Regulation of Distinct Pools of Protein Kinase C δ in Beta Cells

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Abstract   Previous studies from our laboratory have demonstrated the presence of several isoforms of protein kinase C (PKC), Ca^{2+}-independent and Ca^{2+}-dependent, in both whole islets and tumor-derived beta cells. In the basal state, a major proportion of the isoform was found in the crude membrane fraction with smaller amounts found in both the cytosolic and cytoskeletal fractions. Whole islets showed a similar distribution of the isoform. These studies were done to analyze the effects of insulin secretagogues on the distribution of PKC δ to different cellular pools in isolated insulinoma beta cells. The phorbol ester, phorbol 12-myristate 13-acetate (PMA), produced a transient association of PKC δ with the beta cell cytoskeleton along with sustained decreases in cytosolic enzyme and transient increases in membrane enzyme. Neither glucose nor carbachol could acutely affect the subcellular distribution of PKC δ. Oleic acid decreased the amount of the enzyme associated with the cytoskeleton and led to a sustained decrease of cytosolic enzyme and a transient increase in membrane enzyme. Oleic acid was also able to prevent the increase in cytoskeletal enzyme induced by PMA. Both oleic acid and PMA potentiated glucose-induced insulin release but oleic acid, in contrast to PMA, was unable to initiate insulin release in the presence of substimulatory concentrations of glucose. These data demonstrate that different activators of PKC may have different effects on localization of the enzyme within the cells and suggest that there are at least three apparently distinct pools of PKC δ within the beta cell which may be important in insulin secretion or other aspects of beta cell function.   © 1996 Wiley-Liss, Inc.

Key words: islets, oleic acid, cytoskeleton, insulin, free fatty acids

Insulin secretion reflects the response of the islet to multiple incoming signals. Inarguably, glucose is the primary regulator of insulin secretion. However, the ability of glucose to effect insulin secretion is greatly influenced by many other factors, including peptide hormones, amino acids, steroids, free fatty acids, cholinergic agonists, and adrenergic agonists [Karam and Forsham, 1994].

Recently, free fatty acids have become the focus of many investigations into insulin secretion for several reasons, including their actions as insulin secretagogues [Opara et al., 1994], potential second messengers in insulin release [Turk et al., 1993], and their potential to promote beta cell dysfunction in obesity and diabetes [Elks, 1993; Opie and Walfish, 1963; Havel, 1982; Swislocki et al., 1987]. It has been postulated that alterations in cellular function induced by free fatty acids may be in part attribut-

able to their ability to interact and modulate the activity of PKC [Diaz-Guerra et al., 1991; May et al., 1993; Yoshida et al., 1992; Touny et al., 1990; Shinomura et al., 1991].

The role of PKC in fatty acid effects on the beta cell is unknown. Our lab has recently reported that insulinoma beta cells and islets express multiple isoforms of PKC [Knutson and Hoenig, 1994]. In that study it was observed that PKC δ, one of the predominant isoforms, is associated in different pools within the cells. In platelets, PKC δ is potently activated by both diacylglycerol and free fatty acids, including oleic acid [Khan et al., 1993].

The purpose of the following study was to characterize the effects of oleic acid and other insulin secretagogues on the dynamics of PKC δ within the cytosol, membrane, and cytoskeleton of insulinoma beta cells.

MATERIALS AND METHODS
Materials

A polyclonal antibody to the PKC isoform δ was purchased from GIBCO-BRL (Grand Is-

land, NY). This antibody was generated in rabbits against a short peptide sequence that is identical to a short unique variable region on the PKC molecule. Specificity of the antibody was determined by inclusion of this competing peptide to preabsorb the epitope binding sites. Western blotting was performed using the modular mini-electrophoresis system by Bio-Rad (Hercules, CA). Immobilon-P PVDF transfer membranes were obtained from Millipore (Bedford, MA). ^{125}I-protein A (product #68038) was obtained from ICN Biomedicals (Irvine, CA). Unless otherwise specified, all other materials were purchased from Sigma Chemical.

Animals and Transplantation

A radiation-induced transplantable insulinoma was maintained by serial transplantation under the right kidney capsule of male New England Deaconess Hospital rats as previously described [Hoenig et al., 1984].

Cell Preparation and Fractionation

The purification of beta cells from the insulinoma has been described in detail [Hoenig and Sharp, 1986]. In brief, the tumor was removed and minced into fine pieces. The tissue was then subjected to trypsin-collagenase digestion and the beta cells were purified from other cells on discontinuous Ficoll gradients. After rinsing in Hanks' buffer, the cells were resuspended in Krebs buffer (in mM, 25 Hepes, 5 NaCO$_3$, 118 NaCl, 4.7 KCl, 1.19 KH$_2$PO$_4$, 1.19 MgSO$_4$, 5 glucose, and 1.1 CaCl$_2$, pH 7.4) and incubated for 10 min at 37°C prior to addition of secretagogues. PMA and oleic acid were added to the cells from a concentrated stock in dimethyl sulfoxide (DMSO). DMSO itself had no impact on the distribution of PKC δ. Fractionation of the cells was done as previously described [Knutson and Hoenig, 1994].

