Journal of Cellular Biochemistry 62:543–552 (1996)

Arachidonic Acid-Induced Down-Regulation of Protein Kinase C δ in Beta-Cells

Keith L. Knutson and Margarethe Hoenig

Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602

Abstract We have previously identified expression of multiple protein kinase C (PKC) isoforms in insulinoma-derived beta-cells and whole islets. Both PKC δ and PKC α appear to be the more abundantly expressed isoforms. In this report we studied the effects of arachidonic acid (AA) on the subcellular distribution of PKC α and PKC δ. AA has been reported to activate both PKC α and PKC δ and it is thought to be an important second messenger in beta-cells. Here we report that AA interacted with and altered beta-cell pools of PKC δ preferentially over PKC α. AA (100 μM) over the course of 45 min reduced cytosolic levels of PKC δ (to 40 ± 15%, compared to time zero control) leaving membrane- and cytoskeleton-associated levels near control levels. Analysis of whole cell homogenates showed a slight down-regulation of PKC δ indicating proteolysis. The down-regulation of cytosolic PKC δ appeared to be isoform specific since cytosolic PKC α remained at control levels over the time course. The response was dose-dependent and negligible at concentrations below 30 μM and occurred, at least partially, in the cytosolic compartment of the cell. Indomethacin also down-regulated cytosolic PKC δ preferentially over PKC α possibly through accumulation of AA. These findings suggest that cytosolic PKC δ may be a downstream target of this beta-cell second messenger. © 1996 Wiley-Liss, Inc.

Key words: islets, free fatty acids, indomethacin, PKC, arachidonic acid

The signal transduction pathway involved in glucose-mediated insulin release has been exceptionally difficult to characterize. It is clear that the sugar must be metabolized in order to cause secretion, thus it is apparent that some metabolic-coupling factor(s) exists which alters the machinery of the beta-cell. Several hypotheses have been put forward regarding the existence of putative coupling factors transducing the glucose signal to the secretory apparatus, including ATP [Cook et al., 1988], acyl-CoA derivatives [Prentki et al., 1992], free fatty acids [Turk et al., 1993], and DAG [Zawalich and Rasmussen, 1990]. Given the available evidence it would not be difficult to hypothesize that glucose-mediated insulin release is caused by the collective organization of many metabolic events. One particular signal transduction pathway that has generated considerable interest is that involving protein kinase C.

Protein kinase C (PKC) is a family of lipid-dependent serine/threonine protein kinases that are central to signal transduction in virtually all cells. Since Inoue and coworkers discovered the enzyme activity in 1977 [Inoue et al., 1977] it has become increasingly evident that the regulation of this family of enzymes is very complex. The best characterized activator is diacylglycerol (DAG) [Stabel and Parker, 1991]. However, it is now becoming apparent that many other activators and activity potentiators exist within the cell [Nishizuka, 1992]. Some of the more thoroughly studied activators include arachidonic acid and oleic acid. Studies with these fatty acids suggest that they can modulate PKC activity independently of DAG [Aris et al., 1993; Ogita et al., 1992; Koide et al., 1992; Diaz-Guerra et al., 1991; May et al., 1993; Nakanashi and Exton, 1992; Chen and Murakami, 1992; Shinomura et al., 1991; Yoshida et al., 1992; Touny et al., 1990; Khan et al., 1992, 1993].

The role of PKC in nutrient- and hormone-induced insulin release is controversial. Some investigators have suggested that its contribution, if any, is negligible in nutrient-induced

Received November 24, 1995; accepted March 22, 1996.

Address reprint requests to Dr. Margarethe Hoenig, Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia, Athens, GA 30602.

Keith L. Knutson's current address is University of British Columbia, Department of Medicine, Division of Infectious Diseases, 2733 Heather Street, Room 452D, Vancouver, British Columbia, Canada V5Z 3J5.

© 1996 Wiley-Liss, Inc.

insulin secretion. These suggestions arise from several observations such as a lack of significant generation of the endogenous activator DAG [Turk et al., 1993], lack of effect of prior PKC down-regulation on glucose-induced insulin release [Hii et al., 1987; Persuad et al., 1991; Arkhammer et al., 1989], lack of inhibition of insulin release by a PKC inhibitor [Metz, 1988a,b] or lack of significant translocation of the enzymes from the cytosol to the membrane fraction of the cells upon stimulation by glucose [Persuad et al., 1989; Easom et al., 1989]. However, some other studies have suggested a role for the enzymes in nutrient-induced insulin release. For example, it was shown that both glucose and α-ketoisocaproate induced a measurable translocation of the α isoform from the cytosol to the particulate fraction of whole islets [Ganesan et al., 1990], and a PKC inhibitor (albeit non-specific) decreased the secretory response to glucose in perifusion assays [Zawalich et al., 1991]. It was also shown that PKC down-regulation potentiated phase 1 and inhibited phase 2 of glucose-induced insulin release [Thams et al., 1990], and glucose increased the activity of membrane bound PKC [Sahai et al., 1992].

Free fatty acids, most notably arachidonic acid, have recently emerged as potentially important mediators in glucose-induced insulin release. Furthermore, exogenous free fatty acids, such as oleic acid, arachidonic acid, and palmitic acid, have been reported to have effects on both basal and stimulated insulin release [Opara et al., 1994; Sako and Grill, 1990]. Recently, the existence of both Ca^{2+}-independent [Gross et al., 1993] and Ca^{2+}-dependent isoforms of phospholipase A_2 (PLA_2) [Laychock, 1982; Metz et al., 1991] was identified in the pancreatic beta-cell. PLA_2s are enzymes that liberate free fatty acids from the *sn*-2 site of cellular phospholipids. It has been observed that nutrient stimulation of beta-cells resulted in activation of PLA_2 [Laychock, 1982; Wolf et al., 1991]. Therefore it is possible that activation of these enzymes results in the increases in intracellular levels of many free fatty acids observed after exposure to high glucose [Wolf et al., 1991]. Two prominent species generated after exposure of islets to glucose or carbachol are arachidonic and oleic acid [Wolf et al., 1991], suggesting a role for these molecules (or their derivatives) as metabolic coupling factors. Indeed levels of arachidonic acid have been reported to increase by ≈38–75 μM during nutrient-induced insulin release [Wolf et al., 1991] possibly leading to intracellular concentrations as high as 100–200 μM [Metz, 1988a,b]. Further evidence for a potential role of these fatty acids in insulin release is that inhibition of PLA_2 activation attenuated glucose-induced insulin release and mobilization of intracellular Ca^{2+} [Ramanadham et al., 1993].

