Evidence that protein kinase Cδ is not required for palmitate-induced cytotoxicity in BRIN-BD11 β-cells

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Abstract

Chronic exposure of pancreatic β-cells to saturated fatty acids leads to loss of viability, an effect that has been implicated in the process of β-cell 'lipotoxicity' associated with the progression of type 2 diabetes. The mechanisms involved are unknown but recent evidence has implicated the δ isoform of protein kinase C (PKCδ) in mediating fatty acid toxicity. We have investigated this proposition in the clonal insulin-secreting cell line, BRIN-BD11. BRIN-BD11 cells were found to undergo apoptosis when exposed to palmitate and this response was attenuated by the purportedly selective inhibitor of PKCδ, rottlerin. However, activation of PKCδ with the phorbol ester, phorbol-12-myristate-13-acetate (PMA), failed to promote cell death and down-regulation of PKCδ did not prevent the cytotoxic effects of palmitate. Moreover, rottlerin remained effective as a blocker of the palmitate response in cells depleted of PKCδ. Since rottlerin can inhibit various other kinases in addition to PKCδ, a range of additional kinase inhibitors was also tested. Of these, only the putative Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) inhibitor, KN-62, was found to inhibit palmitate-induced cell death. However, this effect was not reproduced by a more selective pseudo-substrate inhibitor of CaM kinase II. Therefore, the present results reveal that palmitate induces cell death in BRIN-BD11 cells and suggest that this may involve the activation of a rottlerin (and KN-62)-sensitive kinase. However, it is clear that PKCδ is not required for this response.

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Introduction

Type II diabetes is characterised by increased peripheral insulin resistance and a progressive decline in insulin secretion which, together, culminate in the appearance of clinical symptoms (reviewed by McGarry & Dobbins 1999, Poitout & Robertson 2002). The deterioration in insulin secretion is caused by an initial loss of β-cell sensitivity to glucose but this is exacerbated by a progressively declining β-cell mass (Butler *et al.* 2003). The latter is likely to reflect an enhancement of β-cell apoptosis and it is important that the underlying causes are defined. Persistent hyperglycaemia has been proposed as one of the factors responsible for β-cell death in type II diabetes but increased levels of free fatty acids (FFA) are also cytotoxic (Efanova *et al.* 1998, Purrello & Rabuazzo 2000, Federici *et al.* 2001, Maedler *et al.* 2001, Mandrup-Poulsen 2001, Lupi *et al.* 2002). In the clinical context, these two factors probably act in combination to mediate β-cell toxicity although elevated fatty acids are likely to play a primary role (Carpentier *et al.* 2000, Poitout & Robertson 2002).

There are conflicting data on the mechanisms by which fatty acids cause β-cell death but one factor that has recently been highlighted as a potentially important contributor is protein kinase C (PKC;

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Roche *et al.* 1999, Eitel *et al.* 2003). FFAs can increase the levels of diacylglycerol in cells to promote the activation of PKC (Yu *et al.* 2001) and some fatty acids may also activate PKC more directly (Kasahara & Kikkawa 1995, Lu *et al.* 2000). PKCδ is one of the isoforms of PKC that could be activated in fatty acid-treated cells, and this enzyme may be of particular importance for β-cell lipotoxicity since it has been implicated as an inducer of apoptosis in a variety of cell types (Alcazar *et al.* 1997, Carpenter *et al.* 2002, Emoto *et al.* 1995, Kikkawa *et al.* 2002, Mizuno *et al.* 1997, Niwa *et al.* 2002). In an attempt to confirm whether PKCδ has a direct role in β-cell lipo-apoptosis, Eitel *et al.* (2003) treated RIN1046-38 cells with palmitate and observed that PKCδ was translocated from the cytosol to the nucleus. They also found that a PKCδ inhibitor (rottlerin) prevented palmitate-induced β-cell death, and that expression of a dominant negative isoform reduced the loss of viability by ∼50%. Thus, they concluded that activation of PKCδ may be involved in the toxic effects of fatty acids.

However, when considering the implications of these data, it is important to note that, although rottlerin is widely employed as a selective inhibitor of PKCδ, it is also known to exert a wide range of additional effects in cells (Soltoff *et al.* 2001, Kayali *et al.* 2002, McGovern & Shoichet 2003). For example, in a recent study, it was revealed that rottlerin is much more effective as an inhibitor of certain MAP kinases and glycogen synthase kinase-3β (GSK3β) than of PKCδ (Davies *et al.* 2000). Therefore, in view of the potential uncertainties surrounding the interpretation of studies with rottlerin, we have performed additional experiments to investigate further the role of PKCδ in mediating β-cell lipotoxicity.

