

Fatty acid and phorbol ester-mediated interference of mitogenic signaling via novel protein kinase C isoforms in pancreatic β -cells (INS-1)

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Abstract

It is possible that activation of protein kinase C (PKC) isoforms by free fatty acids (FFA) plays a role in the failure of pancreatic β -cell mass expansion to compensate for peripheral insulin resistance in the pathogenesis of type-2 diabetes. The effect of lipid moieties on activation of conventional (PKC- α and - β 1), novel (PKC- δ) and atypical (PKC- ζ) PKC isoforms was evaluated in an *in vitro* assay, using biotinylated neurogranin as a substrate. Oleoyl-Coenzyme A (CoA) and palmitoyl-CoA, but not unesterified FFA, significantly increased the activity of all PKC isoforms ($P \leq 0.05$), particularly that for PKC- δ . It was found that FFA (0.4 mM oleate/complexed to 0.5% bovine serum albumin) inhibited IGF-I-induced activation of protein kinase B (PKB) in the pancreatic β -cell line (INS-1), but this was alleviated in the presence of the general PKC inhibitor (Gö6850; 1 μ M). To further investigate whether conventional or novel PKC isoforms adversely affect β -cell proliferation, the effect of phorbol ester (phorbol 12-myristate 13-acetate; PMA)-mediated activation of these PKC isoforms on glucose/IGF-I-induced INS-1 cell mitogenesis, and insulin receptor substrate (IRS)-mediated signal transduction was investigated. PMA-mediated activation of PKC (100 nM; 4 h) reduced glucose/IGF-I mediated β -cell mitogenesis ($>50\%$; $P \leq 0.05$), which was reversible by the general PKC inhibitor Gö6850 (1 μ M), indicating an effect of PKC and not due to a non-specific PMA toxicity. PMA inhibited IGF-I-induced activation of PKB, correlating with inhibition of IGF-I-induced association of IRS-2 with the p85 regulatory subunit of phosphatidylinositol-3 kinase. However, in contrast, PMA activated the mitogen-activated protein kinases, Erk1/2. Titration inhibition analysis using PKC isoform inhibitors indicated that these PMA-induced effects were via novel PKC isoforms. Thus, FFA/PMA-induced activation of novel PKC isoforms can inhibit glucose/IGF-I-mediated β -cell mitogenesis, in part by decreasing PKB activation, despite an upregulation of Erk1/2. Thus, activation of novel PKC isoforms by long-chain acyl-CoA may well contribute to decreasing β -cell mass in the pathogenesis of type-2 diabetes, similar to their inhibition of insulin signal transduction which causes insulin resistance.

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Introduction

The plasticity of pancreatic β -cell mass plays a key role in adapting to certain conditions such as obesity and pregnancy (Lingohr *et al.* 2002a, Rhodes & White 2002). In general, increased β -cell mass and function are able to compensate for the increased insulin demand caused by peripheral insulin resistance found in obesity (Lingohr *et al.* 2002a). However, chronic obesity, especially if untreated, can lead to β -cell dysfunction and a failure of β -cell mass to expand in the face of

insulin resistance resulting in glucose intolerance, insulin insufficiency and eventually obesity-linked type-2 diabetes (Unger & Orci 2001, Lingohr *et al.* 2002a). In the USA, it has been estimated that for the year 2000 around 20% of the population was obese, and between a quarter and a third of these would progress to type-2 diabetes, raising concerns of this growing towards epidemic proportions (Mokdad *et al.* 2001). Similar forecasts are predicted for both Westernized and non-industrialized countries (Fall 2001, Zimmet *et al.* 2001).

There is accumulating evidence to indicate that free fatty acids (FFA), which are chronically elevated in obesity-linked type-2 diabetes, are involved in the pathogenesis of the disease (Unger & Orci 2001, Lingohr *et al.* 2002a). Under normal circumstances, short-term (<1–2 h) exposure to exogenous FFA can have a beneficial effect to stimulate insulin secretion (Warnotte *et al.* 1994, Stein *et al.* 1996); however, chronic exposure (>24 h) of FFA to β -cells can adversely affect glucose-induced insulin secretion contributing to β -cell dysfunction (Bollheimer *et al.* 1998, McGarry & Dobbins 1999). For peripheral insulin resistance in obesity, there is mounting evidence to suggest that increased FFA, which accompanies the accumulation of intracellular triglyceride, interferes with insulin signal transduction pathways, particularly in skeletal muscle (Kraegen *et al.* 2001, Moller 2001). This may be mediated by FFA itself, where it has been shown that FFA and palmitate-derived ceramide can inhibit insulin-induced activation of protein kinase B (PKB; also known as Akt) which leads to decreased GLUT4 translocation and reduced glucose uptake (Kellerer & Haring 1995, De Fea & Roth 1997, Griffin *et al.* 1999, Schmitz-Peiffer *et al.* 1999, Stratford *et al.* 2001). In addition, it has been shown that elevated FFA can chronically activate certain protein kinase C (PKC) isoforms (such as PKC- ϵ , θ and ζ) to serine/threonine phosphorylate insulin receptor substrate-1 (IRS) which, in turn, dampens insulin-induced IRS-mediated signaling, contributing to insulin resistance (Griffin *et al.* 1999, Laybutt *et al.* 1999, Zick 2001).

Although FFA contribute to insulin resistance, increases in pancreatic β -cell mass/function can compensate for peripheral insulin resistance, providing a possible explanation as to why ~70% of obese individuals do not progress to type-2 diabetes (Unger & Orci 2001, Lingohr *et al.* 2002a). Notwithstanding, it should also be noted that IRS-signal transduction pathways in pancreatic β -cells, especially when regulated by insulin-like growth factor-I (IGF-I) and glucose via IRS-2 (Hügl *et al.* 1998, Withers *et al.* 1999, Lingohr *et al.* 2002b), play a prominent role in maintaining β -cell mass (Withers *et al.* 1999, Lingohr *et al.* 2002a). This raises the question as to whether long-term exposure of FFA to β -cells eventually leads to interference of IRS-mediated signaling which, in turn, contributes to decreased β -cell mass associ-

ated with the pathogenesis of obesity-linked type-2 diabetes (Rhodes 2000, Unger & Orci 2001, Lingohr *et al.* 2002a). In this regard, it has been shown that FFA can inhibit IGF-I/glucose-induced β -cell mitogenesis *in vitro* (Cousin *et al.* 2001). In addition, FFA and palmitate-derived ceramide can cause apoptosis in islet β -cells (Shimabukuro *et al.* 1998, Cnop *et al.* 2001, Wrede *et al.* 2002). These observations indicate that FFA can contribute to reducing β -cell mass. It is possible that these adverse effects of FFA on β -cells are mediated, at least in part, by activation of PKC isoforms, similar to that contributing to insulin resistance (Zick 2001).

There are three classes of PKC isoforms, grouped as to their mode of activation (Bell & Burns 1991, Nishizuka 1992, Liu 1996). Conventional PKC isoforms α and β are allosterically activated by Ca^{2+} , phosphatidylserine (PS) and diacylglycerol (DAG), whereas the novel isoforms PKC- δ , θ and ϵ are activated by various lipid moieties independent of Ca^{2+} , and the atypical isoforms, such as PKC- ζ , are Ca^{2+} and DAG independent (Bell & Burns 1991, Nishizuka 1992). In addition, the phosphatidylinositol-3-kinase (PI3-K) pathway can modulate PKC activity in several ways. First, the PI3-K product phosphatidylinositol-3,4,5-trisphosphate (PIP3) has been shown to activate PKC- δ , ϵ and ζ isoforms *in vitro* (Toker *et al.* 1994). Secondly, the PIP3-activated phosphoinositide-dependent kinase-1 (PDK-1) can directly phosphorylate PKC isoforms on the activation loop *in vitro* (Le Good *et al.* 1998), rendering them active. FFA have also been shown to activate several PKC isoforms *in vitro*; however, it is unclear whether fatty acids (Murakami & Routtenberg 1985, Bell & Burns 1991, Chen & Murakami 1992, Nishizuka 1992) or their Coenzyme A (CoA) esters (Bronfman *et al.* 1988, Yaney *et al.* 2000) activate PKC isoforms, and comparative studies regarding the activation of various PKC isoforms by FFA and relative acyl-CoA esters have not been performed. The pancreatic β -cell is known to express at least the PKC isoforms α , β , δ , ϵ , μ , ι and ζ (Knutson & Hoening 1994, Yaney *et al.* 2002). Activation of atypical PKC- ζ , as well as conventional and novel PKC isoforms, have been indicated to partake in the stimulus-coupling mechanism for nutrient-regulated insulin secretion (Tian *et al.* 1996). In addition, it has recently been shown that atypical

PKC- ζ contributes to increasing glucose-dependent incretin-induced β -cell mitogenesis (Buteau *et al.* 2001), but it is unclear whether conventional and/or novel PKC isoforms contribute to control of β -cell growth or survival. In this study, we have compared the ability of FFA, long-chain acyl-CoA as well as certain other lipid moieties to activate conventional PKC isoforms α and $\beta 1$, the novel conventional PKC- δ and atypical PKC- ζ *in vitro*, to address the question as to whether FFA and/or CoA-esterified FFA activate PKC isoforms. By taking advantage of the fact that phorbol esters (such as phorbol 12-myristate 13-acetate (PMA)) only activate conventional and novel PKC isoforms (Kazanietz *et al.* 2000), we have examined whether these particular classes of PKCs affect β -cell proliferation and glucose/IGF-I-induced IRS-mediated signal transduction pathways, in the β -cell line INS-1.