These previous observations have led us to evaluate the effect of arachidonic acid on the subcellular distribution of the δ isoform of PKC. Here we show that arachidonic acid alters the subcellular levels of PKC δ in the beta-cell and suggests that there is an interaction between AA metabolism and cytosolic PKC δ.

MATERIALS AND METHODS

Materials

An isoform-specific antibody to the PKC δ was purchased from GIBCO-BRL (Grand Island, NY). The polyclonal antibody to PKC α was generously provided by Dr. Kirk Ways of East Carolina University (Greenville, NC). Specificity of the antibodies was determined by inclusion of competing peptide to preabsorb the epitope binding sites. Western blotting was performed using the Bio-Rad (Hercules, CA) mini-electrophoresis system. Immobilon-P PVDF transfer membranes were purchased from Millipore (Bedford, MA). ^{125}I-Protein A (68038) was obtained from ICN Biomedicals (Irvine, CA). Unless specified, other chemicals were obtained from Sigma (St. Louis, MO).

Cell Preparation and Fractionation

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]. 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 Hank's buffer, the cells were resuspended in Krebs buffer (in mM, 25 Hepes, 5 $NaHCO_3$, 118 NaCl, 4.7 KCl, 1.19 KH_2PO_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. Arachidonic acid was purchased in its sodium salt form and

solubilized in 95% ethanol at 50 mg/ml. This was further diluted to 100 mM in dimethyl sulfoxide and added to cell incubations. Fractionation of the cells was done as previously described [Knutson and Hoenig, 1994].

In Vitro Down-Regulation of PKC δ

Isolated cells were homogenized in fractionation buffer [Knutson and Hoenig, 1994]. The homogenate was then centrifuged at 100,000*g* to yield cytosol. The cytosol was diluted to 10 mg/ml in the same buffer. Ten microliters of cytosol (i.e., 100 μg) were then added to a buffer containing 20 mM Tris HCl, pH 7.5, 10 μM ATP, 10 mM $MgCl_2$, and either control vehicle or 100 μM AA in a final volume of 100 μL. This reduced the concentrations of the protease inhibitors 10-fold. The mixture was allowed to incubate at 30°C for 30 min and terminated with the addition of 5 × Laemmli buffer, followed by boiling for 5 min. Forty microliters of the mixture was then resolved by SDS-PAGE followed by quantitative Western blotting.

Western Blotting

Blotting was done essentially as previously described [Knutson and Hoenig, 1994]. The autoradiograms of the resultant blots were scanned with a BioRad model 620 video densitometer and statistics performed using unpaired Student's *t*-test.

RESULTS

Differential Effects of Phorbol Ester on PKC α and PKC δ

Phorbol esters induce proteolysis of the enzyme, a manipulation which is sometimes useful in understanding the role that PKC plays in various cellular processes. We have already shown that PKC δ is incompletely down-regulated by chronic treatment of cells with 1 μM phorbol ester for 24 h [Knutson and Hoenig, 1995]. To investigate proteolysis of PKC α, cells were incubated in the presence or absence of 1 μM PMA and sampled at 6, 12, and 24 h and homogenate levels of the isoform were then analyzed. In contrast to the effects seen with PKC δ, PKC α was down-regulated to undetectable levels by 6 h (Fig. 1). Interestingly, PKC α started to reappear by 24 h indicating either catabolism of the phorbol ester and/or increased PKC production. Consistent with the known behavior of PKC α in response to phorbol ester,

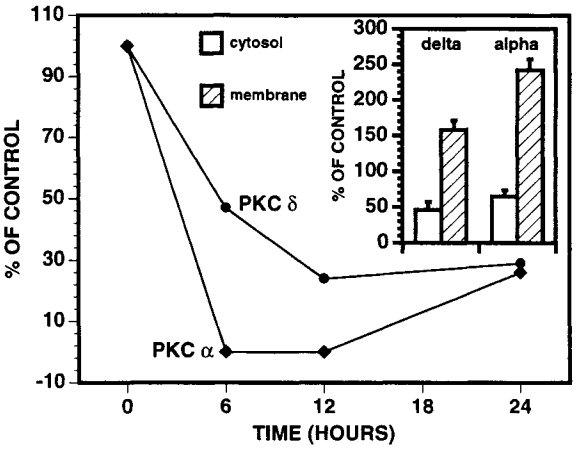

Fig. 1. Differential effects of phorbol esters on PKC α and PKC δ in beta-cells. Cells were incubated for various times in the presence or absence of 1 μM PMA. Forty micrograms of homogenate were examined by blotting as described in Materials and Methods. The results are presented as a percentage of time-matched controls and are the mean of two independent preps which gave nearly identical results. **Inset:** Initial translocations induced by 1 μM PMA. Cells were incubated for 10 (PKC α) or 15 min (PKC δ) and fractionated as described. Forty micrograms of each fraction was analyzed by blotting as described. Shown are the mean ± SEM for 3 independent observations (expressed as a percent of time-matched control). The results for PKC δ have been described elsewhere [Knutson and Hoenig, 1995] and are depicted here for comparison purposes.

this down-regulation was preceded by translocation of the enzyme to the membrane fraction of the cell (Fig. 1, inset). In control cells the levels of each of the enzymes remained at time-zero levels (data not shown).

