PKCα Is Activated But Not Required During Glucose-Induced Insulin Secretion From Rat Pancreatic Islets

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The role of protein kinase C (PKC) in glucose-stimulated insulin secretion (GSIS) is controversial. Using recombinant adenoviruses for overexpression of PKCα and PKCδ, in both wild-type (WT) and kinase-dead (KD) forms, we here demonstrate that activation of these two PKCs is neither necessary nor sufficient for GSIS from batch-incubated, rat pancreatic islets. In contrast, responses to the pharmacologic activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were reciprocally modulated by overexpression of the PKCαWT or PKCαKD but not the corresponding PKCδ adenoviruses. The kinetics of the secretory response to glucose (monitored by perifusion) were not altered in either cultured islets overexpressing PKCαKD or freshly isolated islets stimulated in the presence of the conventional PKC (cPKC) inhibitor Go6976. However, the latter did inhibit the secretory response to TPA. Using phosphorylation state–specific antisera for consensus PKC phosphorylation sites, we also showed that (compared with TPA) glucose causes only a modest and transient functional activation of PKC (maximal at 2–5 min). However, glucose did promote a prolonged (15 min) phosphorylation of PKC substrates in the presence of the phosphatase inhibitor okadaic acid. Overall, the results demonstrate that glucose does stimulate PKCα in pancreatic islets but that this makes little overall contribution to GSIS. *Diabetes* 53:53–60, 2004

The protein kinase C (PKC) family of serine and threonine kinases has 10 members that are characterized by their molecular structure and activation requirements. The subfamilies consist of the conventional PKCs (cPKCs; α, β_I, β_{II}, and γ), which are sensitive to Ca^{2+} and diacylglycerol (DAG); the novel PKCs (δ, ε, η, and θ), which have sensitivity to DAG only; and the atypical PKCs (ζ and ι), which respond to neither DAG nor Ca^{2+}. Various PKC isoforms have been implicated in diverse cellular functions, including growth, differentiation, and apoptosis, as well as specialized responses, such as secretion and muscle contraction (1–3).

Pancreatic islets and β-cell lines contain PKCα and δ (4,5) as major isozymes, but PKCs β_{II}, ε, ζ, and ι are also expressed (5,6). However, the contribution of PKC activation to glucose-stimulated insulin secretion (GSIS) remains extremely controversial. Translocation studies (7–12) have provided indirect evidence that glucose does activate cPKCs, especially PKCα, but even this is not universally observed (13,14). The functional relevance of this activation is even more controversial, with widely varying results reported from studies designed to disrupt PKC function by use of chemical or peptide inhibitors (9,14–18) or by chronic PKC downregulation (6,19–25).

A scarcity of truly isoform-specific inhibitors of PKCs has doubtless contributed to the confusion in the β-cell literature, especially because different PKCs might differentially regulate secretion (21). Moreover, experiments using chronic downregulation are difficult to interpret because of opposing short- and long-term effects (25). Molecular approaches to selectively modulate the expression or function of individual PKC isozymes have not been used to investigate insulin secretion, possibly because the widely studied immortalized β-cell lines are often poorly responsive to glucose and do not have identical PKC complements to normal β-cells (4–6) (C.J.M., T.J.B., unpublished observations). Nonclonal cells have not been amenable to conventional transfection techniques because of their terminally differentiated state. Limitations in tissue availability have probably also contributed to the difficulties in observing direct activation of PKCs using biochemical assays of glucose-stimulated islets (14,20,26,27). Finally, although glucose can increase incorporation of ^{32}P into islet proteins (21,27,28), the contribution to this process of PKC (as opposed to other protein kinases) has been difficult to establish with certainty.

We therefore decided to reexamine the role of PKC in GSIS by taking advantage of two relatively recent technical advances. The first makes use of recombinant adenovirus technology (29) to introduce PKC cDNA into the intact rat pancreatic islet. In particular, we sought to overexpress PKCα and PKCδ in both their wild-type (WT) and kinase-dead (KD) forms because this strategy has been used widely to implicate individual PKC isoforms in

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cPKC, conventional protein kinase C; DAG, diacylglycerol; GSIS, glucose-stimulated insulin secretion; KD, kinase dead; KRB, Krebs-Ringer buffer; PKC, protein kinase C; RIA, radioimmunoassay; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; WT, wild type.

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a host of different cellular functions (30–36). Second, we screened for functional PKC activation in glucose-stimulated islets using specific antisera raised to consensus phosphorylation motifs for cPKC (37). The results show that although glucose results in a transient activation of cPKC, this does not play a major role in the secretory response.

RESEARCH DESIGN AND METHODS

Preparation of recombinant adenovirus. PKCα and PKCδ WT and KD viruses were generated as previously described (33). Briefly, PKCα and PKCδ cDNA were subcloned into pALTER (Promega, Annadale, NSW, Australia) and mutagenesis was performed according to the manufacturer's instructions to produce PKCαKD (K368R substitution) and PKCδKD (K376R substitution). WT and mutant PKC cDNAs were then subcloned into pXCMV (an adenoviral shuttle plasmid generated from pXCX3 [38]). Recombinant adenovirus was then prepared essentially as described by Graham and Prevec (39), by homologous recombination of pXCMV.PKC constructs with pJM17 (virus backbone) in HEK 293 cells. Control virus, designated MX17, was constructed by recombination between pXCX3 and pJM17 and so represents intact virus without a mammalian expression cassette or cDNA.

