# Immunocytochemical Localization of α-Protein Kinase C in Rat Pancreatic β-Cells during Glucose-induced Insulin Secretion

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***Abstract.*** To investigate the role of protein kinase C (PKC) in the regulation of insulin secretion, we visualized changes in the intracellular localization of α-PKC in fixed β-cells from both isolated rat pancreatic islets and the pancreas of awake unstressed rats during glucose-induced insulin secretion. Isolated, perifused rat islets were fixed in 4% paraformaldehyde, detergent permeabilized, and labeled with a mAb specific for α-PKC. The labeling was visualized by confocal immunofluorescent microscopy. In isolated rat pancreatic islets perifused with 2.75 mM glucose, α-PKC immunostaining was primarily cytoplasmic in distribution throughout the β-cells. In islets stimulated with 20 mM glucose, there was a significant redistribution of α-PKC to the cell periphery. This glucose-induced redistribution was abolished when either mannoheptulose, an inhibitor of glucose metabolism, or nitrendipine, an inhibitor of calcium influx, were added to the perifusate. We also examined changes in the intracellular distribution of α-PKC in the β-cells of awake, unstressed rats that were given an intravenous infusion of glucose. Immunocytochemical analysis of pancreatic sections from these rats demonstrated a glucose-induced translocation of α-PKC to the cell periphery of the β-cells. These results demonstrate that the metabolism of glucose can induce the redistribution of α-PKC to the cell periphery of β-cells, both in isolated islets and in the intact animal, and suggest that α-PKC plays a role in mediating glucose-induced insulin secretion.

GLUCOSE is one of the major physiologic regulators of insulin secretion from the β-cells of the pancreatic islets of Langerhans, with increases in ambient glucose leading to a biphasic pattern of insulin release. Glucose is an unusual secretagogue in that there is no membrane receptor responsible for transmembrane signal transduction. Instead the intracellular metabolism of glucose leads to the activation of several second messenger systems and subsequent secretion of insulin (Zawalich and Rasmussen, 1990; Prentki and Matshinsky, 1987).

As extracellular glucose concentrations rise, high capacity glucose transporters in the beta-cell plasma membrane allow the rapid entry of glucose into the cell, where it is rapidly metabolized. This increase in glucose metabolism leads to several intracellular events including: (*a*) depolarization of cell membrane; (*b*) influx of calcium through voltage-dependent $Ca^{2+}$ channels; (*c*) accumulation of cAMP; (*d*) the hydrolysis of membrane phosphatidylinositides (PI)[1] to produce diacylglycerol (DAG) and inositol-triphosphates; and (*e*) the possible de novo synthesis of DAG from glycolytic intermediates (Zawalich and Rasmussen, 1990; Prentki and Matshinsky, 1987; Peter Riesch et al., 1988; Wollheim and Sharp, 1981). The production of membrane DAG, either from PI hydrolysis or de novo synthesis, together with the increase in $Ca^{2+}$ influx can, in principle, activate the protein kinase C (PKC) family of enzymes (Nishizuka, 1986).

Protein kinase C is a family of calcium and phospholipid-dependent kinases which have a widespread tissue distribution and are thought to play a crucial role in calcium-dependent cellular activation in a variety of cell systems (Nishizuka, 1986, 1988). The α-isoenzyme of PKC has been shown to be present in β-cells of pancreatic islets (Onoda et al., 1990), and has been shown to be activated when islets are stimulated with non-fuel secretagogues such as carbachol and cholecystokinin (Persaud et al., 1991; Karlsson and Ahr'en, 1991; Easom et al., 1990). These agonists are thought to work through classic receptor-coupled G-protein to activate PI-specific phospholipase C, leading to DAG production and PKC activation. However, the role of PKC during glucose-induced insulin secretion remains a matter of controversy. We have recently reported that in freshly isolated, perifused rat pancreatic islets, glucose-induced insulin secretion is associated with a marked translocation of α-PKC from the cytosol to the membrane fraction of islet homog-

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1. *Abbreviations used in this paper*: DAG, diacylglycerol; PI, phosphatidylinositide; PKC, protein kinase C.

enates (Ganesan et al., 1990). Inhibition of PKC activity with a low concentration (20 nM) of the PKC inhibitor staurosporine can markedly decrease the sustained insulin secretory response produced by high glucose concentrations (Zawalich et al., 1991). These studies suggest that in freshly isolated islets PKC is involved in glucose-induced insulin secretion. However, several groups have been unable to detect either translocation of PKC activity from cytosolic to membrane fractions (a widely used marker of PKC activation), or an increase in the phosphorylation of the 80-kD putative PKC substrate protein during glucose stimulation in cultured rat pancreatic islets (Easom et al., 1990; Persaud et al., 1989; Easom et al., 1989). Hence there is as yet no consensus as to whether or not fuel agonists such as glucose can induce the translocation and activation of PKC in β-cells (Metz, 1988;Wollheim and Regazzi, 1990).

To further examine the effect of glucose metabolism on PKC in β-cells, we have examined the intracellular localization of α-PKC in β-cells by confocal immunofluorescence microscopy using specific mAbs to α-PKC. These studies were done both in freshly isolated perifused pancreatic islets and in awake, unstressed rats given a glucose infusion. We have been able to demonstrate that α-PKC is redistributed from the cytoplasm to the cell periphery of β-cells during glucose-induced insulin secretion, both in isolated islets and in the intact animal. This redistribution is dependent upon both the metabolism of glucose and the glucose-dependent increase in calcium influx. These results lend further support for the hypothesis that the intracellular metabolism of fuel molecules such as glucose can lead to the activation of PKC in β-cells.

## Materials and Methods

### Islet Isolation and Perifusion

Islets were isolated from anesthetized male Sprague-Dawley rats weighing between 260–360 g that were fed ad libatum. Briefly, the rats were anaesthetized with pentobarbital (50 mg/kg), the bile duct was cannulated, and the pancreas was inflated with ~30 ml of ice-cold Hank's solution. The pancreas was removed by blunt dissection, minced with scissors, and digested with collagenase (1.15 mg/ml Collagenase P; Boehringer-Mannheim Biochemicals, Indianapolis, IN). Islets that were free of exocrine tissue contamination were manually picked under a stereomicroscope with a 23-gauge needle. Groups of 30–50 islets were loaded onto nylon filters and perifused at a flow rate of 1 ml/min with bicarbonate-buffered media containing 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 0.17% BSA, 24 mM $NaHCO_3$, gassed with 95% $O_2$/5% $CO_2$, pH 7.4. The islets were perifused for 30 min with 2.75 mM glucose to establish stable basal insulin secretory rates, and were then perifused with medium supplemented with an appropriate agonist. In control islets the perifusion was continued with 2.75 mM glucose alone. Serial samples of the perifusate were collected for insulin analysis by RIA (Albana et al., 1972) using rat insulin as a standard.

At the end of perifusion, the islets were removed from the perifusion chamber and immediately immersed in ice-cold 4% paraformaldehyde in PBS (100 mM NaCl, 50 mM sodium phosphate, pH 7.4) for 30 min. The islets were washed with ice-cold PBS, and transferred to gelatin-coated glass slides, air dried, and stored at −20°C until processed for immunocytochemistry.

