Protein Kinase Cδ Plays a Non-redundant Role in Insulin Secretion in Pancreatic β Cells*

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Protein kinase C (PKC) is considered to modulate glucose-stimulated insulin secretion. Pancreatic β cells express multiple isoforms of PKCs; however, the role of each isoform in glucose-stimulated insulin secretion remains controversial. In this study we investigated the role of PKCδ, a major isoform expressed in pancreatic β cells on β cell function. Here, we showed that PKCδ null mice manifested glucose intolerance with impaired insulin secretion. Insulin tolerance test showed no decrease in insulin sensitivity in PKCδ null mice. Studies using islets isolated from these mice demonstrated decreased glucose- and KCl-stimulated insulin secretion. Perfusion studies indicated that mainly the second phase of insulin secretion was decreased. On the other hand, glucose-induced influx of Ca2+ into β cells was not altered. Immunohistochemistry using total internal reflection fluorescence microscopy and electron microscopic analysis showed an increased number of insulin granules close to the plasma membrane in β cells of PKCδ null mice. Although PKC is thought to phosphorylate Munc18-1 and facilitate soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors complex formation, the phosphorylation of Munc18-1 by glucose stimulation was decreased in islets of PKCδ null mice. We conclude that PKCδ plays a non-redundant role in glucose-stimulated insulin secretion. The impaired insulin secretion in PKCδ null mice is associated with reduced phosphorylation of Munc18-1.

Altered regulation of insulin secretion is a common feature of type 2 diabetes mellitus, although the underlying mechanism is not fully understood. The mechanism of glucose-stimulated insulin secretion involves closure of ATP-sensitive potassium channels by increased levels of glucose metabolites followed by depolarization of the plasma membrane and increased influx of Ca2+ via voltage-dependent gating of Ca2+ channels. The resultant elevation of intracellular Ca2+ concentration ([(Ca2+)]i) triggers transport of insulin into the plasma membrane and exocytosis (1). Although the events involved in initiating glucose-stimulated insulin secretion are well studied, those of regulated exocytosis are considerably less clear, although it is reported that several molecules expressed in β cells play a crucial role in exocytosis (2).

Various protein kinases are activated by downstream signals of glucose metabolism; however, their precise contribution to insulin secretion is not clear yet (3). Protein kinase C (PKC)2 is one such protein kinase. Previous studies revealed that phorbol 12-myristate 13-acetate, which activates PKC, could trigger insulin secretion (4–10). PKC is a serine/threonine kinase characterized by molecular structure and activation requirement. The isoforms consist of the conventional PKCs (α, β, and γ), which are sensitive to Ca2+ and diacylglycerol (DAG), novel PKCs (δ, ε, η, and θ), which are sensitive to DAG only, and atypical PKCs (ζ, ι), which do not respond to either Ca2+ or DAG (11, 12). The β cells express PKCa and -δ as major isoforms of PKC. However, other isoforms of PKC are also expressed in β cells (7, 13–16). The importance of each PKC isoform in β cell function is not clear yet, mainly due to the scarcity of true isoform-specific inhibitors.

Pancreatic β cells express the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins such as vesicular-associated membrane protein (VAMP), synaptosomal-associated protein of 25 kDa (SNAP25), and syntxin-1A, which form the core machinery for vesicular docking on target membrane and membrane fusion (17, 18). In the machinery of regulated exocytosis, additional families of other proteins function as SNARE regulators, such as Sec/Munc18 proteins (19). Munc18-1, a family member of Munc18, binds with high affinity to syntxin-1A. In the Munc18-1–syntxin-1A complex, syntxin is in a closed conformation, whereby its N-terminal helices are folded back onto the C-terminal SNARE motif. Release of syntxin into an open conformation seems to be required for the association of syntxin with the SNARE complex (19). Thus, it seems that Munc18-1 must be released from syntxin-1A for the fusion event. Previous data showed that PKC could phosphorylate Munc18-1 and allow it to be released for binding with syntxin-1A (20). However, several studies have cast doubt on the role of Munc18-1 and its

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The abbreviations used are: PKC, protein kinase C; SNAP25, synaptosomal-associated protein of 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TIRF, total internal reflection fluorescence; KRS, Krebs-Ringer solution; VAMP2, vesicular-associated membrane protein 2; WT, wild type; KO, knock-out; RT, reverse transcription.