Immunoblotting

Blotting was done essentially as previously described [Knutson and Hoenig, 1994]. In brief, 40 μg of protein of each fraction was applied to a 10% SDS-polyacrylamide gel and resolved at 200 V for approximately 45 min. The protein was then transferred to membranes. After transfer, the membranes were blocked and then incubated with anti-PKC δ isoform-specific antibody. The specificity of the polyclonal antibody used in these studies has been previously described [Knutson and Hoenig, 1994]. After 4 washes the membranes were placed in PBS, pH 8.1, containing ^{125}I-protein A at 0.4 μCi/ml for 3 h at room temperature. After 3 washes in PBS the membranes were dried and exposed to X-ray film (Kodak) for 24–96 h. The autoradiograms were scanned with a Biorad model 620 video densitometer and statistics performed using unpaired Student's t-test.

Kinase Assay of PKC Isoform-Specific Immunoprecipitates

Whole brain from the same species of rats was quickly removed and homogenized at 100 mg (wet weight)/ml in immunoprecipitation (IP) buffer. The protocol for immunoprecipitation was carried out essentially as recommended by GIBCO from whom the antibody was purchased. The IP buffer consisted of 50 mM Tris, pH 7.5, 0.15 M NaCl, 0.5% Triton X-100 (v/v), 25 μg/ml each of leupeptin and aprotinin, 2 mM ethylenediamine-tetraacetic acid (EDTA), and 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA). Following homogenization, the tissue was allowed to solubilize on ice for 30 min and then centrifuged at 16,000g for 15 min to clarify the solution. The supernatant fraction was removed and 2.5 μg of anti-PKC δ antibody was added to 500 μl aliquots. The mixture was incubated overnight while shaking at 4°C. The following day 50 μl of protein A-agarose was added followed by an incubation for 3 h on ice. The immunoprecipitates were then washed once in 1 ml of IP buffer supplemented with 0.4 M NaCl and once in 1 ml of kinase buffer. Kinase buffer contained 5 mM MgCl$_2$, 0.77 mM EGTA, 20 mM Tris-HCl, pH 7.5. Myelin basic protein$_{4-14}$ (MBP$_{4-14}$) was added at a final concentration of 1 mg/ml. The reaction contained a final volume of 100 μl of kinase buffer plus substrate and was initiated by the addition of 10 μM [^{32}P]ATP. The reaction was carried out at 30°C for 10 min. The reaction was stopped by placing the mixture on ice and adding 5× Laemmli buffer. An aliquot was resolved on a 20% polyacrylamide gel exposed to X-ray film. The substrate band (i.e., MBP$_{4-14}$) was then analyzed by densitometry as previously described above.

Cell Perifusion

Approximately 0.5 × 10^6 cells were resuspended in Krebs buffer containing 0.25% fatty acid free BSA, loaded onto cytodex beads in a

plastic column, and perifused by gravity flow at 37°C (95% oxygen/5% CO_2). The flow rate was 0.5 ml/min. Five millimolar glucose was added to all perifusions during the first 30 min, and the cells were then stimulated with 30 mM glucose in the presence or absence of 100 μM oleic acid or 100 nM PMA. Samples were collected in 20 s intervals and assayed for insulin as described [Herbert et al., 1965].

RESULTS

Redistribution of PKC δ in Response to Phorbol Ester

Stimulation of beta cells (time-matched batch incubations of approximately 0.4–0.8 mg cell protein/ml) for 15 min with 1 μM PMA produced redistributions of PKC δ detectable not only to the crude membrane fraction (137 ± 9.5%, n = 7, $P < 0.005$, compared to time-matched control) but also the cytoskeletal fraction (122 ± 2.9%, n = 6, $P < 0.001$) (Fig. 1). This was accompanied by a decrease in the cytosolic fraction (54 ± 8.4%, n = 7, $P < 0.001$). Incubations with a tenfold lower concentration of PMA (100 nM) produced similar results (cytosol 72 ± 4.9%, $P < 0.025$; crude membrane 117 ± 1%, $P < 0.005$; cytoskeleton 134 ± 8.7%, $P < 0.05$; n = 3) (Fig. 1). In shorter-term 3 min incubations (Fig. 1), 1 μM PMA resulted in an increase in crude membrane-associated PKC δ (127 ± 0.8%, $P < 0.001$, n = 3) with a concomitant decrease in the cytosol-associated enzyme (58 ± 5.6%, n = 4, $P < 0.001$). Translocation to the cytoskeletal fraction was not observed (103 ± 15.5%, n = 4).

Over the course of 2 h, 1 μM PMA reduced the levels of cytosolic enzyme to 30–40% of control levels and produced a transient increase in membrane levels which started to return to baseline after 15 min (Fig. 2). The transient effect of phorbol esters on the crude membrane fraction indicates the induction of proteolysis of the enzyme in the membrane fraction. Over this time-course PMA produced a translocation to the cytoskeleton which reached levels of nearly 163% at 30 min but returned to control levels by 2 h. (Fig. 2).

Phorbol-Ester-Induced Down-Regulation of PKC δ in Insulinoma Beta Cells

Consistent with the previously defined behavior of PKC isoforms in response to chronic phorbol ester treatment, PMA induced a down-
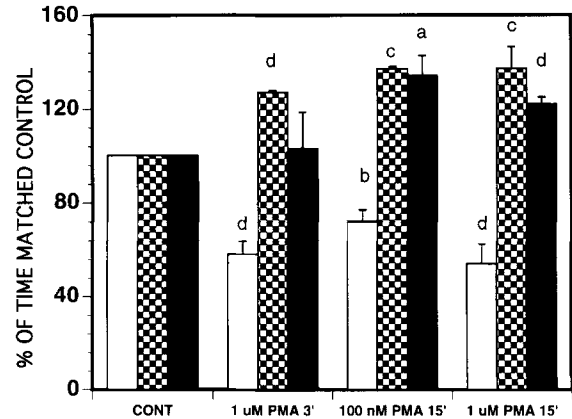