### In Vivo Glucose Infusion

Indwelling catheters were placed in the internal jugular vein and common carotid arteries of male Sprague-Dawley rats, weighing between 300–350 g, under pentobarbital anesthesia (50 mg/kg body weight). The right internal jugular catheter was inserted and advanced to the right atrium and the left carotid catheter was inserted and advanced to the level of the aortic arch. The catheters were filled with a heparin/polyvinylpyrrolidone solution, sealed, and tunneled subcutaneously to the back of the neck, and externalized through a skin incision. After a minimum of a 4-d recovery period, the animals were fasted overnight for 16–20 h, and the indwelling catheters were opened. Catheter patency was maintained with an infusion of a heparin (18 U/ml)-saline solution. The catheters were positioned so as to allow free movement of the rat in the cage throughout the study. After a 30–60-min equilibration period, an infusion of a 25% dextrose solution was begun through the arterial line. The rate of infusion was adjusted to rapidly achieve and maintain a plasma glucose concentration of 300–350 mg/100 ml. Plasma glucose was measured in venous samples by the glucose oxidase method using a glucose analyzer (Beckman Instruments, Inc., Palo Alto, CA). In animals treated with glucose + mannoheptulose, 25% dextrose and 1.38 M mannoheptulose were infused in parallel, with the rate of infusion adjusted to achieve plasma glucose concentration of 300–350 mg/100 ml. Control animals were infused with saline alone. The rate of saline infusion in control rats was adjusted to be similar to the rate of glucose infusion in the rats made hyperglycemic, so that similar volumes were delivered. Venous blood was sampled every 5 min to measure plasma glucose, and aliquots of plasma were saved for determination of plasma insulin concentration by RIA using rat insulin as a standard (Albana et al., 1972). After 30 min of infusion, the animal was quickly rendered unconscious by an intravenous bolus of pentobarbitol. The mediastinum was rapidly exposed, a 16-gauge needle was introduced into the left ventricle, and the right atrium was severed. The animal was perfused through the intracardiac needle, first with 75 ml of ice-cold Hank's solution and then with at least 250 ml of ice-cold 4% paraformaldehyde in PBS to achieve rapid tissue fixation. The fluids were perfused at a pressure of 90–100 mm Hg. The pancreas was removed and immersed in ice-cold 4% paraformaldehyde/PBS for an additional 45 min. The pancreas was then rinsed with PBS and cryoprotected by an overnight incubation in 20% sucrose at 4°C. The pancreas was then cut into small pieces suitable for mounting and 20-μm sections were cut on a cryostat and placed on gelatin-coated microscope slides. These sections were air dried and stored at −20°C until they were processed for immunocytochemistry.

### Immunoblotting

Groups of 80–100 isolated rat pancreatic islets were sonicated in 200 μl of ice-cold homogenization buffer (20 mM Tris, pH 7.4, 0.5 mM EGTA, 50 μM leupeptin, 1 mM PMSF, 0.1% BME, 25 μg/ml aprotinin, 10 μM pepstatin A) and concentrated to 20 μl by agitation under a vacuum (Vortex Evaporator, Buchler Instruments, Fort Lee, NJ). These samples were separated using 10% SDS-PAGE gels and electroblotted onto Immobilon-P membranes (Millipore Corp., Bedford, MA). The membranes were successively incubated with blocking buffer containing 150 mM NaCl, 3% BSA, 0.1% Tween-20, 10 mM $Na_2PO_4$, pH 7.2, for 30 min at room temperature; with PKC isozyme-specific mAb at a final concentration of 2 μg/ml (in incubation buffer containing 10 mM $Na_2PO_4$, pH 7.2, 100 mM NaCl, 0.1% Tween-20, and 0.1% BSA) for 3 h at room temperature; and with $^{125}$I-protein A in incubation buffer (0.5 μCi/ml) for 30 min. The membranes were thoroughly washed after each incubation with a buffer containing 100 mM NaCl, 0.1% Tween-20, 10 mM Tris, pH 7.4. The membranes were then dried and the immunoreactive bands visualized by autoradiography.

Rat brain cytosol was prepared as follows: the brain was dissected from an anesthetized male Sprague-Dawley rat and was rapidly homogenized in 7.2 ml of buffer containing 20 mM Tris, pH 7.4, 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 10 mM β-mercaptoethanol (BME), 50 μM leupeptin, and 1 mM PMSF. The homogenate was centrifuged at 100,000 *g* for 45 min and the supernatant was collected as rat brain cytosol.

The purified α-PKC was prepared as described previously from rat brain cytosol (Ganesan et al., 1990), with final separation of PKC isozymes performed by hydroxyl-apatite high performance liquid chromatography.

### Immunocytochemistry

The samples (either fixed isolated islets or fixed pancreatic sections, prepared as described above) were rehydrated with PBS for 15 min, and then permeabilized by incubating for 15 min in PBS + 0.3% Triton X-100 (PBS-TX). Aldehyde activity was quenched by incubating for 15 min in PBS-TX + 50 mM ammonium chloride. After rinsing thoroughly with PBS, the samples were incubated for 15 min with 1% BSA to block nonspecific binding sites and then incubated with primary antibody diluted in PBS with 0.5% BSA and 0.15% Triton X-100 for at least 2 h at room temperature. The PKC mAbs were purchased from Seikagaku America, Inc., and used

at a final concentration of 5 μg/ml. The anti-insulin antibodies were purchased from Accurate Labs, and used at 1:100 dilution. After rinsing thoroughly with PBS-TX, the samples were incubated with secondary antibody (FITC-conjugated anti-mouse IgG from Sigma Chemical Co. [St. Louis, MO] used at 1:100, Texas-Red conjugated anti-mouse IgG from Molecular Probes, Inc. [Eugene, OR] used at 1:100, FITC-conjugated anti-guinea pig IgG from Sigma Chemical Co. used at 1:100) for at least 1 h at room temperature, rinsed with PBS-TX, and then mounted with 0.1% *p*-phenyldiamine in 75% glycerol in distilled $H_2O$ under a glass coverslip. In double-labeling experiments, sections were incubated first with both primary antibodies and then with both secondary antibodies simultaneously. In the preadsorption experiments, the dilute α-PKC mAb (at a final concentration of 5 μg/ml) was incubated with purified α-PKC (final concentration of ~1 μg/ml) for 30 min at room temperature, and then applied to the samples.

### *Confocal Microscopy*

A Biorad MRC 500 confocal imaging system (Bio-Rad Laboratories, Palo Alto, CA) using an argon laser was used to obtain thin optical slices through the fixed, labeled tissue. Images presented here were obtained at sufficient depth in the individual islets so that the β-cell core is well visualized. Peripheral slices of the islets were dominated by non-β-cells that constitute the mantle.

At least 5–10 islets from each of at least four separate islet preparations were examined for each experimental condition. The results were reproduced by four separate investigators and multiple experiments were done with the observers of the confocal microscopy being blinded to the perifusion conditions of the islets being imaged to avoid observer bias. The patterns of labeling were consistent from cell to cell within the islet, although the total signal intensity could vary across a section as one focused deeper into a given islet. This is probably due to decreasing antibody penetration as one images deeper into the tissue.

Quantitation of signal intensity in peri-plasma membrane and cytosolic regions was done through the use of AREA functions in the MRC software. Peri-plasma membrane and cytoplasmic (excluding the nucleus) areas were outlined manually and the calculated mean fluorescent intensity was obtained for the delineated regions. Optical sections used for quantitation were at sufficient depth in the islets such that the β-cells were the predominant cells in the sections. Images obtained were normalized (using BASE and SCALE functions) before analysis. Only in cells which had a clearly discernible nucleus was quantitation of α-PKC distribution carried out. The peri-plasma membrane regions outlined were ~1–1.5 μm wide. In control (unstimulated) islets, it was difficult to discern peri-plasma membrane regions. Areas of linear staining were used as guides to approximate the peri-plasma membrane region of these cells. Data are presented as the ratio of mean fluorescent intensity in the peri-plasma membrane region to the mean fluorescent intensity of the cytoplasmic region. Results from 10–15 cells from three to five separate isolets from each of at least three separate experiments were averaged for each condition tested. Statistics were done using unpaired two-tailed *t*-test.

## *Results*

### *Immunoblotting of Pancreatic Islets with Monoclonal PKC Antibodies*

The mouse mAbs specific for the α-, β-, and γ-isoenzymes of PKC have been well characterized and used as specific probes to examine the intracellular distribution of PKC in several cell systems (Hidaka et al., 1988; Ito et al., 1988). As shown in Fig. 1, these mAbs all recognized an 80-kD band in rat brain cytosol that is consistent with the molecular weight of PKC. Western blots of homogenized isolated rat pancreatic islets that were probed with these mAbs revealed that the α-PKC antibody detected a single band of 80 kD which comigrates with purified α-PKC. The β-PKC mAb detected a single 80-kD band of low intensity. No bands were visualized in homogenates of pancreatic islets by γ-PKC mAbs. These results indicate that these PKC mAbs are suitable as specific probes for immunocytochemical localization of PKC isoenzymes in pancreatic islets.

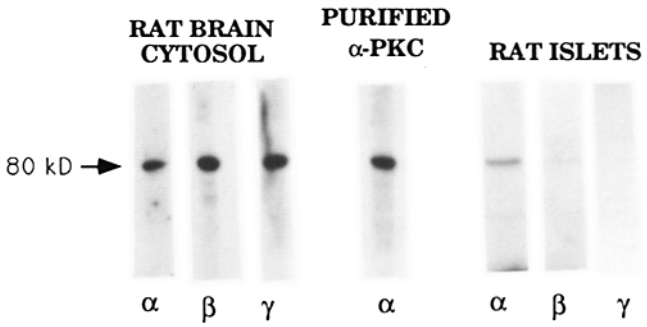



*Figure 1.* Western blots of rat brain cyotsol and homogenized rat pancreatic islets probed with isoenzyme-specific mouse mAbs to PKC. Samples of rat brain cytosol, homogenized rat pancreatic islets, or purified α-PKC were run on SDS/8% polyacrylamide gels, and electroblotted onto Immobilon-P membranes. The membranes were incubated first with mAbs to the α-, β-, or γ-PKC (lanes α, β, and γ, respectively) and then incubated with $^{125}$I-protein A. The immunoreactive bands were visualized by autoradiography.