2 The abbreviations used are: PKC, protein kinase C; SNAP25, synaptosomal-associated protein of 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TIRF, total internal reflection fluorescence; KRS, Krebs-Ringer solution; VAMP2, vesicular-associated membrane protein 2; WT, wild type; KO, knock-out; RT, reverse transcription.

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modulators (19). In addition, the involvement of these events in β cell is not fully elucidated yet.

In the present study, as a first step to elucidate the involvement of PKCd in β cell function, we investigated the function of PKCd in β cells using mice with disruption of the PKCd gene (PKCd−/−). The results showed that disruption of the PKCd gene impaired exocytosis of insulin containing granules, concomitant with decreased glucose-stimulated phosphorylation of Munc18-1.

MATERIALS AND METHODS

Animals—The study protocol was reviewed and approved by the Animal Care and Use Committee of Juntendo University. All mice were housed in specific pathogen-free barrier facilities, maintained 12-h light/dark cycle, and fed a standard rodent food (Oriental Yeast Co., Osaka, Japan) and provided with water ad libitum. In this study, we used 8–20-week-old male mice with C57BL/6J background. We mated PKCd−/− mice to generate PKCd−/− mice, PKCd+/− mice, and wild type mice. Then these mice were used for further analyses. PCR analysis for determination of genotypes was performed as described previously (21).

Measurement of Blood Glucose and Insulin Levels for Phenotypic Characterization—After an overnight fast (16 h), age-matched 8-, 12-, 16-, and 20-week-old male mice were injected intraperitoneally with glucose at 1.0 g/kg of body weight. For the insulin tolerance test, age-matched 12-week-old mice were injected intraperitoneally with insulin (0.75 units/kg). A few microliters of blood samples were taken from the tail vein in awake mice, and glucose levels were measured using whole blood with a compact glucose analyzer (Free Style®, KISSEI Pharmaceutical Co., Nagano, Japan) at the indicated time points. To measure insulin levels, whole blood samples were collected and centrifuged with EDTA and aprotinin. After centrifugation, plasma was stored at −80 °C until analysis. Insulin levels were measured by enzyme-linked immunosorbent assay kit (Morinaga Co., Kanagawa, Japan).

Assay of Insulin Secretion from Isolated Islets and Islet Insulin Content—Pancreatic islets were isolated from 12-week-old mice by collagenase digestion, as described previously (17). The islets were preincubated for 30 min in Krebs-Ringer solution (KRS) at 37 °C under 5% CO2 and saturated humidity. Size-matched 5 islets were incubated in 400 μl of KRS containing various insulinotropic agents for 30 min at 37 °C. For measurement of islet insulin content, islets were solubilized in acid-ethanol solution overnight at 4 °C. All samples were stored at −80 °C until assay. Insulin concentration using isolated islets was analyzed by radioimmunoassay (LINCO insulin kit, LINCO Research Inc., St. Charles, MO). Islet insulin content was calculated after correction by DNA contents.

Islet Perfusion—Pancreatic islets were isolated as mentioned above and preincubated for 30 min in KRS. Size-matched 50 islets were placed in each chamber. Then, the kinetics of insulin release was studied using the perfusion system described previously (22).

Immunohistochemical Analysis and Quantification of β Cell Area—After anesthetization, 12-week-old mice were perfused intracardially with 4% paraformaldehyde, and the pancreas was removed and fixed overnight in a solution of 4% paraformaldehyde at 4 °C. The fixed tissue was embedded in paraffin and then cut into 5-μm-thick sections and mounted on slides. The sections were subjected to immunohistochemical staining for insulin using guinea pig anti-human insulin antibody (Linco Inc.) as described previously (23).