Effects of Arachidonic Acid on PKC δ and PKC α

Over the course of 45 min, exogenous AA (100 μM) promoted the rapid and sustained decrease of cytosol-associated PKC δ (Fig. 2A and B). The levels of the enzyme decreased to 40 ± 15% ($P < 0.05$, compared to time zero control) in the cytosol. The down-regulation was isoform-specific for PKC δ since, in the same experiments, cytosolic PKC α remained near control levels (Fig. 2A). The down-regulation was compartment-specific since the membrane levels of PKC δ remained unchanged during the same period (Fig. 2C). Evidence that down-regulation *may* have been caused by proteolysis was tested by analysis of whole cellular homogenate levels over the same time course where a sustained drop of ≈15% was observed (Fig. 2C). The slight decrease in homogenate levels likely reflects the fact that PKC δ in the cytosolic compartment represents only 25–30% of the total cellular

enzyme. Application of high concentrations of AA to cells can result in lysis followed by leakage of the enzyme into the medium due to the fatty acid's detergent effects, which could explain the decrease. However, these effects were observed in the absence of detectable alterations in the cytosolic/homogenate protein ratios. For example, at 15 min 100 μM AA induced a decrease of cytosolic PKC δ to 66 ± 9% ($P < 0.025$, n = 4, compared to control) but not a decrease in the cytosolic/homogenate protein ratio (control: 44 ± 2% vs. AA: 43 ± 8%, not significant) (Fig. 3). Furthermore, Coomassie analysis of the cytosolic proteins over the time course showed no significant alterations while lysis induced by high concentrations of fatty acids showed dramatic alterations in the cytosolic profile (data not shown). In addition, this compartment-specific down-regulation could be mimicked at least partially in a cell-free cytosolic preparation (Fig. 4). These effects were not accompanied by increases of PKC δ in the cytoskeletal fraction, which did not differ significantly from control over the time course (data not shown). The down-regulation of cytosol-associated PKC δ was dose dependent as seen in Figure 5. At 30 μM AA (30 min) the decrease was slightly detectable and at 60 μM the decrease was intermediate.

Effects of AA Metabolism Inhibitors on PKC δ

In order to determine if the down-regulating effect was due to the metabolism of AA in the beta-cells, we analyzed the effects of cyclooxygenase and lipoxygenase inhibitors in cells exposed to AA and to vehicle. Indomethacin (10 μM), in the absence of AA, was able to reduce the cytosol-associated enzyme to 72 ± 2.9% ($P < 0.001$) compared to time-matched controls in 1 h incubations (Fig. 6). Similar to effects elicited by AA, this was not accompanied by an increase in membrane-associated levels of the enzyme (101 ± 5.6%, NS compared to control). Higher concentrations of the inhibitor were not evaluated because of uncoupling of oxidative phosphorylation. Analysis of the time course of the effects of indomethacin (Fig. 7) on cytosol-associated PKC (α and δ) over 3 h shows that it is remarkably similar to the effect of AA as seen in Figure 2A. The fatty acid, however, exerted its effect sooner. Indomethacin down-regulated cytosol-associated PKC δ in the absence of any sustained effects on cytosol-associated PKC α. It was observed that cytosol-associated PKC α decreased at 1 h to ≈80%, but this drop was not sustained and returned to control levels at 2 and 3 h. The lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 30 μM), in contrast, had no effect on cytosol-associated enzyme (98 ± 8.8%, NS compared to control) in 1 h incubations but was able to slightly but significantly reduce membrane levels of the enzyme to 85 ± 4.5 ($P < 0.05$, compared to control) in the absence of AA (Fig. 6). NDGA did not block AA-induced decrease in cytosol-associated enzyme (Fig. 6, lower graph) and AA did not impact the decline in membrane-associated enzyme observed with NDGA (data not shown). NDGA (30 μM) did however inhibit the decrease in cytosol-associated PKC δ induced by indomethacin in 1 h incubations (Fig. 6). This effect was not observed with 10 μM NDGA (Fig. 7 inset).

CONCLUSIONS

Arachidonic acid has recently been investigated as a possible second messenger in insulin release since concentrations of the free fatty acid have been shown to rise when islets are exposed to high glucose [Wolf et al., 1991] and could potentially reach an intracellular concentration as high as 100–200 μM [Metz, 1988a,b]. There are a number of identified pathways leading toward the generation of AA in cells. In addition to cleavage of AA from membrane phospholipids by the action of PLA_2, AA can be generated from the hydrolysis of DAG [Konrad et al., 1992]. The generation of AA in islets stimulated with glucose or glucose plus carbachol is very rapid and appears to peak between 10 and 15 min [Jolly et al., 1993]. Activation of the Ca^{2+}-dependent PLA_2 appears to occur no earlier than 10 min in islets stimulated with glucose and carbachol [Jolly et al., 1993] suggesting that the initial fluxes of AA are either the result of Ca^{2+}-independent PLA_2 activation or hydrolysis of DAG. Activation of Ca^{2+}-independent PLA_2 is thought to be mediated by an ATP-regulatory protein, such that changes in ATP levels during glucose stimulation may lead to AA generation via this mechanism [Gross et al., 1993]. It is the inhibition of this PLA_2 by a selective inhibitor that suppresses glucose-induced insulin release and Ca^{2+} entry, suggesting that early glucose-recognition is mediated by AA generation [Ramanadham et al., 1993].