Isolation and infection of islets with recombinant adenoviruses. Pancreatic islets were isolated from 220- to 250-g male Wistar rats by ductal infusion of collagenase (Serva Feinbiochemica, Heidelberg, Germany), purified on a Histopaque 1077 gradient, and then handpicked under a binocular microscope. Batches of 300 to 600 islets were infected with CsCl-purified recombinant adenovirus in RPMI culture medium (Gibco BRL, Melbourne, Australia) and 20 mmol/l polyethylene glycol (PEG_{6000}; Merck, Kilsyth, Victoria, Australia). Islets were routinely incubated for 1 h at 37°C with virus at $\sim 2 \times 10^6$ plaque-forming units/islet. Islets were maintained in culture for 48 to 72 h in RPMI containing 5.5 mmol/l glucose, 10% (vol/vol) heat-inactivated FCS, 500 units/ml penicillin G sodium, 50 μg/ml streptomycin, and 2 mmol/l glutamine (reagents from Gibco BRL, and plasticware from Becton and Dickinson and Co, Lincon Park, NJ).

PKC activity assays. Crude lysates from islets infected with recombinant adenovirus were prepared by resuspending islets in lysis buffer (PBS, 1% Triton X-100, 2 mmol/l phenylmethylsulfonyl fluoride, 200 μg/ml leupeptin, 2 mmol/l benzamidine, 100 mmol/l sodium pyrophosphate, 10 mmol/l sodium orthovanadate, 10 mmol/l sodium fluoride, 1 mmol/l EDTA, 1 mmol/l EGTA, and 0.5% [wt/vol] Mega 10). Islets were sonicated on a Branson Sonifier with three rounds of 10 cycles at low power. The detergent soluble fraction was isolated after centrifugation at 13,000*g* for 10 min. PKC was partially purified from these fractions by loading onto 1-ml Macro-prep DEAE columns (Bio-Rad, Regents Park, NSW, Australia) pre-equilibrated with a buffer containing 20 mmol/l NaCl, 20 mmol/l MOPS (pH 7.5), 4% glycerol, 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l DTT, 0.1% (wt/vol) Mega 10, 2 mmol/l phenylmethylsulfonyl fluoride, 200 μg/ml leupeptin, 2 mmol/l benzamidine, 100 mmol/l sodium pyrophosphate, 10 mmol/l sodium orthovanadate, and 10 mmol/l sodium fluoride. After two rounds of washing with the equilibration buffer containing first 20 mmol/l and second 100 mmol/l NaCl, PKC was then eluted by application of equilibration buffer containing 300 mmol/l NaCl (40). Kinase activity assays were performed essentially as described (41). Briefly, PKC extracts were incubated in kinase assay buffer containing 24 mmol/l MOPS (pH 7.5), 0.04% (vol/vol) Triton X-100, 120 mmol/l IP_{20} (cAMP-dependent protein kinase inhibitor peptide), 100 μmol/l [γ-^{32}P]ATP (100–200 cpm/pmol), 5 mmol/l $Mg(CH_2COO)_2$, 5 μmol/l modified (19–31, Ser 25) PKC pseudosubstrate peptide ± 125 μg/ml phosphatidylserine, and 2.5 μg/ml di-octanoylglycerol. Incubations were allowed to proceed for 7.5 min at 30°C and terminated with 10 μl of 150 mmol/l "cold" ATP. A total of 40 μl of assay sample was spotted onto P81 paper (Whatman, Maidstone, England), washed five times in 1% (vol/vol) phosphoric acid, dried, resuspended in water, and Cerenkov radiation counted using a Beckman β counter. Results are expressed as amount of ATP incorporated ($\text{pmol} \cdot \text{islet}^{-1} \cdot \text{min}^{-1}$).

Measuring insulin secretion. Freshly isolated or cultured islets were picked in groups of 15 for batch incubations and washed with a modified Krebs-Ringer buffer (KRB; containing 5 mmol/l $NaHCO_3$, 1 mmol/l $CaCl_2$, 2.8 mmol/l glucose, 10 mmol/l HEPES [pH 7.4], and 0.5% (wt/vol) BSA. Batch incubations were first preincubated at 37°C for 30 min in 2.8 mmol/l glucose KRB and then incubated in 1 ml of KRB containing 2.8 mmol/l glucose, 16.7 mmol/l glucose, or 500 nmol/l 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 1 h. The incubation was terminated upon removal of KRB for analysis. Islets for perifusion studies were loaded into Swinex chambers (Millipore, North Ryde, NSW, Australia) and perifused at 37°C at 0.5 ml/min with 2.8 mmol/l glucose in KRB for 20 min before experimental additions as described. In some instances, 0.1 μmol/l Go6976 (Biomol, Plymouth Meeting, PA) was included with 0.03% DMSO used as a vehicle in the control. Insulin content from batch supernatants and perifusates was determined by radioimmunoassay (RIA; Linco Research, St. Louis, MO) with rat insulin standard.