Fig. 1. Redistribution of PKC δ at 3 and 15 min in response to phorbol ester. Fifteen minute incubations with either 100 nM PMA or 1 μM TPA. Forty micrograms of each fraction were immunoblotted and the resultant autoradiograms were scanned by densitometry. The O.D. of treatment bands was expressed as a percentage of time-matched control bands for each experiment. Each graph represents 3–7 independent experiments. a, $P < 0.05$, b, $P < 0.025$, c, $P < 0.005$, d, $P < 0.001$. Open bars = cytosol; solid bars = cytoskeleton; checkered bars = membrane.

regulation of total cellular PKC δ. In response to 1 μM PMA, the down-regulation of total cellular PKC δ was detectable as early as 6 h and the maximal effect was seen at 12 h when the enzyme reached a level of 25–30% compared to time zero control (Fig. 3). Measurement at 24 h
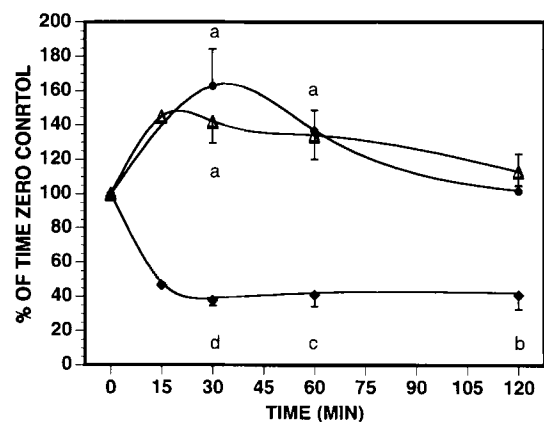

Fig. 2. Time-course of cytosolic to membrane/cytoskeleton translocation of PKC δ induced by 1 μM PMA. Cells were aliquoted and exposed for the depicted times in the presence of the phorbol ester. After incubation the cells were fractionated into cytosol and membrane fractions. Forty micrograms of each fraction were analyzed by western analysis and densitometry. Each point represents the mean of 3 independent observations compared to time zero control (except 15, n = 2). a, $P < 0.05$; b, $P < 0.025$; c, $P < 0.01$; d, $P < 0.005$. Circles = cytoskeleton; diamonds = cytosol; triangles = membrane.
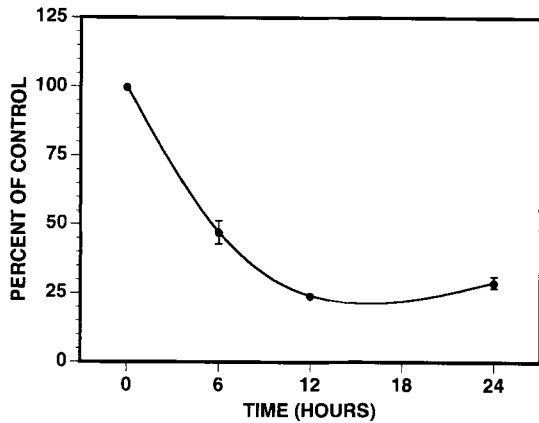
Fig. 3. Down-regulation of PKC δ in response to chronic PMA treatment. Cells were aliquoted and exposed for the depicted times in the presence of the 1 μM PMA. After incubation the cells were homogenized. Forty micrograms of the homogenate was analyzed by western analysis and densitometry. Each point represents the mean of 2 independent observations compared to time-matched controls. The standard deviations were less than 10%.
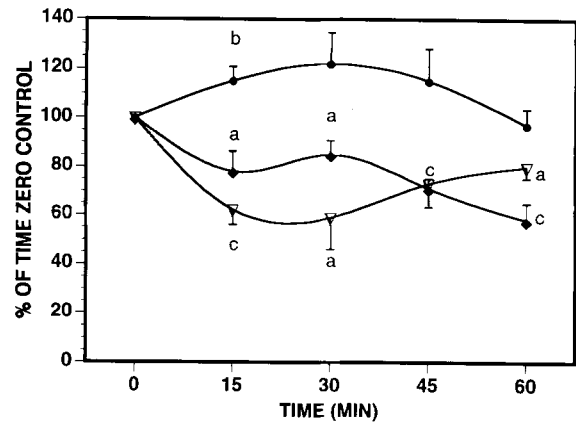

Fig. 4. Time-course of cytosolic to membrane translocation of PKC δ induced by 100 μM oleic acid. Cells were aliquoted and exposed for the depicted times in the presence of the phorbol ester. After incubation the cells were fractionated. Forty micrograms of each fraction were analyzed by western analysis and densitometry. Each point represents the mean ± S.E. of 3–5 independent observations compared to time zero control. a, $P < 0.05$; b, $P < 0.025$; c, $P < 0.005$. Circles = membrane; diamonds = cytosol; triangles = cytoskeleton.

showed levels similar to those obtained at 12 h and in control cells the levels of the enzyme remained at time zero levels (data not shown).