Materials and methods

Cell culture

The insulin secreting cell line, BRIN-BD11 (McClenaghan *et al.* 1996) was grown in RPMI-1640 medium containing 11 mM glucose, 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37 °C in 5% CO_2 and were grown and maintained in 75-cm^3 flasks. They were used in experiments or passaged when approximately 80% confluent.

Exposure of BRIN-BD11 cells to fatty acids

Palmitate (Sigma) was dissolved in 50% ethanol by heating to 70 °C and then bound to fatty acid-free bovine serum albumin (10% vol:vol) at 37 °C for 1 h. This mixture was then added to serum-free medium (modified RPMI-1640 containing 5·5 mM glucose) to give final concentrations of 0·5% ethanol and 1% BSA. Cells were treated with the albumin-bound palmitate in the presence or absence of appropriate test compounds, 24 h after seeding into 6-well plates (1×10^5 cells/well). All control wells received 0·5% ethanol and 1% BSA alone.

Vital dye staining

For routine determination of the proportion of cell death, vital dye staining was used. Floating and attached cells were collected individually from each well and stained with Trypan blue. This is actively excluded from living cells, but can penetrate dead cells staining them blue. The number of unstained (live) and blue (dead) cells were counted using a haemocytometer and the percentage dead cells calculated for each experimental condition.

Insulin secretion assays

BRIN-BD11 cells were seeded into 24-well plates at $2·5 \times 10^4$ cells per well. They were pre-incubated for 40 min in 0·5 ml bicarbonate buffered physiological saline (Gey & Gey 1936) containing 6 mM glucose, 1 mM $CaCl_2$ and 0·1% BSA, pH 7·4. Cells were then washed and acutely stimulated with 100 nM phorbol-12-myristate-13-acetate (PMA) for 1 h at 37 °C, prior to the measurement of insulin secretion by radioimmunoassay.

Protein extraction

Cells were washed in ice-cold PBS before addition of 0·5 ml lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100, with 10 μl/ml protease inhibitor cocktail added just before use) per 75-cm^3 flask, for 10 min, on ice. The flask was then scraped and the contents transferred to a microfuge tube, vortexed (4 × for 15 s), centrifuged at 13 000 ***g*** for 10 min at 4 °C and the supernatant was stored at − 80 °C.

Western blotting

Equal amounts of denatured protein were loaded per well onto 12% pre-cast polyacrylamide gels (Invitrogen). A prestained marker set (Amersham) was included to allow the sizes of relevant bands to be determined. Gels were run at 200 V in MOPS-SDS running buffer (50 mM 3-(N-morpholino) propane sulphonic acid, 50 mM Tris base, 3·5 mM SDS, 1 mM EDTA). Proteins were transferred to PVDF membranes and these were blocked with 5% goat serum. The primary anti-PKCδ antibody (Sigma) was diluted 1:2000 in the presence of 1% goat serum and blots incubated for 4 h at room temperature. Anti-rabbit IgG-alkaline phosphatase conjugate (1:30 000; Sigma) was added and incubated for 1 h. Immunoreactive bands were visualised after addition of chemiluminescent substrate, CDP-*Star* (Sigma) and exposure to X-ray film (Fuji medical film).

Statistical analysis

Individual experiments were repeated on at least two separate occasions and the results were analysed by ANOVA. Differences were considered significant when $P<0·05$.