### *Localization of α-PKC to the β-Cells of Isolated Rat Pancreatic Islets*

Isolated rat pancreatic islets that had been perifused with substimulatory glucose concentrations (2.75 mM) were fixed in 4% paraformaldehyde, detergent-permeabilized, labeled with the α-PKC mAb and a fluorescently tagged secondary antibody, and the staining was visualized by immunofluorescent confocal microscopy. These islets showed an intense, predominantly cytoplasmic staining of most of the cells in the core of the islet (Fig. 2 *A*). A layer of cells in the periphery of the islets is unlabeled (data not shown). This pattern of staining is consistent with the α-PKC being localized to the β-cell of pancreatic islets. Double-labeling experiments using a polyclonal antibody to insulin as well as the α-PKC mAb reveal that both antibodies label the same population of cells in the islet, confirming that α-PKC immunoreactivity is localized to the β-cells (Fig. 2, *A* and *D*). To ensure that the apparent colabeling was not artifactual, it was confirmed that labeling of islets with either the insulin or the α-PKC antibody alone gave a pattern of labeling similar to that seen by that same antibody in the double-labeling experiment (data not shown). When the α-PKC mAb was preincubated with purified α-PKC, the labeling of cells in the pancreatic islets was greatly reduced, indicating that the antibody binding is specific for α-PKC (Fig. 2 *E*). Preincubation of α-PKC mAb with unrelated proteins did not affect labeling of the islets (data not shown). Isolated rat pancreatic islets incubated with β-PKC mAb display intense labeling of a small population of cells located mainly in the periphery of the islet (Fig. 2 *B*). The anatomical localization of these cells is consistent with the localization of the glucagon secreting α-cells of the pancreatic islet. The β-PKC mAb also labels occasional cells scattered throughout the islet (data not shown). The identity of these cells is unclear but they may be nonendocrine mesenchymal cells or endothelial cells. There is no labeling of any cells in the islet with the γ-PKC mAb (Fig. 2 *C*). These results are consistent with previous studies showing that α-PKC is localized to β-cells and β-PKC is localized to the α-cells of pancreatic islets (Onoda et al., 1990).

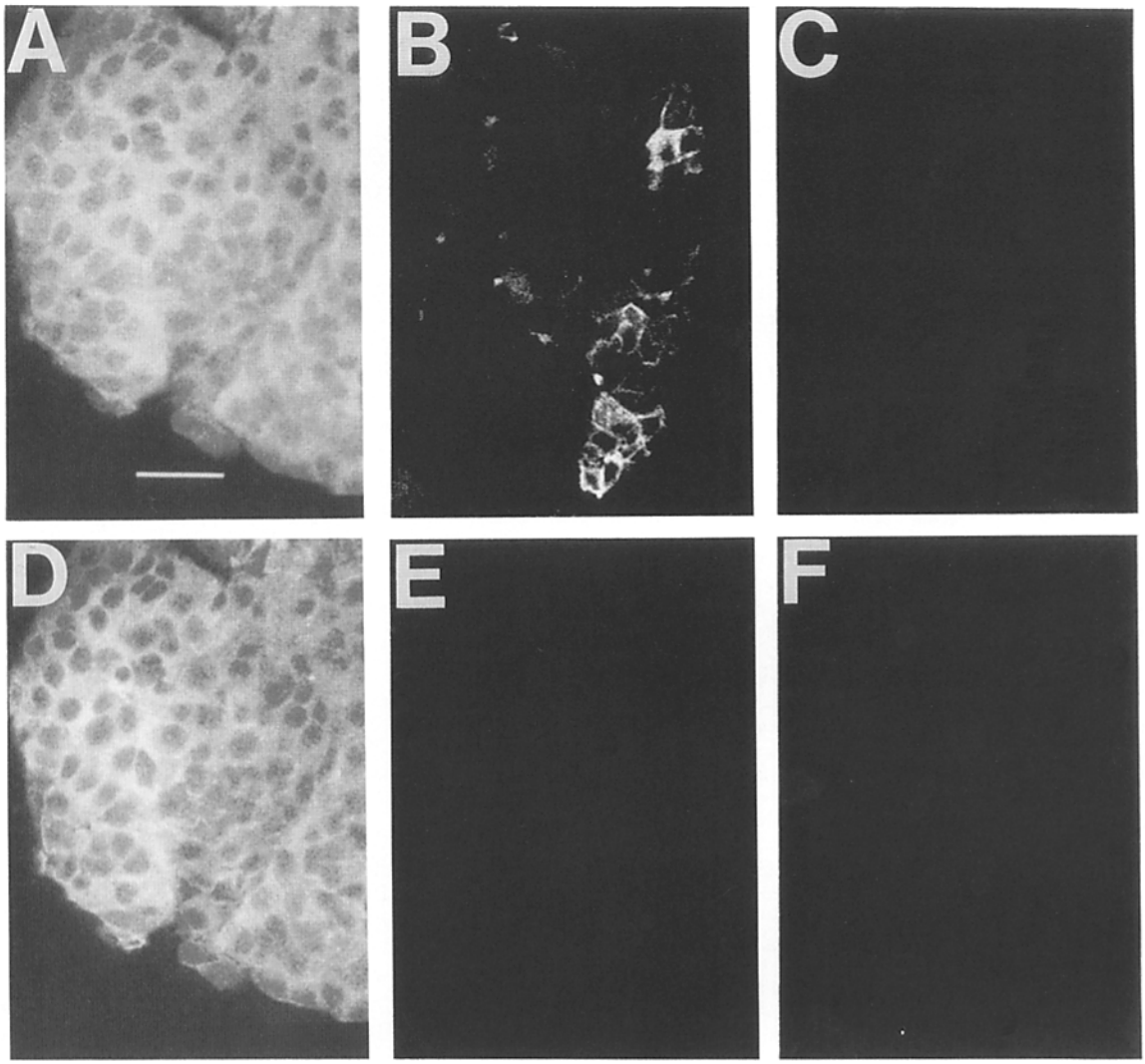
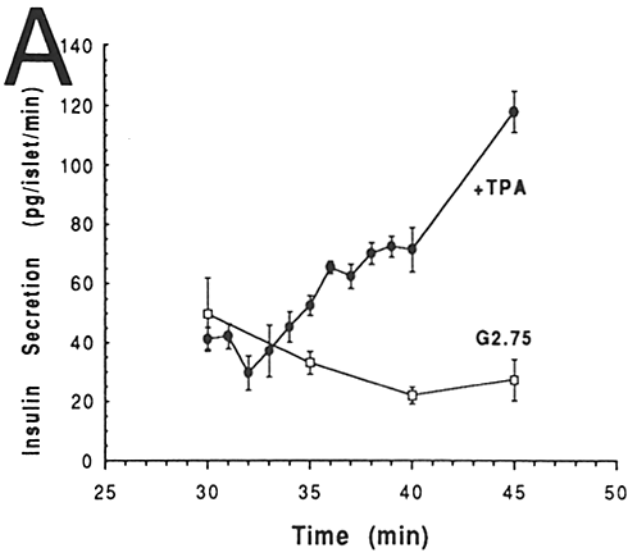
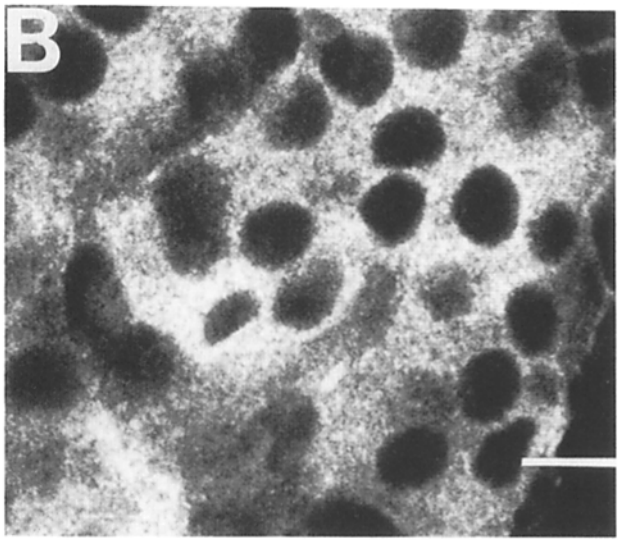



*Figure 2.* Immunofluorescence localization of α-, β-, and γ-PKC in rat pancreatic islets. Freshly isolated rat pancreatic islets were perifused in media supplemented with 2.75 mM glucose, fixed in 4% paraformaldehyde, detergent permeabilized, and labeled with mAbs specific to α-PKC (*A*), β-PKC (*B*), and γ-PKC (*C*). The labeling was visualized by confocal immunofluorescent microscopy. *D* shows the same field as *A*, labeled with a polyclonal anti-insulin antibody. *E* shows labeling of an islet with the mAb to α-PKC that had been preincubated with an excess of purified α-PKC. *F* shows a negative control in which the islet was not exposed to the primary antibody. All images shown represent optical slices taken at sufficient depth in the isolated islets such that the majority of cells visualized should be β-cells. Bar, 25 μM.