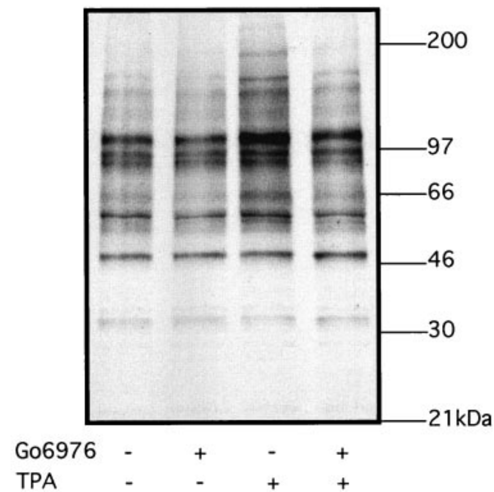

FIG. 1. Characterization of cPKC substrate antisera using freshly isolated pancreatic islets. Batches of islets were incubated for 2 min in KRB with 2.8 mmol/l glucose plus or minus 500 nmol/l TPA and in the presence or absence of 1 μmol/l Go6976. Extracts (equivalent to 10 islets/lane) were subjected to SDS-PAGE using 4–15% gradient gels. Protein bands were detected by immunoblotting on nitrocellulose membranes using primary rabbit anti-phosphoserine cPKC substrate antisera, followed by horseradish peroxidase–conjugated secondary antibody and visualized by enhanced chemiluminescence. For further details, see RESEARCH DESIGN AND METHODS. The figure is a representative immunoblot from three independent experiments.

Immunoblot analysis. Batches of 30 to 75 freshly isolated or cultured islets were incubated in modified KRB for secretory experiments. Reactions were terminated by removal of KRB and addition of 1 ml of ice-cold PBS. Whole-cell lysates were prepared after removal of PBS by resuspension of the islets in 60 to 150 μl of Laemmli sample buffer. Islets were disrupted by sonication and subjected to SDS-PAGE using 10% gels (PKC expression) or 4 to 15% gradient gels (phosphorylation studies). Reagents for SDS-PAGE and immunoblotting (Ready Gels [4–15%], 2% bis-acrylamide, 40% acrylamide, mercaptoethanol, ammonium persulfate, and nitrocellulose membrane) were from Bio-Rad. Proteins were electroblotted onto nitrocellulose membrane and probed with primary antisera and followed by horseradish peroxidase–conjugated secondary antibody (Jackson Immuno Research, West Grove, PA). Sources of primary antibodies were mouse anti-PKCα and -PKCδ (BD Biosciences, San Diego, CA); mouse anti-PKCβII, -PKCε, and -PKCζ (Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit anti-phosphoserine PKC substrate (Cell Signaling Technology, Beverly, MA). Bands were visualized by incubation with ECL detection kit (Amersham, Little Chalfont, Buckinghamshire, U.K.) and exposure to X-ray film (Fuji, Tokyo, Japan) with images stored using a Molecular Dynamics Personal Densitometer SI and visualized using software by IPLab-Gel (Signal Analytics, VA).

Statistics. Results are presented as mean ± SE. Statistical significance was determined using unpaired Student's *t* test.

RESULTS

cPKC substrate phosphorylation in freshly isolated islets. To provide direct functional evidence of cPKC activation, we used an antibody raised against phosphorylated Ser residues surrounded by Arg or Lys at the −2 and +2 positions and a hydrophobic residue at +1 (37). This forms a consensus phosphorylation motif for cPKCs but not the novel or atypical PKC isozymes (42,43). As shown in Fig. 1, this antiserum detects a number of protein bands in lysates of freshly isolated islets. The intensity of many of these bands is increased in islets treated for 2 min with TPA, a pharmacologic activator of novel and cPKCs. Conversely, TPA-stimulated phosphorylation was de-

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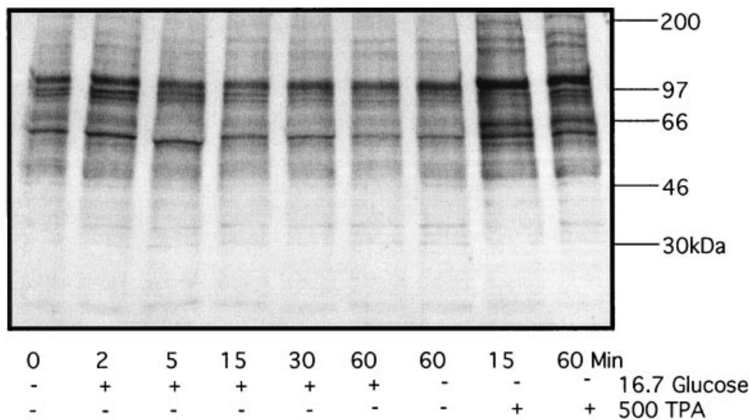

FIG. 2. Effect glucose and TPA on cPKC substrate phosphorylation in freshly isolated pancreatic islets. Batches of islets were incubated for times as indicated with either 2.8 mmol/l glucose plus or minus 500 nmol/l TPA or with 16.7 mmol/l glucose. Islets were lysed and cPKC substrate phosphorylation was determined by immunoblotting as described in the legend of Fig. 1. Shown is a representative immunoblot of a total of five (glucose) or four (TPA) independent experiments.

creased by pretreatment with Go6976. This compound inhibits cPKCs with a Ki several orders of magnitude below that required for inhibition of the other PKC isozymes (42,44) but can also act on other (non-PKC) protein kinases. Our results are very consistent with those of a recent study using breast cancer cells (37) and confirm the specificity of the antiserum in detecting cPKC substrates. Phosphorylation as a result of TPA was maintained for at least 15 min but returned toward baseline by 60 min (Fig. 2). In contrast, 16.7 mmol/l glucose elicited, at best, a very minor and transient increase in cPKC substrate phosphorylation (Fig. 2). When analyzed by densitometry and averaged from five independent experiments, phosphorylation caused by glucose was not significantly increased at any measured point over the entire time course of 0 to 60 min (results not shown).