The mechanism by which AA is involved in insulin secretion has been a source of debate. Exogenous AA induces insulin secretion in the

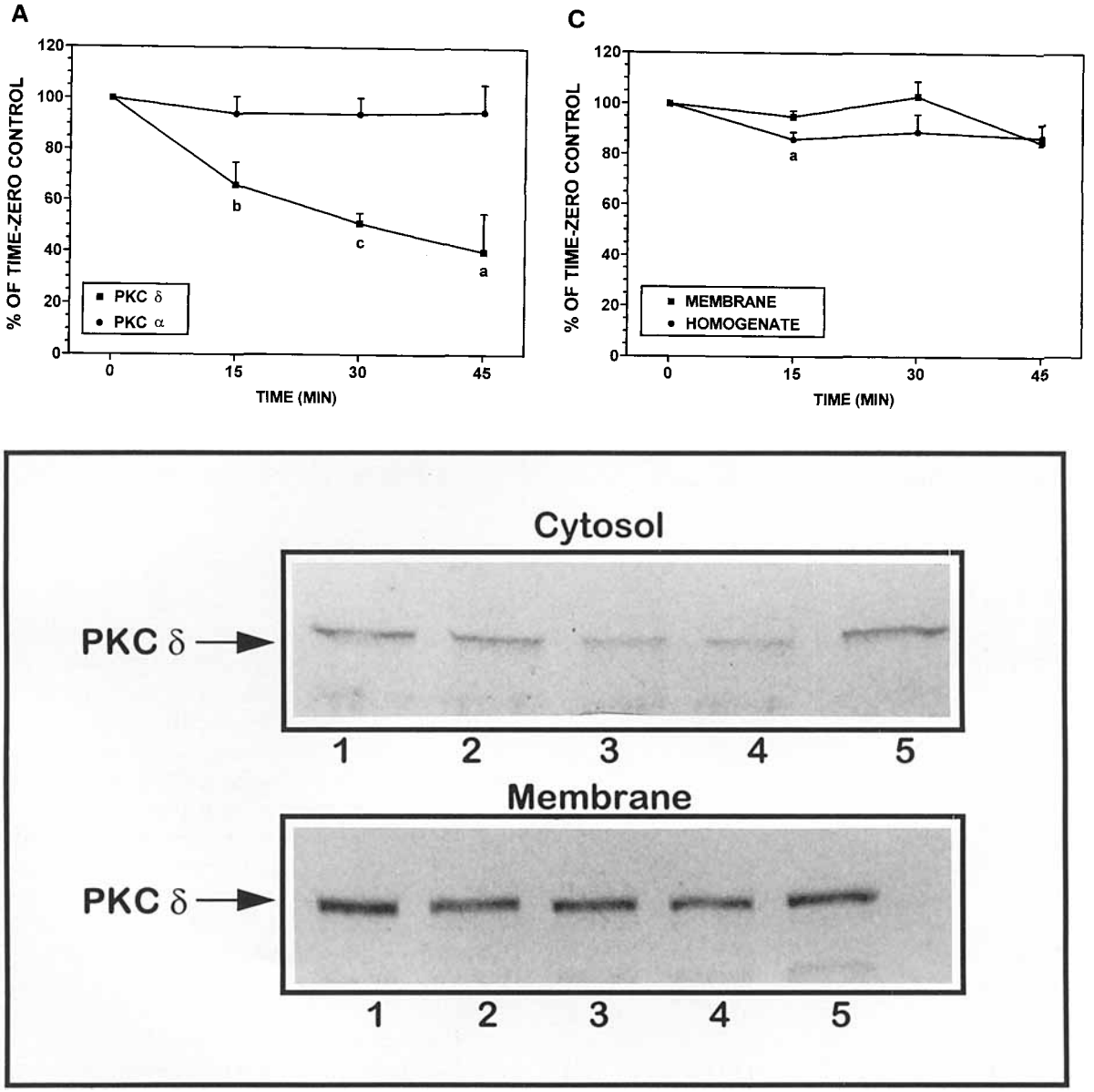

Fig. 2. PKC isoform- and compartment-selective down-regulation by AA. **A:** Cells were incubated in a time-course over 45 min in the presence of 100 μM AA. At the indicated times, cells were fractionated and 40 μg of cytosolic protein was subjected to blotting as described in Materials and Methods with anti-PKC α or anti-PKC δ. Circles = cytosolic PKC α; squares = cytosolic PKC δ. **B:** Representative blot showing a time course of the effects of AA on cytosolic and membrane PKC δ. **Lanes 1, 5:** Control; **2:** 15 min; **3:** 30 min; **4:** 45 min. AA treatment. **C:** Effects of AA on membrane and total cellular PKC δ. See A legend for details. Squares = membrane PKC δ; circles = total cellular PKC δ. a = $P < 0.05$. Results are the mean ± SEM of 3 independent observations (expressed as a percent of time-zero control. a = $P < 0.05$, b = $P < 0.025$, c = $P < 0.005$).

presence of substimulatory concentrations of glucose [Band et al., 1993] but has been shown to inhibit glucose-induced insulin release at concentrations at or above 20 mM glucose [Landt et al., 1992; Wolf et al., 1986]. Because AA can stimulate basal release of insulin it may function as a fusogen rather than mediating its secretory activity through protein phosphorylation or as a calcium mobilizer [Band et al., 1993]. The ability of AA to inhibit glucose-induced insulin release argues that the fusogenic effects, if any, may be limited. However, it

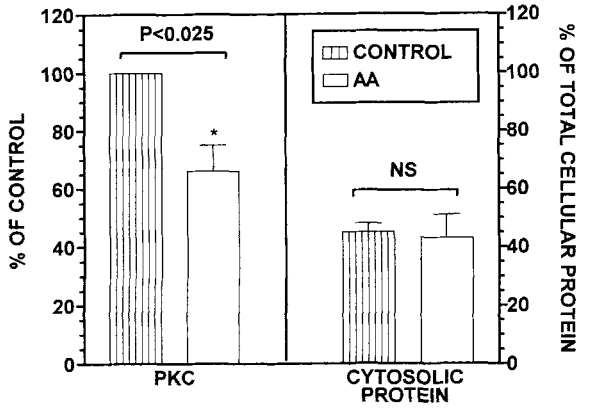

Fig. 3. Dissociation of cytolysis and PKC δ down-regulation. Cells were incubated in the presence of 100 μM AA for 15 min followed by fractionation and blotting for PKC cytosol-associated PKC δ as described in Figure 2. Total cytosolic protein was then quantitated and expressed as a percentage of total cellular homogenate protein and compared to a similar procedure for control cells. There is no difference in cytosol/homogenate ratios between the two groups, although there is a significant decline in cytosol-associated PKC δ ($P < 0.025$) in cells incubated in AA.