Materials and methods

Materials

Total PKB, phospho-PKB Thr³⁰⁸ and phospho-PKB Ser⁴⁷³ antibodies were from New England Biolabs Inc. (Beverly, MA, USA). The SignaTECT PKC assay system, anti-phospho-extracellular regulated kinase (Erk1/2) and total-Erk1/2 antibodies were from Promega Corporation (Madison, WI, USA). Recombinant PKC- α , - $\beta 1$, - δ and - ζ and the PKC inhibitors Gö6850 (bisindolylmaleimide I), Gö6976 and Rottlerin were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). PIP3 (1- α -stearoyl-2- α -arachidonoyl-sn-glycer-3-yl-D-myo-inositol-3,4,5-trisphosphate) was from AG Scientific, Inc. (San Diego, CA, USA). DAG (1,2-dioleoyl-sn-glycerol) and PS (1- α -phosphatidylserine) were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). PKC- α , - $\beta 1$, - δ and - ζ antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The p70 kDa S6 protein kinase (p70^{S6K}) antiserum was a gift from Dr M G Myers (Joslin Diabetes Center, Boston, MA, USA). The anti-rabbit IgG horseradish peroxidase (HRP) conjugate was from Jackson Immuno Research (West Grove, PA, USA). IGF-I was obtained from GroPep Pty Ltd (Adelaide, Australia). PMA, oleoyl-CoA and palmitoyl-CoA were from Sigma (St Louis, MO, USA). Oleic acid (OE) and palmitic acid (PA) were purchased from

Alltech (Deerfield, IL, USA). Recombinant inactive PKB and recombinant active phosphoinositide-dependent kinase-1 (PDK-1) were from Upstate Biotechnology (Lake Placid, NY, USA). Protein A Sepharose CL-4B, methyl- $[^3\text{H}]$ thymidine and $[^{32}\text{P}]\gamma$ -ATP were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). The bicinchoninic (BCA) protein assay kit was from Pierce (Rockford, IL, USA) and the chemiluminescence reagent was from NEN (Boston, MA, USA). The Ready Safe scintillation fluid was from Beckman Instruments Inc. (Fullerton, CA, USA). All other chemicals were of analytical grade either from Sigma or Fisher Scientific (Pittsburgh, PA, USA).

In vitro PKC assay

The assay was adapted from the Promega SignaTECT PKC assay protocol with major changes to measure PKC activity under different conditions, using neurogranin as a PKC-specific substrate (Chakravarthy *et al.* 1999). PS and DAG in chloroform were dried under N₂ and sonicated in H₂O in order to form micelles. The final concentrations in the assay were 50 μM for PS and 13 μM for DAG. Forty nanograms of recombinant PKC- α or 100 ng recombinant PKC- $\beta 1$, - δ or - ζ were incubated for 10 min at 30 °C with 100 μM biotinylated neurogranin as PKC substrate, 100 μM ATP, 0.5 μCi $[^{32}\text{P}]\gamma$ -ATP (3000 Ci/mM) and 20 mM Tris-Cl, pH 7.5, 2 mM MgCl₂, 100 mM NaCl and 250 μM EGTA. Depending on the experiment, 400 μM CaCl₂, 100 nM PMA, 10 μM PIP3, 10 μM FFA or 10 μM FFA-CoA were added. The reaction was stopped with 7.5 M guanidine hydrochloride and an equivalent of the reaction mix was spotted onto a streptavidin-coated membrane (Promega SAM² biotin capture membrane). The membrane was washed four times in 2 M NaCl and in 2 M NaCl 1% H₃PO₄, once in H₂O and rinsed with 95% ethanol. After drying, scintillation fluid was added and the samples were counted in a Beckman LS6500 scintillation counter. The data are presented as the average fold increase over H₂O \pm S.E. of three to ten independent experiments.

Cell culture

The insulin-secreting, glucose-sensitive β -cell line, INS-1 (Asfari *et al.* 1992), was cultured in

RPMI1640 media containing 11.2 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin, as previously described (Asfari *et al.* 1992). The cells were maintained at 37 °C with 5% CO₂ and subcultured at 70% confluence.

[³H]-Thymidine incorporation

INS-1 cells were plated at a density of 15 000 cells/well in 96-well plates and allowed 24 h to attach to the plate. After that, cells were made quiescent in starvation media (RPMI1640 media containing 0.5 mM glucose, 0.1% bovine serum albumin (BSA), 100 U/ml penicillin and 100 μ g/ml streptomycin) overnight. The cells were treated for 24 h with RPMI1640 media containing 0.5 mM, 3 mM and 15 mM glucose, with or without 10 nM IGF-I. For the final 4 h of this incubation period, 5 μ Ci/ml [³H]thymidine and, in some experiments, 100 nM PMA, were added. The cells were lysed by transferring to UniFilter-96 GF/C filter plates (Packard Instruments, Meriden, CT, USA) with the Packard filter mate cell harvester (Cambridge Technology Inc.). Packard microscint 20 scintillation fluid (25 μ l) was added per well and the radioactivity incorporated into DNA was determined with a Packard TopCount NXT counter, as described (Hügl *et al.* 1998, Cousin *et al.* 2001).

Stimulation and cell lysis conditions

Cells grown to 70% confluence were made quiescent in starvation media for 18 h. All inhibitors were used at given concentrations, using stock solutions that had been prepared in dimethyl sulfoxide (DMSO) with a final DMSO concentration up to 0.5%. The preincubation period was 20 min with Rottlerin and 2 h with Gö6850 and Gö6976. Treatment was performed with fresh starvation media containing 0.5 mM, 3 mM or 15 mM glucose \pm 10 nM IGF-I with or without 100 nM PMA for the indicated time at 37 °C. After the incubation period, the medium was completely removed and the cells were lysed in ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 1% NP-40, 4 mM EDTA, 2 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 2 μ M leupeptin, 100 μ M phenylmethylsulfonyl

fluoride and 1 μ g/ml aprotinin. The lysate was sonicated, insoluble material removed by centrifugation at 16 000 g and the samples were stored at -80 °C.

Immunoprecipitation

Cell lysates were adjusted to 1 mg/ml total protein in lysis buffer after measuring protein concentration with the BCA protein assay kit. Samples were precleared for 1 h at 4 °C with 25 μ l protein A Sepharose to eliminate unspecific binding. The beads were then removed, IRS-2 antibody added and the supernatant rotated overnight at 4 °C. This was followed by addition of 25 μ l protein A Sepharose for 2 h and the lysate was washed four times with lysis buffer. Prior to gel loading, 30 μ l 5 \times SDS gel loading buffer was added and the samples were boiled for 5 min.

Protein immunoblot analysis

Protein levels of cell lysates were determined using the BCA protein assay kit. Aliquots with 40 μ g protein were separated by SDS-polyacrylamide electrophoresis. After transferring to nitrocellulose, membranes were blocked in 5% non-fat dry milk in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1% Tween-20) for 1 h. The incubation with the primary antibody (1:1000–1:10 000) was performed in 5% BSA/TBST overnight, and with the HRP-conjugated secondary antibody at 1:10 000 in 5% BSA/TBST for 1 h, as described (Hügl *et al.* 1998, Cousin *et al.* 2001, Dickson *et al.* 2001, Lingohr *et al.* 2002b). For detection, chemiluminescence reagent and X-ray film were used.

PDK-1 assay

Unactivated recombinant PKB (100 ng) was incubated with 5 ng active recombinant PDK-1 for 20 min at 37 °C in the presence of 50 μ M ATP, 250 μ M PS, 25 μ M PIP₃, 50 μ M EGTA, 5 mM MgCl₂ and 10 mM Hepes, pH 7.4. Rottlerin concentrations were adjusted to 1–15 μ M. The reaction was stopped by the addition of 5 \times SDS gel loading buffer. Proteins were separated by SDS gel electrophoresis and PKB phosphorylation was determined by immunoblotting with phosphospecific antibodies for PKB Ser³⁰⁸ and PKB Ser⁴⁷³, as described (Dickson *et al.* 2001).

