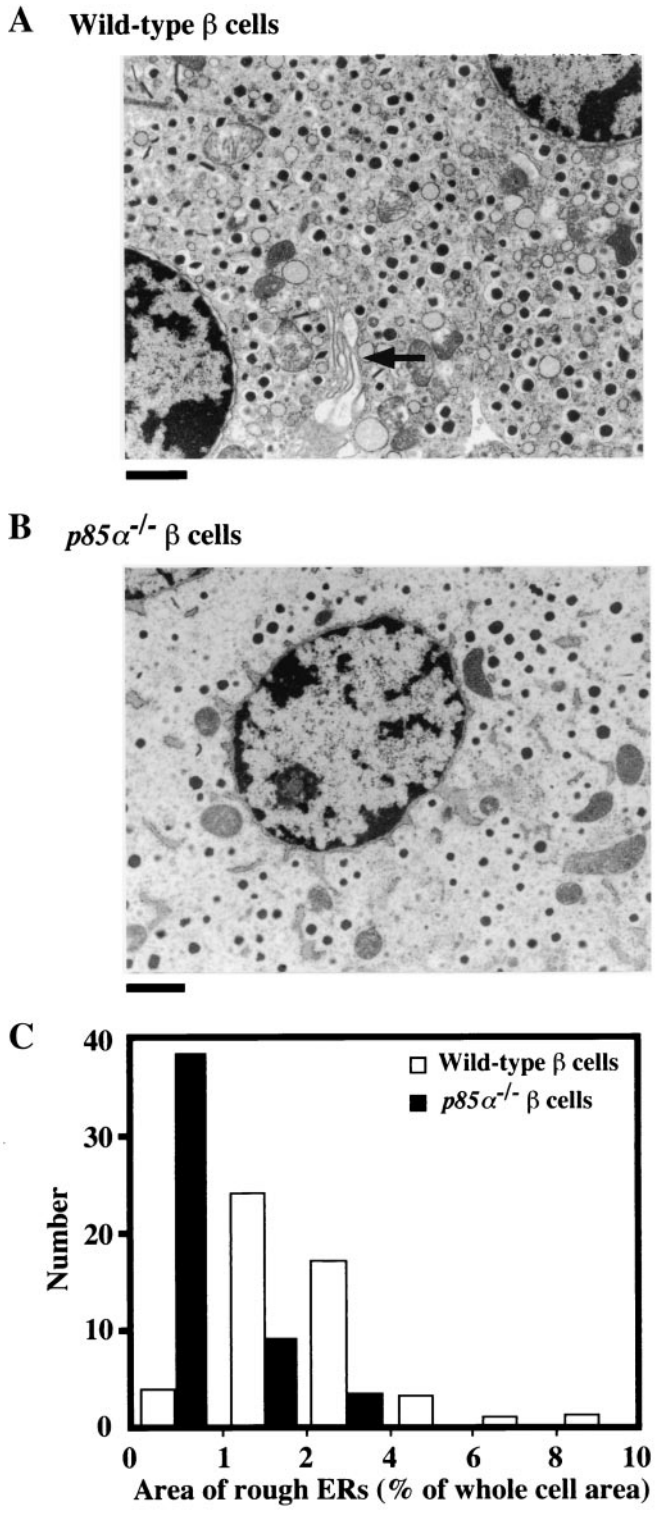


**FIG. 1. Electron microscopic examination of β-cells. Islets were isolated from 8-week-old male mice and immersed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) overnight and then subjected to fixation with 2.5% glutaraldehyde and 1% osmic acid. Thereafter, the islets were embedded in Epon 812 and cut into ultrathin sections. The sections were doubly stained with uranyl acetate and lead acetate and examined under a Hitachi HU-11B electron microscope. The photographs shown are representative of 50 observations. *A*: Wild-type β-cells. An arrow indicates rough ER. *B*: $p85\alpha^{-/-}$ β-cells. Horizontal bars beneath the photographs indicate 1 μm (*A* and *B*). *C*: Histogram of rough ER areas in each β-cell. Rough ER areas were traced and calculated on 150 β-cell sections using ×4,000 photographs. They were expressed as percentage of whole β-cell areas.**

area; $P < 0.01$ versus wild-type β-cells) (Fig. 1*C*). These data suggested decreased insulin synthesis in $p85\alpha^{-/-}$ β-cells, which may reflect decreased demand for insulin secretion as a result of increased insulin sensitivity in $p85\alpha^{-/-}$ mice.

**Expression of PI 3-kinase regulatory subunits and PI 3-kinase activity in $p85\alpha^{-/-}$ β-cells.** To confirm abrogation of the p85α molecule in islets from $p85\alpha^{-/-}$ mice, we performed Western blot analysis using an anti-$p85\alpha^{PAN}$ antibody that recognized full-length p85α protein (36). We detected no p85α in islets from the knockout mice (Fig. 2*A*, left panel). When PI 3-kinase activities after incubation with 2.8 mmol/l glucose were assayed in islet homogenates immunoprecipitated with the anti-$p85\alpha^{PAN}$ antibody, the activity in $p85\alpha^{-/-}$ islets was not abolished, but rather remained at 21.2 ± 3.7% ($n = 3$) of that of wild-type islets (Fig. 2*B*). With 22.2 mmol/l glucose, the PI 3-kinase activity was increased by 24.6 ± 4.1% ($n = 3$) as compared with 2.8 mmol/l glucose in wild-type islets. A similar slight activity increase in response to 22.2 mmol/l glucose (20.2 ± 2.9%, $n = 3$) was also observed in $p85\alpha^{-/-}$ islets, maintaining the activity ratio between $p85\alpha^{-/-}$ and wild-type islets unchanged at 22.2 mmol/l glucose. In skeletal muscle and adipocytes of $p85\alpha^{-/-}$ mice, p50α (43), a truncated splice variant of p85α with higher PI 3-kinase activity, was overexpressed so as to play a role in overcompensating for the lack of p85α, leading to rather increased $PI(3,4,5)P_3$ levels and enhanced whole-body insulin sensitivity (36). Thus, we presumed that this variant contributed to the remaining PI 3-kinase activity in $p85\alpha^{-/-}$ mice. In fact, we observed p50α to be expressed in wild-type islets (Fig. 2*A*, left panel). The expression of p50α in wild-type islets was far lower than that of p85α, as was reported in skeletal muscle and adipocytes (36). In $p85\alpha^{-/-}$ islets, p50α was expressed to the same level as in wild-type islets (Fig. 2*A*, left panel). We confirmed that the immunoprecipitated sample from $p85\alpha^{-/-}$ islets for PI 3-kinase activity measurement contained p50α that was comparable in amount to that from wild-type islets (Fig. 2*A*, right panel). These results demonstrated that p50α contributed to the residual PI 3-kinase activity and that the mechanism of compensatory overexpression of p50α was not operative in islets that lacked p85α, unlike the situation in skeletal muscle and adipocytes. The failure of p50α overexpression in tissues from $p85\alpha^{-/-}$ mice was also observed in B-lymphocytes (44).

