Glucose-dependent exocytosis of insulin requires activation of protein kinase C (PKC). However, because of the great variety of isoforms and their ubiquitous distribution within the β-cell, it is difficult to predict the importance of a particular isoform and its mode of action. Previous data revealed that two PKC isoforms (α and ε) translocate to membranes in response to glucose (Zaitzev, S. V., Efendic, S., Arkhammar, P., Bertorello, A. M., and Berggren, P. O. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9712-9716). Using confocal microscopy, we have now established that in response to glucose, PKC-ε but not PKC-α associates with insulin granules and that green fluorescent protein-tagged PKC-ε changes its distri-
bution within the cell periphery upon stimulation of β-cells with glucose. Definite evidence of PKC-ε require-
ment during insulin granule exocytosis was obtained by using a dominant negative mutant of this isoform. The presence of this mutant abolished glucose-induced insulin secretion, whereas transient expression of the wild-
type PKC-ε led to a significant increase in insulin exo-
cytosis. These results suggest that association of PKC-ε with insulin granule membranes represents an impor-
tant component of the secretory network because it is essential for insulin exocytosis in response to glucose.

In pancreatic β-cells, glucose metabolism generates a great variety of intracellular signals that in concert promote insulin exocytosis (for review see Refs. 1 and 2). There are other insulin secretagogues; however, their mode of action occasionally involves the regulation of signaling molecules that are different from the ones regulated in response to glucose. Among those signals, particular attention has been focused on the role of protein kinase C (PKC) because it is known that protein phosphorylation/dephosphorylation can rapidly affect the function of a given protein or signaling molecule in response to a given agonist (3, 4). Definite proof of the role that PKC may have in the process of insulin exocytosis has been difficult to obtain, primarily because of the great diversity of PKC isoforms, the lack of specific inhibitors, and the use of different experimental designs and cell models to study their functions (for review see Ref. 5).

Previous attempts to establish the identity of the PKC iso-
forms that are responsive to glucose in pancreatic β-cells, although complex, indicated that the α and ε isoforms are likely candidates (6, 7). Moreover, cell fractionation assays of intact islets exposed to high glucose confirm that both PKC-α and PKC-ε isoforms indeed translocate to membranes, an event that was even fast enough to coincide with the initial phase of insulin secretion (8). Using antibodies against specific PKC isoforms and selective anchoring peptides that block their ac-
tivation, it was possible to assess the role of different isoforms during glucose-stimulated insulin secretion (9). Although the results of that study (9) further supported a pivotal role of PKC-α and PKC-ε by their translocation to the plasma membrane upon glucose stimulation, it failed to accurately identify the targets of the different PKCs within β-cell membranes. Furthermore, functional studies are lacking that examine the relevance of those isoforms at early time points, matching glucose metabolism and early phases of insulin exocytosis. The present study was designed to examine whether PKC-α and PKC-ε associate with insulin granule membranes and thereby translocate to the plasma membrane during insulin exocytosis and to determine the relevance of such an event for insulin release.

EXPERIMENTAL PROCEDURES

Materials—The cDNAs for PKC-ε and PKC-βI were kindly provided by Dr. Y. Nishizuka (Kobe University, Kobe, Japan). A dominant nega-
tive mutant of PKC-ε was obtained from Dr. K. Ridge (Northwestern University, Chicago, IL). INS-IE cells were obtained from Dr. C. B Wollheim (University Medical Center, Geneva, Switzerland).

Isolation of Pancreatic β-Cells from ob/ob Mice—Pancreatic β-cells were isolated from ob/ob mice. Mice were obtained from a locally bred colony at the animal facility within the Karolinska Hospital, Stockholm, Sweden. The animals (starved overnight) were decapitated, the pancreas was excised from the abdominal cavity and pancreatic islets, and β-cells were isolated after collagenase digestion as previously described (10, 11). The cells were kept overnight in RPMI 1640 medium supple-
mented with 11 mM glucose, 10% fetal calf serum, 100 μg/ml strepto-
mycin, and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO2 in air.