Statistics

Means and s.e. were calculated using standard procedures. For significance levels, Student's *t*-test was applied, when $P < 0.05$ was considered statistically significant.

Results

In vitro PKC activity assay

Various lipid moieties can differentially activate PKC isoforms (Bell & Burns 1991, Nishizuka 1992, Liu 1996). In this study, we compared which particular lipid moieties were capable of activating, and to what extent, PKC- α and - β (examples of conventional PKC isoforms), PKC- δ (a model novel PKC isoform) and PKC- ζ (a model atypical PKC isoform) *in vitro*. Since neurogranin was used as a substrate in this assay it was relatively PKC specific (Chakravarthy *et al.* 1999). Nonetheless, in initial *in vitro* experiments it was found that neither recombinant active PDK-1 nor immunoprecipitated activated PKB (from INS-1 cell lysates incubated at 15 mM glucose + 10 nM IGF-I, as previously described (Cousin *et al.* 2001, Dickson *et al.* 2001)) were able to significantly phosphorylate neurogranin (data not shown), reaffirming this assay as relatively specific for PKC activities.

The conventional PKC isoforms PKC- α (Fig. 1A) and PKC- β (Fig. 1B) were best activated using a combination of PS, DAG and Ca^{2+} . The product of PI3-K, PIP3, led to around a threefold increase for PKC- α and PKC- β activity, independent of PS, DAG and/or Ca^{2+} (Fig. 1A and B; $P \leq 0.05$). FFA OA and PA did not increase the activity of conventional PKCs *in vitro*, but their CoA esters significantly increased PKC- α and PKC- β activity by about twofold (Fig. 1A and B; $P \leq 0.05$).

The combination of PS and DAG, in the absence of Ca^{2+} , led to the highest activation of the novel PKC- δ , as expected (approximately eightfold; Fig. 1C). In contrast to conventional PKCs, addition of Ca^{2+} to PS and/or DAG reduced PKC- δ activity (Fig. 1C). PIP3 alone increased PKC- δ activity (approximately sixfold; Fig. 1C). Addition of OA-CoA and PA-CoA, but not their unesterified forms, resulted in significant activation of PKC- δ (approximately sixfold; Fig. 1C).

Atypical PKC- ζ activity was modestly activated by PS and DAG, in the absence of Ca^{2+} (<twofold; $P < 0.05$; Fig. 1D), whereas PIP3 was the best

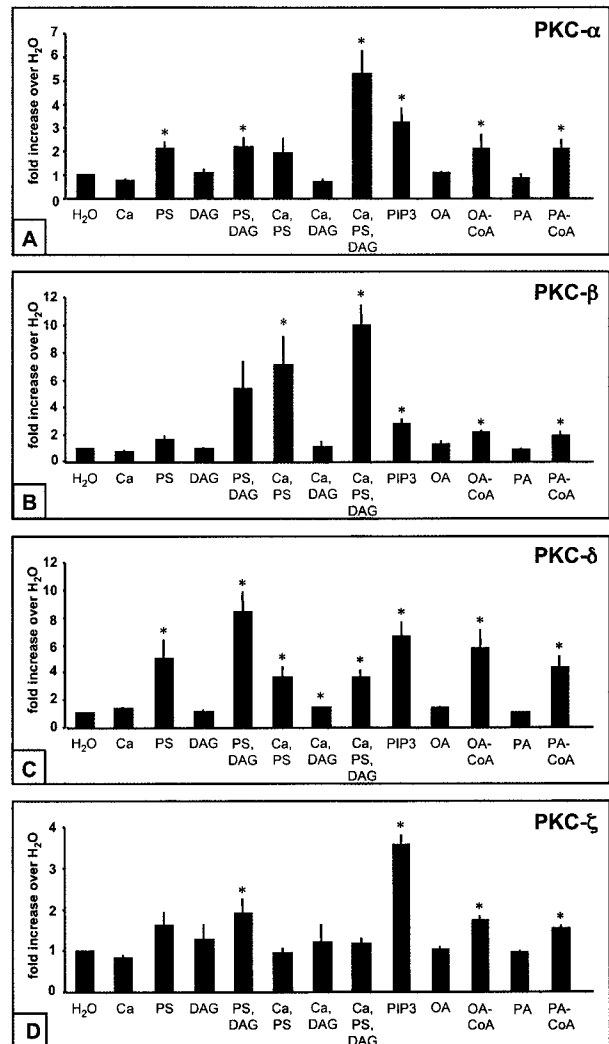


Figure 1 PKC activity assay *in vitro*. PKC activity assays with 40 ng recombinant PKC- α (A) or 100 ng recombinant PKC- β (B), PKC- δ (C) or PKC- ζ (D) were incubated in the presence of known allosteric PKC activators (Ca^{2+} (Ca), DAG, PS) or potential candidate PKC activators (PIP3, OA, oleoyl-CoA (OA-CoA), PA or palmitoyl-CoA (PA-CoA)) for 10 min at 30 °C with [^{32}P]-ATP and biotinylated neurogranin as substrate. Incorporated radioactivity was measured as described in Materials and methods. Data represent the mean \pm s.e. of at least three independent experiments. * $P \leq 0.05$ compared with H₂O control.

activator of PKC- ζ activity *in vitro* (approximately fourfold; $P \leq 0.05$; Fig. 1D). PKC- ζ activity was also significantly ($P \leq 0.05$) increased, albeit modestly, by OA-CoA and PA-CoA, but not by unesterified FFA (Fig. 1D).

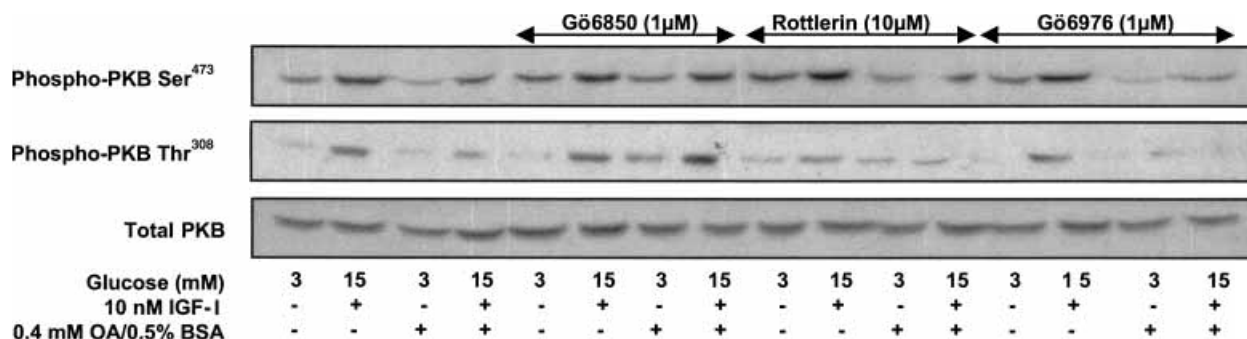


Figure 2 FFA-mediated inhibition of IGF-I-induced PKB phosphorylation activation is alleviated by the general PKC inhibitor, Gö6850. INS-1 cells were incubated at a basal 3 mM glucose with either 0.5% (w/v) BSA or 0.4 mM OA complexed to 0.5% (w/v) BSA for 18 h, as previously described (Wrede *et al.* 2002). Then, the cells were preincubated for 2 h with a general PKC inhibitor (Gö6850; 1 µM) or a conventional PKC isoform inhibitor (Gö6976; 1 µM) or for 20 min with Rottlerin (10 µM), in the same media. INS-1 cells were then incubated for 10 min at a basal 3 mM glucose or at stimulatory 15 mM glucose+10 nM IGF-I in the continued presence of FFA and/or PKC inhibitor as indicated. Cell lysates were prepared and immunoblotted with antibodies for phospho-PKB Ser⁴⁷³, phospho-PKB Thr³⁰⁸, and total PKB, as described in Materials and methods. A representative immunoblot of at least three independent experiments is shown.

PMA (100 nM) alone did not affect the activity of any tested PKC isoform in the *in vitro* assay (data not shown), in accordance with the function of phorbol esters to increase PKC membrane affinity (Mosior & Newton 1995). Autophosphorylation of all PKC isoforms was found to be quite low and did not correlate with allosteric PKC activation (data not shown).