Effects of Oleic Acid on the Subcellular Distribution of PKC δ

In time course studies of 60 min, 100 μM oleic acid also produced a translocation but more moderate than that induced by phorbol ester (Fig. 4). PKC δ decreased in the cytosol over 60 min to 58 ± 6.9% ($P < 0.005$, compared to time zero control). Oleic acid also produced an increase to the membrane fraction first observable at 15 min which peaked at 30 min and returned to control levels at 60 min (97 ± 6.7, NS compared to time zero control). Analysis of whole cell homogenate levels of the enzyme over this time-course showed a steady decline in total levels of the enzyme but only reached significance at 60 min (78 ± 3.4%, $P < 0.005$, compared to time zero control, data not shown). This decrease in total cellular levels coincided with the return of membrane levels of the enzyme back to control levels at 60 min. Figure 4 also shows the changes of the levels of the enzyme associated with cytoskeleton over a 60 min time-course. In contrast to the effects of PMA on cytoskeletal levels of PKC δ, oleic acid decreased the amount associated with this fraction, which was maximal at 30 min, reaching a nadir of 59 ± 13.0% ($P < 0.05$). The levels then returned toward control levels and reached 80 ± 4.9%

($P < 0.05$) at 60 min. Longer term analysis was not done because cellular viability after long-term incubation was compromised. The net effect of oleic acid appears to be a preferential down-regulation of both cytosolic and cytoskeletal pools. This is corroborated by a moderate down-regulation of the whole cell homogenate levels at 60 min. The extent of the decrease in whole cell homogenate levels is expected given that 65–70% of the enzyme is associated with the membrane in the basal state in our estimate.

Effects of High Glucose or PMA on Oleic Acid-Induced Translocation of PKC δ

We then tested the effects of high (30 mM) glucose on the oleic acid-induced redistribution of PKC δ since glucose may stimulate the production of activators or inhibitors of PKC. Similar changes were seen with oleic acid in the presence of high (30 mM) or low (5 mM) glucose (cytosol: 65 ± 9% high [$P < 0.025$ compared to control] vs. 67 ± 7.3% low; membrane: 124 ± 3% high glucose [$P < 0.01$ compared to control] vs. 121 ± 4.1% low glucose; cytoskeleton: 79 ± 13.9% high [NS compare to control] vs. 67 ± 8.0% low) (Fig. 5). High (30 mM) glucose alone had no effect on the distribution of PKC δ when measured at 3, 15, or 120 min (data not shown). In the presence of both 1 μM PMA and oleic acid the amount associated with the cytosol dropped even further to 44 ± 8% ($P < 0.005$

compared to control) but was similar to that seen with PMA alone (46 ± 11.3%, $P < 0.01$ compared to control) (Fig. 5). However, the amount associated with the membrane in cells incubated with both oleic acid and PMA (128 ± 0.3%, $P < 0.001$ compared to control) was significantly less than seen in cells treated with PMA alone (158 ± 13.5%, $P < 0.05$ vs. oleic acid + PMA treatment, $P < 0.05$ compared to control). This latter observation indicates that the combination of both activators enhanced proteolysis of the enzyme. Perhaps one of the most striking effects of oleic acid was its ability to reverse the increase in enzyme associated with the cytoskeleton in cells treated with PMA alone. In the presence of oleic acid the increase usually induced by PMA was not observed and the amount of enzyme associated with this pool was actually less than in control cells (87 ± 14.0%), albeit not significantly.

Oleic Acid-Induced Activation of Rat PKC δ Immunoprecipitates

In order to determine if oleic acid induced translocation could be correlated with an increase in activity of the enzyme, rat brain PKC δ was immunoprecipitated with polyclonal anti-PKC δ antibody and protein A-agarose beads, followed by kinase activity of the immobilized enzyme as described in Materials and Methods.
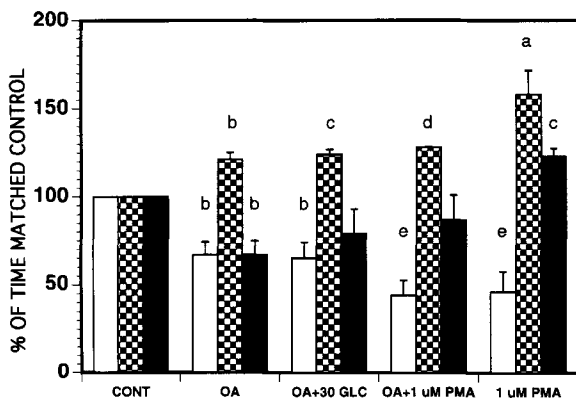
Immunoprecipitation of PKC δ was verified by subsequent western blotting of the eluted immunoprecipitates (data not shown). As shown in Figure 6, the phosphorylating activity of the immobilized enzyme was increased in the presence of oleic acid but in the absence of PMA and PS, which is consistent with previous reports that oleic acid can activate PKC independent of DAG or phorbol esters [Khan et al., 1993]. As expected PMA and PS also increased the activity of the enzyme [Stabel and Parker, 1991]. Oleic acid produced a level of activation similar to that seen in with PMA and PS.

Effects of Phorbol Ester and Oleic Acid on Glucose-Induced Insulin Release From Perifused Insulinoma Beta Cells

Oleic acid potentiated second phase insulin release (Fig. 7) in response to 30 mM glucose by 37% ($P < 0.03$, comparison of area under the curve). In the presence of 5 mM glucose, oleic acid had no effect on insulin release. PMA also potentiated second phase insulin release in response to 30 mM glucose by 26% ($P < 0.05$), similar to the response seen with oleic acid.