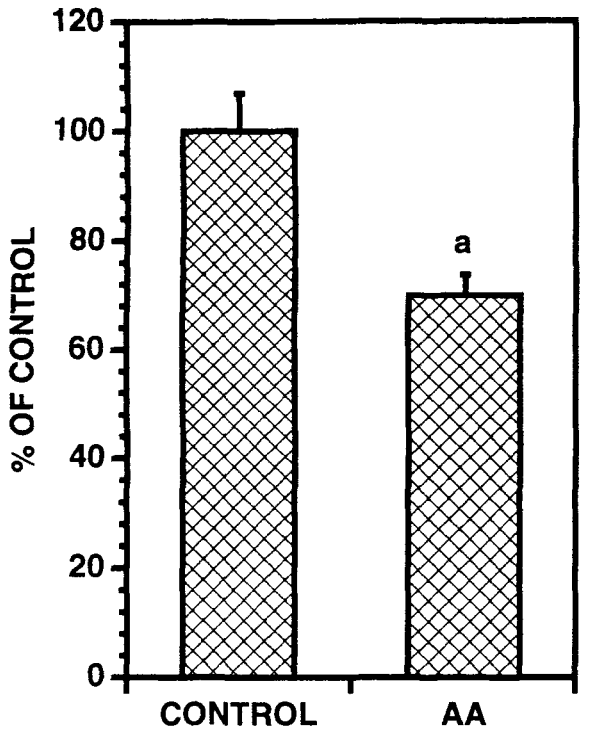

Fig. 4. Cell-free down-regulation of cytosolic PKC δ. Cytosol was incubated in the absence or presence of 100 μM AA as described in Materials and Methods. The cytosol was then blotted and probed. a = $P < 0.02$.

has also been suggested that AA induces insulin release by two mechanisms, Ca^{2+} mobilization and activation of PKC [Metz, 1988c; Ramanadham et al., 1992]. The secretory response to AA in the presence of low glucose is slow in

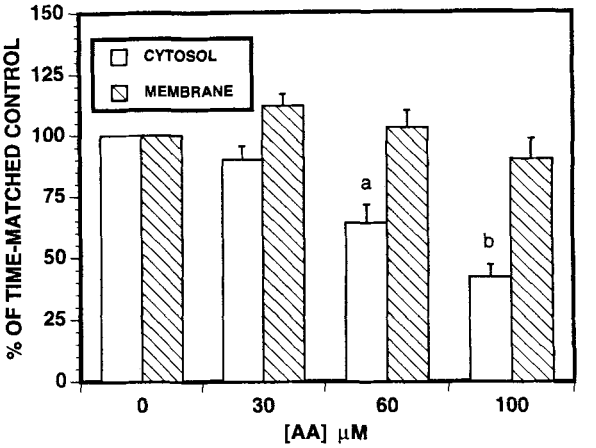

Fig. 5. Dose-dependency of AA-induced down-regulation of cytosolic PKC δ. See details of Figure 2. Cells were incubated for indicated times in the absence or presence of varying concentrations of AA. Results are the mean ± SEM of 3 independent observations (expressed as a percent of time-matched control). a = $P < 0.025$; b = $P < 0.005$.

onset and small compared to that seen with high glucose [Ramanadham et al., 1992]. An inhibitory effect of AA was seen only on the second phase of glucose-induced insulin release and it was suggested that AA may be important in modulating the temporal pattern of sustained stimulation [Jolly et al., 1993]. Previous studies have shown that islet cytosol-associated PKC activity can be stimulated by AA in a Ca^{2+}-dependent manner, indicating that the activity being measured was most likely that of a Ca^{2+}-dependent isoform such as PKC α [Thams et al., 1993; Landt et al., 1992]. Furthermore the application of AA to whole islets did not increase the phosphorylation state of the 80 kD PKC substrate, suggesting that AA does not activate the enzymes [Landt et al., 1992].

Metabolites of AA may also participate in modulating insulin secretion, although their importance is unknown [reviewed in Turk et al., 1993; Metz, 1988; Prentki and Matschinsky, 1987]. In whole islets less than 5% of the total AA is metabolized via a cyclooxygenase or lipoxygenase pathway and very little is thought to be used as fuel [Metz, 1988]. Some studies have shown that inhibition of cyclooxygenase metabolism leads to amplification of glucose-induced insulin release lending speculation that these metabolites may be negative modulators of insulin release [reviewed in Metz, 1988c; Prentki and Matschinsky, 1987]. However, others have found that under conditions where cyclooxygenase was completely inhibited by indomethacin, glucose-induced insulin release was unaffected [Turk et