FFA-induced inhibition of IGF-I-induced PKB activation in pancreatic β -cells (INS-1) can be averted by a general PKC inhibitor (Gö6850)

It has been previously demonstrated that FFA can inhibit IGF-I-induced PKB activation in β -cells which may be a key to FFA-induced inhibition of β -cell mitogenesis and survival (Cousin *et al.* 2001, Wrede *et al.* 2002). An indication of PKB phosphorylation activation can be ascertained by examining the phosphorylation at Thr³⁰⁸ and Ser⁴⁷³ of PKB by specific immunoblot analysis as previously described (Dickson *et al.* 2001, Wrede *et al.* 2002). INS-1 cells were incubated at a basal 3 mM glucose with either 0.5% (w/v) BSA or 0.4 mM OA complexed to 0.5% (w/v) BSA for 18 h, as previously described (Wrede *et al.* 2002). Then, the cells were preincubated for 2 h with a general PKC inhibitor (Gö6850; 1 µM) (Toullec *et al.* 1991) or a conventional PKC isoform inhibitor (Gö6976; 1 µM) (Martiny-Baron *et al.* 1993), or for 20 min with Rottlerin (10 µM), a relatively

specific inhibitor of PKC- δ (Gschwendt *et al.* 1994), in the same media. INS-1 cells were then incubated for 10 min at 37 °C at a basal 3 mM glucose or at stimulatory 15 mM glucose+10 nM IGF-I in the continued presence of FFA and/or PKC inhibitor as indicated (Fig. 2). In the presence of 0.4 mM OA/0.5% BSA, but in the absence of PKC inhibitors, an FFA-mediated inhibition of IGF-I-induced PKB Thr³⁰⁸ and Ser⁴⁷³ phosphorylation activation was observed (Fig. 2), as previously demonstrated (Wrede *et al.* 2002). However, in the presence of the general PKC inhibitor (Gö6850), this FFA-mediated inhibition of IGF-I-induced PKB activation was alleviated (Fig. 2). In contrast, the conventional PKC isoform inhibitor (Gö6976) was unable to prevent FFA-mediated inhibition of PKB activation (Fig. 2). In the absence of FFA, Rottlerin appeared to have a general effect on inhibiting IGF-I-induced PKB Thr³⁰⁸ phosphorylation but not IGF-I-induced PKB Ser⁴⁷³ phosphorylation (Fig. 2), perhaps consistent with Rottlerin also being able to inhibit PDK-1 (Davies *et al.* 2000) which, in turn, would reduce PDK-1-mediated PKB Thr³⁰⁸ phosphorylation (Dickson *et al.* 2001). Nonetheless, Rottlerin was unable to alleviate the FFA-mediated inhibition of IGF-I-induced PKB Thr³⁰⁸ or Ser⁴⁷³ phosphorylation (Fig. 2). These data suggest the possibility that FFA-mediated inhibition of PKB activation in pancreatic β -cells (INS-1) may, at least in part, be mediated by FFA-induced activation of a novel or atypical PKC isoform.

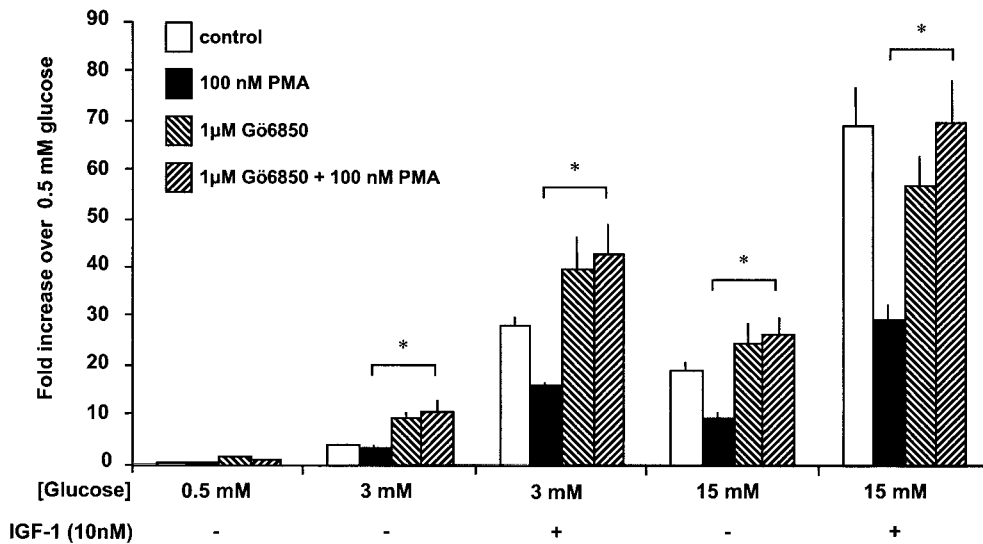


Figure 3 [^3H]thymidine incorporation assays in INS-1 cells with the PKC activator PMA. INS-1 cells were cultured in 96-well plates for 24 h and made quiescent by incubating for 18 h in starvation media (0.5 mM glucose) overnight. The cells were subsequently cultured for 24 h in RPMI1640 medium containing the indicated glucose concentrations with or without 10 nM IGF-I. In the last 4 h, [^3H]thymidine or [^3H]thymidine with PMA (100 nM) was added \pm 1 μM Gö6850 (the bisindolylmaleimide general PKC inhibitor) as indicated. The data represent at least five independent experiments done in triplicate. Means \pm S.E. are shown. * $P \leq 0.001$ between the indicated groups.

Short-term PMA treatment decreased pancreatic β -cell (INS-1) mitogenesis, via activation of PKC

It has recently been indicated that the atypical PKC isoform, PKC- ζ , plays a positive role in glucagon-like peptide-1-induced β -cell proliferation and is unlikely to have any negative effect on mitogenesis (Buteau *et al.* 2001). In order to examine the role that conventional and novel PKC isoforms might play in the control of β -cell proliferation, we took advantage of the characteristic that phorbol esters (such as PMA) preferentially activate conventional and novel PKC isoforms, with little effect on activating atypical PKC isoforms (Liu 1996, Kazanietz *et al.* 2000). However, it should be noted that long-term exposure of high concentrations of PMA (>1 μM) to pancreatic β -cells reduces the expression of several PKC isoforms (Yaney *et al.* 2000, 2002), and thus these series of experiments were restricted to a relatively short-term 4-h incubation with PMA. In preliminary dose-response experiments, it was found that a maximal effect of PMA to inhibit glucose/IGF-I-induced β -cell mitogenesis in 4 h

(see below; Fig. 3) occurred between 50 and 100 nM PMA although significant inhibition was also observed at ≥ 5 nM PMA (data not shown). A stimulatory glucose concentration (15 mM) promoted [^3H]thymidine incorporation in INS-1 cells above that at basal 3 mM glucose ($P \leq 0.01$; Fig. 3), as previously shown (Hügl *et al.* 1998). IGF-I (10 nM) further increased INS-1 cell proliferation at both basal 3 mM glucose and stimulatory 15 mM glucose ($P \leq 0.01$; Fig. 3). PMA (100 nM) had no effect on INS-1 cell mitogenesis at basal 3 mM glucose. However, PMA caused a significant $\sim 50\%$ reduction in [^3H]thymidine incorporation at stimulatory 15 mM glucose ($P \leq 0.01$; Fig. 3). PMA also significantly inhibited IGF-I-induced INS-1 cell mitogenesis at both 3 mM and 15 mM glucose, also by about 50% ($P \leq 0.005$; Fig. 3). The presence of bisindolylmaleimide Gö6850 (1 μM), a general PKC inhibitor (Toullec *et al.* 1991), during the [^3H]thymidine incorporation assay completely prevented the inhibitory effect of PMA on INS-1 cell proliferation at 15 mM glucose and in the presence of IGF-I at both 3 mM or 15 mM glucose (Fig. 3). This reaffirmed that the PMA effect was

not due to non-specific cytotoxicity. In contrast, the conventional PKC isoform inhibitor (Gö6976; 1 μ M) or Rottlerin did not appear to significantly alleviate PMA-induced inhibition of glucose/IGF-I-induced INS-1 cell proliferation (data not shown).