Role of cPKC in insulin secretion from freshly isolated islets. We next investigated whether cPKC activation was necessary for GSIS. This did not seem to be the case, as secretion from islets that were batch-incubated with either maximal (16.7 mmol/l) or submaximal (9.8 mmol/l) glucose concentrations was totally unaffected by Go6976 (Fig. 3*A*). In contrast, this compound dose-dependently inhibited TPA-stimulated insulin secretion, with complete inhibition occurring at 1 μmol/l (Fig. 3*A*). This was further confirmed by the more sensitive perifusion analysis showing that biphasic GSIS was completely unaltered by Go6976, under conditions in which the response to TPA was markedly inhibited (Fig. 3*B*). When analyzed over the TPA treatment period, the area under the curve amounted to 957 ± 60 vs. 357 ± 62 pg · islet^{-1} · 35 min^{-1} in the absence and presence of Go6976, respectively ($P <$ 0.005).

Activation of cPKC in cultured islets. Most of the previous studies demonstrating glucose-dependent translocation of cPKC (9–12) were conducted with cultured islets, primary β-cells, or clonal cell lines, rather than the freshly isolated islets used for the experiments reported in Figs. 1–3. We therefore examined functional activation of cPKC in islets after 48-h tissue culture (Fig. 4). Under these conditions, the increase in cPKC substrate phosphorylation caused by 16.7 mmol/l glucose was more pronounced than had been seen in fresh islets and peaked consistently at 2 to 5 min after simulation. This transient peak, followed by a decline to baseline by 30 min, was confirmed by densitometric analysis of the phosphorylation of a 95-kDa protein from four experiments (Fig. 4, *bottom*). Essentially similar results were obtained from analyses of other major phosphorylation bands (results not shown). As was the case with freshly isolated islets, TPA also promoted a larger and more prolonged phos-

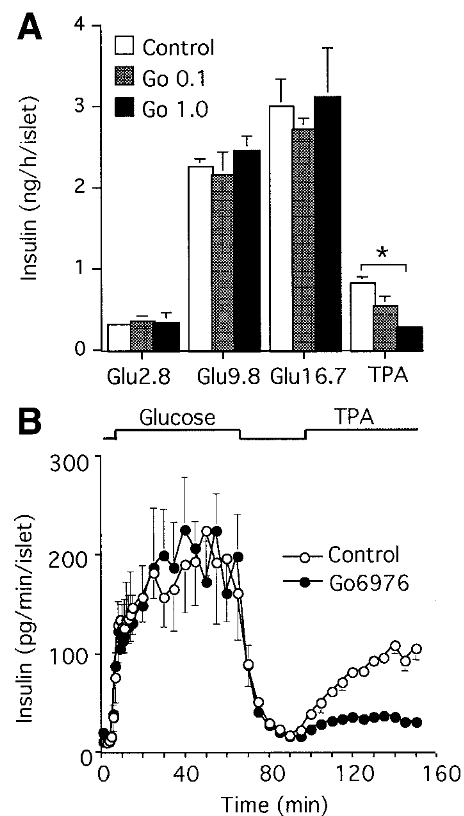

FIG. 3. Effect of Go6976 on glucose- and TPA-induced insulin secretion from batch incubations (*A*) or perifusions (*B*) of freshly isolated rat islets. *A*: Batches of 15 islets were isolated, washed in modified KRB, and incubated for 1 h at 37°C in KRB containing glucose (mmol/l as indicated) or 2.8 mmol/l glucose plus 500 nmol/l TPA, in the presence or absence of Go6976 (Go) at 0.1 or 1.0 μmol/l. Insulin was analyzed by RIA. Results are from two to three independent experiments each performed in quadruplicate. *$P < 0.005$ where indicated. *B*: Groups of 25 islets were perifused in KRB containing 2.8 mmol/l glucose for 20 min before a stimulatory period of 60 min with KRB containing 16.7 mmol/l glucose. The perifusate was then switched back to 2.8 mmol/l glucose for 30 min to allow insulin secretion to return to basal levels before exposure to 500 nmol/l TPA for 60 min (at 2.8 mmol/l glucose). Where indicated, Go6976 (0.1 μmol/l) was included throughout the perifusion period. One-minute fractions of the perifusate were collected for the first 15 min, then 5-min fractions for all following time points at a flow rate of 0.5 ml/min. Insulin was analyzed by RIA. Mean data are presented from five to six separate experiments.

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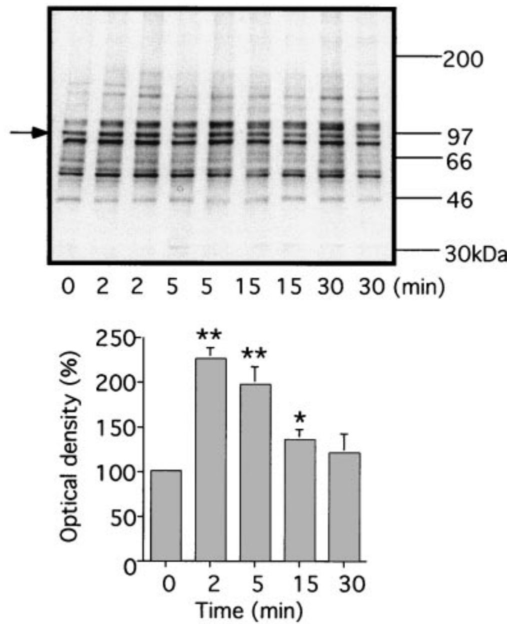