Plasmid Construction—To generate pRCMViPKC-ε and pRCMViPKC-βI, both PKC-ε and PKC-βI cDNA were subcloned into a pRCMVi backbone plasmid, i.e. pRC/CMV (Invitrogen) shortened by BamHI digestion and religation (12). To obtain pRCMViPKC-ε-GFP, we first subcloned the cDNA for the humanized and enhanced version of the GFP gene into pRCMVi. Thereafter, the PKC-ε fragment lacking the stop codon obtained by digesting with EcoRI was sub-
cloned in-frame in front of the GFP gene following digestion of
PCMV-GFP with EcoRV and EcoRI. To obtain pCMV-PCK-
βI-GFP, we first introduced a ClaI site in front of the stop codon of
PKC-βI, using the QuickChange site-directed mutagenesis kit (Strat-
agene, La Jolla, CA), which then allowed in-frame fusion with the GFP
gene via ClaI and XhoI sites. All vector constructions were verified by
DNA sequence analysis.

Culturing of INS-1E Cells—INS-1E cells were cultured in RPMI
1640 medium containing 11 mM glucose and supplemented with 10%
fetal bovine serum, penicillin/streptomycin (100 IU/ml and 100
μg/ml, respectively), 2 mM glutamine, 50 mM sodium pyruvate, 0.5 M HEPES,
and 50 mM 2-mercaptoethanol in a 5% CO2 incubator at 37
°C.

Cell Transfection—Transfection of ob/ob and INS-1E cells was per-
formed by lipofection using LipofectAMINE (Invitrogen). For transfec-
tion, 1 × 106 cells were treated with ~3 μg of DNA and LipofectAMINE
overnight according to the manufacturer’s instructions. Following
transfection, cells were cultured in RPMI 1640 medium supplemented
as described for ob/ob and INS-1 cells, respectively, at 37°C for at least
24 h. All experiments were performed 1 day after transfection.

Confocal Microscopy—Primary cultures of ob/ob β-cells were grown
on glass coverslips and incubated in the presence of either 3 or 16 mM
glucose for 3 min at room temperature. The incubation was terminated
by fixation of the cells with 4% formaldehyde in phosphate-buffered
saline for 10 min at room temperature, as previously described (13).

After rinsing twice with phosphate-buffered saline, β-cells were incu-
bated for 5 min in acetone at −20°C and then quenched with phos-
phate-buffered saline containing 1% bovine serum albumin for 30 min.

Staining with primary antibodies against insulin (BioGenex, San
Ramón, CA) and against PKC-α and PKC-ε (Santa Cruz Biotechnology,
Santa Cruz, CA) was performed at room temperature for 1 h. Detection
of insulin and of PKC-α and PKC-ε was performed by incubation with
anti-mouse Oregon green-labeled and anti-rabbit rhodamine-labeled
secondary antibodies (Molecular Probes, Eugene, OR), respectively,
for 1 h at room temperature. After rinsing with phosphate-buffered saline,
the coverslips were mounted (SlowFade light; Molecular Probes) and
examined using a confocal laser scanning microscope (Leica TCS NT;
Leica Lasertechnik GmbH, Heidelberg, Germany). Excitation wave-
lengths of 488 nm for Oregon green and 568 nm for rhodamine were
used. The confocal microscope was equipped with an Ar/Kr laser, a
double dichroic mirror for rhodamine/fluorescein and a ×63 objective
(Leica PL APO 63×/1.32–0.6 oil). Analysis of the data was performed
with IMARIS and COLOCALISATION software (Bitplane, Zurich,
Switzerland).

Fluorescence Imaging in Living Cells—INS-1E cells expressing either
PKC-ε-GFP or PKC-βI-GFP were grown on 24-mm glass coverslips
in RPMI 1640 medium supplemented as described under “Culturing of
INS-1E Cells.” The medium was changed to low glucose (2 mM) RPMI
1640 medium two hours before the experiment. For fluorescence imag-
ing, the coverslip was placed in a perfusion chamber and mounted on
an inverted fluorescence microscope Leica DMRB (Leica Lasertechnik
GmbH). The cells were maintained throughout the experiment in low
glucose RPMI 1640 at 37 °C. The fluorescence of GFP-tagged PKCs was
monitored by confocal laser scanning fluorescence microscopy, using an
argon/krypton laser at 488 nm (Leica TCS NT; Leica Lasertechnik
GmbH). The following filter settings were used: dichroic mirror TK 500,
emission filter BP525/50, with ×63 (Leica PL APO ×63/1.32–0.6 oil)
and ×100 (Leica PL APO ×100/1.40–0.7 oil) objectives.

Analysis of Translocation Kinetics—To analyze translocation kinet-
icS, cells were imaged at 4 frames/min for 3 min, and glucose (16 mM)
was added as a puff immediately after the first frame was recorded.