Phorbol ester inhibited PKB phosphorylation and p85 kDa regulatory subunit of PI3-K (p85)/IRS-2 association, but increased Erk1/2 phosphorylation

We, and others, have previously shown the importance of IRS-mediated signal transduction pathways (especially that via IRS-2) contributing to control of β -cell proliferation (Withers *et al.* 1997, 1999, Hügl *et al.* 1998, Schupp *et al.* 1998). As such, it was examined whether PMA modulated glucose/IGF-I-induced IRS signaling in INS-1 cells. Treatment of quiescent INS-1 cells for 10 min with 15 mM glucose alone resulted in increased Erk1/2 phosphorylation activation; however, glucose (15 mM) did not independently increase PKB phosphorylation activation (Fig. 4A), in agreement with previous observations (Dickson *et al.* 2001, Lingohr *et al.* 2002b). Notwithstanding, the addition of IGF-I (10 nM) promoted PKB phosphorylation both at Ser⁴⁷³ and Thr³⁰⁸ residues, and further increased Erk1/2 phosphorylation (Fig. 4A). In the presence of PMA, IGF-I-induced phosphorylation of PKB on both Ser⁴⁷³ and Thr³⁰⁸ residues was significantly reduced by $\geq 75\%$ ($P \leq 0.05$; Fig. 4A). In contrast, the phosphorylation activation of Erk1/2 was markedly enhanced in the presence of PMA in a glucose-independent manner (approximately eightfold at 0.5 or basal 3 mM glucose; $P \leq 0.001$; Fig. 4A), and further increased 15 mM glucose/IGF-I-induced Erk1/2 phosphorylation three- to fourfold ($P \leq 0.005$; Fig. 4A). Phosphorylation activation of p70^{S6K} in INS-1 cells was promoted by 15 mM glucose and IGF-I over a 30-min period, resulting in an increased mobility shift in polyacrylamide gel electrophoresis (Fig. 4B), as previously recognized (Dickson *et al.* 2001, Lingohr *et al.* 2002b). In contrast to the diverse effects of PMA on Erk1/2 and PKB activation, PMA treatment of INS-1 cells did not appear to have a significant effect on glucose/IGF-I-induced p70^{S6K} phosphorylation (Fig. 4B). It was also examined whether PMA had an adverse effect upstream of IGF-I-induced PKB phosphorylation at the level of IGF-I-induced IRS-2 association

with the p85 regulatory subunit of PI3-K. Co-immunoprecipitation analysis showed that at a stimulatory 15 mM glucose IGF-I induced a significant $\sim 50\%$ increased IRS-2/p85 PI3-K interaction ($P \leq 0.05$), that was significantly reduced in the presence of PMA (Fig. 4C).

The general PKC inhibitor Gö6850 prevented the PMA-mediated changes in PKB and Erk1/2 phosphorylation

The bisindolylmaleimide Gö6850 is a general PKC isoform inhibitor, which has improved selectivity compared with staurosporine regarding the inhibition of protein kinases other than PKC (Toullec *et al.* 1991). Moreover, Gö6850 alleviated FFA-induced inhibition of IGF-I-induced PKB activation (Fig. 2), as well as reversed PMA-induced inhibition of glucose/IGF-I-induced β -cell (INS-1) mitogenesis (Fig. 3), suggesting an effect mediated by a conventional or novel PKC isoform. We examined whether these PKC isoforms were implicated in PMA-mediated inhibition of glucose/IGF-I-induced PKB phosphorylation activation and PMA-induced activation of Erk1/2 (Fig. 5A), using a titration inhibition analysis of Gö6850 (0–5 μ M). The Gö6850 did not affect glucose/IGF-I-induced PKB or Erk1/2 phosphorylation activation in the absence of PMA (Fig. 5). However, Gö6850 completely alleviated the PMA-induced inhibition of IGF-I-induced PKB phosphorylation activation $> 1 \mu$ M Gö6850 ($IC_{50} \sim 200$ nM Gö6850; Fig. 5A). Likewise, Gö6850 also inhibited the PMA-induced activation of Erk1/2 which was maximal at $> 1 \mu$ M Gö6850 ($IC_{50} \sim 200$ nM Gö6850; Fig. 5B). This implied that the contrasting PMA effects on PKB and Erk1/2 activation were mediated by conventional and/or novel PKC isoforms (note that PMA is not effective in activating atypical PKC isoforms (Kazanietz *et al.* 2000)), and not by other phorbol receptors (Ron & Kazanietz 1999).

The relative isoform-specific PKC inhibitors, Rottlerin and Gö6976, had contrasting effects on Erk1/2 and PKB phosphorylation

In order to narrow down which particular PKC isoforms might be mediating the PMA-induced effects in β -cells, a titration inhibition analysis of PMA induced activation of Erk1/2 and alleviation

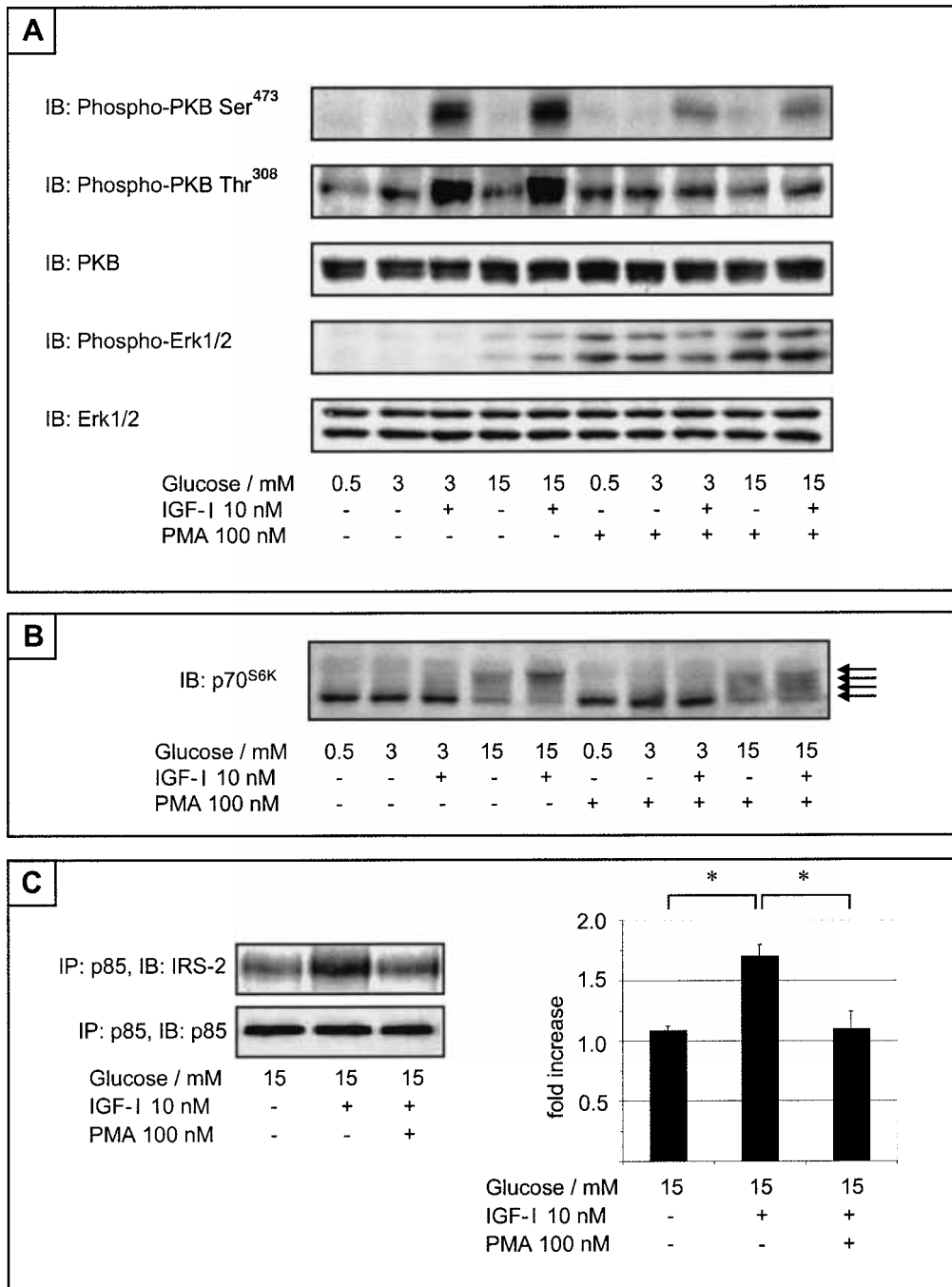


Figure 4 The effect of PMA on mitogenic signaling pathways in INS-1 cells. INS-1 cells were made quiescent for 18 h then incubated for 10 min (A and C) or 30 min (B) with the indicated glucose concentrations \pm 10 nM IGF-I with or without 100 nM PMA. Cell lysates were prepared and immunoblotted (IB) with antibodies for phospho-PKB Ser⁴⁷³, phospho-PKB Thr³⁰⁸, total PKB, phospho-Erk1/2 and total Erk1/2 (A) or p70^{S6K} (B), as described in Materials and methods. For the co-immunoprecipitation (IP) analysis (C), 1 mg cell lysates were immunoprecipitated with p85 antibody and immunoblotted for p85 and IRS-2. A representative immunoblot of at least three individual experiments is shown (A–C) and means \pm s.e. of at least three independent experiments are shown for IRS-2/p85 PI3-K co-immunoprecipitation analysis (C). * $P \leq 0.05$.