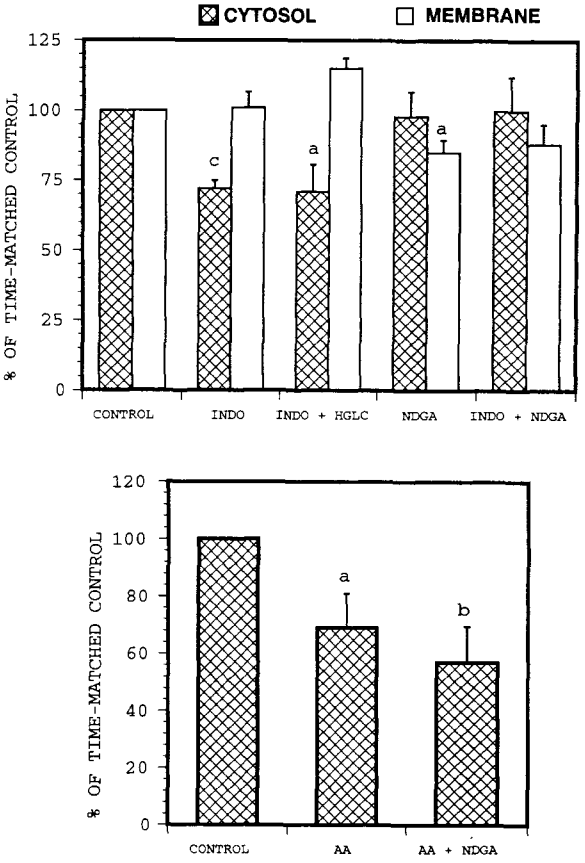

Fig. 6. Effects of oxygenase inhibitors on PKC δ in beta-cells. Cells were incubated for 60 min in the presence or absence of listed drugs. See Figure 2 for details. **Top:** Results are the mean ± SEM for 3–4 independent experiments. INDO = 10 μM indomethacin; NDGA = 30 μM NDGA; HGLC = 30 mM glucose. **Bottom:** AA (50 μM) was added 15 min into the incubation in cells treated with NDGA and incubated for an additional 45 min. Cytosol was extracted from cells and subjected to blotting. Results are the mean ± SEM for 4 independent experiments. a = $P < 0.05$; b = $P < 0.025$; c = $P < 0.001$.

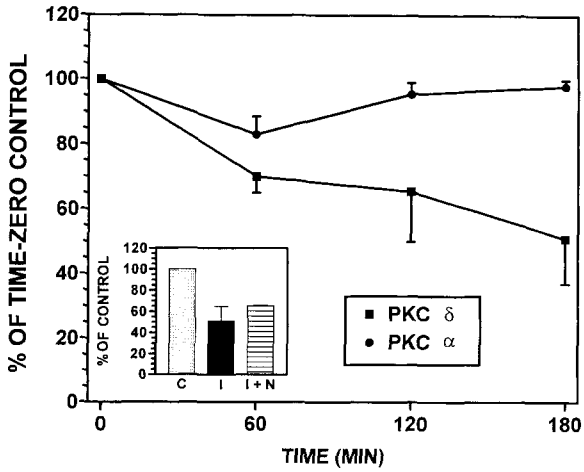

Fig. 7. Effects of indomethacin on cytosolic levels of PKC δ and PKC α. Cell were incubated for various times in the presence or absence of 10 μM indomethacin, followed by fractionation. Forty to fifty micrograms of cytosolic protein were analyzed by immunoblotting with antibodies to either PKC α or PKC δ. **Inset:** Groups of cells were incubated in the presence or absence of 10 μM indomethacin and 10 μM NDGA for 3 h. The NDGA was added 10 min before the addition of the indomethacin. Results are expressed as a percent of control cells incubated without any agents (mean ± SD) and show that indomethacin still down-regulated cytosolic PKC even in the presence of the lipoxygenase inhibitor (C = control; I = 10 μM indomethacin; N = 10 μM NDGA). Similar results were obtained in two independent cell preparations.

al., 1984]. Studies have also shown that inhibition of the lipoxygenase pathway inhibited glucose-induced insulin release, suggesting that this pathway is a positive modulator of insulin release [reviewed in Metz, 1988c; Prentki and Matschinsky, 1987].

AA has been studied extensively as an activator and modulator of PKC activity [Aris et al., 1993; Ogita et al., 1992; Koide et al., 1992; Nakanashi and Exton, 1992; Chen and Murakami, 1992; Shinomura et al., 1991; Wang et al., 1993]. Although our studies clearly indicate that AA can modulate cytosolic levels of the enzyme, it is unknown if this is a direct effect (i.e., activational down-regulation) or secondary to activation of a protease. Aris et al. [1993] and Ogita et al. [1992] have shown that arachidonic acid inhibited DAG/PS-activated PKC δ in vitro, suggesting that the fatty acid does indeed interact with the kinase. However, there are conflicting reports as to whether the fatty acid can activate the enzymes. Aris et al. [1993] have reported that arachidonic acid alone (i.e., in the absence of DAG and PS) was incapable of activating human PKC δ. This, however, is in contrast to other reports where the porcine and murine enzymes were strongly activated by the fatty acid [Leibersperger et al., 1990; Wang et al., 1993]. These results suggest that AA may be, in some cases, a physiological inhibitor of DAG-activated PKC δ. However, there may be substrate and species differences for AA-induced activation of PKC δ.

In these studies, we showed that phorbol ester selectively down-regulated PKC α over PKC δ. PKC α was rapidly down-regulated within 6 h while PKC δ could not be fully down-regulated over a 24 h period. This may reflect the greater affinity of phorbol esters for PKC α or different susceptibilities of each isoform to proteases. Nonetheless, this is interesting in view of the fact that PKC δ is preferentially down-regulated in the presence of AA. However, this effect could also be due to inaccessibility of PKC α to AA.

Selective down-regulation, therefore, may be useful to study the dependency of insulin release on PKC in freshly isolated islets which express both PKC α and PKC δ in amounts similar to that of tumor-derived beta-cells.