DISCUSSION

As previously reported by us and other investigators rat insulinoma beta cells and whole islets express several isoforms of PKC [Knutson

Fig. 5. Influence of glucose and PMA on oleic acid-induced redistribution of PKC δ. Cells were aliquoted and exposed for 15 min in the presence and absence of 100 μM oleic acid (OA), 30 mM glucose plus OA, 1 μM PMA plus OA, or 1 μM PMA. After incubation the cells were fractionated. Forty micrograms of the was fraction was analyzed by western analysis and densitometry. Each point represents the mean ± S.E. of 4 independent observations compared to time-matched control. a, $P < 0.05$; b, $P < 0.025$; c, $P < 0.01$; d, $P < 0.005$; e, $P < 0.001$. Open bars = cytosol; solid bars = cytoskeleton; checkered bars = membrane.
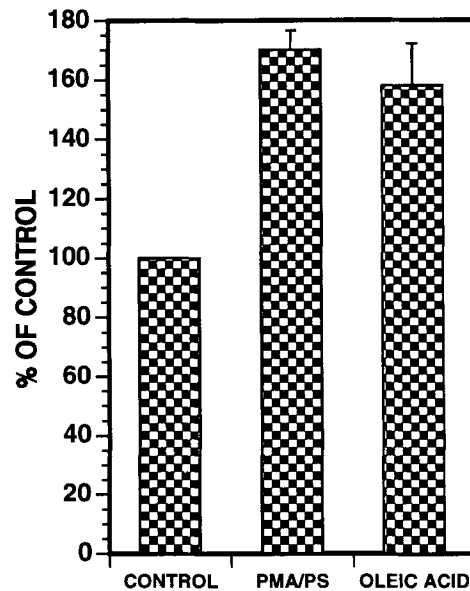

Fig. 6. Oleic acid-induced activation of PKC δ immunoprecipitates. Rat brain PKC δ was immunoprecipitated and subjected to a kinase assay as described in Materials and Methods. Results are mean ± SD of two independent experiments.
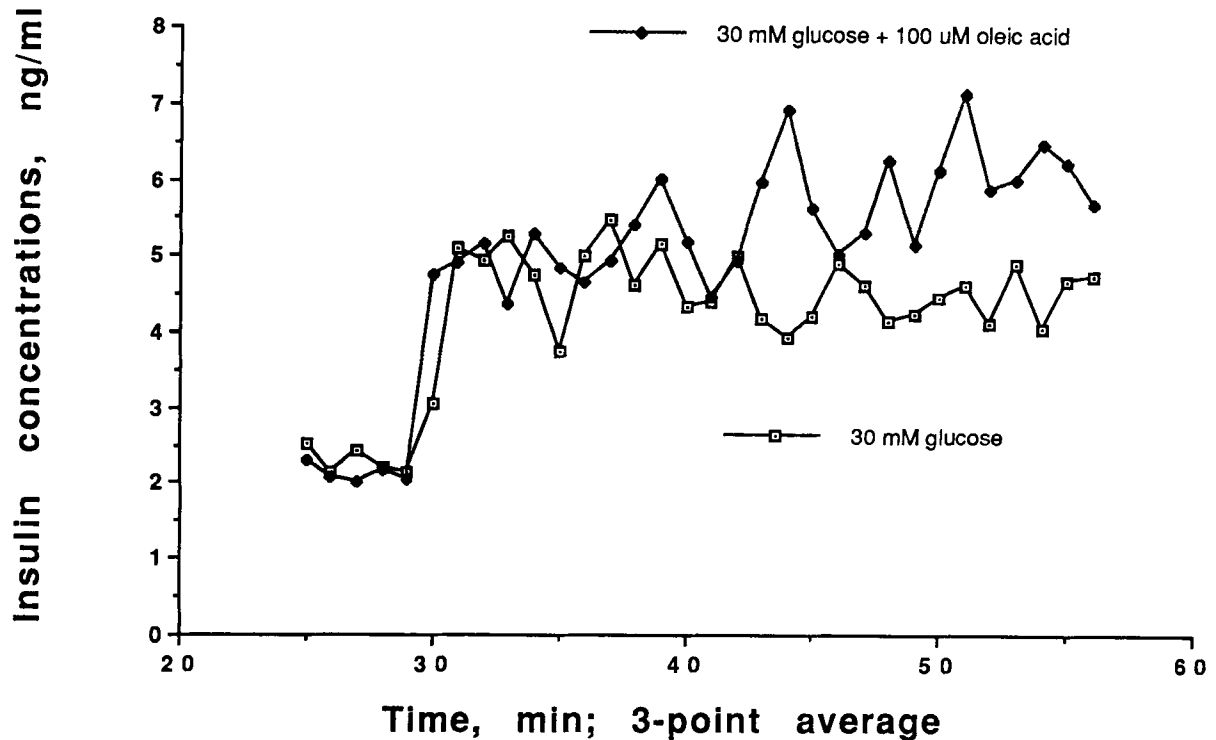
Fig. 7. Insulin secretion in response to 30 mM glucose in the absence or presence of oleic acid. Cells were perifused as described in Materials and Methods. Samples were collected at 20 s intervals. Each data point reflects the average of insulin concentration from 3 × 20 s fractions.

and Hoenig, 1994; Ganesan et al., 1990, 1992]. Two of the more predominant isoforms are PKC α and PKC δ. PKC α in the beta cell is localized to the cytosolic pool [Knutson and Hoenig, 1994; Ganesan et al., 1990]. However, PKC δ is localized, in the basal state, to multiple pools in both insulinoma beta cells and whole islets with a predominant fraction (65–70%) localized to the membrane fraction [Knutson and Hoenig, 1994]. Based on the data presented here, each pool of PKC δ within the beta cell is unique and subject to distinct regulation. This suggests that each pool may have separate functions within the beta cell. The notion of distinct localization and multiple functions of one protein within a cell has been postulated previously [Neubig, 1994]. Other investigators studying localization and activation of PKC isoforms have also described discrete pools of certain isoforms and have designated specific activators for each pool [Khan et al., 1993]. Studies on the effects of arachidonic and oleic acid on PKC in platelets has led to the hypothesis that the membrane-associated PKC is the target for DAG and cytosol-associated enzyme is the target for free fatty acids [Khan et al., 1993].