The β cell area was determined in 10 insulin-stained sections separated by 200 μm by evaluating the mean area of the individual cell. Images were captured with a microscope (E800; Nikon, Tokyo) connected to a digital camera (Sony, Tokyo). The area of insulin-stained pancreatic islets was determined automatically using image analysis software Pro4.5J (Planerotron (Tokyo)). β cell area was calculated using the following formula: islet β cell area (%) = (area stained by insulin antibody/whole pancreatic area) × 100. Nonspecific insulin staining was excluded from quantification of β cell area.

Measurement of [Ca2+]i in Single Mouse β Cells—Isolated islets were placed in 100 μl of Ca2+-free KRS containing 1 mM EGTA and gently agitated till turbidity due to the floating dispersed islets. After centrifugation at 500 rpm for 1 min, 50 μl of the supernatant was discarded, and 150 μl fresh KRS was decanted for washing. Finally, 50 μl of residual dispersed islets were again gently agitated and incubated on 0.04 mg/ml fibronectin-coated (Koken Co., Tokyo) Petri dishes with a 35-mm cover glass in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum at 37 °C and under 5% CO2 for 3 days. Cells were loaded with 2 μM Fura2/AM (Molecular Probes, Eugene, OR) in 200 μl of KRS containing 2.8 mM glucose for 20 min at 37 °C. After incubation, Fura2-loaded cells were placed in KRS supplemented with 2.8 or 16.7 mM glucose on a micro-warm plate (Kitazato Co., Shizuoka, Japan) maintained at 37 °C and mounted on the stage of TE300 microscope (Nikon, Tokyo). The loaded cells were excited at 340 and 380 nm; the florescence emitted at 510 nm was captured by an intensified charge-couple device camera. The images were analyzed by ARGUS/HiSCA system (Hamamatsu Photonics, Shizuoka).

Electron Microscopy—Each mouse at 12 weeks of age was anesthetized with pentobarbital sodium, and the pancreas was removed after cardiac perfusion and fixed in a solution of 2.5% glutaraldehyde. The pancreas was cut into pieces of ~1 mm3. The pieces were fixed for 2 h in 2.5% glutaraldehyde buffered to pH 7.4 with phosphate buffer and treated with osmium tetroxide for 2 h at 4 °C. The tissues were dehydrated with graded concentrations of ethanol solutions and then embedded with Epon 812. Thin sections were cut with a Leica Ultracut UCT using a diamond knife and then stained with uranyl acetate followed by lead citrate. Electron micrographs were taken with a JEOL JEM-1200EX electron microscope operated at 80 kV.

To measure the number of insulin granules according to their distance from the granule center, isolated islets obtained from 5 mice were incubated in KRBB (2.8 or 16.7 mmol/liter glucose) at 37 °C for 60 min. After incubation, islets were fixed with 2% paraformaldehyde, 2% glutaraldehyde, 0.2% picric acid in 0.1 M cacodylate buffer, pH 7.4, for 90 min at room temperature. Fixed islets were further treated by osmium tetroxide and then embedded in epoxy-resin. Micrographs were taken randomly at a ×3000 magnification. Using image analysis software (Image-Pro Plus Version 4.0, Planerotron), we counted the total number of insulin granules that resided within 500 nm of the plasma membrane. Then, the insulin granules were categorized according to their distance from the granule center to the
plasma membrane (0–100, 100–200, 200–300, 300–500 nm), and the number of insulin granules in each fraction was counted. Data were presented as % distribution below the plasma membrane. In these experiments, 100% corresponds to total counts of insulin granules that resided within 500 nm of the plasma membrane.