Sequences of GFP images were processed using the Leica Confocal
software v.2.437. Analysis of moving structures between successive
frames was done using the subtraction function of the three-dimen-
sional module of the Leica Confocal Software v.2.437. The running
subtraction shown is the actual mathematical subtraction performed on
the corresponding pixels (pixels with identical locations) of two succes-
sive images. Thus, the program subtracts the pixel value in an image
from the corresponding pixel in the previous frame, canceling out those
pixels with identical values and showing only those structures that
either move or experience a change in fluorescence intensity in the
second image.

Determination of β-Cell Capacitance—Pipettes were pulled from
borosilicate glass, coated with Sylgard near their tips, and fire polished.

When filled with the pipette solution, the electrodes had a resistance
of 3–4 MΩ. Exocytosis was measured as changes in cell capacitance using
an EPC-9 patch clamp amplifier and pulse software (version 8.30;
HEKA Elektronik, Lambrecht/Pfalz, Germany) (11). The interval be-
tween two successive points was 0.2 s. The measurements were initi-
ted <5 s after establishment of the standard whole-cell configuration.
The extracellular solution consisted of (in mM) 138 NaCl, 5.6 KCl, 2.6
CaCl2, 1.2 MgCl2, 5 HEPES (pH 7.4 with NaOH), and 5 glucose.

The pipette solution consisted of (in mM) 125 potassium glutamate, 10 KCl
bodies to evaluate a possible co-localization of PKC-α included confocal microscopy and PKC isoform-specific antibodies to molecules to the periphery of INS-1E cells. This fast translocation was directed to selected areas of the membrane and seemed to peak at ~45 s after glucose stimulation of the cells and to decrease thereafter until a second association event can be observed occurring at ~2 min (Fig. 3) and in conjunction with changes in membrane morphology. This effect of glucose on PKC-ε motion was not exclusively observed in INS-1E cells; it also occurred in an insulin-secreting cell line (MIN6) of mouse origin (not shown).

To further prove the specificity of this response, we transfected INS-1E cells with a fluorescent construct (PKC-β-GFP) of a PKC isoform not implicated in glucose-induced insulin secretion (PKC-βI). As predicted, the distribution of PKC-βI-GFP remained unchanged in response to 16 mM glucose (up移至胰岛细胞膜，故推测PKC-ε在胰岛细胞膜内移位，可能与胰岛素的分泌有关。

RESULTS AND DISCUSSION

Previous experiments (6–9) demonstrated that glucose promotes the association of two distinct PKC isoforms (PKC-α and PKC-ε) with membranes obtained from pancreatic islets. However, those experiments did not reveal the identity of the membranes to which these isoforms translocate. Therefore, we examined whether PKC-α and PKC-ε associate with insulin granule membranes in response to glucose, thereby playing an important regulatory role in insulin exocytosis. Our approach included confocal microscopy and PKC isoform-specific antibodies to evaluate a possible co-localization of PKC-α and/or PKC-ε with insulin granules in a glucose-dependent manner. Primary ob/ob β-cells grown on glass coverslips were incubated with either 3 or 16 mM glucose for 5 min. At submaximal glucose concentrations (3 mM), insulin granules (green) and PKC-ε (red) are depicted as punctuated structures of homogeneous distribution in scans along the surface of the cell (Fig. 1a). Incubation with 16 mM glucose, however, promoted the co-localization of PKC-ε and insulin granules, revealed by the yellow intensity seen in overlay images and co-localization analysis of the data. Middle scans of the same β-cells (Fig. 1b) indicated the polarized distribution of the co-localization between PKC-ε and insulin granules in response to 16 mM glucose, most prominent in the periphery of the β-cell. By contrast, using the same strategy as above it was found that 16 mM glucose did not promote the co-localization of PKC-α with insulin granules (Fig. 2, a and b). Taken together, these results indicate that although the PKC-ε isoform translocates to membranes, the structures are likely to be part of the insulin-containing granules, whereas the PKC-α isoform might indeed translocate to the plasma membranes or to membranes of other intracellular organelles.