**Enhanced glucose-stimulated insulin secretion in $p85\alpha^{-/-}$ islets.** We next studied glucose-stimulated insulin release from $p85\alpha^{-/-}$ islets during static incubation (Fig. 3*A*). Glucose-stimulated insulin releases did not differ from those of wild-type islets at 2.8 and 5.6 mmol/l glucose. However, they were increased by 27% at 11.1 mmol/l glucose ($P < 0.05$, $n = 4$) and by 53% at 22.2 mmol/l glucose ($P < 0.01$, $n = 4$) as compared with wild-type islets. Because the average cell number that constituted the $p85\alpha^{-/-}$ islets that we isolated and used for experiments was not different from that of wild-type islets, glucose-stimulated insulin releases per cell were clearly increased at 11.1 and 22.2 mmol/l glucose as compared with wild-type islet cells. Insulin contents per islet for 2.8, 5.6, 11.1, and 22.2 mmol/l glucose conditions were 42.1 ± 3.9, 44.2 ± 4.2, 50.0 ± 3.5, and 48.5 ± 5.1 ng for wild-type

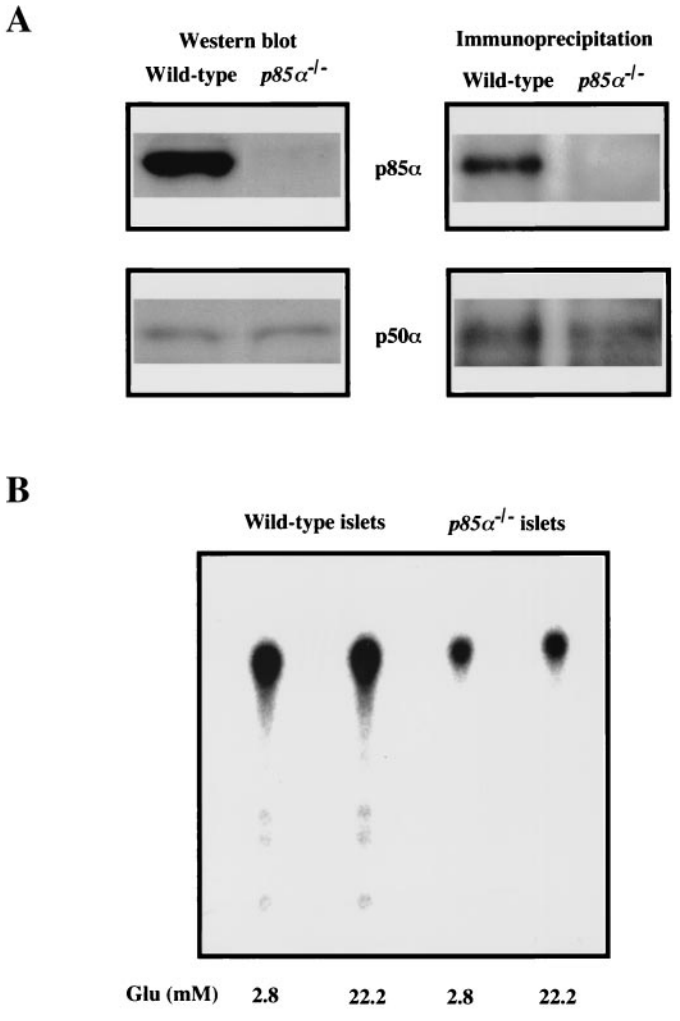


**FIG. 2. Western blot and immunoprecipitation analysis of PI 3-kinase regulatory subunits and assay of PI 3-kinase activities in $p85\alpha^{-/-}$ islets. *A*: For Western blot analysis, islets were homogenized in ice-cold buffer A and boiled in Laemmli's buffer for 5 min. Samples (30 μg of protein) were separated on SDS–polyacrylamide gels and transferred to nitrocellulose membranes, followed by detection with an ECL kit with anti-p85α$^{PAN}$ antibody. For immunoprecipitation analysis, islet homogenates (100 μg of protein) were incubated in buffer A with anti-p85α$^{PAN}$ antibody and protein G agarose for 2 h at 4°C. The immune complex was precipitated, washed with buffer A and PI 3-kinase buffer, and Western-blotted with anti-p85α mouse monoclonal antibody. *B*: For measurement of PI 3-kinase activity, islets were incubated in KRB buffer with the indicated concentrations of glucose for 60 min at 37°C. The islet homogenates (100 μg of protein) were then immunoprecipitated as in *A* and used for the PI phosphorylation reaction. The reaction products were applied to chromatography and quantified with an image analyzer. Results shown are representative of three experiments.**

islets, and 29.4 ± 4.7, 37.9 ± 3.6, 31.7 ± 4.0, and 30.1 ± 4.0 ng for $p85\alpha^{-/-}$ islets, respectively. When secretion was expressed per insulin content, fractional insulin releases from individual $p85\alpha^{-/-}$ β-cells were increased by 110% ($P < 0.01$, $n = 4$) and 130% ($P < 0.01$, $n = 4$) at 11.1 and 22.2 mmol/l glucose, respectively, as compared with wild-type controls (Fig. 3*B*). Perifusion experiments revealed that knockout of the p85α molecule failed to affect the kinetics of biphasic insulin release in response to 22.2 mmol/l glucose as compared with wild-type controls (data not shown), although glucose-responsive secretion from $p85\alpha^{-/-}$ islets was greater by 35% in the first phase (0–12 min after stimulation) and by 50% in the second phase (12–60 min after stimulation) of secretion as compared with that of wild-type islets (Table 1).