Total Internal Reflection Fluorescence (TIRF) Imaging Study—The Olympus total internal reflection system (Olympus, Tokyo) was used for counting the number of insulin granules 80 nm from the plasma membrane in β cells after immunostaining of insulin as described previously (24).

Tissue RNA Isolation and Real-time Quantitative RT-PCR—Total RNA was isolated from ~200 islets using TRIzol reagent (Invitrogen), and first strand cDNA was synthesized using 3 µg of total RNA with Oligo(dT) primers and Superscript reverse transcriptase (Invitrogen). For quantitative SYBR Green real-time PCR, cDNA was analyzed by ABI 7700 PCR with 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and specific primers. Thermal cycle for amplification was set as follows; 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. Using this protocol, neither nonspecific primer-dimer amplification nor PCR products were observed in no-template control samples. Single-band PCR products were detected by 1% agarose gels with end reaction products. The relative mRNA expression levels were calculated by the cycle threshold (Ct) method with standard cDNA. As an internal invariant standard, β-actin mRNA. The primers used were as follows: PKCa, forward, 5′-GAAGGTGATGCTTGTGCTACA-3′, reverse, 5′-CTTGACGTATTCCTATGAC-3′; PKβ2, forward, 5′-CTTGATTTCTAGGGAATCC-3′, reverse, 5′-AGCTGACAACGAGGACGACT-3′; Rab3a, forward, 5′-AGCCACACCTTCACATAAGG-3′; Rab27a, forward, 5′-GTCGCTCAGCTCTTT-3′, reverse, 5′-CTTGATCTTCTAGGGAATCC-3′; synaptotagmin 3, forward, 5′-ACTCCGCTGACTGCTTT-3′, reverse, 5′-GGGGTGTGACTGCTCTGTT-3′; syntaxin 1A, forward, 5′-GGGGTGTGACTGCTCTGTT-3′; Munc18-1, forward, 5′-CTCTCAGCTGTGGTGGTGAA-3′, reverse, 5′-AGTGGGTCATGGCTTGGTAG-3′; SNAP25, forward, 5′-CAAAGTGCAAACAGATCTC-3′, reverse, 5′-GGGGTGTGACTGCTCTGTT-3′; anti-syntaxin-1a antibody was from BD Transduction Laboratories. Anti-syntaxin-1a antibody was purchased from Sigma. To detect phosphorylated Munc18-1 and SNAP25, we collected phosphorylated protein using PhosphoProtein purification kit (Qiagen), which is based on affinity chromatography, a technique that delivers complete separation of phosphorylated and unphosphorylated proteins from a cell lysate. Approximately 1000 islets were used to collect phosphorylated proteins as a sample. Then, 20 µg of each sample was used for immunoblotting using anti-Munc18-1 or anti-SNAP25 antibody. Immunoreactivity was visualized and quantitated using Fuji LAS 1000 (Fuji Film, Tokyo).

Data Analysis—Results are presented as the mean ± S.E. Differences between groups were examined for statistical significance using the unpaired Student’s t test. A p value less than 0.05 denoted the presence of a statistically significant difference.

**RESULTS**

**PKCop−/− Mice Exhibit Glucose Intolerance and Impaired Insulin Secretion**—As reported previously (21), PKCop−/− mice are born normal and fertile with no apparent abnormalities in general appearance or behavior, but they start to develop B lymphocytic autoimmune disease after the age 24 weeks. Body weight of PKCop−/− mice increased with age, but the gain was modestly lower than that of PKCop+/− and wild type mice (Fig. 1a). Although fasting glucose level was comparable among the three groups (Fig. 1b), intraperitoneal glucose challenge at the age of 12 weeks identified glucose intolerance with reduced glucose-stimulated insulin secretion in PKCop−/− mice (Fig. 1, c and d). The glucose intolerance was continuously observed from 8 to 20 weeks of age, but no obvious deterioration was observed with aging (Fig. 1e). An insulin tolerance test did not demonstrate a significant decrease in insulin sensitivity in PKCop−/− mice (Fig. 1f). These results suggest that the impaired glucose tolerance observed in PKCop−/− mice is mainly due to impaired glucose-stimulated insulin secretion.