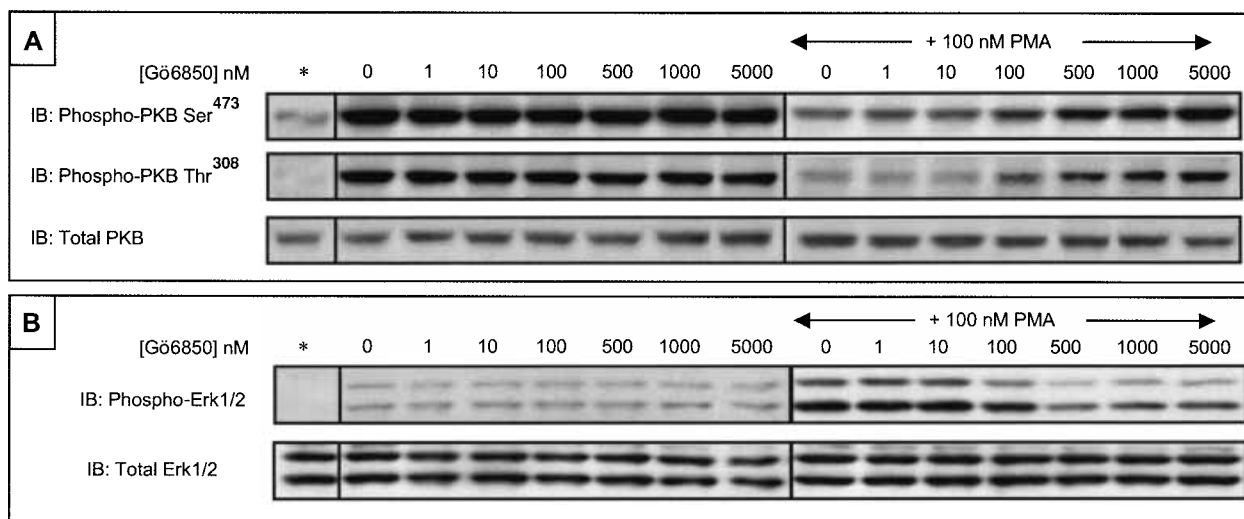


Figure 5 Immunoblot (IB) analysis of INS-1 cells treated with Gö6850. Quiescent INS-1 cells were pretreated for 2 h with Gö6850 (0–5 μ M) in media containing 0.5 mM glucose, followed by a 10-min incubation at 15 mM glucose+10 nM IGF-I with or without 100 nM PMA. Control cells (*) were incubated in 0.5 mM glucose. Cell lysates were subjected to immunoblot analysis with antibodies for phospho-PKB Ser⁴⁷³, phospho-PKB Thr³⁰⁸ and total PKB (A) or antibodies for phospho-Erk1/2 and total Erk1/2 (B), as described in Materials and methods. A representative immunoblot of at least three independent experiments is shown.

of PMA-mediated inhibition of IGF-I-induced PKB phosphorylation was examined using the relatively PKC isoform-specific inhibitors Rottlerin and Gö6976.

In cell culture experiments, Rottlerin has a lower IC_{50} for the novel PKC- δ than for conventional and atypical PKC isoforms (Gschwendt *et al.* 1994). In this study, we found that Rottlerin dose dependently inhibited glucose/IGF-I-induced Erk1/2 phosphorylation in the absence or presence of PMA, although the effect was greater in the presence of PMA (Fig. 6B). The calculated IC_{50} for Rottlerin-mediated inhibition of PMA-induced Erk1/2 phosphorylation was $3.7 \pm 0.4 \mu$ M ($n=4$) and that for glucose/IGF-I-induced Erk1/2 phosphorylation in the absence of PMA was $4.3 \pm 0.3 \mu$ M ($n=4$) equivalent to that for the Rottlerin IC_{50} to inhibit PKC- δ activity between 3 and 5 μ M in intact cells (Gschwendt *et al.* 1994). These data might be suggestive of a PMA-induced phosphorylation activation of Erk1/2 in INS-1 cells mediated by PKC- δ . In contrast to effects on Erk1/2, Rottlerin failed to alleviate the PMA-mediated inhibition of IGF-I-induced PKB phosphorylation in INS-1 cells (Fig. 6A), suggesting that it was unlikely that the PMA-mediated inhibition of

IGF-I-induced PKB phosphorylation in β -cells was via PKC- δ . However, a proviso should be made over the interpretation that the effects of Rottlerin are all attributable to PKC- δ inhibition. Rottlerin may not directly inhibit PKC- δ (Davies *et al.* 2000, Soltoff 2001) and is capable of inhibiting a few other lipid-activated protein kinase activities (Davies *et al.* 2000). In this latter regard, it was noted that Rottlerin inhibited IGF-I-induced PKB phosphorylation in INS-1 cells (Fig. 6A), albeit at a significantly higher concentration (IC_{50} of $9.4 \pm 0.6 \mu$ M ($n=3$); $P<0.05$) than that required to inhibit PKC- δ activity in β -cells (IC_{50} between 3 and 5 μ M (Gschwendt *et al.* 1994)), or PMA-induced phosphorylation of Erk1/2 (Fig. 6B). It has previously been reported that Rottlerin can also inhibit PDK-1 activity *in vitro* (Davies *et al.* 2000) and, in an *in vitro* assay as previously described (Dickson *et al.* 2001), using recombinant PDK-1, we also found that Rottlerin inhibited PDK-1 activity with an IC_{50} between 9 and 11 μ M (data not shown). This would be consistent with the observed Rottlerin-mediated inhibition of IGF-I-induced PKB-Thr³⁰⁸ phosphorylation (Fig. 2), which is catalyzed by PDK-1 (Dickson *et al.* 2001). Notwithstanding, due to the questionable specificity

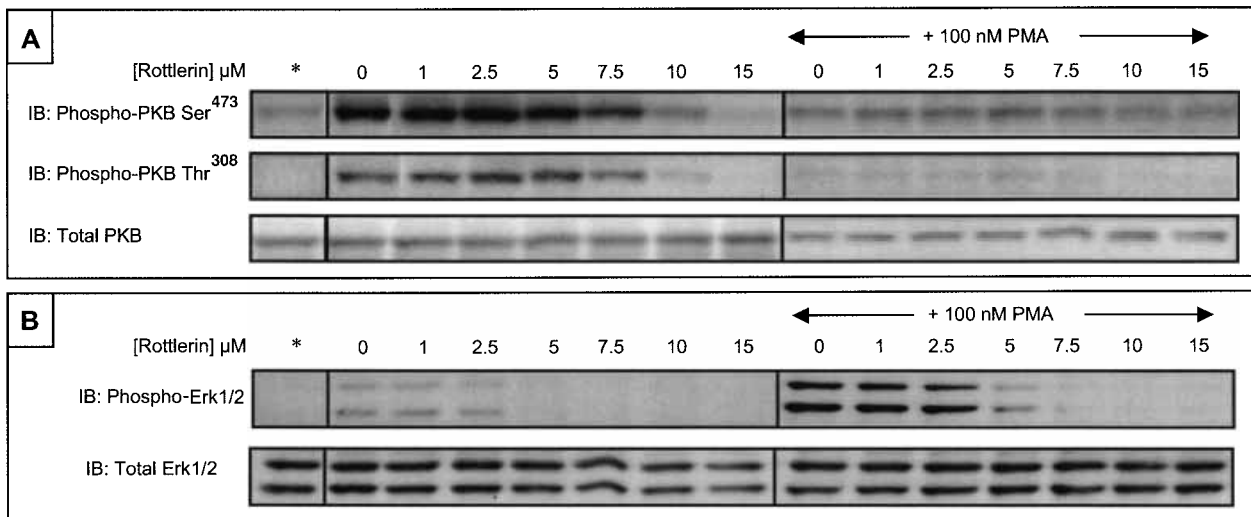


Figure 6 The effect of the novel PKC inhibitor Rottlerin on PKB and Erk1/2 phosphorylation in INS-1 cells. Quiescent INS-1 cells were preincubated for 20 min with the indicated concentrations of Rottlerin (0–15 μ M) in media containing 0.5 mM glucose, followed by a 10-min incubation at 15 mM glucose+10 nM IGF-I with or without 100 nM PMA. Control cells (*) were incubated in 0.5 mM glucose. Cell lysates were subjected to immunoblot (IB) analysis with antibodies for phospho-PKB Ser⁴⁷³, phospho-PKB Thr³⁰⁸ and total PKB (A) or antibodies for phospho-Erk1/2 and total Erk1/2 (B), as described in Materials and methods. A representative immunoblot of at least three independent experiments is shown.

of Rottlerin for PKC- δ (Davies *et al.* 2000, Soltoff 2001), it would be premature to conclusively state that PKC- δ mediates PMA-induced Erk1/2 phosphorylation, and although this remains a possibility other experiments are required to substantiate this notion.