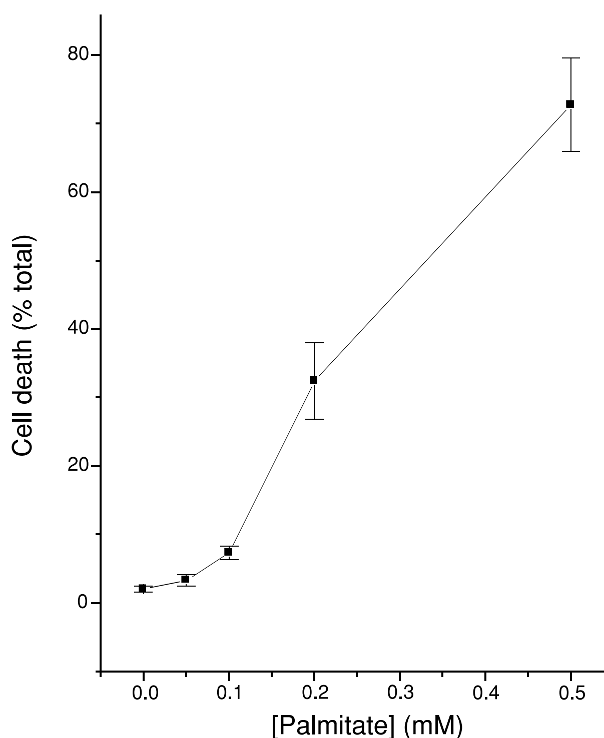

Figure 1 Effects of palmitate on the viability of BRIN-BD11 cells. BRIN-BD11 cells were treated with increasing concentrations of palmitate for 18 h and the extent of viability determined by vital dye staining. Results are mean values±S.E.M. (n=4).

Results

The rat β-cell line, BRIN-BD11, was chosen as the model system for the present work since preliminary data revealed that, unlike certain other widely employed β-cell lines, their responses to fatty acids were similar to those reported in primary rat and human β-cells. In addition, a single fatty acid (palmitate) was selected as the principal cytotoxic molecule since, as in the case of primary islet cells, combinations of fatty acids were found to elicit markedly different responses according to the components used (H J Welters, unpublished data).

To establish the effect of palmitate on the viability of BRIN-BD11 cells, the cells were treated with increasing concentrations of palmitate (between 0·1 and 0·5 mM) complexed to bovine serum albumin for 18 h (Fig. 1). This was associated with a marked loss of viability, such that up to 80% of the cells were killed during incubation with 0·5 mM palmitate. Staining of palmitate-treated cells with a fluorescently labelled caspase substrate revealed that the loss of viability was accompanied by caspase activation suggesting that the mode of cell death was primarily apoptotic (not presented).

Initially, we investigated the possibility that palmitate-induced cell death may involve the formation of ceramide by the use of two selective inhibitors of ceramide formation, fumonisin B1 and ISP-1. However, neither of these agents attenuated the response to palmitate in BRIN-BD11 cells (not shown), suggesting that ceramide formation is not important for this response.

Involvement of protein kinase Cδ in palmitate-induced cell death

Rottlerin has recently been shown to inhibit palmitate-mediated toxicity in RIN 1046-38 cells (Eitel *et al.* 2003), suggesting a role for PKCδ in palmitate-induced cell death in β-cells. A similar effect was also seen in BRIN-BD11 cells, where 5 μM rottlerin significantly attenuated the loss of viability caused by exposure to palmitate (Fig. 2).

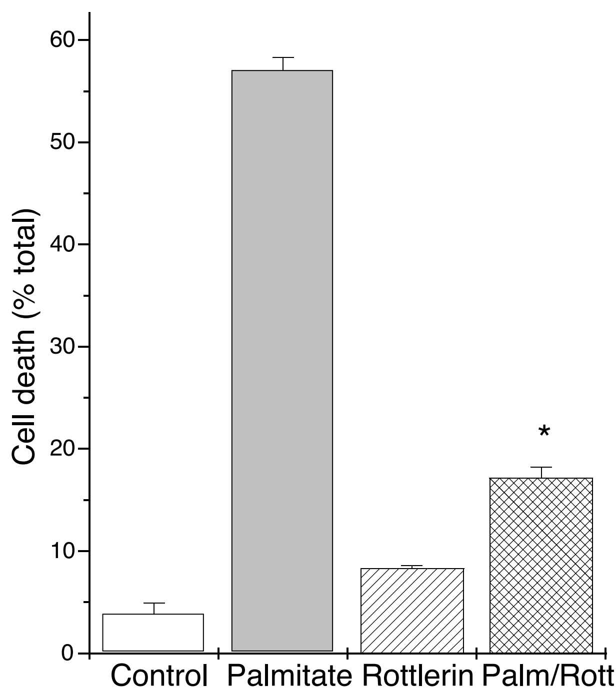

Figure 2 Effect of rottlerin on palmitate-induced cell death. BRIN-BD11 cells were treated with 0·25 mM palmitate in the presence or absence of 5 μM rottlerin for 20 h. The extent of cell viability was determined by vital dye staining. Results are shown as means ± S.E.M. (n=4). *P<0·001 compared with palmitate in the absence of rottlerin. Palm/Rott, palmitate/rottlerin.