To search for the mechanism of impaired glucose-stimulated insulin release in PKCop−/− mice, we investigated islet morphology and β cell area by immunohistochemistry. However, we could not find any obvious changes in islet morphology or significant differences in β cell area between PKCop−/− mice and wild type (Fig. 2, a and b). The insulin content of isolated islets was also comparable (Fig. 2c). Furthermore, electron micro-
FIGURE 1. Impaired glucose tolerance and impaired insulin secretion in PKCδ−/− mice. a, changes in body weight of wild type, PKCδ+/−, and PKCδ−/− mice between 8 and 20 weeks of age. b, changes in fasting glucose levels in mice between 8 and 20 weeks of age. c, blood glucose concentrations measured during intraperitoneal glucose tolerance test in each group at the age of 12 weeks. d, serum insulin levels measured during intraperitoneal glucose tolerance test in each group at the age of 12 weeks. e, changes in glucose tolerance in each group at the age of 8, 16, 20 weeks. f, results of intraperitoneal insulin tolerance test at the age of 12 weeks. Open circles, wild type mice (n = 6); solid triangles, PKCδ+/− mice (n = 6); solid circles, PKCδ−/− mice (n = 7). Data are the mean ± S.E. $, p < 0.05$ versus WT.
scopical analysis demonstrated no apparent abnormalities of β cell and endothelial cells in PKCδ−/− islets (Fig. 2d) (28). Therefore, the reduced glucose-stimulated insulin secretion in PKCδ−/− mice was not associated with any apparent reduction in insulin-containing granules or abnormal β cell morphology.

Characterization of Impaired Insulin Secretion in Islets of PKCδ−/− Mice—To elucidate whether the deletion of PKCδ gene in islets resulted in compensatory increases in the expression of other PKC family members, we investigated mRNA and protein expression levels of conventional and novel PKC family members that are known to be expressed in β cells (29). However, we could not find any significant increase in the expression of PKCα, PKCβ2, and PKCε investigated in the present study (Fig. 3, a and b). In further search for the mechanism of decreased glucose-stimulated insulin secretion in PKCδ−/− mice, we investigated insulin secretion using several secretagogues in isolated PKCδ−/− islets. Insulin secretion stimulated by high glucose was modestly but significantly lower in PKCδ−/− islets than the wild type (Fig. 3c). We further investigated glucose-stimulated insulin secretion in PKCδ−/− islets in a set of perfusion studies (Fig. 3d). The second phases of insulin secretion by stimulation with high glucose (16.7 mM) was significantly decreased in PKCδ−/− islets (Fig. 3e). In addition, insulin secretion in response to depolarization induced by KCl was also reduced in PKCδ−/− islets (Fig. 3c). Arginine, an insulinotropic agent thought to depolarize β cell membrane to activate voltage-dependent Ca2+ channel by its positively charged form (30, 31), also failed to stimulate insulin secretion in PKCδ−/− islets to levels comparable with those in wild type islets. These results suggest impairment of glucose-stimulated insulin secretion in PKCδ−/− islets after membrane depolarization. Insulin secretion was also significantly decreased in PKCδ−/− islets in response to phorbol 12-myristate 13-acetate, an activator of PKC, and to phorbol 12-myristate 13-acetate and forskolin, activators of adenylate cyclase. Thus, the decrease in insulin secretion in PKCδ−/− islets is observed even after application of various secretagogues.

Characterization of Insulin Granules in PKCδ−/− Islets—Next, we determined changes in [Ca2+]i in response to glucose stimulation in single β cells by using fura-2, a Ca2+ indicator. As shown in Fig. 4, the amplitude and time course of a glucose-induced rise in Ca2+ concentration was not different between PKCδ−/− β cells and wild type. These results suggest normal glucose metabolism and ATP production in PKCδ−/− β cells.