**Effects of PI 3-kinase inhibitors on glucose-stimulated insulin secretion in wild-type islets.** Glucose-stimulated insulin secretion was increased at higher glucose concentrations in $p85\alpha^{-/-}$ β-cells. However, the $p85\alpha^{-/-}$ mice also exhibited systemic insulin sensitivity, and the supernormal secretory phenotype thus might be a change secondary to this sensitivity, rather than a primary result of decreased PI 3-kinase activity itself in β-cells. Moreover, $p85\alpha^{-/-}$ islets still had ~20% of the residual PI 3-kinase activity, and this incomplete abrogation of the activity should be cautiously taken into account when those results from the animal model islets are interpreted. To address these issues, we performed complementary experiments to analyze the effect of pharmacological inhibition of PI 3-kinase with wortmannin on glucose-stimulated insulin secretion in wild-type islets, resulting in confirmation of previous reports (29,34,35). We first confirmed that wortmannin dose-dependently suppressed PI 3-kinase activities in wild-type islet homogenates (Fig. 4). Its $IC_{50}$ was below 10 nmol/l, and PI 3-kinase activity was

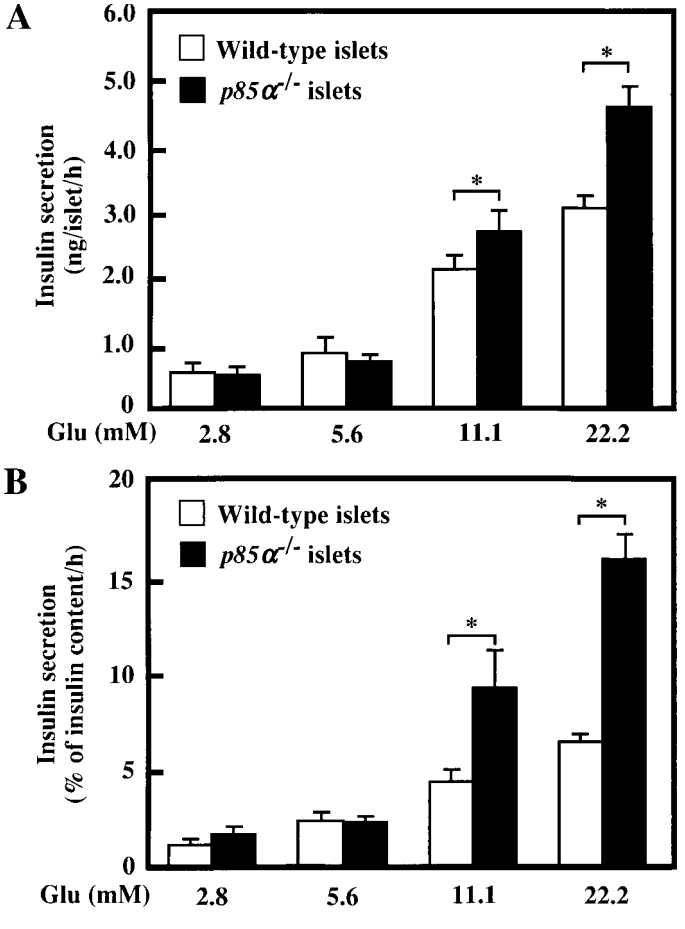


**FIG. 3. Glucose-stimulated insulin secretion in $p85\alpha^{-/-}$ islets. *A*: In static incubation, batches of 10 freshly isolated islets were preincubated at 37°C for 30 min in KRB buffer containing 2.8 mmol/l glucose, followed by incubation with the indicated concentrations of glucose for 60 min ($n = 4$). *B*: Insulin release was expressed as percentage of insulin contained in islets. After incubation at 37°C for 60 min, the media were stored for later measurement of secreted insulin. Pelleted islets were solubilized in 74% ethanol and 1.4% HCl solution at 4°C overnight, and insulin liberated into the solution was measured ($n = 4$). *$P < 0.05$.**

TABLE 1
Glucose-stimulated insulin secretion from islets in perifusion experiments

| | Wortmannin (−) | | Wortmannin 100 nmol/l | |
|---|---|---|---|---|
| | 0–12 min | 12–60 min | 0–12 min | 12–60 min |
| Wild-type islets (pg/islet) | 359.9 ± 24.2 | 2,134.9 ± 87.2 | 763.4 ± 55.4 | 5,114.5 ± 259.1 |
| $p85\alpha^{-/-}$ islets (pg/islet) | 487.5 ± 38.2* | 3,194.7 ± 146.3† | 843.2 ± 40.6 | 5,779.8 ± 295.9 |

The insulin release in response to 22.2 mmol/l glucose for 60 min was quantified. The release during 0–12 min after glucose stimulation corresponded to the first-phase secretion, and the release during 12–60 min corresponded to the second-phase secretion. Results were expressed as means ± SE ($n = 4$). *$P < 0.05$, †$P < 0.01$ as compared with wild-type controls.

progressively suppressed by up to 100 nmol/l wortmannin, at which concentration the residual activity was 6.8% of the original activity. Wortmannin dose-dependently increased 22.2 mmol/l glucose–stimulated insulin release (Fig. 5*A*). The secretion peaked at 100–300 nmol/l wortmannin but decreased slightly at 1 μmol/l wortmannin. Thus, the insulin secretion curve (Fig. 5*A*) and the PI 3-kinase inhibition curve (Fig. 4) in response to up to 100 nmol/l wortmannin were well correlated in a reciprocal manner. At 100 nmol/l, wortmannin's potentiating effect on glucose–stimulated insulin secretion was also clearly observed at 8.3 and 11.1 mmol/l glucose but not at 2.8 or 5.6 mmol/l glucose (Fig. 5*B*). This potentiating effect took 20 min to reach maximum levels, as shown in perifusion experiments (Fig. 5*C*) in which an additional stimulation with 100 nmol/l wortmannin was applied to wild-type islets during the second-phase secretion attained with 22.2 mmol/l glucose. When islets were exposed to wortmannin also during the preincubation period for 30 min, the drug potentiated not only the second-phase secretion but also the first-phase secretion (Table 1). The acute onset of wortmannin's effect (Fig. 5*C*) suggested that a transcriptional regulatory mechanism was not involved in the potentiation of glucose–stimulated insulin secretion. Supporting this, the transcriptional inhibitor actinomycin D (5 μg/ml) (45) did not affect insulin secretion in response to 22.2 mmol/l glucose or 100 nmol/l wortmannin–induced potentiation of the secretion by 22.2 mmol/l glucose (data not shown).

Essentially identical effects on glucose–stimulated insulin releases were observed with LY294002, another inhibitor of PI 3-kinase (46,47). The 22.2 mmol/l glucose-stimulated insulin secretion was potentiated by ~100% with 25–50 μmol/l LY294002 (data not shown). With 50 μmol/l LY294002, the potentiating effect was not observed at 2.8 or 5.6 mmol/l glucose but was evident at 8.3 and 11.1 mmol/l glucose (Fig. 5*D*). The common results from $p85\alpha^{-/-}$ islets and wild-type islets treated with pharmacological inhibitors of PI 3-kinase that glucose–stimulated insulin secretion was increased at higher glucose concentrations under those conditions enabled us to propose that PI 3-kinase normally plays a suppressive role in glucose-stimulated insulin secretion. Neither rapamycin, an inhibitor of p70 ribosomal S6 kinase located downstream from PI 3-kinase (47–49), nor PD98059, an inhibitor of mitogen-activated protein (MAP) kinase kinase (50,51), showed such potentiating effects in static incubations with 22.2 mmol/l glucose (data not shown).