To study the real-time changes in PKC-ε distribution in response to high glucose, we developed a fluorescent form of the kinase by adding a GFP tag to its C-terminal region. The presence of the tag did not affect its ability to translocate or to phosphorylate histone (not shown). An insulin-secreting cell line derived from rat insulinoma (INS-1E) was transiently transfected with the vector containing the PKC-ε-GFP cDNA, and the cells were analyzed using a laser scanning confocal microscope (Fig. 3). To effectively study the movement of PKC molecules, the cells were maintained in low glucose (2 mM) RPMI 1640 medium for 2 h prior to the experiment. The cells were imaged at 4 frames/min over a period of 2.5 min, and glucose stimulation was achieved by raising the sugar concentration in the microscope chamber to 16 mM immediately after the first frame was recorded. To maximize the detection of moving fluorescent structures, we performed a running subtraction of the successive frames taken after glucose was introduced to the microscope chamber. This subtraction cancels out stationary structures to reveal only moving elements within the picture (16). The analysis of a representative xy subtracted image shows the tracks followed by GFP-tagged PKC-ε molecules (Fig. 3). Glucose promoted a rapid translocation of PKC-ε molecules to the periphery of INS-1E cells. This fast translocation was directed to selected areas of the membrane and seemed to peak at ~45 s after glucose stimulation of the cells and to decrease thereafter until a second association event can be observed occurring at ~2 min (Fig. 3) and in conjunction with changes in membrane morphology. This effect of glucose on PKC-ε motion was not exclusively observed in INS-1E cells; it also occurred in an insulin-secreting cell line (MIN6) of mouse origin (not shown).

To further prove the specificity of this response, we transfected INS-1E cells with a fluorescent construct (PKC-β-GFP) of a PKC isoform not implicated in glucose-induced insulin secretion (PKC-βI). As predicted, the distribution of PKC-βI-GFP remained unchanged in response to 16 mM glucose (up
successive frames of a movie taken at 15 s intervals. Glucose (16 mM) was added as a puff to the microscope chamber immediately after the first intracellular dialysis with a Ca\(^{2+}\) negative mutant. Increases in cell capacitance occurring during transfected with either the PKC-\(\varepsilon\)-isoform during exocytosis was further studied. Although inhibitors of different PKC isoform groups have been described, the lack of specific isoenzyme inhibitors makes it difficult to assess with certainty their specific role in diverse cellular functions. Therefore, we took advantage of the use of dominant negative mutants. A mutant of PKC-\(\varepsilon\) was constructed in which a single amino acid mutation (Arg \(\rightarrow\) Lys) was introduced in its catalytic domain that abolished the ATP binding ability (18). Efficient transient transfection of these constructs has been established previously (18).

The role of PKC-\(\varepsilon\) on insulin secretion was evaluated by measuring membrane capacitance in INS-1E cells transiently transfected with either the PKC-\(\varepsilon\) wild-type or a dominant negative mutant. Increases in cell capacitance occurring during intracellular dialysis with a Ca\(^{2+}\) buffer containing 0.2 \(\mu\)M [Ca\(^{2+}\)]\(_{i}\) are illustrated in Fig. 5. Under control conditions, exocytosis was observed as a gradual capacitance increase that reached a new steady-state level within 2–4 min. It is clear that the exocytotic response was stimulated in cells transfected with the wild-type isoform of PKC-\(\varepsilon\), whereas the dominant negative PKC-\(\varepsilon\) isoform significantly reduced the rate of exocytosis (Fig. 5a). On average (Fig. 5b), wild-type PKC-\(\varepsilon\) evoked a 202\% stimulation of the exocytotic rate \((p < 0.01; n = 5)\), whereas the dominant negative mutant of PKC-\(\varepsilon\) induced a reduction of 58\% \((p < 0.05; n = 5)\). Transient expression of the PKC-\(\beta\)-isoform did not influence exocytosis (Fig. 5). The latter was anticipated because this isoform did not translocate in response to glucose (Fig. 4).

We have previously demonstrated that sulfonylureas stimulate insulin exocytosis in pancreatic \(\beta\)-cells in a PKC-dependent manner (19). This also applies to INS-1E cells, as suggested by the 3-fold stimulation \((p < 0.01; n = 10)\) of exocytosis following inclusion of 100 nM glibenclamide in the pipette-filling solution (Fig. 5). As expected, glibenclamide produced a dose-dependent increase in exocytosis in INS-1E cells (Table I). Thus, the involvement of PKC-\(\varepsilon\) in the secretory pathway evoked by glibenclamide was evaluated through the use of the dominant negative mutant PKC-\(\varepsilon\). The effect of glibenclamide (100 nM) was significantly increased in wild-type PKC-\(\varepsilon\)-transfected cells as the combination of glibenclamide and wild-type PKC-\(\varepsilon\) produced an additive increment of cell capacitance (Table I).