This finding together with the experiments of insulin secretion indicates that the decrease in insulin secretion in PKCδ−/− islets might be simply due to a decrease in the releasable pool of insulin granules. Thus, to investigate the number of insulin granules that are located close to the plasma membrane, pancreatic β cells isolated from PKCδ−/− mice were immunostained with insulin and visualized by TIRF microscopy. TIRF imaging can identify single insulin granules within <100 nm from underneath the plasma membrane (32). Counting the number of insulin granules in the obtained image indicated that the density of insulin granules in PKCδ null β cells was not decreased but, rather, was increased (Fig. 5, a and b).

To confirm this finding, we counted the relative number of insulin granules according to their distance from the granule center to the plasma membrane in wild type and PKCδ−/− islets after incubation with low and high glucose. As shown in Fig. 5c, the number of granules residing within 100 nm of the plasma membrane was much lower than those located at other distances from the membrane as described previously (33). The number of insulin granules in this fraction was significantly increased in PKCδ−/− islets treated with low glucose. In addition, the number of insulin granules located 100–200 nm from the membrane was increased in PKCδ−/− islets treated with low glucose. On the other hand, the number of insulin granules in other fractions was not significantly different between wild type and PKCδ−/− islets. Probably the increased number of insulin granules close to the plasma membrane reflects the increase in granular backup in trafficking. Next, we investigated
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FIGURE 3. Impaired glucose-stimulated insulin secretion in isolated PKCδ−/− islets. a, abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis using total RNA extracted from wild type and PKCδ−/− islets. The amounts of the mRNAs in PKCδ−/− islets are expressed relative to those in wild type islets. Data are the mean ± S.E. of five independent experiments for each group. b, abundance of the indicated proteins was determined by Western blotting analysis. The amounts in PKCδ−/− islets are expressed relative to those in wild type islets. Data are the mean ± S.E. of five independent experiments for each group. KO, knock out. c, insulin secretion induced by various insulinotropic agents in batch-incubated pancreatic islets of wild type (open bars) and PKCδ−/− mice (solid bars) at the age of 12 weeks. Data were derived from at least six independent experiments for each group. Data are the means ± S.E. §, p < 0.05 versus wild type. PMA, phorbol 12-myristate 13-acetate. IRI, immunoreactive insulin. d, insulin secretory responses to glucose in perfused pancreatic islets of wild type (open circles) and PKCδ−/− (solid circles) islets. Data were obtained from each of five independent experiments for wild type and PKCδ−/− islets. §, p < 0.05 versus wild type. e, the area under the curve (AUC) of the first phase (0–2 min after stimulation with high glucose) and second phase of insulin secretion (2–20 min after stimulation by high glucose) for wild type (open bars) and PKCδ−/− (solid bars) islets. Data are the mean ± S.E. of five independent experiments for wild type and PKCδ−/− islets. §, p < 0.05 versus wild type.

Reduced Phosphorylation of Munc18-1 in PKCδ−/− Islets—PKCδ is a serine/threonine kinase; thus, a decreased phosphorylation state of target molecules might be involved in impaired insulin secretion in PKCδ−/− islets. Among the molecules involved in exocytosis, VAMP2, synaptotagmin 3 and 7, Rab3a, SNAP25, and Munc18-1 (20, 38, 42–45) have been identified as substrates of PKCs. Previous data demonstrated that Ser residue at position 189 of SNAP-25, a known PKC phosphorylation site, is phosphorylated in islets in response to glucose. However, this phosphorylation does not seem to be related to glucose-sensitive insulin secretion (46). On the other hand, Munc18-1 was reported to bind to syntaxin-1A to inhibit the formation of SNARE complex (19). The PKC-catalyzed phosphorylation of Munc18-1 inhibits its interaction with syntaxin-1A (20); thus, the reduced phosphorylation state might be the underlying mechanism of decreased insulin secretion. However, the association of Munc18-1 and PKCδ in β cells has not been studied.