These studies demonstrate that AA (or a metabolite) interacts and selectively down-regulates PKC isoforms in beta-cells. The down-regulation appeared to be pool-specific since cytosol-associated PKC δ was more sensitive than membrane-associated enzyme. These results are consistent with a recent model proposing that cytosolic PKC is an important target for fatty acids while membrane bound PKC is a target for diacylglycerols [Khan et al., 1993]. Furthermore, down-regulation of PKC by fatty acids has just been recently demonstrated although the physiological importance is unknown [Diaz-Guerra et al., 1991]. This down-regulation induced by AA in beta-cells was not inhibitable by NDGA, suggesting that the down-regulation was not due to a lipoxygenase or cyclooxygenase metabolite since this compound has been reported to inhibit both pathways although showing preference for lipoxygenase [Salari et al., 1984]. However, the effects of AA could be mimicked by indomethacin (albeit over a longer time period) which possibly increases the bioavailability of AA. In the resting beta-cell the levels of AA are maintained by release from membranes and degradation by oxygenases. Thus, by inhibiting pathways of degradation it would be possible that new resting levels of AA would be higher. If NDGA was inhibiting both pathways then it would have been expected to behave like indomethacin. However, the effects of NDGA in whole cell metabolism appear to be quite complex and evidence suggests that it may inhibit many cellular processes. For example, NDGA is more potent in inhibiting endogenous metabolism of AA than exogenously supplied AA, suggesting it has multiple effects within the cell in addition to inhibiting lipoxygenase [Salari et al., 1984]. NDGA also inhibits fuel metabolism in islets and would thus likely decrease availability of factors (e.g., ATP) which may be required for the generation of free AA [Metz, 1988c]. The inhibition of indomethacin-induced down-regulation of cytosol-associated PKC δ by NDGA was therefore probably not related to inhibition of the lipoxygenase pathway since indomethacin has been observed to inhibit both cyclooxygenase and lipoxygenase pathways in beta-cells [Turk et al., 1984] and platelets [Siegel et al., 1979].

The time course of the effects of indomethacin was much slower, which is possibly due to a slow transition to a new steady state level of AA within the beta-cell. Interestingly, over the time course, PKC α remained near control levels similar to that seen with AA, further suggesting that the effects of indomethacin are mediated by increases in AA. Although this may be the first report of isoform-specific depletion of PKC by the fatty acid, others have observed that free fatty acids including AA can decrease total cellular PKC activity [May et al., 1993; Thams et al., 1993]. In mouse pancreatic islets, the addition of concentrations of AA similar to those used in this study, over 60 min decreased total cellular PKC activity [Thams et al., 1993]. This effect was seen in both the cytosolic and membrane compartments and could be reproduced in lysed cell preparations. The isoform expression in mouse pancreatic islets, however, is currently unknown but worthy of investigation.

In conclusion we have shown that AA promotes the down-regulation of PKC δ in a time- and dose-dependent fashion. These effects are pool specific, showing preference for the cytosol-associated enzyme, and occur at concentrations observed in normal islets during nutrient-induced insulin release. Furthermore, these studies establish a link between AA generation and PKC in beta-cells and support the notion that cytosolic PKC may be a downstream target in AA-induced insulin release [Metz, 1988]. The role of the enzyme in glucose-induced insulin release remains to be determined, but the rapid effects of AA may indicate that it is involved in some aspects of beta-cell memory, such as sensitization. Aside from the ability of AA to activate PKC, the rapid down-regulation may explain why the free fatty acid has inhibitory effects on glucose-induced insulin release. These studies also support our previous reports suggesting that PKC δ is a multifunctional enzyme in the beta-cell [Knutson and Hoenig, 1995]. Future studies using isoform specific down-regulation of PKC δ should clarify the role of this enzyme in beta-cell functions.

REFERENCES

Aris JP, Basta PV, Holmes WD, Ballas LM, Moomaw C, Rankl WD, Blobel G, Loomis CR, Burns DJ (1993): Molecular and biochemical characterization of a recombinant human PKC-δ family member. Biochim Biophys Acta 1174:171–181.

Arkhammer P, Nilsson T, Welsh M, Welsh N, Berggren P-O (1989): Effects of protein kinase C activation on regulation of stimulus-secretion coupling in pancreatic β-cells. Biochem J 264:207–15.

Band AM, Jones PM, Howell S (1993): The mechanism of arachidonic acid-induced insulin secretion from rat islets of Langerhans. Biochim Biophys Acta 1176:64–68.

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

Cook DL, Satin LS, Ashford MLJ, Hales CN (1988): ATP-sensitive K^+ channels in the pancreatic β-cells: Spare channel hypothesis. Diabetes 37:495–498.

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.

Easom RA, Hughes JH, Landt M, Wolf BA, Turk J, McDaniel ML (1989): Comparison of effects of phorbol esters and glucose on protein kinase C activation and insulin secretion in pancreatic islets. Biochem J 264:27–33.

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

Gross RW, Ramanadham S, Kruszka KK, Han X, Turk J (1993): Rat and human pancreatic islet cells contain a calcium ion independent phospholipase A_2 activity selective for hydrolysis of arachidonate which is stimulated by adenosine triphosphate and is specifically localized to islet β-cells. Biochemistry 32:337–346.

Hii CS, Jones PM, Persuad SJ, Howell SL (1987): A reassessment of the role of protein kinase C in glucose-stimulated insulin secretion. Biochem J 246:825–30.

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.

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

Inoue M, Kishimoto A, Takai Y, Nishizuka Y (1977): Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II: Proenzyme and its activation by calcium-dependent protease from rat brain. J Biol Chem 252:7610–7616.

Jolly YC, Major C, Wolf BA (1993): Transient activation of calcium-dependent phospholipase A_2 by insulin secretagogues in isolated pancreatic islets. Biochemistry 32: 12209–12217.

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

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.

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

Knutson KL, Hoenig M (1995): Regulation of distinct pools of protein kinase C δ in beta-cells. J Cell Biochem 60: 130–138.

Koide H, Ogita K, Kikkawa U, Nishizuka Y (1992): Isolation and characterization of the ϵ-subspecies of protein kinase C from rat brain. Proc Natl Acad Sci USA 89:1149–1153.

Konrad RJ, Jolly YC, Major C, Wolf BA (1992): Carbachol stimulation of phospholipase A_2 and insulin secretion in pancreatic islets. Biochem J 287:283–290.