FIG. 4. Time course of glucose-stimulated cPKC substrate phosphorylation in cultured pancreatic islets. Batches of islets that had been cultured for 48 h were incubated in KRB medium for the indicated times. Islets were lysed, and cPKC substrate phosphorylation was determined by immunoblotting as described in the legend to Fig. 1. *Top*: Representative immunoblot of four similar experiments. *Bottom*: Mean of densitometric analysis of the intensity of a 95-kDa band (arrow in *top*) from four independent experiments, each determined in duplicate. Results are expressed as a percentage of the time 0 response. *$P < 0.05$, **$P < 0.001$ vs. time 0.

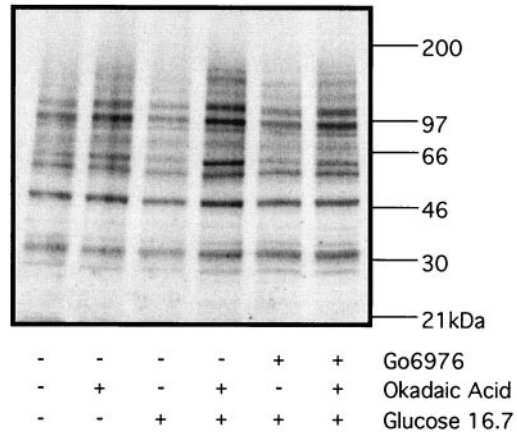

FIG. 5. Effect of glucose okadaic acid and Go6976, alone or in combination, on cPKC substrate phosphorylation in cultured islets. Batches of islets that had been cultured for 48 h were incubated in KRB medium for 15 min with either 2.8 mmol/l glucose (first two lanes) or 16.7 mmol/l glucose. Go6976 (1 μmol/l) and okadaic acid (1 μmol/l) were also present where indicated. Islets were lysed and cPKC substrate phosphorylation determined by immunoblotting as described in the legend to Fig. 1. The figure is a representative immunoblot of three similar experiments.

phorylation than did glucose in the cultured islets (results not shown).

An explanation for the transient phosphorylation of cPKC substrates caused by glucose in cultured islets might be the concomitant activation of serine/threonine protein phosphatases (45–47). To address this possibility, we made use of the inhibitor okadaic acid at a concentration (1 μmol/l) known to attenuate the activities of both protein phosphatase 1 and 2A (47). This study was undertaken using islets stimulated for 15 min, by which time the transient increase in cPKC substrate phosphorylation caused by glucose is returning toward baseline levels (Fig. 5). Under these conditions, basal phosphorylation was slightly elevated by okadaic acid. Most important, the combination of 16.7 mmol/l glucose plus okadaic acid resulted in a phosphorylation pattern markedly greater than that caused by okadaic acid alone, and this was inhibited by Go6976 (Fig. 5). This strongly suggests that, provided phosphatase activity is inhibited, glucose can provoke a prolonged and substantial phosphorylation of cPKC substrates but that the latter is usually counteracted by a concomitant activation of serine/threonine phosphatases directed toward cPKC substrates.

PKC overexpression by adenoviral-mediated gene transfer. Instead of relying solely on pharmacologic inhibitors, the responsiveness of cultured islets to cPKC phosphorylation now made possible a more rigorous molecular approach in which recombinant adenovirus was used to reinvestigate the potential role cPKC activation in GSIS. Figure 6*A* confirms that exposure of islets to PKCαWT, PKCαKD, PKCδWT, and PKCδKD viruses led to a marked increase in expression of the relevant isoform compared with islets infected with MX17 control virus (containing viral genes and expression cassette but no transgene). Endogenous levels of other isoforms known to be expressed in islet β-cells (PKCβ_{II}, PKCε, and PKCζ) were also analyzed and found to be similar to levels observed in the MX17 control and therefore unaffected by PKCα or PKCδ overexpression. This argues against nonspecific effects of the transgene being mediated by alterations in the protein levels of other endogenously expressed isoforms. To confirm the activity of the various overexpressed PKC constructs, we performed kinase assays on purified extracts of cell lysates from islets infected with MX17 or appropriate PKC adenoviruses (Fig. 6*B*). Relative kinase activities from lysates of islets infected with PKCαWT or PKCδWT were ~50- and 20-fold higher, respectively, than those seen with MX17-infected islets. This suggests that PKCαWT and PKCδWT are overexpressed to similar levels because the substrate used in these assays is known to display a three- to fivefold higher affinity for PKCα over PKCδ (42,48). PKCαKD and PKCδKD were also shown to possess no kinase activity as previously documented (30,31,49). In other experiments, using confocal microscopy, we determined that transgene expression (green fluorescent protein or PKCα) was readily and uniformly detected throughout the islet core containing β-cells (results not shown). Finally, analysis of insulin secretion indicates that cellular responses are not adversely affected by exposure to adenovirus, because islets infected with the control MX17 virus displayed basal and stimulated secretory responses that were not significantly different from those of uninfected islets (Fig. 6*C*).