We have found in this and a previous study [Knutson and Hoenig, 1994] that in the insulinoma beta cell as well as whole islets a predominant fraction of PKC δ resides in the basal state in the membranous (i.e., 0.5% Triton X-100 soluble) fraction. The activation state of this pool is unknown. It has been suggested that the association of PKC isoforms with the membrane in the basal state may represent a constitutive activation [Stabel and Parker, 1991], which is possible given that endogenous levels of diacylglycerols in whole islets have been shown to be as high as 200 μM [Turk et al., 1993]. Alternatively, it has been postulated that the basal state association with the membrane may be the result of some modification (e.g., phosphorylation) that gives the enzyme a localization different from the cytosol [Olivier et al., 1992]. This localization could in theory permit the enzyme access to discrete and different substrates within the cell.

It is estimated from our studies that cytosol-associated PKC δ in the beta cell accounts for approximately 25–30% of the total cellular enzyme. It is probable that this pool represents a target for fatty acids that are generated within

the beta cell or supplied exogenously. The effects of oleic acid on PKC have been studied extensively and are thought to be an important regulator of its activities within the cell [Khan et al., 1992, 1993; Shinomura et al., 1991; Diaz-Guerra et al., 1991; May et al., 1993; Nakanashi and Exton, 1992; Chen and Murakami, 1992; Yoshida et al., 1992; Touney et al., 1990]. Previous studies have shown that cytosolic Ca^{2+}-independent PKC (predominantly δ) in platelets is activated by oleic acid with an EC_{50} of 5 μM. Oleic acid appeared to preferentially activate cytosolic over membrane bound PKC [Touney et al., 1990]. Our experiments suggest as well that PKC δ is activated by oleic acid. In platelets, oleic acid promoted about a 16% decrease is cytosolic Ca^{2+}-independent activity (primarily PKC δ) with a 15% increase in membrane activity, which is in contrast to phorbol ester which produced a 65% decrease in cytosolic Ca^{2+}-independent activity accompanied by an increase by 35% in the membrane activity [Khan et al., 1993]. These changes appeared to roughly parallel the changes noted by immunoblotting with anti-PKC δ antibodies. In our studies oleic acid also produced smaller redistributions compared to phorbol ester treatment. In studies done in platelets it was suggested that proteolysis occurred rapidly after administration of oleic acid which explained why only marginal increases of PKC δ were seen in the membrane as compared to the more dramatic decreases seen in the cytosol [Khan et al., 1993]. However, in our studies we found that at least over 45 min there was no detectable change in the whole cell homogenate levels suggesting little or no proteolysis. It should be emphasized that since membrane levels may approximate 65–70% of the total cellular enzyme, downregulation of cytosolic levels by proteolysis would be difficult to measure given the inherent error levels in western blotting analysis.

A previous study has shown that oleic acid is generated during exposure of islets to high glucose [Wolf et al., 1991], which raises the question of why we were unable to measure a translocation of PKC δ when insulinoma cells were stimulated with 30 mM glucose. Several possibilities exist: a) that oleate generation after glucose stimulation was insufficient to translocate the enzyme but high enough to activate it. This is plausible since a major proportion of PKC δ in these cells is associated with the membrane which may in turn bind to the available oleic acid. b) Western blotting does not provide a

sensitive enough measurement to detect translocation. c) The insulinoma cells used in these studies did not respond with hydrolysis of oleate from cellular lipids to the same extent that normal islets do in their natural surroundings. This is consistent with the fact that some RIN cells do not contain an ATP-activatible calcium-independent phospholipase A_2, whereas normal islet-derived beta cells do [Gross et al., 1993]. Free fatty acid can be supplied to beta cells by other mechanisms besides the cleavage of membrane phospholipids by the activity of phospholipase A_2, including increases induced by fasting [Opie and Walfish, 1963], capillary lipid cleavage [Havel, 1982], and abnormal lipid metabolism (i.e., in diabetes and obesity) [Havel, 1982; Swislocki et al., 1987].

Of particular interest is the finding that the association of PKC δ with the cytoskeleton is dynamic and that it can be influenced by activators of the enzyme. The presence of cytoskeleton-associated PKC has been reported but the activation state is currently unknown [Stabel and Parker, 1991]. Kiley and coworkers have recently shown that in GH_4C_1 cells both phorbol ester and thyrotropin releasing hormone increase the amount of PKC δ (as well as other isoforms) associated with the cytoskeleton [Kiley et al., 1992]. Many cytoskeletal substrates of PKC have been identified, such as vinculin [Werth and Pastan, 1984], actin filaments [Zalewski et al., 1991], intermediate filament proteins cytokeratin 8 and 18 [Chou and Bishr Omary, 1991], focal contact protein talin [Litchfield and Ball, 1986], and the actin-binding protein caldesmon$_{77}$ [Litchfield and Ball, 1987]. In addition, Papadopoulos and Hall [1989] have demonstrated the association of PKC activity with the cytoskeleton in the Y-1 adrenal tumor cell line but did not identify the expressed isoforms.