### *Intracellular Localization of α-PKC in Pancreatic Islets Stimulated with a Phorbol Ester*

Phorbol esters are pharmacologic activators of PKC that cause the membrane association and catalytic activation of PKC (Nishizuka, 1986). To ensure that our immunocytochemical technique could detect significant changes in the intracellular distribution of α-PKC, we examined α-PKC localization in control and phorbol ester–stimulated islets. Islets that were perifused with bicarbonate-buffered medium supplemented with 2.75 mM glucose alone showed stable baseline rates of insulin secretion. At the end of the perifusion, the islets were rapidly fixed by immersion in 4% paraformaldehyde, and were processed for immunocytochemistry with a mouse mAb specific for α-PKC. Visualization of the α-PKC signal by confocal immunofluorescent microscopy showed that the unstimulated islets had a diffusely cytoplasmic distribution of the α-PKC signal (Fig. 3 *B*). Islets that were perifused for 15 min with bicarbonate-buffered medium supplemented with 2.75 mM glucose + 200 nM of the phorbol ester TPA showed a slowly rising pattern of insulin secretion (Fig. 3 *A*). Confocal immunofluorescent microscopy using an α-PKC mAb showed that there was marked redistribution of the α-PKC immunoreactivity to the cell periphery of the β-cells of the islets that were perifused with TPA (Fig. 3, *A* and *B*). This redistribution of α-PKC was consistent in cells throughout a given islet (although the intensity of overall labeling decreased as one imaged the center of the islets secondary to decreased antibody penetration) and was consistent between different islets from the same rat and between different islet preparations. There was no increase in α-PKC immunoreactivity either in the nucleus or the perinuclear region. Quantitative analysis was performed by calculating the ratio of the mean fluorescent intensity of a peri-plasma membrane region to the mean fluorescent intensity of the cytoplasmic region in groups of β-cells. This analysis revealed that there was a significant increase in the peri-plasma membrane α-PKC signal in the β-cells of islets



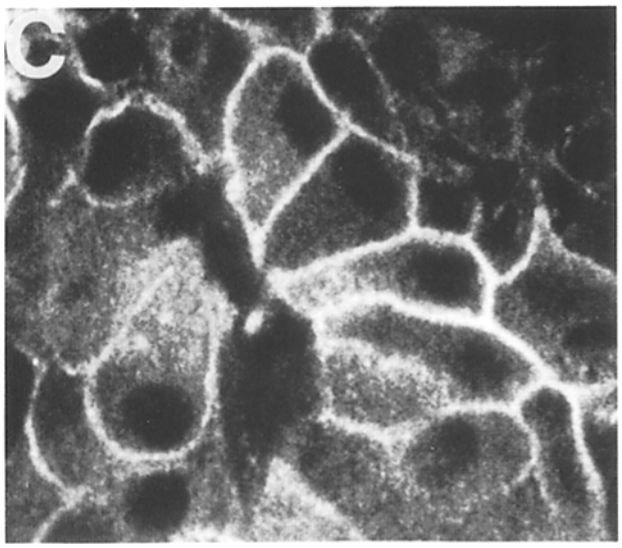




*Figure 3.* Confocal immunofluorescence localization of α-PKC in pancreatic islets stimulated with phorbol ester. Freshly isolated pancreatic islets were initially perifused for 30 min with a bicarbonate-buffered media supplemented with 2.75 mM glucose, and then perifused for an additional 15 min with media supplemented with either 2.75 mM glucose + 200 nM TPA or with 2.75 mM glucose alone. At the end of the perifusion, the islets were immersed in 4% paraformaldehyde and processed for confocal immunofluorescence microscopy using the α-PKC mAb. (*A*) Insulin secretory responses of islets perifused with either 2.75 mM glucose + 100 nM TPA (●—●) or with 2.75 mM glucose alone (○—○). (*B* and *C*) Confocal immunofluorescent images of α-PKC labeling in islets perifused for 15 min with either 2.75 mM glucose (*B*) or with 2.75 mM glucose + 100 mM TPA (*C*). Bar, 10 μM.

stimulated with TPA as compared to control islets (see Fig. 8). These results also validate our immunocytochemical protocol as being sensitive enough to detect changes in the intracellular distribution of α-PKC.

### *Intracellular Localization of α-PKC in Pancreatic Islets Stimulated with Glucose*

Islets that were perifused with 2.75 mM glucose alone showed low stable rates of insulin secretion as well as a predominantly cytoplasmic pattern of α-PKC immunoreactivity (Fig. 4, *A* and *B*). Islets that were perifused for 15 min with either 10 or 20 mM glucose exhibited a biphasic insulin secretory response with a greater than 15- or 50-fold increase, respectively, in the secretory rate over control islets perifused with 2.75 mM glucose alone (Fig. 4 *A*). Rapid fixation and immunocytochemical analysis revealed that there was a marked redistribution of α-PKC immunoreactivity to the periphery in islets stimulated with either 10 or 20 mM glucose (Fig. 4, *C* and *D*). This redistribution was consistently observed in beta-cells throughout a given islet, and was consistent from islet to islet in a given preparation, and between islets from different preparations. This redistribution has been observed by four separate investigators in over 100 islets from over 10 separate islet preparations. Multiple experiments were also performed with the islet perifusion conditions being kept blinded from the observers of the islet immunocytochemistry to eliminate observer bias in the interpretation of the results. Quantitative analysis of the fluorescent images showed that there was a significant increase in the relative signal intensity in the peri-plasma membrane region of the islets stimulated with either 10 or 20 mM glucose as compared to control islets perifused with 2.75 mM glucose alone (see Fig. 8). There was also a significant increase in the relative peri-plasma membrane intensity of α-PKC labeling in islets stimulated with 20 mM glucose as compared to islets stimulated with 10 mM glucose (see Fig. 8).

There was maximal redistribution of α-PKC in response to 20 mM glucose as early as 5 min after stimulation (Fig. 5 *B*). (Time points earlier than 5 min after stimulation cannot be accurately assessed due to the dead space of the perifusion system.) When the perifusion with 20 mM glucose was extended to 30 min, the α-PKC redistribution was also maintained showing that α-PKC translocation persists as long as the high glucose stimulus is present (Fig. 5 *D*).

Double-labeling studies using both an antibody to insulin and the α-PKC mAb revealed that in islets perifused with 20 mM glucose for 15 min, the insulin labeling remained largely cytoplasmic while the α-PKC signal was largely redistributed to the cell periphery (Fig. 6).