Analysis of the role of PKCα and PKCδ glucose and TPA-induced insulin secretion. The rationale underlying these experiments is that overexpression of a WT PKC enzyme will upregulate a physiologic response mediated by that isozyme. Conversely, the KD mutant acts as a dominant negative, most probably by competition for binding proteins that regulate the localization and cellular function of endogenous isozyme. This approach has been

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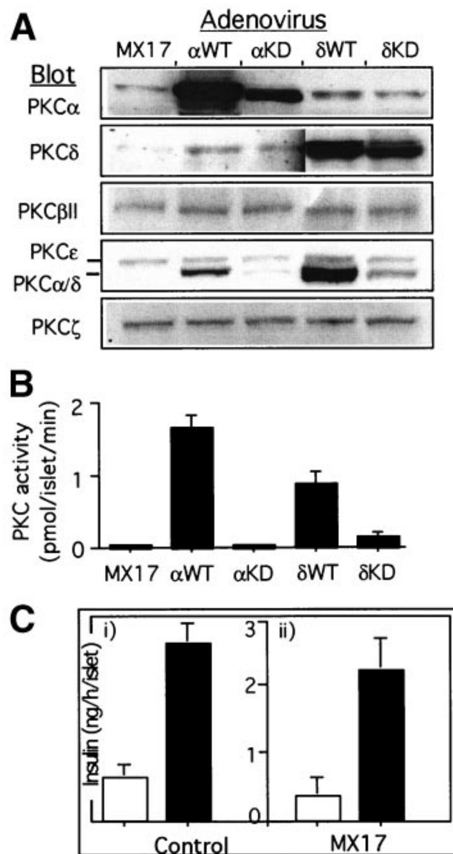

FIG. 6. *A*: Analysis of PKCα and PKCδ overexpression and effects on other endogenously expressed isoforms. Relative levels of PKCαWT or PKCαKD overexpression in rat pancreatic islets was determined by infecting islets with the appropriate recombinant adenovirus under routine conditions. Whole-cell lysates were prepared at day 3 as described in RESEARCH DESIGN AND METHODS with protein concentrations standardized by Bradford assay. Proteins were separated by SDS-PAGE on 10% gels, electroblotted onto nitrocellulose, and immunoblotted for PKCα and PKCδ using appropriate antibodies. Analysis of relative levels of endogenously expressed PKC isoforms, namely $PKC\beta_{II}$, PKCε, and PKCζ, were also performed by analysis of separate blots with appropriate antibodies. Note that the PKCε antisera cross-reacted with the overexpressed PKCα/δ as well as the slower migrating endogenous PKCε band. *B*: Protein kinase activity in islets infected with PKCα and PKCδ adenovirus. Kinase activity from lysates of islets infected with MX17, PKCαWT, PKCαKD, PKCδWT, or PKCδKD adenovirus was determined after partial purification of PKC on DEAE columns as described in RESEARCH DESIGN AND METHODS. Purified PKC extracts were then assayed for kinase activity by incubating for 7.5 min at 30°C in the presence of 1 mmol/l Ca^{2+} (for PKCα lysates) and phosphatidylserine (125 μg/ml) and di-octanylglycerol (12.5 μg/ml; for both PKCα and PKCδ lysates) with modified pseudosubstrate peptide (5 μmol/l) as the substrate. Results are expressed as picomoles ATP incorporated into substrate per islet per minute. Mean data from three (MX17, PKCαWT, and PKCδKD) or two separate experiments (PKCαKD and PKCδWT) are shown. *C*: Glucose responsiveness of islets exposed to adenovirus. Islets were preexposed to medium (*i*) or infected with MX17 adenovirus (*ii*) as described in RESEARCH DESIGN AND METHODS. At day 3 postinfection, batches of 15 islets were incubated in KRB with 2.8 mmol/l glucose (□) or 16.7 mmol/l glucose (■) for 60 min. Supernatant was removed, and RIA was performed to determine insulin secreted. Mean data of three separate experiments are presented.