The data presented here provide the first documentation that cytoskeleton-associated PKC is influenced by fatty acids. It is possible that the influence is a direct interaction of oleic acid (or a metabolite) with the enzyme or related to a distal event such as depolymerization of potential PKC substrates in the cytoskeleton. The reason that oleic acid down-regulated cytoskeleton-associated PKC δ rather than increasing it, as did PMA, is unknown since both are potent activators of the isoform. It is not well understood how activation of PKC promotes its association with cellular targets, but it is possible

that oleic acid can only activate it once it is associated with it cellular substrates. This activation then could lead to release of the enzyme from its substrate. This is supported by our observation that oleic acid prevented the accumulation of PKC δ in the cytoskeleton induced by phorbol ester. Therefore, it is possible that once PMA associated the enzyme with its substrate oleic acid enhanced its release.

A number of earlier studies with insulin secreting beta cells have suggested that insulin secretion is dependent on the reorganization of the cytoskeleton. The microtubule-microfilamentous hypothesis of insulin secretion was first put forth in 1968 by the experimental findings of Lacy et al. [1968] who described that colchicine, a microtubule disrupting agent, was able to inhibit insulin release from isolated rat islets. This hypothesis has since given rise to a number of studies that have corroborated the importance of the cytoskeleton in insulin biosynthesis and release [Van Obberghan et al., 1973, 1974, 1975; Malaisse-Lagae et al., 1979; McDaniel et al., 1975; Lacy et al., 1973]. Our observations that acute exposure of beta cells to oleic acid, at concentrations that mimic normal physiological concentrations, enhanced glucose-induced insulin release is consistent with previous reports of the effects of lipids on insulin release [Sako and Grill, 1990]. In our studies we were unable to show an increase in basal levels of insulin release when cells were exposed to oleic acid acutely. Other reports have shown that free fatty acids (including oleic acid) can increase basal secretion of insulin but in these studies the concentrations of the fatty acids have been pharmacological [Opara et al., 1994; Elks, 1993] or required prolonged incubation [Zhou and Grill, 1994]. Future studies should be directed at analyzing the effects of the fatty acid on glucose-induced insulin release using different temporal and dosage parameters and analyzing the effects of different albumin concentrations.

In conclusion, these results suggest that there are at least three discrete pools of PKC δ and that each of the pools may have different functions. These studies also show the effects of various insulin secretagogues on the subcellular distribution of PKC δ in the beta cell. The interaction and potential for down-regulation of PKC δ by oleic acid suggests that some effects of the fatty acid, whether acute or chronic, may be mediated through the kinase. Future studies should be directed at analyzing the effects of PKC δ down-regulation on beta cell function.

REFERENCES

Bishr Omary M, Baxter GT, Chou CF, Riopel CL, Lin WY (1992): PKCε-related kinase associates with and phosphorylates cytokeratins 8 and 18. J Cell Biol 117:583–593.

Chen SG, Murakami K (1992): Synergistic activation of type III protein kinase C by cis-fatty acid and diacylglycerol. Biochem J 282:33–39.

Chou CF, Bishr Omary M (1991): Phorbol acetate enhances the phosphorylation of cytokeratins 8 and 18 in human colonic epithelial cells. FEBS Lett 282:200–204.

Diaz-Guerra MJM, Junco M, Bosca L (1991): Oleic acid promotes changes in the subcellular distribution of protein kinase C in isolated hepatocytes. J Biol Chem 266: 23568–23576.

Elks ML (1993): Chronic perifusion of rat islets with palmitate suppressed glucose-stimulated insulin release. Endocrinology 133:208–214.

Ganesan S, Calle R, Zawalich KC, Smallwood JI, Zawalich WS, Rasmussen H (1990): Glucose-induced translocation of protein kinase C-α in rat pancreatic islets. Proc Natl Acad Sci U S A 87:9893–9897.

Ganesan S, Calle R, Zawalich K, Greenwalt K, Zawalich W, Shulman GI, Rasmussen H (1992): Immunocytochemical localization of α-protein kinase C in rat pancreatic β-cells during glucose-induced insulin release. J Cell Biol 119:313–324.

Gross RW, Ramanadham S, Kruszka KK, Han X, Turk J (1993): Rat and human pancreatic islets contain a calcium ion independent phospholipase A2 activity selective for hydrolysis of arachidonate which is stimulated by adenosine triphosphate and is specifically localized to islet β-cells. Biochemistry 33:327–336.

Havel RJ (1982): Symposium on lipid disorders: approach to the patient with hyperlipidemia. Med Clin North Am 66:319–333.

Herbert V, Lau K, Gottlieb CW, Bleicher SJ (1965): Coated charcoal immunoassay of insulin. J Clin Endocrinol 25: 1375–1384.

Hoenig M, Ferguson DC, Matschinsky FM (1984): Pharmacological modifications of insulin release in vitro from fuel-responsive transplantable insulinomas. Endocrinology 115:1496–1499.

Hoenig M, Sharp GWG (1986): Glucose induced insulin release and a rise in cytosolic calcium concentration in a transplantable rat insulinoma. Endocrinology 119:2502–2507.

Karam JH, Forsham PH (1994): Pancreatic hormones and diabetes mellitus. In Greenspan FS, Baxter JD (eds): "Basic and Clinical Endocrinology." Connecticut: Appleton and Lange.

Khan WA, Blobe G, Halpern A, Taylor W, Wetsel WC, Burns D, Loomis C, Hannun Y (1993): Selective regulation of protein kinase C isoenzymes by oleic acid in human platelets. J Biol Chem 268:5063–5068.

Khan WA, Blobe GC, Hannun YA (1992): Activation of protein kinase C by oleic acid. J Biol Chem 267:3605–3612.