Moreover, we studied the effect of wortmannin on glucose–stimulated insulin secretion in $p85\alpha^{-/-}$ islets with perifusion experiments to evaluate the effect of further reducing the residual PI 3-kinase activity of the islets. Without wortmannin, 22.2 mmol/l glucose–stimulated insulin secretion from $p85\alpha^{-/-}$ islets was nearly 50% greater than that from wild-type islets during 60 min of incubation (Table 1). After treatment with 100 nmol/l wortmannin, the secretion from $p85\alpha^{-/-}$ islets was increased but became comparable to that of wild-type islets (Table 1). Thus, we assumed that wortmannin further reduced the residual PI 3-kinase activity in $p85\alpha^{-/-}$ islets, which led to an enhancement of glucose–stimulated insulin secretion even in the islets, and that the blunted potentiating effect of wortmannin on secretion in $p85\alpha^{-/-}$ islets was consistent with the idea that the increased insulin secretion in the islets may indeed be causally related to reduced PI 3-kinase activity.

**Glucose metabolism and cytosolic [$Ca^{2+}$] in wortmannin-treated wild-type islets.** In an attempt to identify sites at which wortmannin exerted its potentiating effect, we analyzed glucose metabolism in wild-type islets with [U-$^{14}$C]glucose oxidation (41). Application of 100 nmol/l wortmannin did not change [U-$^{14}$C]glucose oxidation at either 22.2 mmol/l or 2.8 mmol/l glucose (Fig. 6*A*). ATP contents were also comparable between control and wortmannin-treated islets at both basal and stimulating concentrations of glucose (Fig. 6*B*). We next studied the effect of wortmannin on cytosolic [$Ca^{2+}$], which is regulated by

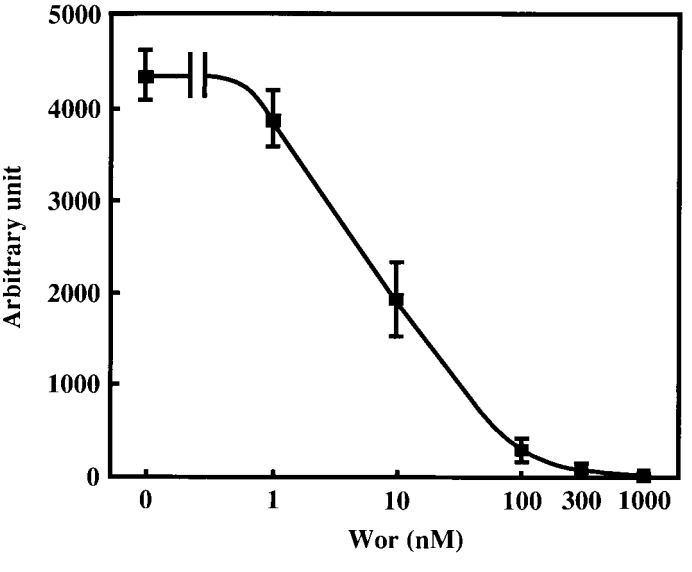


**FIG. 4. Dose-dependent inhibition by wortmannin of PI 3-kinase activity in wild-type islet homogenates. Wild-type islet homogenates (100 μg of protein) were incubated in buffer A with anti-p85α$^{PAN}$ antibody and protein G agarose for 2 h at 4°C. The immune complex was precipitated, washed with buffer A and PI 3-kinase buffer, and used for the PI phosphorylation reaction with the indicated concentrations of wortmannin. The reaction products were applied to chromatography and quantified with an image analyzer ($n = 3$).**