Taken at face value, these results suggest that PKCδ may become activated in palmitate-treated cells and that blockade of this enzyme attenuates the apoptotic response. In an attempt to confirm this, cells were also exposed to a direct activator of PKC, PMA. Treatment of cells with PMA leads to activation of a variety of PKC isoforms, including PKCδ; thus it would be expected that exposure to PMA would also promote cell death. However, this was not the case. Exposure of BRIN-BD11 cells to PMA for up to 48 h failed to reduce their viability (Fig. 3).

More prolonged treatment of cells with PMA has an additional consequence in that it leads to the down-regulation of those isoforms of PKC that are activated in response to PMA. Thus, we used this technique to deplete BRIN-BD11 cells of PKC in order to examine whether this would modify the cytotoxicity of palmitate. To confirm the loss of functional PKC after chronic PMA treatment, insulin secretion experiments were performed. Exposure of BRIN-BD11 cells to 100 nM PMA for 1 h resulted in a large increase in insulin secretion when cells were cultured under control conditions, but this effect was completely lost following 24-h pre-culture with PMA (Fig. 3A) consistent with the expected reduction in PKC activity under these conditions.

Having confirmed that pre-culture with PMA leads to loss of functional PKC, BRIN-BD11 cells were then chronically exposed to PMA prior to the addition of palmitate (in the continued presence of PMA; Fig. 3B). In control cells (not pre-cultured with PMA) exposure to palmitate caused the expected large-scale loss of viability. Significantly, this response was entirely unaffected in cells that had been pre-cultured with PMA to down-regulate PKC activity. This suggests that PMA-sensitive PKCs (such as PKCδ) are not required for palmitate toxicity. However, in drawing this conclusion, it was also important to confirm that PKCδ had been lost from the cells during the initial 24-h period of exposure to PMA. This was achieved by Western blotting of cell extracts with an antibody specific to PKCδ (Fig. 3C). In control cells, an 80 kDa band corresponding to PKCδ was strongly expressed but this was absent from cells chronically exposed to PMA, confirming that down-regulation of PKCδ had occurred.

Since it was suspected that the effects of rottlerin on palmitate-induced apoptosis were independent of PKCδ, cells were cultured with PMA to down-regulate PKCδ, then exposed to palmitate in the absence or presence of rottlerin (Fig. 4). As observed previously, palmitate reduced the viability of the cells irrespective of the status of PKCδ and, even more strikingly, rottlerin was equally effective as an inhibitor of this response in control cells and in those where PKCδ had been down-regulated.

Involvement of other protein kinases in mediating palmitate cytotoxicity

In view of the finding that rottlerin is an effective inhibitor of palmitate-induced cytotoxicity in BRIN-BD11 cells (Figs 2 and 4) and that this compound can inhibit a range of other protein kinases (Davies *et al.* 2000), we used a further set of inhibitors in an attempt to identify the critical kinase involved. Initial studies focussed on calcium/calmodulin-dependent protein kinases (CaM kinase) since some isoforms of CaM kinase are effective targets for rottlerin (Gschwendt *et al.*

1994). KN-62 is an inhibitor of CaM kinase II (Tokumitsu *et al.* 1990) that has been shown to inhibit responses mediated by this enzyme in β-cells (Aucouturier *et al.* 1994). Moreover, this reagent significantly attenuated palmitate-induced death in BRIN-BD11 cells (Fig. 5) suggesting the possibility that CaM kinase II might be a target for palmitate in β-cells. However, in order to verify this observation, we also employed a second, more specific, inhibitor of CaM kinase II, autocamtide-2 related inhibitory peptide of CaM kinase II (AIP; Ishida *et al.* 1995). This peptide is a highly selective and potent pseudo-substrate inhibitor of CaM kinase II that is able to gain entry to intact cells by virtue of an N-terminal myristoyl moiety. BRIN-BD11 cells were treated with AIP in the absence or presence of 0·25 mM palmitate but the peptide failed to alter the extent of cell death at concentrations up to 20 μM (cell death: control, 6·6 ± 0·4%; AIP alone, 6·8 ± 0·8%; 0·25 mM palmitate, 66·5 ± 4%; palmitate plus 20 μM AIP, 69·0 ± 3%). This suggests that CaM kinase II is unlikely to be involved in the activation of cell death by palmitate, and that the attenuation of the palmitate response by KN-62 (and rottlerin) is not due to inhibition of CaM kinase II.