Kiley SC, Parker PJ, Fabbro D, Jaken S (1992): Hormone- and phorbol ester-activated protein kinase C isozymes mediate a reorganization of the actin cytoskeleton associated with prolactin secretion in GH$_4$C$_1$ cells. Mol Endocrinol 6:120–131.

Knutson K, Hoenig M (1994): Identification and subcellular characterization of protein kinase C (PKC) isoforms in insulinoma beta-cells and whole islets. Endocrinology 135:881–886.

Lacy PE, Howell SL, Young DA, Fink CJ (1968): New hypothesis of insulin secretion. Nature 219:1177–1179.

Lacy PE, Klein NJ, Fink CJ (1973): Effect of cytochalasin B on the biphasic release of insulin in perifused rat islets. Endocrinology 92:1458–1468.

Litchfield DW, Ball EH (1987): Phosphorylation of caldesmon$_{77}$ by protein kinase C in vitro and in intact human platelets. J Biol Chem 262:8056–8060.

Litchfield DW, Ball ER (1986): Phosphorylation of the cytoskeletal protein talin by protein kinase C. Biochem Biophys Res Commun 134:1276–1283.

Malaisse-Lagae F, Amherdt M, Ravazzola M, Sener A, Hutton JC, Orci L, Malaisse WJ (1979): Role of microtubules in the synthesis, conversion, and release of (pro)insulin. A biochemical and radioautographic study in rat islets. J Clin Invest 63:1284–1296.

May CL, Southworth AJ, Calder PC (1993): Inhibition of lymphocyte protein kinase C by unsaturated fatty acids. Biochem Biophys Res Commun 195:823–828.

McDaniel M, Roth C, Fink CJ (1975): Effects of cytochalasins B and D on alloxan inhibition of insulin release. Biochem Biophys Res Commun 66:1089–1096.

Nakanashi H, Exton J (1992): Purification and characterization of the ζ isoform of protein kinase C from bovine kidney. J Biol Chem 267:16347–16354.

Neubig RR (1994): Membrane organization in G-protein mechanisms. FASEB J 8:939–946.

Olivier AR, Kiley SC, Pears C, Schaap D, Jaken S, Parker PJ (1992): The molecular biology of nervous system-specific proteins. Protein kinase C-δ and -ϵ: a functional appraisal. Biochem Soc Trans 20:603–607.

Opara EC, Garfinkel M, Hubbard VS, Burch WM, Akwari OE (1994): Effect of fatty acids on insulin release: role of chain length and degree of unsaturation. Am J Physiol 266:E635–E639.

Opie LH, Walfish PG (1963): Plasma free fatty acid concentrations in obesity. N Engl J Med 268:757–760.

Papadopoulos V, Hall P (1989): Isolation and characterization of protein kinase C from Y-1 adrenal cell cytoskeleton. J Cell Biol 108:553–567.

Sako Y, Grill V (1990): A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B-cell oxidation through a process likely coupled to fatty acid oxidation. Endocrinology 127:1580–1589.

Shinomura T, Asaoka Y, Oka M, Yoshida K, Nishizuka Y (1991): Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: its possible implications. Proc Natl Acad Sci U S A 88:5149–5153.

Stabel S, Parker PJ (1991): Protein kinase C. Pharmacol Ther 51:71–95.

Swislocki ALM, Chen YDI, Golay A, Chang MO, Reaven GM (1987): Insulin suppression of plasma-free fatty acid concentrations in normal individuals and patients with type 2 (NID) diabetes. Diabetologia 30:622–626.

Touny SE, Khan W, Hannun Y (1990): Regulation of platelet protein kinase C by oleic acid. J Biol Chem 265:16437–16443.

Turk J, Gross RW, Ramanadham S (1993): Amplification of insulin secretion by lipid messengers. Diabetes 42:367–374.

Van Obberghan F, Somers G, Devis G, Ravazzola M, Malaisse-Lagae F, Orci L, Malaisse WJ (1974): Dynamics of insulin release and microtubular-microfilamentous system. VI. Effects of D$_2$O. Endocrinology 95:1518–1528.

Van Obberghen E, Somers G, Devis G, Ravazzola M, Malaisse-Lagae F, Orci L, Malaisse WJ (1975): Dynamics of insulin release and microtubular-microfilamentous system. VII. Do microfilaments provide the motive force for the translocation and extrusion of beta granules? Diabetes 24:892–901.

Van Obberghen E, Somers G, Devis G, Vaughan GD, Malaisse-Lagae F, Orci L, Malaisse WJ (1973): Dynamics of insulin release and microtubular-microfilamentous system. 1. Effect of cytochalasin B. J Clin Invest 52:1041–1051.

Werth DK, Pastan I (1984): Vinculin phosphorylation in response to calcium and phorbol esters in intact cells. J Biol Chem 259:5264–5270.

Wolf BA, Pasquale SM, Turk J (1991): Free fatty acid accumulation in secretagogue-stimulated pancreatic islets and effects of arachidonate on depolarization-induced insulin secretion. Biochemistry 30:6372–6379.

Yoshida K, Asaoka Y, Nishizuka Y (1992): Platelet activation by simultaneous actions of diacylglycerol and unsaturated fatty acids. Proc Natl Acad Sci U S A 89:6443–6446.

Zalewski PD, Forbes IJ, Giannakis C, Betts WH (1991): Regulation of protein kinase C by Zn^{2+}-dependent interaction with actin. Biochem Int 24:1103–1110.

Zhou Y-P, Grill VE (1994): Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. J Clin Invest 93:870–876.