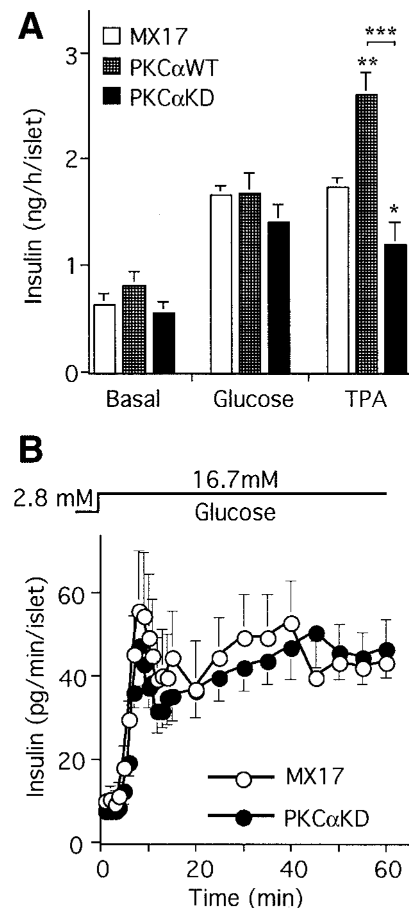

FIG. 7. Glucose- and TPA-induced insulin secretion from batch incubations (*A*) or perifusions (*B*) of cultured rat islets overexpressing PKCα adenovirus. *A*: Islets were infected with recombinant MX17, PKCαWT, and PKCαKD adenovirus as described in RESEARCH DESIGN AND METHODS. Batches of 15 islets were isolated, washed in modified KRB, and incubated for 1 h at 37°C in KRB containing 2.8 mmol/l glucose (basal), 16.7 mmol/l glucose, or 500 nmol/l TPA. Mean data of eight separate experiments, each performed in at least quadruplicate, are shown. $*P < 0.05$, $**P < 0.001$, and $***P < 0.001$. Effects of WT and KD are compared with the MX17-infected islets within that condition; however, the bar represents significance between PKCαWT and PKCαKD. *B*: Islets were infected with MX17 or PKCαKD virus as described in RESEARCH DESIGN AND METHODS. Groups of 25 islets were perifused in KRB containing 2.8 mmol/l glucose for 20 min before a stimulatory period of 60 min with KRB containing 16.7 mmol/l glucose. One-minute fractions of the perifusate were collected for the first 15 min, then 5-min fractions for all following time points, all at a flow rate of 0.5 ml/min. Insulin was analyzed by RIA. Mean data from nine separate experiments are presented.

used in a host of studies (30–32), including some with β-cells (33–36). We now show that overexpression of PKCαWT and PKCαKD protein had no significant effect on either basal secretion or GSIS from batch-incubated pancreatic islets (Fig. 7*A*). In contrast, PKCαWT overexpression led to an 80% incremental rise in TPA-stimulated insulin secretion relative to that of the MX17 control, whereas PKCαKD overexpression significantly inhibited this by almost 50% when compared with the control. This confirms that the PKCα viruses were functioning as expected according to the overexpression strategy. Because it is possible that PKCα might exert an effect on the kinetics of GSIS, which would not be apparent in batch incubations, perifusion analysis was also performed on islets infected with either control or PKCαKD viruses. However, both groups of islets displayed an equivalent biphasic secretory response in the presence of 16.7 mmol/l glucose (Fig. 7*B*). Thus, there was no significant difference between the areas under the curves for islets infected with MX17 and PKCαKD viruses when analyzed for first-phase secretion (0.37 ± 0.09 vs. 0.29 ± 0.04 $ng \cdot islet^{-1} \cdot 10\ min^{-1}$, respectively) or second-phase secretion (2.12 ± 0.47 vs. 2.07 ± 0.31 $ng \cdot islet^{-1} \cdot 50\ min^{-1}$).

Corresponding experiments undertaken to assess in-

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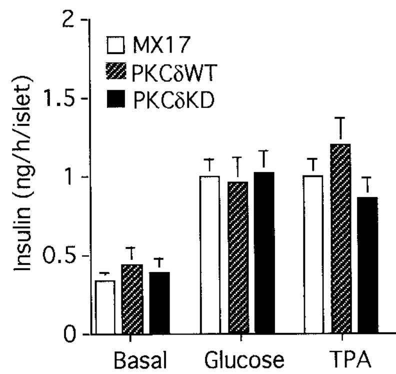

FIG. 8. Glucose- and TPA-induced insulin secretion from batch incubations of cultured rat islets overexpressing PKCδ WT and KD mutants. Islets were infected with recombinant MX17, PKCδWT, and PKCδKD adenovirus as described in the legend to Fig. 7. Batches of 15 islets were isolated, washed in modified KRB, and incubated for 1 h at 37°C in KRB containing 2.8 mmol/l glucose (basal), 16.7 mmol/l glucose, or 500 nmol/l TPA. Mean data of seven separate experiments, each performed in at least quadruplicate, are shown. No statistically significant differences were found between the viral treatments for each secretary stimulus.

volvement of PKCδ revealed that overexpression of PKCδWT and PKCδKD protein did not significantly affect the secretory responses to either glucose or TPA in batch incubations (Fig. 8). Similarly, no significant modulation of secretory dynamics was observed in perifusion experiments making use of the PKCδKD adenovirus (results not shown). As well as suggesting that PKCδ is unlikely to play a major role in controlling the exocytosis of insulin, these results serve as a negative control and attest to the specificity of the effects of PKCα overexpression on TPA-stimulated secretion shown above (Fig. 7*A*).

DISCUSSION

The results of the current study do not support a major role for cPKC activation in the regulation of GSIS. This conclusion is based on the application of two technologies that had not previously been used in this context but that now, in addition to helping resolve the issue, potentially explain some of the controversies arising from earlier studies. The first technology relies on detection of cPKC activation via its most direct and important physiologic readout: substrate phosphorylation. This represents an advance on PKC translocation assays, which although very sensitive when used in conjunction with confocal microscopy, are only indirect measures of activation (50). Moreover net protein phosphorylation represents a balance between the activities of both protein kinases and phosphatases, so kinase activation might be of little functional consequence if countered by a concomitant rise in phosphatase activity. Indeed, a glucose-stimulated phosphatase activation seems to be a major contributor to the transient and relatively modest nature of the cPKC substrate phosphorylation observed here. Although a detailed investigation of the phosphatases underlying this mechanism is beyond the scope of the current study, it is noteworthy that other recent work has also provided evidence of phosphatase activation by glucose and has pointed to important functional roles for this phenomenon (45,46).