Interestingly, cell capacitance was significantly reduced to almost control levels in cells expressing the dominant negative form of the PKC-\(\varepsilon\) isomer (74\% inhibition; \(p < 0.01; n = 5)\), (Table I). The degree of stimulation in cell capacitance
Exocytosis Requires PKC-ε Association with Insulin Granules

Figure 5. Exocytosis of insulin in β-cells transiently expressing PKC-ε. a, increases in cell capacitance elicited by intracellular infusion with a Ca²⁺-EGTA buffer with a free Ca²⁺ concentration of 0.2 μM in non-transfected INS-1E cells in the absence (control) or the presence of 100 nM glibenclamide or in INS-1E cells transfected with either wild-type PKC-ε (PKC-ε wt), dominant negative PKC-ε form (PKC-ε DN), or the wild-type PKC-β isoform (PKC-β wt). Increases in cell capacitance were observed during the first 2 min after establishment of the standard whole-cell configuration. Throughout the recordings, the cells were clamped at −70 mV in order to avoid activation of the voltage-dependent Ca²⁺ channels that would otherwise interfere with the measurements. b, histograms depicting average rates of increases in cell capacitance in non-transfected cells in the absence (control) or the presence of 100 nM glibenclamide or in cells transfected with the wild-type (PKC-ε wt) or the dominant negative form (PKC-ε DN) of PKC-ε and in cells expressing PKC-β wt or wild-type PKC-ε plus 100 nM glibenclamide (PKC-ε wt + gli). Data are mean values ± S.E. of 5–20 different cells. *p < 0.01.

Table 1

| Treatment                  | Cell capacitance (pF/s) |
|----------------------------|-------------------------|
| None                       | 4.7 ± 0.5 (n = 14)      |
| PKC-ε wt                   | 14.1 ± 1.6 (n = 5)      |
| 10⁻⁷ M Gli                 | 17.7 ± 0.7 (n = 10)     |
| 10⁻⁶ M Gli                 | 28.3 ± 1.7 (n = 5)      |
| 10⁻⁵ M Gli                 | 27.9 ± 2.2 (n = 5)      |
| 10⁻⁷ M Gli + PKC-ε wt      | 27.8 ± 2.1 (n = 5)      |
| 10⁻⁶ M Gli + PKC-ε wt      | 28.9 ± 2.1 (n = 6)      |
| 10⁻⁵ M Gli + PKC-ε DN      | 7.3 ± 1.3 (n = 7)       |
| 10⁻⁶ M Gli + PKC-ε DN      | 6.5 ± 1.7 (n = 5)       |
| PKC-β wt                   | 5.0 ± 0.4 (n = 20)      |
| 10⁻⁷ M Gli + PKC-β wt      | 16.7 ± 0.7 (n = 15)     |

* p < 0.01 versus untreated cells.
† p < 0.01 versus 10⁻⁷ M Gli + PKC-ε wt.
‡ p < 0.01 versus 10⁻⁶ M Gli + PKC-ε wt.
§ p < 0.01 versus PKC-β wt.

induced by glibenclamide was not affected by the PKC-β isoform, again suggesting a specific response mediated by the PKC-ε isoform (Table 1). A recent work reported the translocation of the PKC-β isoform to the plasma membrane and intracellular organelles, suggested to be mature insulin granules, in response to glucose (20). Although that report eloquently demonstrates the motion of the βII isoform in living cells, the functional significance of such motion (potential targets) remains to be addressed. The results of the present study show for the first time that PKC-ε associates with insulin granules in response to glucose, thereby indicating a specific role of PKC-ε in insulin secretion in pancreatic β-cells. Our data also indicate that glibenclamide promotes insulin secretion in INS-1E cells by a mechanism of action that involves PKC-ε. Thus, the interaction of PKC-ε with the insulin granule membrane appears to be a mechanism not only associated with glucose-induced insulin secretion but also with other secretagogues such as sulfonylureas, because these compounds directly stimulate insulin exocytosis in a PKC-dependent manner (19) apart from their known action on ATP-dependent K⁺ channels. The molecular mechanisms for the association of PKC-ε and insulin granule membranes as well as the potential PKC-ε targets within that membrane remain to be explored.

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