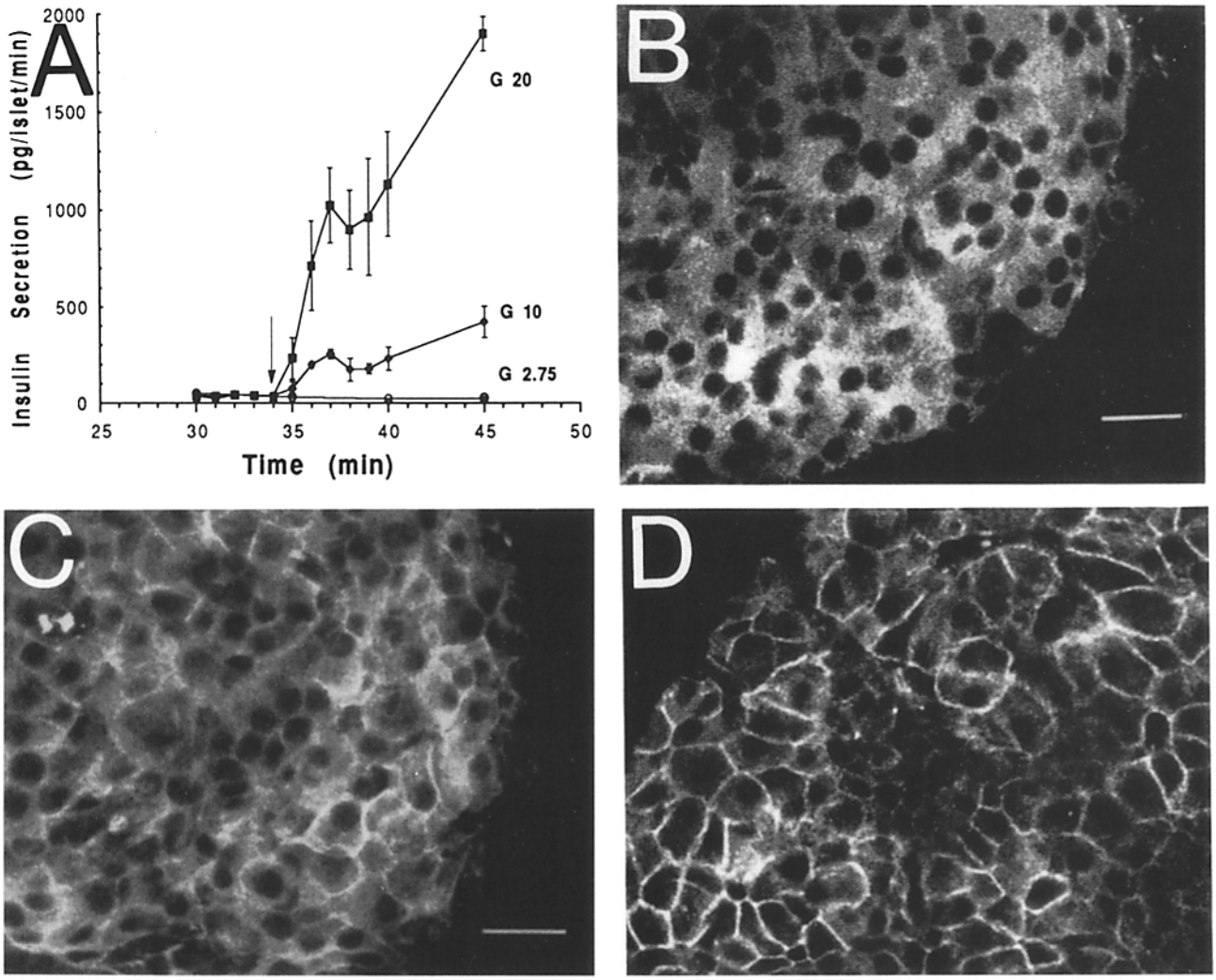



*Figure 4.* Confocal immunofluorescence localization of α-PKC in pancreatic islets perifused with stimulatory glucose concentrations. Freshly isolated rat pancreatic islets were initially perifused for 30 min in a bicarbonate-buffered media supplemented with 2.75 mM glucose, and then perifused for an additional 15 min with either 2.75 mM glucose, 10 MM glucose, or 20 mM glucose. At the end of the perifusion, the islets were processed for confocal immunofluorescence microscopy using the α-PKC mAb. (*A*) Insulin secretory responses of islets stimulated with either 2.75 mM glucose (○—○), 10 mM glucose (●—●), or 20 mM glucose (■—■). The arrow marks the beginning of the high glucose perifusion. (*B–D*) Confocal images showing α-PKC labeling in islets perifused for 15 min with 2.75 mM glucose (*B*), 10 mM glucose (*C*), or 20 mM glucose (*D*). Bar, 25 μM.

### *Inhibition of Glucose-induced Insulin Secretion and α-PKC Redistribution by Inhibiting either Glucose Metabolism or Calcium Influx*

Mannoheptulose has been established to be a potent and specific inhibitor of glucokinase activity (Coore and Randle, 1964; Matschinsky, 1990). Inhibition of glucokinase activity with mannoheptulose will prevent the increased glucose metabolism induced by elevated glucose levels, and thus prevent glucose-induced insulin secretion (Zawalich et al., 1978). To investigate whether the redistribution of α-PKC seen during glucose-induced insulin secretion is dependent upon the metabolism of glucose, we examined the intracellular distribution of α-PKC of islets exposed to high glucose concentrations in the presence of mannoheptulose. In islets perifused with 20 mM glucose + 30 mM mannoheptulose, both the biphasic insulin secretory response and the translocation of α-PKC immunoreactivity to the cell periphery seen in islets stimulated with 20 mM glucose were abolished (Fig. 7, *A–C*). Quantitative analysis showed no significant difference in the relative peri-plasma membrane intensity of the α-PKC signal in islets stimulated with 20 mM glucose + 30 mM mannoheptulose as compared with control islets perifused with 2.75 mM glucose alone (Fig. 8).

The metabolism of glucose during glucose-induced insulin secretion is known to cause an increase in calcium influx through L-type calcium channels in the β-cell. To investigate whether the redistribution of α-PKC that we observed during glucose-induced insulin secretion is dependent upon this increase in calcium influx, we examined the intracellular α-PKC distribution in glucose-stimulated islets in the presence of the calcium channel blocker nitrendipine. In islets perifused with 20 mM glucose + 500 nM nitrendipine both the biphasic insulin secretory response as well as the associ-