Studies of PKC-substrate phosphorylation in islets have been very limited, but there are a few reports of glucose-stimulated ^{32}Pi incorporation into known PKC substrates such as MARCKS protein (15,21,27) and a Na^+,K^+-ATPase subunit (28). However, both of these proteins are phosphorylated by multiple kinases, so it difficult to attribute these results unequivocally to glucose-stimulated PKC activation. In addition, incorporation of ^{32}Pi could represent enhanced turnover of phosphorylated residues, rather than a net increase in phosphorylation. The use of antisera raised against known PKC phosphorylation sites overcomes both of these potential problems. In the single, previous application of such an approach to pancreatic islets, an antiserum raised against a phosphorylated Ser residue at position 189 of SNAP-25 (a known PKC site) revealed a modest increase in phosphorylation in response to glucose, but this seemed unrelated to GSIS (51). Our results extend this lack of correlation between secretory capacity and PKC activation. First, increased substrate phosphorylation with glucose was more readily observed in cultured islets compared with freshly isolated islets, although the latter responded better in terms of secretion. The reason for the apparently decreased sensitivity to glucose-stimulated cPKC substrate phosphorylation in noncultured islets is unknown, but this does not detract from the loss of correlation with secretion. Second, Go6976 markedly decreased phosphorylation of several cPKC substrates in the presence of either glucose or TPA but inhibited only secretion in response to TPA. Third, the phorbol ester was less potent than glucose in promoting secretion from freshly isolated islets but enhanced phosphorylation to a much greater extent. It is thus apparent that activation of cPKC can be dissociated from GSIS at a variety of levels.

The second technology used here is that of recombinant adenovirus for overexpression of PKC isozymes in both WT and KD forms. The time required for transgene expression necessarily restricts this approach to use with cultured cells. However, under our culturing conditions, perifused islets still displayed a robust biphasic secretion in response to 16.7 mmol/l glucose, albeit three to five times lower than that seen with freshly isolated islets. That TPA elicited similar secretory responses in fresh and cultured islets further attests to the suitability of this model for analyzing PKC function. Although the overexpression strategy has recently been used for investigation of other aspects of β-cell function (33–36), it has not been previously applied to the role of PKCs in regulating insulin secretion. This study therefore represents an important advance on the use of pharmacologic inhibitors for which specificity is always a concern. This is true even of an agent such as Go6976, which, although very selective for cPKCs versus other PKC isozymes (42,44), does also inhibit some other protein kinases unrelated to the PKC family (52). Nevertheless, it is reassuring that the results obtained in islets overexpressing PKCαKD were consistent with those treated with Go6976, in that both approaches inhibited the secretory response to TPA but not glucose. However, whereas Go6976 completely blocked TPA-stimulated secretion, the adenovirus inhibited it by ~50%. This is not surprising given that PKCα is unlikely to account for all of the secretion caused by TPA but that other cPKCs, such as PKCβ, might also contribute. In any event, our results suggest that activation of cPKCs (in particular PKCα) clearly does contribute to the secretory

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response, provided that the activation is strong and sustained, such as occurs with TPA (but not glucose). This raises the possibility that cPKCs might mediate secretion stimulated by other more physiologic agonists, such as acetylcholine (13,53), where phospholipase C activation is more pronounced than with glucose (54,55) and/or where phosphatase induction might be weaker. However, it should be stressed that PKC would also be expected to exert a feedback inhibition on phospholipase C under these conditions (56), and so the roles of individual PKC isozymes in acetylcholine-stimulated insulin secretion are likely to be complex and their elucidation would require extensive, further investigation.

The specificity of the PKCα overexpression strategy is witnessed by the lack of effect of the corresponding PKCδ viruses on TPA-induced insulin secretion. Indeed, our studies further suggest that PKCδ activation is not a major player in GSIS. This conclusion is more tentative than that for PKCα in that it is based solely on the adenoviral strategy. Because TPA-stimulated secretion was also unaffected by PKCδ overexpression, there was no internal control to verify that these kinase constructs functioned correctly inside in the β-cell. However, the identical viral strategy has been used successfully in pancreatic β-cells when measuring other functional end points for PKCδ, namely mRNA stabilization and apoptosis (33,34,36), so there seems to be no reason for why it could not also be used successfully to study secretion. However, a proapoptotic role for PKCδ in β-cells (34,36), as widely observed in many other models (57), would probably make it unlikely that this PKC isozyme is either activated by glucose or plays any major role in promoting secretion. Note also that although rottlerin is widely used as a pharmacologic inhibitor of PKCδ, we were unable to show this directly in assays of extracts of PKCδ-overexpressing islets (L.C., T.J.B., unpublished observations). This is in keeping with other in vitro studies (52) and the demonstration of nonspecific effects of this compound in intact cells (57). We therefore avoided using rottlerin as a pharmacologic confirmation of our PKCδ adenoviral strategy.

In summary, our data demonstrate that TPA promotes a large and relatively prolonged phosphorylation of cPKC substrates and that TPA-stimulated insulin secretion is inhibited by cPKC inhibitors or overexpression of a PKCαKD mutant. In contrast, glucose induced a secretory response that was not inhibited by these pharmacologic or molecular interventions and evoked a correspondingly smaller and shorter-lived phosphorylation of cPKC substrates. The simplest interpretation consistent with these multiple strands of evidence is that activation of PKCα is not a major contributor to GSIS but does play a role in the secretory response to TPA. We do not exclude that a small, transient phosphorylation of cPKC substrates might not have long-lived effects (and indeed our data show there is no moment-to-moment correlation between substrate phosphorylation and secretion, even in response to TPA). A short-lived phosphorylation triggered by glucose might still, for example, play a role in priming the β-cell for enhanced responsiveness to incretins or, alternatively, be implicated in functional responses unrelated to secretion. Analysis of these potential roles would be a worthy subject for further investigation.

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