In experiments to determine the specificity of protein kinase inhibitors (Davies *et al.* 2000), rottlerin and KN-62 were shown to inhibit a common group of protein kinases, including MAPKAP-K2 (mitogen-activated protein kinase-activated protein kinase 2) and PRAK (p38 regulated/activated kinase). These kinases are both regulated by the upstream kinase p38 (Rouse *et al.* 1994, New *et al.* 1998), a member of the mitogen activated protein kinase (MAPK) family. Since the MAPK pathway regulates cell growth, differentiation and apoptosis, we also studied the potential involvement of p38 in the response to palmitate. SB203580 is a selective inhibitor of p38 (Cuenda *et al.* 1995) but it had no effect on the ability of 0·25 mM palmitate to induce cell death in BRIN-BD11 cells (control, 6 ± 1%; 20 μM

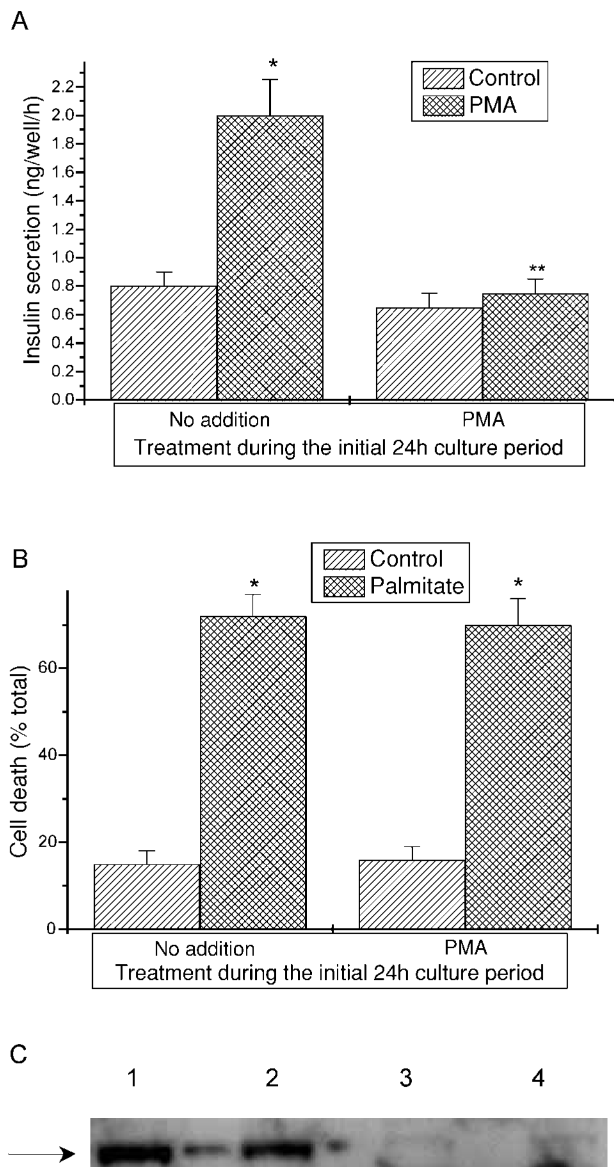

Figure 3 Effect of PMA-induced PKCδ down-regulation on palmitate-induced cell death and insulin secretion from BRIN-BD11 cells. Cells were cultured for 24 h either in the absence (no addition) or presence of 100 nM PMA. (A) After the initial period of culture in either the absence (no addition) or presence of PMA, cells were washed and either left untreated (control) or were stimulated with 100 nM PMA. Insulin secretion was measured after incubation for 1 h. *$P<0·001$ compared with control, ** $P<0·001$, significantly less than control cultured cells exposed to PMA. (B) After the initial culture period, cells were washed and either left untreated (control) or exposed to 0·25 mM palmitate. Cell death was determined after a further 18 h by vital dye staining. *$P<0·01$ compared with the relevant control. (C) BRIN-BD11 cells were treated for 24 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 100 nM PMA and protein extracts prepared. Thirty micrograms of each sample were analysed for PKCδ expression by Western blotting. PKCδ was identified as an ~80 kDa protein (arrow) by reference to markers run in parallel with the experimental samples.