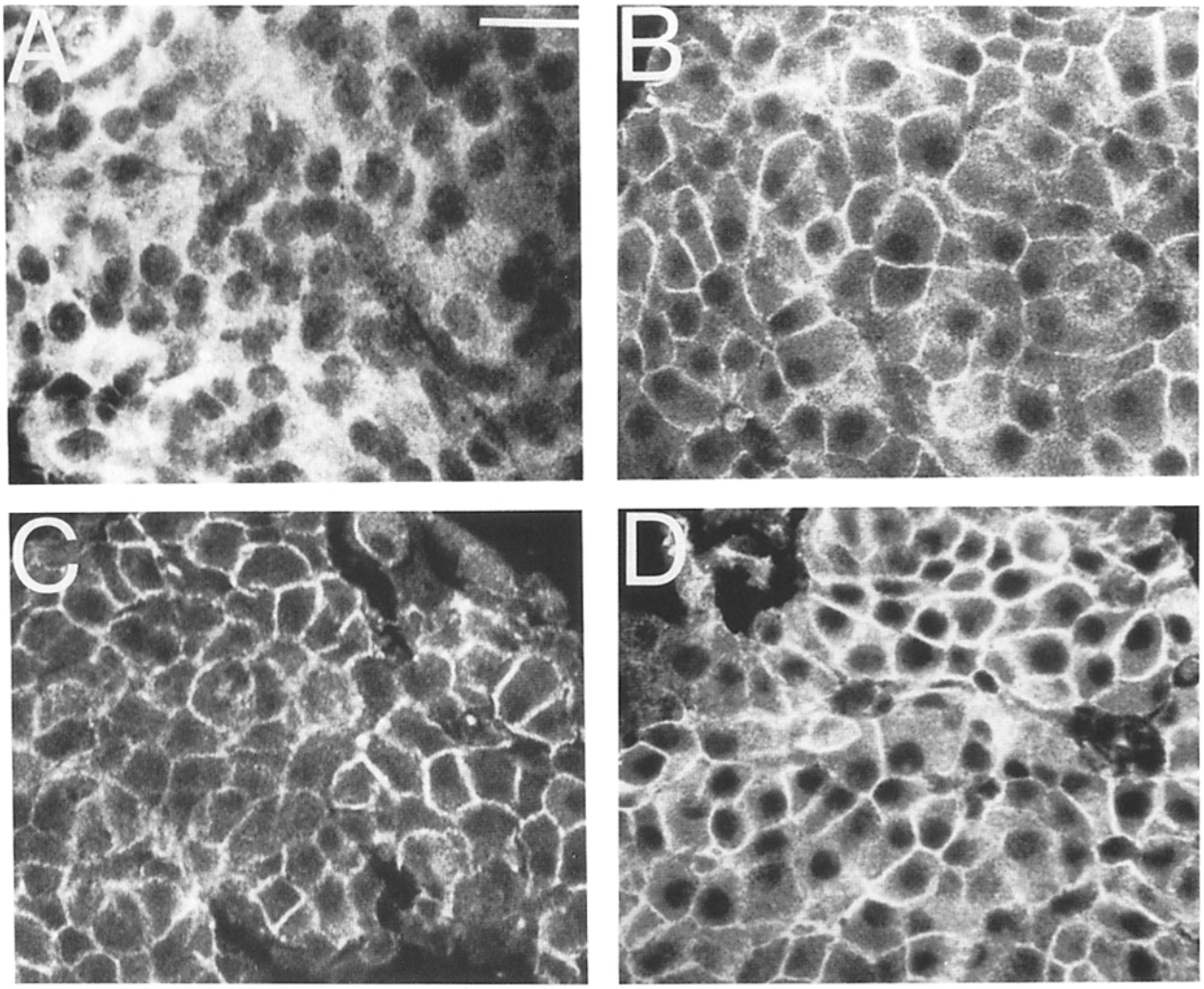



*Figure 5.* Time course of redistribution of α-PKC during glucose-induced insulin secretion. Freshly isolated rat pancreatic islets were initially perifused for 30 min in a bicarbonate-buffered media supplemented with 2.75 mM glucose, and then perifused with media supplemented with 20 mM glucose. After perifusion with 20 mM glucose for 5, 15, or 30 min, islets were immersed in 4% paraformaldehyde and processed for immunocytochemistry using the α-PKC mAb as described in Materials and Methods. (*A*) Confocal secretion showing immunofluorescence labeling of α-PKC in an islet perifused with 2.75 mM glucose for 30 min. (*B–D*) Confocal images of α-PKC labeling in islets perifused with 20 mM glucose for 5 (*B*), 15 (*C*), or 30 min (*D*). Time matched controls for these three time points that were perifused with 2.75 mM glucose demonstrated patterns of α-PKC labeling that were identical to that seen in *A* (not shown). Bar, 25 μm.

ation of α-PKC immunoreactivity with the plasma membrane were markedly reduced (Fig. 7, *A* and *D*). Quantitative analysis showed no significant difference in the relative periplasma membrane intensity of the α-PKC signal in islets exposed to 20 mM glucose + 500 nM nitrendipine as compared with islets perifused with 2.75 mM glucose alone (Fig. 8).

### *Intracellular Localization of α-PKC in the β-Cells of Awake Rats Given an Intravenous Infusion of Glucose*

To investigate whether the glucose-induced redistribution of α-PKC that we observed in isolated rat pancreatic islets was also seen in β-cells of intact animals made hyperglycemic, we examined the intracellular distribution of α-PKC in the β-cells of awake, unstressed rats given an intravenous infusion of glucose.

Adult Sprague-Dawley rats weighing between 250–350 g had chronic indwelling venous and arterial catheters placed. Rats that were then subjected to a 16–20-h overnight fast and infused with normal saline for 30 min showed both stable plasma glucose concentrations of 105–115 mg/dl (5–5.5 mM) and stable plasma insulin concentrations of 2.5–3.5 ng/ml (Fig. 9 *A*). The rats were then rapidly anaesthetized and perfused with 4% paraformaldehyde to achieve rapid tissue fixation. Immunocytochemical analysis of sections of fixed pancreata from these rats showed that the islets of Langerhans were intensely labeled with the α-PKC mAb (Fig. 9 *B*). In the β-cells, the α-PKC immunoreactivity was distributed mostly in the cytosol with little or no linear staining at the cell peripheries (Fig. 9 *B*).

Fasted rats that were given an arterial glucose infusion adjusted to rapidly achieve and maintain a plasma glucose concentration of 300–350 mg/dL (16.7–19.5 mM) exhibited a biphasic pattern of insulin secretion with mean plasma insulin levels of 19.2 ± 2.0 ng/ml after 30 min of glucose infusion (Fig. 9 *C*). Rapid fixation of the animal tissues and im-

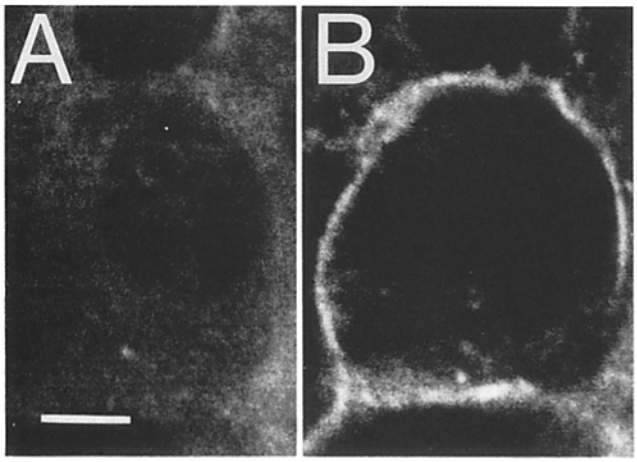



*Figure 6.* Localization of insulin and α-PKC in glucose-stimulated islets. Freshly isolated rat pancreatic islets were initially perifused for 30 min in a bicarbonate-buffered media supplemented with 2.75 mM glucose, and then perifused for an additional 15 min with media supplemented with 20 mM glucose. After the perifusion the islets were immersed in 4% paraformaldehyde and processed for immunocytochemistry, double-labeling with both an anti-insulin antibody and the α-PKC mAb. Confocal images of the insulin labeling (*A*) and the α-PKC labeling (*B*) are shown for a representative β-cell. Bar, 5 μM.

munocytochemical analysis of the pancreas from these animals showed that there was a redistribution of α-PKC to the periphery of β-cells as seen by a marked increase in linear staining at the periphery of these cells (Fig. 9 *D*). These experiments were performed in at least three separate animals and identical results were obtained each time. Quantitation of the fluorescent images revealed a significant increase in the relative fluorescent intensity of the peri-plasma membrane region in β-cells from rats infused with glucose as compared to that from rats infused with saline (the average ratio of mean fluorescent intensity of the peri-plasma membrane region to the mean fluorescent intensity of the cytosolic region was 1.06 ± 0.03 in β-cells from control rats as opposed to 1.43 ± 0.07 in β-cells from rats given a glucose infusion, $P < 0.005$).

When fasted rats were infused with equimolar quantities of glucose and mannoheptulose, with the rate of infusion adjusted to rapidly achieve and maintain a plasma glucose concentration of 300–350 mg/dL (16.7–19.4 mM), the insulin secretory response normally induced by these elevated plasma glucose concentrations was abolished (data not shown). Immunocytochemical analysis of the pancreata of these rats revealed that there was no redistribution of α-PKC in the β-cells, with the α-PKC remaining predominantly cytoplasmic (data not shown).

## *Discussion*

We have demonstrated that the intracellular metabolism of glucose can induce the translocation of α-PKC from the cytosol to the cell periphery in β-cells of the pancreatic islets of Langerhans during glucose-induced insulin secretion, both in isolated rat pancreatic islets and in intact animals (Figs. 4 and 9). These data are consistent with our earlier observation that glucose metabolism can induce the translocation of α-PKC from a soluble (cytosolic) to a particulate (membrane) fraction as assessed by Western blotting of subcellular fractions of freshly isolated, perifused rat islets (Ganesan et al., 1990). These results establish that PKC can be translocated by certain agonists through mechanisms that do not require activation of a transmembrane receptor. We have also demonstrated that this redistribution of α-PKC during glucose-induced insulin secretion is dependent upon both glucose metabolism and calcium influx (Figs. 7 and 8). These results are in agreement with earlier studies showing that the hydrolysis of phosphatidylinositides stimulated during glucose-induced insulin secretion is dependent upon both the metabolism of glucose and the influx of calcium (Zawalich and Zawalich, 1988). Given these results, it is reasonable to propose the following chain of events as linking glucose metabolism to α-PKC redistribution: as glucose is metabolized, ATP levels rise leading to inhibition of ATP-sensitive $K^+$ channels (Ashcroft et al., 1973; Arkhammar et al., 1987), cell membrane depolarization, and influx of calcium through voltage-gated L-type calcium channels (Cook et al., 1988). This increased calcium influx leads to activation of the $Ca^{2+}$-dependent PI-specific PLC, with the subsequent generation of IP3 and DAG (Peter Riesch et al., 1988; Zawalich and Zawalich, 1988; Best, 1986; Turk et al., 1986). Additional DAG may be generated by de novo synthesis from glycolytic intermediates (Peter Riesch et al., 1988). The combination of increased $Ca^{2+}$ influx and increased DAG in the plasma membrane induces the redistribution of α-PKC to the cell periphery.