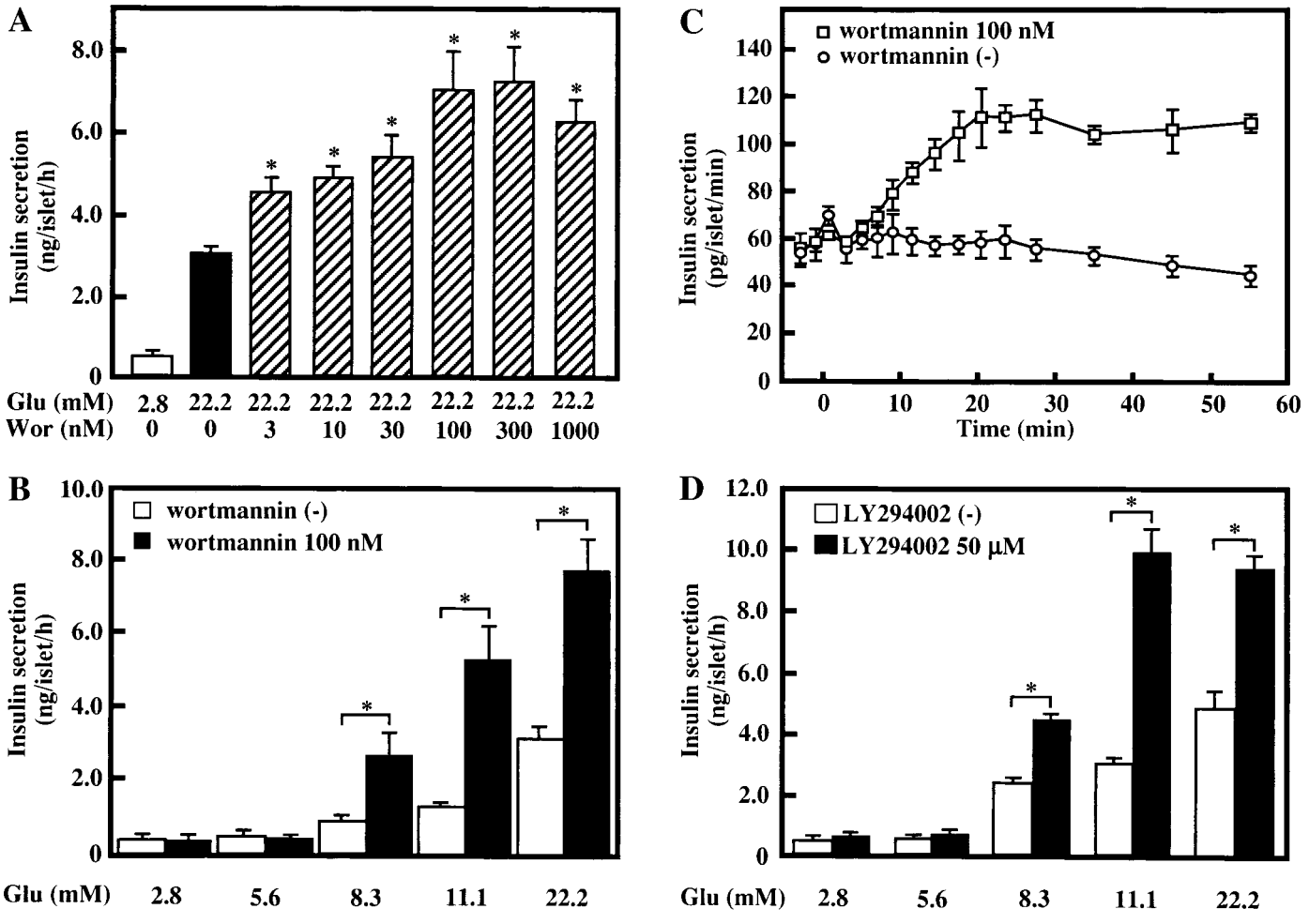


**FIG. 5. Effects of wortmannin (*A–C*) or LY294002 (*D*) on glucose–stimulated insulin release in wild-type islets. *A*: The dose-dependence of 22.2 mmol/l glucose–stimulated insulin secretion on wortmannin was measured under static incubation conditions ($n$ = 4). Statistical significance versus 22.2 mmol/l glucose stimulation without wortmannin is indicated. *B*: Glucose concentration–dependent potentiation of secretion by 100 nmol/l wortmannin ($n$ = 4). *C*: In the perifusion study, islets were stimulated with 22.2 mmol/l glucose for 30 min to obtain steady second-phase secretion states. Then, at 0 min, 100 nmol/l wortmannin was applied to the media (□) ($n$ = 4); ○, control without wortmannin application ($n$ = 4). *D*: Glucose concentration–dependent potentiation of insulin secretion by 50 μmol/l LY294002 ($n$ = 4). *$P < 0.05$.**

cellular ATP levels through $K_{ATP}$ channels (9). The cytosolic [$Ca^{2+}$] was monitored with the fura-2 fluorescence ratio at 355: 380 nm (42). Stimulation by 22.2 mmol/l glucose triggered a sharp cytosolic [$Ca^{2+}$] rise (the first phase) followed by a sustained plateau (the second phase) (Fig. 6*C*). When 100 nmol/l wortmannin was applied to islets 10 min after 22.2 mmol/l glucose stimulation during the second-phase plateau, this treatment did not change cytosolic [$Ca^{2+}$] levels (Fig. 6*C*). At 20 min after wortmannin application, increases in the fura-2 fluorescence ratio (0.25 ± 0.03, $n$ = 6) were not different from those of 22.2 mmol/l glucose stimulation without wortmannin at the corresponding time (0.27 ± 0.04, $n$ = 6).

**Effects of modulating cytosolic [$Ca^{2+}$] on insulin secretion.** Finally, we studied the relationship between wortmannin's effect and cytosolic [$Ca^{2+}$] in β-cells. For this purpose, cytosolic [$Ca^{2+}$] was modified with diazoxide, an opener of $K_{ATP}$ channels, plus a depolarizing concentration of KCl (Fig. 7) (11,12). Insulin contents per islet just after secretion experiments under four conditions (from the left in Fig. 7*A*) were 56.3 ± 4.2, 53.8 ± 4.6, 43.1 ± 6.2, and 50.4 ± 4.6 ng for wild-type islets, and 47.7 ± 5.3, 32.2 ± 3.0, 30.5 ± 3.7, and 37.8 ± 4.5 ng for $p85\alpha^{-/-}$ islets, respectively. When $p85\alpha^{-/-}$ islets were stimulated by $Ca^{2+}$ influx with 250 μmol/l diazoxide and 30 mmol/l KCl in the presence of 2.8 mmol/l glucose, secretion was significantly less ($P < 0.05$, $n$ = 4) than that of wild-type islets (Fig. 7*A*, columns 3 and 4). However, when it was corrected by insulin content, fractional secretion did not differ between wild-type and $p85\alpha^{-/-}$ islets (Fig. 7*B*, columns 3 and 4). These results suggested that $Ca^{2+}$ influx stimulation without glucose only induced insulin secretion proportional to stored amounts of insulin, not evoking a potentiation effect as seen in stimulation with high glucose concentrations under decreased PI 3-kinase activities. Next, the effects of combined application of diazoxide and KCl were studied on 22.2 mmol/l glucose–stimulated insulin secretion (Fig. 7*A*, columns 5 and 6). Although this treatment further enhanced the insulin secretion in both wild-type and $p85\alpha^{-/-}$ islets, the secretion from $p85\alpha^{-/-}$ islets was significantly higher ($P < 0.05$, $n$ = 4) than that from wild-type islets (Fig. 7*A*, columns 7 and 8). When expressed as fractional insulin secretion, secretion by 22.2 mmol/l glucose from $p85\alpha^{-/-}$ islets was higher by 46% ($P < 0.01$) than that from wild-type islets in the presence

of 250 μmol/l diazoxide and 30 mmol/l KCl (Fig. 7*B*, columns 7 and 8).

The same series of experiments was performed in wild-type islets using wortmannin. Pharmacological inhibition of PI 3-kinase activity with wortmannin failed to modify insulin secretion in response to $Ca^{2+}$ influx stimulation induced by 250 μmol/l diazoxide and 30 mmol/l KCl at 2.8 mmol/l glucose (Fig. 7*C*, columns 3 and 4), as was consistent with the results in columns 3 and 4 of Fig. 7*B*. Next, 250 μmol/l diazoxide and 30 mmol/l KCl were added to incubation media containing 22.2 mmol/l glucose. By this addition, 22.2 mmol/l glucose–stimulated insulin secretion in either the absence or the presence of wortmannin (Fig. 7*C*, columns 5 and 6) was further augmented (Fig. 7*C*, columns 7 and 8). However, the secretion in the presence of wortmannin was significantly higher ($P < 0.01$, $n = 4$) than that in the absence of this drug, which was also consistent with the results in columns 7 and 8 of Fig. 7*B*. These results, together with the results of cytosolic [$Ca^{2+}$] measurements, indicated the effect of PI 3-kinase inhibition on glucose–stimulated insulin secretion to be exerted largely at sites distal to the cytosolic [$Ca^{2+}$] elevation.

## DISCUSSION

In the present study, we investigated the role of PI 3-kinase in β-cell functions, such as insulin storage and insulin secretion in response to glucose, using $p85\alpha^{-/-}$ islets and wild-type islets treated with PI 3-kinase inhibitors.