Landt M, Easom RA, Colca JR, Wolf BA, Turk J, Mills LA, McDaniel ML (1992): Parallel effects of arachidonic acid on insulin secretion, calmodulin-dependent protein kinase and protein kinase C activity in pancreatic islets. Cell Calcium 13:163–172.

Laychock SG (1982): Phospholipase A_2 activity in pancreatic islets is Ca^{2+}-dependent and stimulated by glucose. Cell Calcium 3:43–54.

Leibersperger H, Gschwendt M, Marks F (1990): Purification and characterization of a calcium-independent, phorbol ester-activated protein kinase from porcine spleen. J Biol Chem 265:16108–16115.

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

Metz SA (1988a): Is protein kinase C required for physiological insulin release? Diabetes 37:3–7.

Metz SA (1988b): Exogenous arachidonic acid promotes insulin release from intact or permeabilized rat islets by dual mechanisms. Diabetes 37:1453–1469.

Metz SA (1988c): Arachidonic acid and its metabolites: Evolving role as transmembrane signals for insulin release. Prostagland Leukotriene Essent Fatty Acids 1988:187–202.

Metz S, Holmes D, Robertson RP, Leitner W, Draznin B (1991): Gene expression of type I phospholipase A_2 in pancreatic β-cells. FEBS Lett 295:110–112.

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

Nishizuka Y (1992): Membrane phospholipid degradation and protein kinase C for cell signaling. Neurosci Res 15:3–5.

Ogita K, Miyamoto S-I, Yamaguchi K, Kiode H, Fujisawa N, Kikkawa U, Sahara S, Fukami Y, Nishizuka Y (1992): Isolation and characterization of δ-subspecies of protein kinase C from rat brain. Proc Natl Acad Sci USA 89:1592–1596.

Opara EC, Garfinkle 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.

Persuad SJ, Jones PM, Sugden D, Howell SL (1989): Translocation of protein kinase C in rat islets of Langerhans: Effects of a phorbol ester, carbachol, and glucose. FEBS Lett 245:80–84.

Persuad SJ, Jones PM, Howell SL (1991): Activation of protein kinase C is not required for glyceraldehyde-stimulated insulin secretion from rat islets. Biochim Biophys Acta 1095:183–85.

Prentki M, Matschinsky FM (1987): Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. Pharmacol Rev 67:1185–1248.

Prentki M, Vischer S, Gleeno MC, Regazzi R, Deeney JT, Corkey B (1992): Malonyl-CoA and long chain acyl-CoA derivatives as metabolic coupling factors in nutrient-induced insulin secretion. J Biol Chem 267:5802–5810.

Ramanadham S, Gross R, Turk J (1992): Arachidonic acid elevates the cytosolic calcium concentration in individual pancreatic β-cells. Biochem Biophys Res Commun 184: 647–653.

Ramanadham S, Gross RW, Han X, Turk J (1993): Inhibition of arachidonate release by secretagogue-stimulated pancreatic islets suppresses both insulin secretion and the rise in β-cell cytosolic calcium ion concentration. Biochemistry 32:337–346.

Sahai A, Fadda GZ, Massry SG (1992) Parathyroid hormone activates protein kinase C of pancreatic islets. Endocrinology 131:1889–1894.

Sako Y, Grill (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.

Salari H, Braquet P, Borgeat P (1984): Comparative effects of indomethacin, acetylenic acids, 15-hete, nordihydroguaiaretic acid, and BW755C on the metabolism of arachidonic acid in human leukocytes and platelets. Prost Leuk Med 13:53–60.

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 USA 88:5149–5153.

Siegel MI, McConnell RT, Cuatrecasas P (1979): Aspirin-like drugs interfere with arachidonate metabolism by inhibition of the 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid peroxidase activity of the lipoxygenase pathway. Proc Natl Acad Sci USA 76:3774–3778.

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

Thams P, Capito K, Hedeskov CJ, Kofod H (1990): Phorbol-ester-induced down-regulation of protein kinase C in mouse pancreatic islets. Potentiation of phase 1 and inhibition of phase 2 of glucose-induced insulin release. Biochem J 265:777–787.

Thams P, Hedeskov CJ, Capito K (1993): Exogenous arachidonic acid inactivates protein kinase C in mouse pancreatic islets. Acta Physiol Scand 149:227–235.

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

Turk J, Colca JR, McDaniel ML (1985): Arachidonic acid metabolism in isolated pancreatic islets. III. Effects of exogenous lipoxygenase products and inhibitors on insulin secretion. Biochim Biophys Acta 834:23–36.

Turk J, Gross RW, Ramanadham S (1993): Amplification of insulin secretion by lipid second messengers. Diabetes 42:367–74.

Wang X-J, Warren BS, Beltran LM, Fosmire SP, DiGiovanni J (1993): Further identification of protein kinase C isozymes in mouse epidermis. J Cancer Res Clin Oncol 119: 279–287.

Wolf BA, Turk J, Sherman WR, McDaniel ML (1986): Intracellular Ca^{2+} mobilization by arachidonic acid. J Biol Chem 261:3501–3511.

Wolf BA, Pasquale SM, Turk J (1991): Free fatty acid accumulation in secretagogues-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 USA 89:6443–6446.

Zawalich WS, Rasmussen H (1990): Control of insulin secretion: A model involving Ca^{2+}, cAMP and diacylglycerol. Mol Cell Endocrinol 70:119–137.

Zawalich W, Zawalich W, Rasmussen H (1984): Insulin secretion: combined tolbutamide, forskolin, and TPA mimic action of glucose. Cell Calcium 5:551–558.

Zawalich WS, Zawalich KC, Ganesan S, Calle R, Rasmussen H (1991): Influence of staurosporine on glucose-mediated and glucose-conditioned insulin release. Biochem J 279: 807–813.