The PKC inhibitor Gö6976, an indolocarbazole derivative, selectively inhibits the conventional PKC isoforms α and β (Martiny-Baron *et al.* 1993). The Gö6976 had no effect on IGF-I-induced PKB at Ser⁴⁷³ or Thr³⁰⁸ phosphorylation in the absence of PMA, neither did it alleviate PMA-mediated inhibition of IGF-I-induced PKB phosphorylation (Fig. 7A). This indicated that conventional PKC isoforms are not necessarily involved in PMA-induced inhibition of IGF-I-induced PKB phosphorylation activation in β -cells. The Gö6976 also appeared to have no effect on independent PMA-induced phosphorylation of Erk1/2 (Fig. 7B), indicating that it is not conventional PKC isoforms that mediate PMA-induced Erk1/2 phosphorylation in β -cells. However, glucose/IGF-I-induced Erk1/2 phosphorylation, in the presence or absence of PMA, was enhanced by approximately fivefold at $>1\mu$ M Gö6976 (half maximal activation = 93 ± 12 nM Gö6976 ($n=3$); Fig. 7B). This suggested a possible inhibitory effect of

conventional PKC isoforms on glucose/IGF-I-induced Erk1/2 activation.

Discussion

Several signaling molecules involved in mitogenic pathways, especially certain lipid moieties (Bell & Burns 1991, Nishizuka 1992), have been described to activate PKC isoforms that may have a possible role in β -cell mitogenesis and/or survival (Lingohr *et al.* 2002a). However, such signaling molecules can differentially activate different PKC isoforms (Bell & Burns 1991, Nishizuka 1992, Liu 1996). Thus, a comparison of the effects of the potentially relevant PKC activators in the β -cell, PIP₃, oleate, palmitate, oleoyl-CoA and palmitoyl-CoA with the known allosteric activators, PS, DAG and Ca²⁺, was investigated in an *in vitro* assay. As anticipated, the conventional PKCs (PKC- α and - β 1) were most effectively activated by the combination of Ca²⁺, DAG and PS, the novel PKC- δ was best activated by DAG and PS in the absence of Ca²⁺, and atypical PKC- ζ activity was increased by PIP₃ as previously described (Bell & Burns 1991, Nishizuka 1992, Toker *et al.* 1994, Liu 1996). It was also found that the activated FFA, oleoyl-CoA and

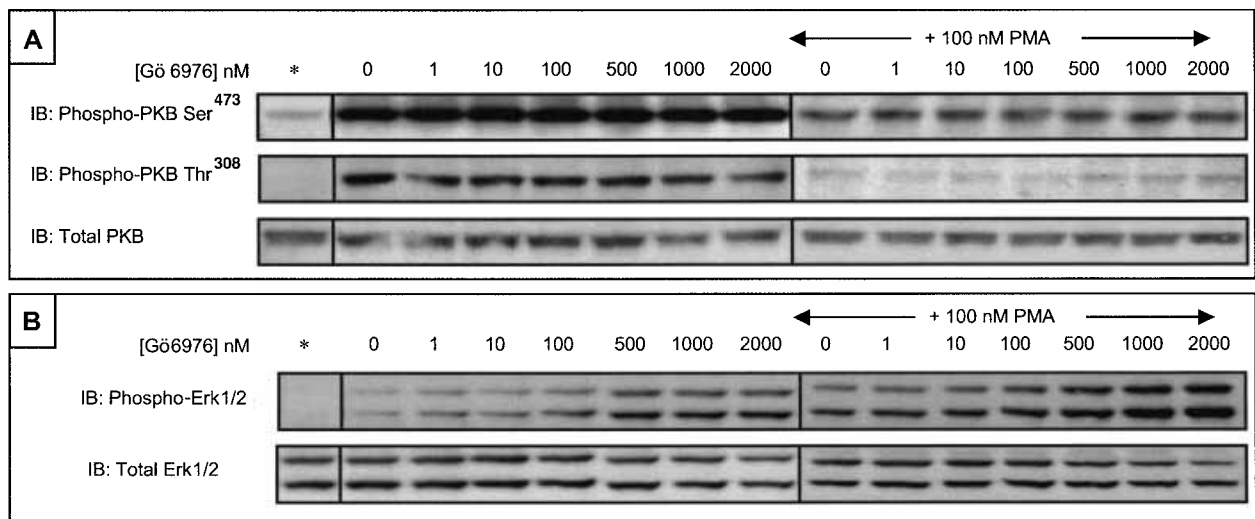


Figure 7 The effect of the conventional PKC inhibitor Gö6976 on PKB and Erk1/2 phosphorylation in INS-1 cells. Quiescent INS-1 cells were preincubated for 2 h with the indicated concentrations of Gö6976 (0–2 μ M) in media containing 0.5 mM glucose, followed by a 10-min incubation at 15 mM glucose+10 nM IGF-I with or without 100 nM PMA. Control cells (*) were incubated in 0.5 mM glucose. Cell lysates were subjected to immunoblot (IB) analysis with antibodies for phospho-PKB Ser⁴⁷³, phospho-PKB Thr³⁰⁸ and total PKB (A) or antibodies for phospho-Erk1/2 and total Erk1/2 (B), as described in Materials and methods. A representative immunoblot of at least three independent experiments is shown.

palmitoyl-CoA, but not unesterified FFA, independently increased the activity of all PKC isoforms examined *in vitro*, particularly that of the novel PKC- δ (Fig. 1C). As such, it can be envisaged that accumulation of long-chain acyl-CoA, but not unesterified FFA, can result in increased PKC activity in β -cells and particularly novel PKC isoform activation. In addition to FFA-CoA activation, all PKC isoforms, but predominantly PKC- δ and PKC- ζ , were activated by the product of PI3-K activation, PIP₃. A transient PIP₃-induced activation of PKC- ζ would be consistent for a role of PKC- ζ in glucose/GLP-1-increased β -cell mitogenesis (Buteau *et al.* 2001). Thus, one can envisage that certain PKC isoforms can play a positive role in influencing β -cell mitogenic signal transduction pathways. It should be noted that autophosphorylation of PKC isoforms was relatively modest in this *in vitro* assay, and that the protein kinases found downstream of PI3-K, PDK-1 and PKB, in constitutively active forms had no significant effect on PKC activation (data not shown). This emphasized the importance of lipid-mediated allosteric activation of PKC isoforms (Bell & Burns 1991, Nishizuka 1992, Liu

1996), although it is realized that *in vitro* observations do not always translate into the *in vivo* context so that phosphorylation of certain PKC isoforms should not be fully ruled out as an additional regulator of PKC activity *in vivo* (Le Good *et al.* 1998).

FFA, which are chronically elevated in the course of obesity-linked type-2 diabetes, have been proposed to play a role in the failure of β -cell mass to increase in compensation of the peripheral insulin resistance (Shimabukuro *et al.* 1998, Rhodes 2000, Unger & Orci 2001, Lingohr *et al.* 2002a). This FFA-mediated impediment in β -cell growth likely occurs via a combination of inhibiting β -cell mitogenesis (Cousin *et al.* 2001) and increasing β -cell apoptosis (Shimabukuro *et al.* 1998, Cnop *et al.* 2001, Wrede *et al.* 2002). It has been postulated, although not extensively examined, that these adverse effects of chronic FFA on maintaining a β -cell population might, in part, be mediated via activation of certain, yet to be identified, PKC isoforms (Unger & Orci 2001, Lingohr *et al.* 2002a). The observation that fatty acyl-CoA, rather than FFA, activates PKC isoforms (Fig. 1) would be consistent with this idea, considering that FFA-

induced β -cell apoptosis and inhibition of mitogenesis is also dependent on fatty acyl-CoA formation (Cousin *et al.* 2001, Wrede *et al.* 2002). In addition, one should consider that IRS-2 signal transduction pathways, especially via PI3-K/PKB, play an important role in promoting β -cell growth and survival (Lingohr *et al.* 2002a, Rhodes & White 2002). The observation that FFA-induced inhibition of PKB activation can be alleviated by the general PKC inhibitor (Gö6850) (Fig. 2) also implicated chronic FFA-mediated activation of certain PKC isoforms having a negative effect on β -cell growth/survival. Neither Rottlerin nor the conventional PKC isoform inhibitor, Gö6976, could alleviate FFA-induced inhibition of PKB activation in β -cells (Fig. 2), suggestive that this might be mediated via certain novel or atypical PKC isoforms.