Insulin content was significantly decreased in $p85\alpha^{-/-}$ β-cells possessing only ~20% of the PI 3-kinase activity of wild-type β-cells. Hypersecretion of insulin and the resulting depletion of stored insulin seemed not to be responsible for the decrease because $p85\alpha^{-/-}$ mice were insulin-sensitive and their serum insulin levels were low in both fasting and glucose-loaded states (36). The decrease in cellular insulin content may be attributed to lowered biosynthesis of the hormone, which would be consistent with the markedly reduced proliferation of rough ER in the β-cells (Fig. 1*B*), although we have not directly addressed the rate of proinsulin biosynthesis and its conversion to insulin, or the rate of insulin degradation. This speculation was supported by the data that insulin gene transcription was markedly suppressed by the PI 3-kinase inhibitor LY294002 (31,32). However, it is unknown whether signals to stimulate insulin gene transcription via PI 3-kinase is mainly originated from ligand binding to insulin receptor because β-cell–specific knockout of insulin receptor did not lead to a reduction in pancreatic insulin content (52).

Rough ER changes in $p85\alpha^{-/-}$ β-cells were thought possibly to represent adaptation to the increased insulin sensitivity and hence the decreased insulin demand on β-cells. Thus, we observed that rough ER proliferation was very striking in β-cells obtained from *insulin receptor substrate-1 (IRS-1)* $^{-/-}$ and *IRS-2*$^{-/-}$ mice showing insulin resistance and compensatory hyperinsulinemia (K.E. and T.K., unpublished observations). The whole-body insulin sensitivity or resistance may adjust capacity of insulin-synthesizing machinery represented by rough ER.

Insulin secretion was studied in the two β-cell models of decreased PI 3-kinase activities: the β-cells that were genetically devoid of p85α regulatory subunit and the

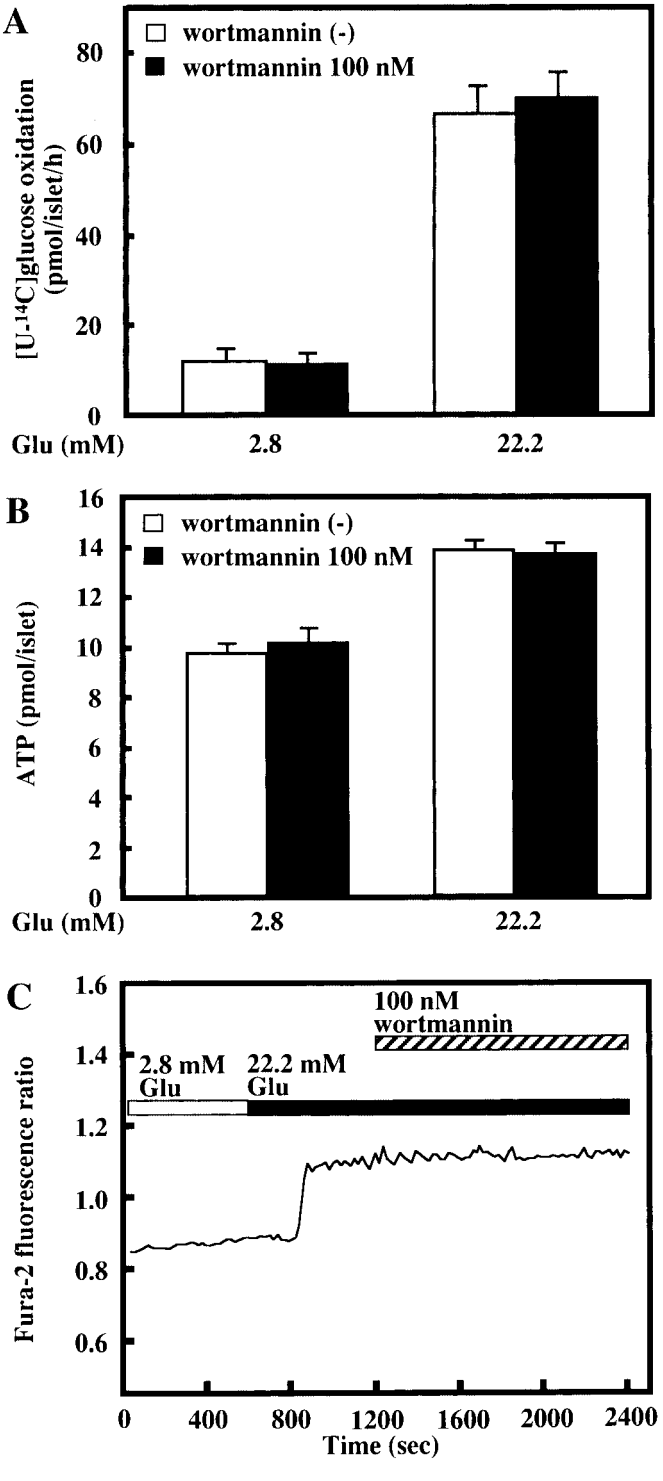


**FIG. 6. Effects of wortmannin on glucose oxidation, ATP content, and cytosolic [$Ca^{2+}$] in wild-type islets. Islets were cultured at the indicated concentrations of glucose in the absence or presence of 100 nmol/l wortmannin. *A*: Glucose oxidation was measured by the generation of $^{14}CO_2$ from D-[U-$^{14}$C]glucose ($n = 4$). *B*: Cellular ATP content was measured with a luciferase assay kit after homogenization of islets ($n = 4$). *C*: Cytosolic [$Ca^{2+}$] was monitored with a fura-2 fluorescence ratio at 355 nm/380 nm. The traces shown are representative of six experiments. The glucose concentration was changed from 2.8 to 22.2 mmol/l as indicated. Then, 100 nmol/l wortmannin was added to perifusion media 10 min after changing the glucose concentration from 2.8 to 22.2 mmol/l.**

β-cells that were treated with the pharmacological PI 3-kinase inhibitors. In the former, the decrease in PI 3-kinase activity was chronic and not complete, whereas it was acute and more complete in the latter (33). Glucose-stimulated insulin secretion from $p85\alpha^{-/-}$ β-cells with lower insulin contents was increased at higher glucose concentrations as compared with the wild-type controls (Fig. 3*A*), and the increase became more striking when it was normalized by cellular insulin content (Fig. 3*B*). Both the PI 3-kinase inhibitors wortmannin and LY294002 were found to enhance potently glucose–stimulated insulin secretion from intact mouse islets at higher glucose concentrations (Fig. 5*B* and *D*). Although these drugs at the doses currently used are believed to be specific to PI 3-kinase activity (30,33,46,47), the similar effect of the genetically downregulated model of PI 3-kinase activity with that of the pharmacological downregulation in wild-type controls further reinforced the idea that the potentiating effects seen in both models stemmed from specific suppression of the PI 3-kinase activity. It must be noted that the residual PI 3-kinase activity in $p85\alpha^{-/-}$ islets may partly be attributed to class II PI 3-kinase-C2β, which is also sensitive to low nanomolar levels of wortmannin (53), although its expression or activity in β-cells has not been addressed so far.