The exact localization of α-PKC when it redistributes to the cell periphery remains unclear. One possibility is that α-PKC binds directly to the plasma membrane as has been demonstrated in some cell systems (Ito et al., 1988). Other possibilities include association with specific PKC receptor proteins (Mochly-Rosen et al., 1991), with cytoskeletal elements at the cell periphery (Jaken et al., 1989; Mochly-Rosen et al., 1990), or with marginated vesicles. Experiments in progress to localize α-PKC by immunocytochemistry at the electron microscope level may help differentiate among these possibilities. Also of note is the fact that phorbol esters and glucose both cause α-PKC to redistribute to the cell periphery in β-cells, but nuclear labeling is unchanged. It has been recently reported that phorbol esters can induce the translocation of α-PKC to the perinuclear region in NIH 3T3 cells (Leach et al., 1989). These differences in phorbol ester–induced redistribution of α-PKC suggest that there may be distinct mechanisms to target α-PKC to either the cell periphery, as in β-cells, or to the perinuclear region, as in NIH 3T3 cells. One possibility is that PKC receptor proteins are differentially distributed in different cell types, and thus dictate the localization of activated PKC. There is as yet no experimental evidence to support this hypothesis.

It is of note that the degree of redistribution to the cell periphery of α-PKC seen in β-cells in intact animals in response to an intravenous infusion of glucose, is less than that seen in freshly isolated islets perifused with similar high glucose concentrations. This difference is probably due to several factors. In the intact animal, the presence of other counter-regulatory neural and hormonal signals, e.g., epinephrine, may act upon the β-cell and cause a reduction in

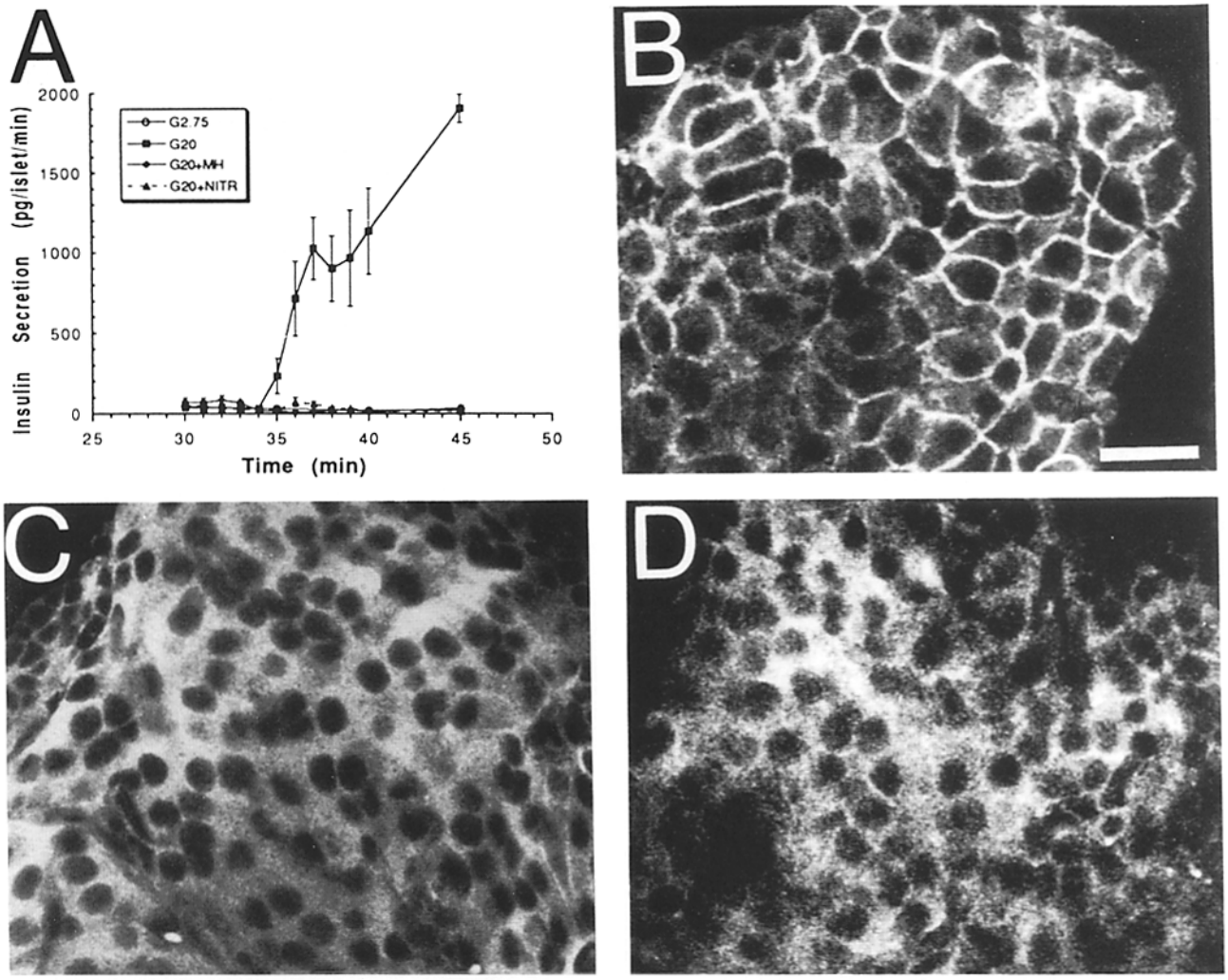



*Figure 7.* Effect of mannoheptulose or nitrendipine on glucose-induced redistribution of α-PKC. Freshly isolated rat pancreatic islets were initially perifused for 30 min in a bicarbonate-buffered media supplemented with 2.75 mM glucose, and then perifused for an additional 15 min with either 2.75 mM glucose, 20 mM glucose, 20 mM glucose + 30 mM mannoheptulose, or 20 mM glucose + 200 nM nitrendipine. At the end of the perifusion, the islets were immersed in 4% paraformaldehyde and processed for confocal immunofluorescence microscopy using the α-PKC mAb. (*A*) Insulin secretory response of islets perifused with 2.75 mM glucose (○—○), 20 mM glucose (■—■), 20 mM glucose + 30 mM mannoheptulose (◆—◆), or 20 mM glucose + 200 nM nitrendipine (▲- -▲). (*B–D*) Confocal images showing α-PKC labeling in islets perifused with 2.75 mM glucose (not shown), 20 mM glucose (*B*), 20 mM glucose + 30 mM mannoheptulose (*C*), or 20 mM glucose + 500 nM nitrendipine (*D*). Bar, 25 μM.

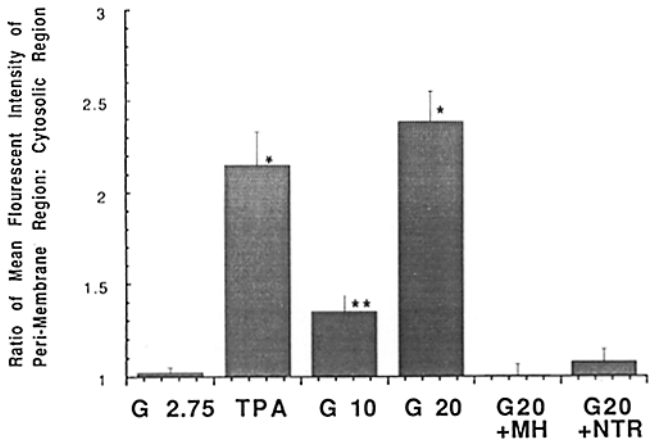



*Figure 8.* Quantitation of relative membrane-associated α-PKC immunofluorescence in isolated islets. Fluorescence images obtained on the BioRad MRC 500 Confocal Imaging System were quantitated using the AREA function. Peri-plasma membrane and cytosolic areas of labeled β-cells were manually outlined, and the mean fluorescent intensities of the delineated regions were calculated. These calculations were performed for at least 10–15 cells from each of three to five islets from at least three separate islet preparations for each of the conditions shown. Results are presented as the average ratio of the mean fluorescent intensity of the peri-plasma membrane region to the mean fluorescent intensity of the cytosolic regions for islets perifused for 15 min with 2.75 mM glucose (*G2.75*), G2.75 + 100 nM TPA (*TPA*), 10 mM glucose (*G10*), 20 mM glucose (*G20*), 20 mM glucose + 30 mM mannoheptulose (*G20 + MH*), 20 mM glucose + 500 nM nitrendipine (*G20 + NTR*). (*$P < 0.005$, **$P < 0.05$ when compared to G 2.75; $P < .005$ for G10 compared to G20.)