However, we have previously demonstrated that the atypical PKC- ζ is involved in upregulating β -cell mitogenesis (Buteau *et al.* 2001). To restrict an evaluation of whether there were adverse effects of conventional or novel PKC isoform activation on pancreatic β -cell proliferation, we examined whether the phorbol ester, PMA, affected glucose/IGF-I-induced β -cell proliferation. Phorbol esters activate conventional or novel, but not atypical, PKC isoforms (Ron & Kazanietz 1999, Kazanietz *et al.* 2000). We found that PMA caused a significant reduction in glucose/IGF-I-induced β -cell mitogenesis, which was reversed in the presence of the general PKC isoform inhibitor, Gö6850 (Fig. 3), but not Rottlerin or the conventional PKC isoform inhibitor, Gö6976. It might be considered that 4 h of treatment with PMA, as used in this study, could conceivably lead to some downregulation of certain PKC isoforms (Ways *et al.* 1991, Ozawa *et al.* 1993, Yaney *et al.* 2002), which in turn contributed to the apparent inhibition of β -cell mitogenesis. However, in β -cells only conventional PKC isoforms are rapidly downregulated within a 4-h period of PMA exposure (Yaney *et al.* 2002). Moreover, since the inhibitory effect of PMA on β -cell mitogenesis was reversed by Gö6850, it was unlikely due to a PMA-induced downregulation of a PKC isoform. This reversibility by Gö6850 also indicated that the PMA inhibitory effect was not due to non-specific cytotoxic effects or the activation of other phorbol ester binding proteins (Kazanietz *et al.* 2000). Together, these observations tended to suggest that it was most likely a

novel PKC isoform activity that had a negative effect on glucose/IGF-I-induced β -cell mitogenesis, but this needs to be substantiated.

IRS-2 signal transduction is important for β -cell growth and survival (Lingohr *et al.* 2002a, Rhodes & White 2002). As such, the influence of PMA-activated PKC isoforms on glucose/IGF-I-induced IRS-2-mediated signaling in INS-1 cells was investigated, using a pharmacological approach that might also better indicate which particular PKC isoforms could mediate the negative effect on β -cell growth. In the presence of PMA, IGF-I-induced association of IRS-2 with the p85 regulatory subunit of PI3-K, which results in PI3-K activation (Benito *et al.* 1996, Rhodes & White 2002), was significantly inhibited in β -cells correlating with PMA-induced inhibition of β -cell mitogenesis. This, in turn, brought about a PMA-induced inhibition of PKB phosphorylation activation, which is downstream of IRS-2 and PI3-K in the signal transduction cascade (Benito *et al.* 1996, Dickson *et al.* 2001). The PMA-induced inhibition of IGF-I-induced PKB phosphorylation was reversed by the general PKC inhibitor, Gö6850, but not by Rottlerin or the conventional PKC isoform inhibitor, Gö6976, similarly to FFA-induced inhibition of PKB phosphorylation activation (Fig. 2). Since phorbol esters are not particularly effective at activating atypical PKC isoforms (Liu 1996, Kazanietz *et al.* 2000), our data are consistent with the notion that a novel PKC isoform was involved in FFA/PMA-induced inhibition of PKB activation. As Rottlerin has been considered a PKC- δ inhibitor (Gschwendt *et al.* 1994), and was not particularly effective in rescuing FFA/PMA-induced inhibition of PKB phosphorylation activation, it might be suggested that alternative novel PKC isoforms to PKC- δ contributed to FFA/PMA-induced inhibition of β -cell growth. However, such a conclusion is only tentative since Rottlerin is capable of inhibiting other protein kinases (Davies *et al.* 2000), and not very effective at inhibiting PKC- δ *in vitro* (Davies *et al.* 2000, Soltoff 2001). As such, alternative, more specific experimental approaches are required to pin-point which particular novel PKC isoform can contribute to FFA/PMA-induced inhibition of PKB activation. Despite a marked PMA-induced inhibition of PKB phosphorylation activation, PMA did not appear to affect downstream phosphorylation of p70^{S6K}. However, it has

previously been shown that glucose can activate p70^{S6K} directly via mammalian target of rapamycin (mTOR), bypassing a requirement for upstream PKB activation (Dickson *et al.* 2001). As such, it appears that alternative PKB targets, other than mTOR/p70^{S6K}, are more pertinent to glucose/IGF-I-mediated control of β -cell mitogenesis.

In contrast to PMA inhibitory effects on PKB activation, a PMA-induced Erk1/2 phosphorylation activation was observed, independent of glucose and IGF-I. This perhaps provides an explanation as to why PMA-induced inhibition of β -cell mitogenesis was only partial, since glucose/IGF-I-induced Erk1/2 activation has previously been indicated to contribute to β -cell mitogenesis in addition to the PI3-K signaling pathway (Hügl *et al.* 1998, Dickson *et al.* 2001). The PMA-induced Erk1/2 phosphorylation was inhibited by the general PKC inhibitor, Gö6850, suggesting that it was mediated by a PKC isoform. This is consistent with previous observations that Erk1/2 can be activated via PKC-induced phosphorylation activation of Raf-1, independent of Ras, upstream of mitogen activated protein kinase kinase (MEK) and Erk1/2 (Ueda *et al.* 1996, Pearson *et al.* 2001). Alternatively, since PMA-inhibited PKB activation, these observations might also reflect an alleviation of PKB-mediated inhibition of Erk1/2 phosphorylation activation, previously observed in β -cells (Dickson *et al.* 2001). However, the preferential conventional PKC isoform inhibitor, Gö6976, further increased glucose/IGF-I- and PMA-induced Erk1/2 phosphorylation in a dose-dependent manner without affecting PKB activation, suggesting that conventional PKC isoforms can negatively affect Erk1/2 activation independent of PKB. It has been previously shown in other cell types that PMA-mediated Erk1/2 activation was reduced when constitutively active conventional PKCs (α and β 1) were expressed, whereas expression of novel and atypical PKCs (δ , ϵ and ζ) potentiated PMA-mediated Erk1/2 activation (Ueda *et al.* 1996, Schönwasser *et al.* 1998). Titration inhibition of PMA-induced Erk1/2 phosphorylation by Rottlerin suggested that it might be mediated by PKC- δ in β -cells, as found in other cell types (Ueda *et al.* 1996, Pearson *et al.* 2001). However, given the reservation about Rottlerin's specificity and ability to directly inhibit PKC- δ (Davies *et al.* 2000, Soltoff 2001), this would be a premature and tentative conclusion. While

PKC- δ -mediated phosphorylation activation of the Raf1/Mek/Erk1/2 pathway in β -cells remains a possibility, further experimentation is required to better demonstrate this. Notwithstanding, it should be noted that Erk1/2 activation in β -cells is complex and, as well as activation by a novel PKC isoform, it can be influenced by elevation in Ca²⁺ and/or cAMP via increased glucose metabolism (Frodin *et al.* 1995, Khoo & Cobb 1997, Benes *et al.* 1999) as well as via tyrosine phosphorylation of IRS-2 and Shc (Hügl *et al.* 1998). In this latter regard, it is interesting to note that despite PMA-mediated inhibition of IGF-I-induced IRS-2/p85-PI3-K association, a PMA-induced increased Erk1/2 phosphorylation activation was still observed. This indicates the importance of alternative signaling pathways in the β -cell, independent of IRS-2 (e.g. via Shc, Ca²⁺ and/or cAMP), in mediating glucose/IGF-I-induced Erk1/2 phosphorylation activation.

In summary, we postulate that FFA/PMA-induced inhibition of PKB phosphorylation activation leads to a decreased glucose/IGF-I-induced β -cell mitogenesis and might be contributed via chronic activation of a novel PKC isoform. Admittedly, this supposition is mostly based on observations derived from pharmacological titration inhibition experiments, and the putative role of a novel PKC isoform in mediating FFA/PMA-induced inhibition of PKB phosphorylation in β -cells will have to be confirmed using a complementary molecular approach. Nonetheless, it is known that of the family of novel PKC isoforms, PKC- δ , ϵ and μ are expressed in β -cells (Yaney *et al.* 2002), and this initial characterization study has narrowed the field to these candidate novel PKC isoforms to interfere with IRS-2-mediated mitogenic signal transduction pathways in β -cells. Intriguingly, FFA-induced activation of novel PKC isoforms in skeletal muscle have been shown to play a negative role on insulin signal transduction pathways contributing to the pathogenesis of insulin resistance *in vivo* (Griffin *et al.* 1999, Laybutt *et al.* 1999, Patti 1999, Kraegen *et al.* 2001, Moller 2001). If, in future studies, it becomes apparent that the same novel PKC isoforms which cause peripheral insulin resistance also have a negative effect on β -cell growth, then one might envisage these as novel pharmacological targets that could both alleviate insulin resistance and preserve compensating increases in β -cell mass to

slow the pathogenic course of obesity-linked type-2 diabetes.

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