Thus, we investigated first the interaction between PKCδ and Munc18-1. Although there are no data on the exact PKC isoform(s) that can bind to Munc18-1, we demonstrated that PKCδ directly bound with Munc18-1 by in vitro pulldown assay (Fig. 6a). In addition, co-immunoprecipitation experiments revealed that PKCδ tightly bound to Munc18-1 (Fig. 6, b and c) in HIT cells and mouse islets. This interaction was not altered by glucose stimulation and was not observed in PKCδ−/− islets (Fig. 6c).

Next, we investigated the phosphorylation of Munc18-1 by glucose stimulation. In HIT cells, 30 min of stimulation with high glucose concentration increased the phosphorylation of Munc18-1. The increase in Munc18-1 phosphorylation level was suppressed by rottlerin (Fig. 7a). Furthermore, time-series studies showed that the increase in Munc18-1 phosphorylation...
to high glucose concentration (data not shown). In addition, we also found that isolated PKCδ−/− islets secreted lower amounts of insulin in response to KCl and arginine. Thus, the activation of PKCδ by the metabolic change is not likely to be involved in the mechanism of glucose-stimulated insulin release. Instead, PKCδ might be activated by the stimulation of exocytosis by various insulin secretagogues and forms an essential component of the regulated insulin exocytosis. A very recent study reported the unique structural feature of PKCδ among the members of PKC regarding with the independence of its activity from activation loop phosphorylation (50). Such unique structure might be involved in the manner of the activation.

Recently, Carpenter et al. (7) reported that overexpression of kinase dead PKCδ in rat islets did not affect glucose-stimulated insulin secretion assessed by batch incubation study. The conflict with our experiments might be due to several reasons. First, species differences might explain this finding. It is possible that PKCδ could play a nonspecific role in insulin secretion in mice but not in rats. Second, it is also possible that deletion of PKCδ could have deleterious effects on the proper formation of β cells during the developmental stage. However, we found that rotterin, a PKCδ inhibitor, also decreased glucose-stimulated insulin secretion in wild type mice (data not shown). In addition, we did not find obvious morphological changes in PKCδ knock-out islets using light and electron microscopy. Thus, it is unlikely that PKCδ plays major roles in islet formation during the differentiation of β cells. Third, the adenoviral infection used in the previous study might change the natural properties of glucose-stimulated insulin secretion through certain factors such as adenovirus-activated cytokines (51). Fourth, although we used a model of complete disruption of PKCδ gene, infection with a dominant negative adenovirus approach often does not completely suppress the activity of PKCδ. Differences in suppression level might explain the discrepancy.