the extent of α-PKC translocation in response to glucose. Also α-PKC may dissociate from the membrane during the several minutes between the time when the animal is rendered unconscious and fixation of the tissues with parafor-

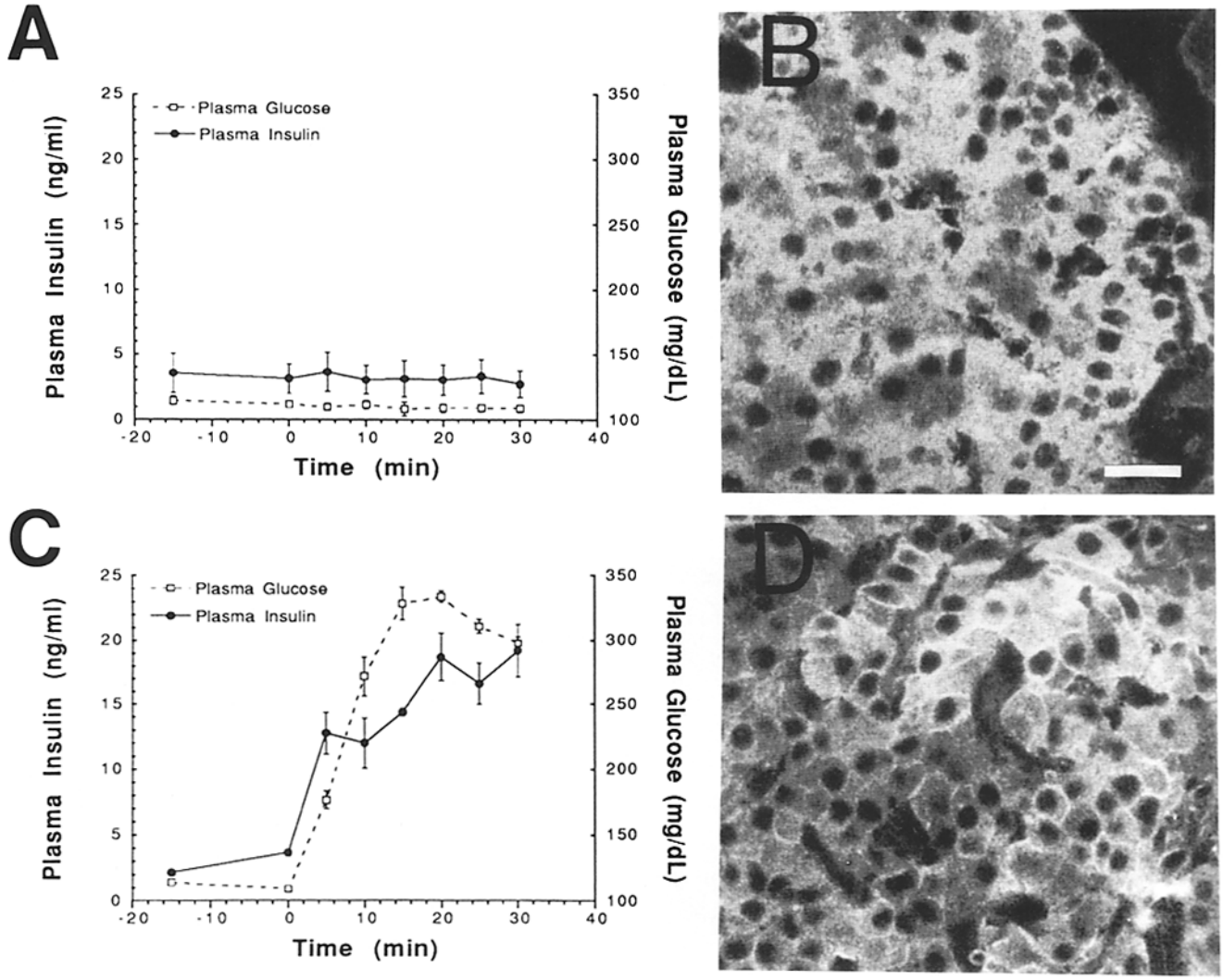



*Figure 9.* Immunofluorescence localization of α-PKC in β-cells during glucose-induced insulin secretion in awake rats. Rats in which indwelling venous and arterial catheters were placed were subjected to an overnight fast and given a 30-min infusion of either normal saline (*A* and *B*) or 25% glucose (*B* and *C*) as described in Materials and Methods. The glucose infusion was adjusted to rapidly achieve and maintain a plasma glucose concentration of 300–350 mg/100 dl (16.7–19.4 mM). Plasma glucose and insulin concentrations were measured every 5 min and are plotted in *A* and *C* (data shown is the mean of at least three experiments ± SE). At the end of the infusion, the animals were rapidly anesthetized and perfused with 4% paraformaldehyde. Sections of the pancreas were labeled with a mouse mAb specific for α-PKC and the labeling was visualized by immunofluorescent confocal microscopy. *B* and *D* are representative confocal sections showing α-PKC labeling in pancreatic sections taken from rats given an infusion of normal saline (*B*) or glucose (*D*). The experiments have been repeated at least three times for each condition with identical results. Bar, 25 μM.

maldehyde is achieved, thereby reducing the amount of plasma membrane associated α-PKC measured.

There was some heterogeneity in the degree of glucose-induced α-PKC translocation in different β-cells in a given islet, notable especially in the pancreatic slices from the intact animal (Figs. 4 and 9). One explanation for this observation is that the heterogeneity of α-PKC redistribution may reflect the intrinsic heterogeneity of the response of individual β-cells to glucose. This heterogeneity of β-cell response has been reported for glucose-induced insulin biosynthesis (Kiekens et al., 1992). Alternately, this apparent heterogeneity may simply reflect variable antibody penetration through the islet.

The data presented in this study also demonstrate that there is no simple correlation between the extent of α-PKC translocation and magnitude of insulin secretory rates induced by an agonist. Although glucose stimulates both insulin secretion and α-PKC translocation in a dose-dependent fashion, 100 nm TPA induces approximately the same amount of α-PKC translocation as 20 mM glucose, but stimulates insulin secretion to a far lesser degree (Figs. 3, 4, and 8). This difference in the effectiveness of glucose and TPA as secretagogues is probably due to the fact that unlike TPA, high glucose stimulates a variety of signal transduction events, such as the accumulation of cAMP and increases in calcium influx, that can act synergistically with PKC to stimulate insulin secretion (Rasmussen et al., 1990). This hypothesis is supported by studies showing that pharmacologic activation of calcium influx (tolbutamide or ionophores), and pharmacologic activation of cAMP accumula-

tion (forskolin) can interact synergistically with phorbol esters to enhance insulin secretion (Zawalich et al., 1983, 1984).

We have recently reported that the extent of phosphorylation of the 80-kD putative PKC substrate protein MARCKS is increased in isolated islets stimulated with high glucose, implying that PKC is activated during glucose-induced insulin secretion (Calle et al., 1992). The results of this study and our present study differ from the results of several recent studies using cultured rat islets, wherein it has been reported that there was neither detectable translocation of PKC activity to membrane fractions nor phosphorylation of the 80-kD putative PKC substrate protein in response to high glucose concentrations (Easom et al., 1989; Persaud et al., 1989; Easom et al., 1990). However, these cultured islets display a markedly attenuated insulin secretory response to high glucose, having ~10% of the secretory response to high glucose normally seen in either freshly isolated islets in the perfused pancreas or in the intact animal (Ganesan et al., 1990; Curry et al., 1968; Gerich et al., 1974; Grill et al., 1978). One explanation for these discrepancies is that there may be fundamental alterations in the signal transduction mechanisms activated by glucose in cultured islets, as opposed to those activated in freshly isolated islets. The process of culturing islets may uncouple glucose metabolism from the activation of PKC, leading both to the markedly reduced insulin secretory response and lack of detectable PKC translocation or PKC activation upon stimulation with glucose. As $\alpha$-PKC translocation in response to high glucose has been demonstrated both in freshly isolated islets and in the intact animal, but not in cultured islets, freshly isolated islets may more closely reflect islet physiology in the intact animal. Another explanation for the difference in results may be that the measurement of enzyme activity used in these other studies is less sensitive than the immunocytochemical techniques used in our present study.

The data presented in this paper demonstrate that the intracellular metabolism of glucose that occurs during glucose-induced insulin secretion can cause the redistribution of $\alpha$-PKC to the cell periphery of $\beta$-cells, both in isolated islets and in the intact animal. These results suggest that $\alpha$-PKC may play an important role in the cellular events underlying glucose-induced insulin secretion.

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