The acute onset of wortmannin's effect on secretion (Fig. 5*C*) suggested that the effect might be mediated by inhibition of metabolite production by PI 3-kinase via its acute metabolic effects, rather than through inhibition of gene transcription or de novo protein synthesis. Inability of actinomycin D or rapamycin to affect glucose–stimulated insulin secretion supported this speculation. We assumed that time lag for ~20 min to reach the maximum effect of wortmannin might be time required for attaining a new equilibrium of PI(3–5)P3 levels after the drug stopped synthesis of the lipid molecules. Similar time course of wortmannin's effect on glucose–stimulated insulin secretion was also shown by Zawalich and Zawalich (35). Specifically, the importance of PI 3-kinase in the suppression of glucose–stimulated insulin secretion was reinforced by the observation that inhibition of the MAP kinase pathway by PD98059 (54–56) had no effect on secretion, as reported previously (50,51).

Essentially identical consequences that wortmannin potentiated glucose–stimulated insulin secretion at intermediate glucose concentrations can be extracted from data by us and others (29,34,35). Wortmannin at 100 nmol/l potentiated 15–25 mmol/l glucose–stimulated insulin secretion by ~100% in MIN6 cells (29), which was in good agreement with our results. However, minor differences among them should be pointed out. Thus, Nunoi et al. (34) reported that the potentiating effect of 10 nmol/l wortmannin was not significant at 8.3 mmol/l glucose but was at 16.7 mmol/l glucose, whereas Zawalich and Zawalich (35) reported that the potentiating effect of 50 nmol/l wortmannin observed at 8.3 mmol/l glucose was no longer evident at 20 mmol/l glucose. These may originate from the differences in species used for experiments or in incubation conditions. It is widely known that progression of the second-phase insulin secretion after glucose challenge was substantially smaller in mouse islets than in rat islets (57). We might speculate that PI 3-kinase activity in mouse islets was high relative to rat islets, which led to seemingly stronger effects of PI 3-kinase downregulation in mouse islets.

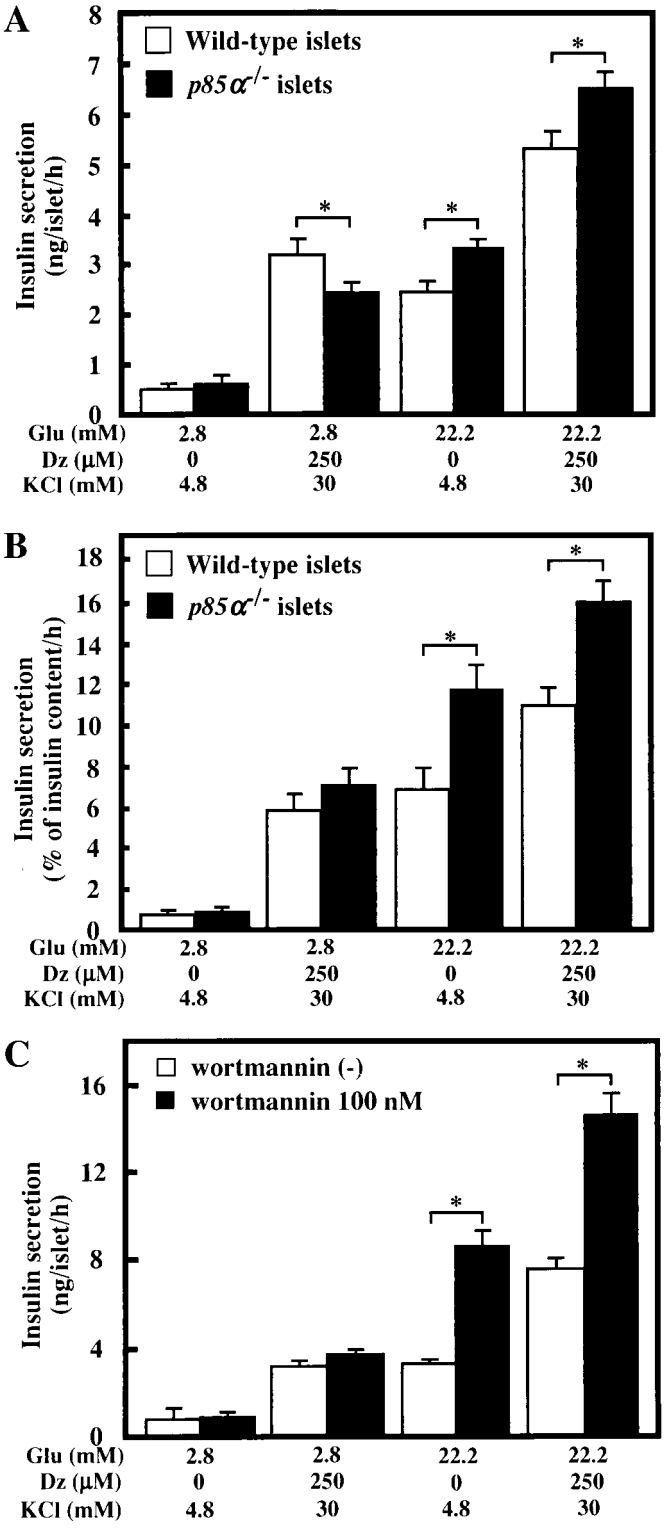


**FIG. 7. Effects of cytosolic [$Ca^{2+}$] modulation on insulin secretion. *A*: Effects of 250 μmol/l diazoxide plus 30 mmol/l KCl on insulin secretion at 2.8 or 22.2 mmol/l glucose in $p85\alpha^{-/-}$ islets ($n = 4$). *B*: The results in *A* are expressed as fractional secretion ($n = 4$). *C*: Effects of 250 μmol/l diazoxide plus 30 mmol/l KCl on insulin secretion stimulated by glucose or glucose plus 100 nmol/l wortmannin in wild-type islets ($n = 4$). *$P < 0.05$.**

Aspinwall et al. (58) proposed that insulin stimulated

insulin secretion in single β-cells, using amperometric detection of exocytosis. This insulin-stimulated insulin exocytosis was completely abolished by an application of wortmannin (59). These results seemingly argued the opposite of our results. However, we assumed that these two findings were observing different effects of wortmannin on insulin secretion at different sites at different time points. Thus, Aspinwall and colleagues. observed exocytotic processes nearby the plasma membrane during the very short time span of ~30 s, whereas we observed insulin secretion events that should include translocation of insulin-containing vesicles to the plasma membrane, conditioning of them to a readily releasable pool, and final exocytotic processes during a relatively long time span of ~20 min. Molecular mechanisms of these dual effects of wortmannin, which presumably have distinct molecular targets, require further investigation.