We observed that Ca2+ influx after glucose stimulation was not altered in PKCδ null β cells. In addition, the number of insulin granules located close to plasma membrane in PKCδ null β cells was not decreased but, rather, increased compared with wild type β cells. The increased docked insulin granules might represent the increase in granular backup in trafficking. These results suggest that the decreased insulin release in PKCδ null islets occurs mainly after the process of docking of insulin granules to the plasma membrane. Thus, we investigated the phosphorylation state of molecules involved in exocytosis. The SNARE proteins play a major role in intracellular membrane fusion events. An additional family of proteins that acts as SNARE regulators, the Sec1/Munc18 proteins, is also an essential component of the fusion machinery (19). Previous data demonstrated that Munc-18-1 protein is present in HIT cells and functions as a negative regulator of the insulin secretory machinery via a mechanism that does not involve syntaxin-associated calcium channels (52). In adrenal chromaffin cells, Munc-18-1 is phosphorylated on Ser-313 by phorbol 12-myristate 13-acetate treatment. The phosphorylated Munc-18-1 reduces its affinity for syntaxin (42). Thus, this event allows syntaxin to be released into an open conformation and associate into the SNARE complex. Although the involvement of Munc18-1 in insulin secretion has not been elucidated in islets,
it is likely that a similar mechanism is also involved in the process of insulin secretion. Although HIT cells do not always represent the normal machinery of insulin secretion, a strong association between Munc18-1 and PKC/H9254 was observed in our study. This interaction was not affected by glucose concentration. On the other hand, high glucose stimulation increased the phosphorylation of Munc18-1 within 5 min, which was suppressed by rottlerin. Because the amount of phosphoproteins harvested from mouse islets is limited, we could not investigate the phosphorylation state of Munc18-1 in islets in detail. However, we found 2.5-fold increase in Munc18-1 induced by high glucose. This increase was markedly suppressed in PKC/H9254/H11002 islets. Thus, it seems that PKC/H9254 null islets have impaired phosphorylation of Munc18-1. Considering the phenotype of PKC/H9254/H11002 islets, our data suggest the importance of Munc18-1 as a substrate of PKC/H9254 in the fusion event of insulin-containing granules. However, there are no data on the importance of the phosphorylation status of Munc18-1 in insulin secretion in islets. Thus, further assessment will be needed to complete our understanding of this process.

Regulated exocytosis is a process of the fine-tuning of secretion in response to external stimuli. Other than molecules investigated in this study, recent data demonstrate the implication of several molecules such as Noc2 (53), collectrin (54), and granuphilin (55) in regulated exocytosis in HIT cells. Whereas in this study we suggested the implication of Munc18-1 as a target molecule of PKC/H9254, it is also possible that PKC/H9254 modifies such molecules and, thus, is involved in insulin secretion.

FIGURE 5. Increased insulin granules docked to the plasma membrane in PKC/H9254/KO cells. a, typical TIRF images of docked insulin granules in wild type and PKC/H9254/KO cells. Cells were fixed with paraformaldehyde, then immunostained for insulin. Scale bars = 5 μm. b, number of granules morphologically docked to the plasma membrane (per 200 μm²) in wild type and PKC/H9254/KO cells by TIRF images (n = 12 cells, each). §, p < 0.05 versus wild type. c, relative number of insulin granules categorized according to the distance from the granule center to the plasma membrane. Data are expressed as percent distribution below the plasma membrane (100% corresponds to total counts of insulin granules that resided within 500 nm of the plasma membrane) (n = 9 cells each). §, p < 0.05 versus wild type. d, abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis using total RNA extracted from wild type and PKC/H9254/KO islets. The amounts of the mRNAs in PKC/H9254/KO islets are expressed relative to those in wild type islets. Data were obtained from five independent experiments for each group. Data are the mean ± S.E.

FIGURE 6. PKC/H9254 binds to Munc18-1 in HIT cells and islets. a, [35S]methionine-labeled PKC/H9254 were incubated with His6 or His6 fusion Munc18-1 protein in interaction buffer. After washing, the samples were applied to SDS-PAGE with 50% amount of [35S]methionine-labeled PKC/H9254 used in a pulldown assay. b and c, after 30 min of starvation, HIT cells (b) and mouse islets (c) were incubated in KRS with the indicated glucose concentration at 37.0 °C for 60 min. Then 1 mg of sonicated cell lysates was used for each immunoprecipitation (IP) using anti-PKC/H9254 antibody. The samples were immunoblotted (IB) by anti-Munc18-1 antibody. Data are obtained from four independent experiments for each group.
In conclusion, our data clearly demonstrated that PKCδ is an essential player in glucose-stimulated insulin secretion. Impaired glucose-stimulated insulin release is considered one of the possible genetic mechanisms of type 2 diabetes mellitus. Thus, it is important to identify each player involved in the mechanism of glucose-stimulated insulin release. Accumulation of such knowledge will finally contribute to isolation of candidate genes of type 2 diabetes and drug targets for its treatment.

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