The effect of wortmannin to potentiate glucose–stimulated insulin secretion was undoubtedly exerted distal to the cytosolic [$Ca^{2+}$] elevation. Thus, under conditions in which $K_{ATP}$ channels were fixed open with diazoxide and a depolarizing concentration of KCl was applied to wild-type islets, wortmannin further augmented this secretion (Fig. 7*C*, columns 7 and 8). This was also the case in the β-cell model of genetically downregulated PI 3-kinase activity (Fig. 7*B*, columns 7 and 8). It is noteworthy that $Ca^{2+}$ influx–stimulated insulin secretion at basal glucose concentration was indeed decreased in $p85\alpha^{-/-}$ islets (Fig. 7*A*, columns 3 and 4) and became comparable to that of wild-type controls after calibration by cellular insulin content (Fig. 7*B*, columns 3 and 4). The pharmacological inhibition of PI 3-kinase was also unable to potentiate $Ca^{2+}$ influx–stimulated insulin secretion in wild-type islets (Fig. 7*C*, columns 3 and 4). These results demonstrated the potentiating effect of PI 3-kinase inhibition on insulin secretion to be specific to glucose stimulation accompanied by $Ca^{2+}$ influx but not relevant to "pure" $Ca^{2+}$ influx stimulation without glucose. This mechanism should work in the first phase as well as the second phase of glucose-stimulated insulin secretion and actually enhance both of these phasic secretions (Table 1).

$PI(4,5)P_2$ has been shown to activate $K_{ATP}$ channels by competing with ATP (60,61). Moreover, LY294002 completely blocked the activation of the $K_{ATP}$ channels by $PI(4,5)P_2$, and application of $PI(3,4,5)P_3$ directly increased the channel currents in a rat insulinoma cell line (62). These results suggested $PI(3,4,5)P_3$ to be a candidate for direct channel activation and that wortmannin's potentiating effect on glucose–stimulated insulin secretion might be mediated through the closure of these channels. Nevertheless, wortmannin did not further augment the cytosolic [$Ca^{2+}$] elevation induced by glucose in our study (Fig. 6*C*). These results indicated that the increased $K_{ATP}$ channel sensitivity to ATP contributed only slightly, if at all, to the mechanism of potentiation by wortmannin.

$Ca^{2+}$-dependent release of neurotransmitters from synaptic vesicles requires an ATP-dependent priming step (23,63). The PI(4) 5-kinase requirement of this step suggested that $PI(4,5)P_2$ plays a role in the process. Synaptotagmins and rabphilin-3A, which contain $Ca^{2+}$/phospholipid binding domains, are among the candidates for $PI(4,5)P_2$ effector molecules (64,65). By analogy with synaptic vesicles, the insulin-containing secretory vesicles may require $PI(4,5)P_2$ for their ATP-dependent priming step. Inhibition of PI 3-kinase activity should lead to accumulation of this phosphoinositide form. Thus, an increase in $PI(4,5)P_2$, rather than a decrease in $PI(3,4,5)P_3$, at the site of exocytosis might be responsible for the potentiation of insulin secretion in response to glucose.

In adipocytes, Akt kinase, a downstream effector of PI 3-kinase (66,67), was demonstrated to activate phosphodiesterase (PDE) 3B directly (68), which facilitates degradation of cAMP (69,70). If this cascade is operative in β-cells, then wortmannin may cause an increase in cellular cAMP content, thereby activating protein kinase A and resulting in the potentiation of glucose–stimulated insulin secretion. In fact, we observed that 100 nmol/l wortmannin augmented cAMP content by ~30% in islets incubated for 60 min in the presence of 22.2 mmol/l glucose. However, the increase in cAMP content was too small to account solely for wortmannin's potentiating effect on glucose–stimulated insulin secretion, as compared with a ~10-fold increase in cAMP content induced by 1 mmol/l of the nonspecific PDE inhibitor 3-isobutyl-1-methylxanthine, which potentiated as much secretion as 100 nmol/l wortmannin in the presence of 22.2 mmol/l glucose (K.E. and T.K., unpublished observations). These results suggested that increased cellular cAMP is not the principal mediator of wortmannin's effect, although it is possible that wortmannin changes sensitivity of cAMP-dependent steps to a small increase in cAMP.

It has been shown that leptin and insulin-like growth factor-1 suppress glucose plus cAMP-stimulated insulin secretion (71,72). One of the responsible mechanisms seems to involve PI 3-kinase, Akt kinase, and PDE3B activation and a subsequent reduction in cAMP content. These changes in response to leptin were reversed by treatment with wortmannin or LY294002 and actually resulted in recovery of insulin secretion. Thus, wortmannin's effect of sustaining higher levels of cAMP through deactivation of PDE3B may contribute considerably to increased insulin secretion under conditions in which the cAMP concentration becomes high with adenylyl cyclase agonists or PDE inhibitors (71,72), in contrast to the situation of glucose stimulation alone, which is accompanied by a minimal, at most, increase in the cAMP concentration.

Mouse knockout technology has provided us with several model islets that were defective in components of insulin signal transduction pathways. In β-cell–specific insulin receptor knockout, a selective loss of acute-phase insulin secretion in response to glucose was exhibited. It is interesting that insulin content was not impaired in those β-cells. Roles of IRS-1 and IRS-2, immediately downstream molecules of the insulin receptor, in the proliferation and function of β-cells differed with a marked contrast (5,6,73). Comprehensive understanding of the relationship between insulin secretion/biosynthesis and insulin signal transduction pathways or the autocrine regulation by insulin of β-cell functions needs much more accumulation of experimental evidence. Our present results also should be reevaluated from a viewpoint of signal transduction cascades proximal and distal to PI 3-kinase.

In conclusion, decreased PI 3-kinase activities that were

induced both genetically and pharmacologically were associated with increased insulin secretory responses to higher concentrations of glucose, indicating that PI 3-kinase normally plays a suppressive role in the regulation of glucose–stimulated insulin secretion. The suppressive effect on secretion seems to be exerted not through glucose-metabolizing steps that lead to ATP generation and $Ca^{2+}$ influx, but through steps distal to the cytosolic [$Ca^{2+}$] elevation. Clarification of the mechanism by which PI 3-kinase modulates β-cell secretory function will shed light on new aspects of glucose–stimulated insulin secretion and provide clues to the development of therapeutic agents that are capable of potentiating insulin secretion through a novel mode of action.

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