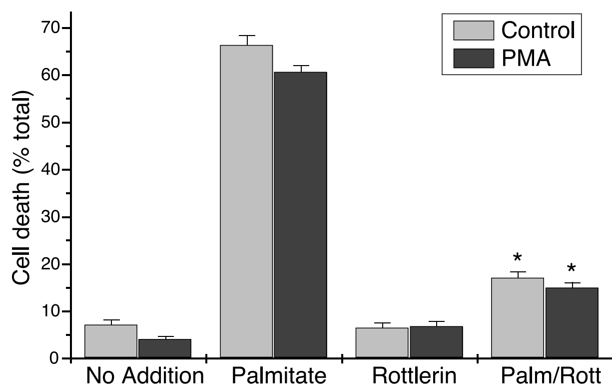

Figure 4 Effect of PMA-induced down-regulation of PKCδ on the protective effects of rottlerin. BRIN-BD11 cells were treated with 100 nM PMA for 24 h to down-regulate PKCδ. Cells were then washed and exposed to 0·25 mM palmitate in the presence or absence of 5 μM rottlerin and in the continued presence of 100 nM PMA. Cell death was determined by vital dye staining 20 h after addition of palmitate. Results shown are mean values±S.E.M. (*n*=5). **P*<0·001 compared with palmitate alone. Palm/Rott, palmitate/rottlerin.

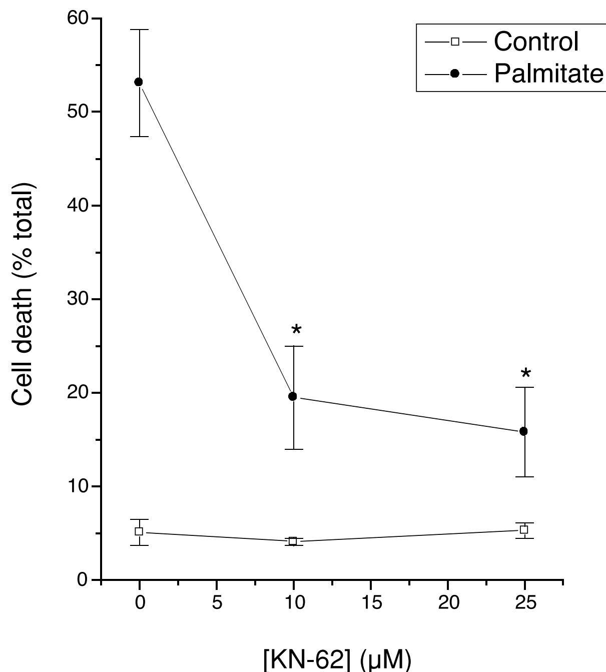

Figure 5 Effect of KN-62 on palmitate-induced cell death. BRIN-BD11 cells were treated with 0, 10 and 25 μM KN-62 for 1 h before the addition of 0·25 mM palmitate in the continued presence of KN-62. Cells were then incubated for a further 20 h before the extent of viability was determined by vital dye staining. Results are shown as mean values±S.E.M. (*n*=4). **P*<0·001 compared with palmitate alone.

SB203580, 7·5 ± 1%; 0·25 mM palmitate, 78 ± 4%; palmitate plus SB203580, 83 ± 4%).

Finally, we tested the effects of an inhibitor of GSK3β, SB216763, on the response to palmitate, since Davies *et al.* (2000) reported that rottlerin and KN-62 can also inhibit this enzyme. However, when BRIN-BD11 cells were treated with SB216763 at concentrations shown to inhibit the enzyme in intact cells (Coghlan *et al.* 2000, Cross *et al.* 2001) it failed to alter palmitate-induced cell death (control, 6 ± 1%; 5 μM SB216763, 7 ± 1·8%; 0·25 mM palmitate, 77 ± 4%; palmitate plus SB216763, 65 ± 5%).

A summary of the data generated with the various kinase inhibitors used in these studies is provided in Table 1. This reveals that none of the identified kinases can account fully for the inhibition of palmitate-mediated cytotoxicity by rottlerin and KN-62.

Discussion

There is considerable uncertainty about the mechanisms by which saturated fatty acids promote β-cell death and it is important that these are defined since this may then suggest a means to slow the rate of β-cell loss in patients with type II diabetes. In the present work, we have employed the saturated fatty acid, palmitate, to induce cell death in BRIN-BD11 β-cells since this model system recapitulates many of the features of lipotoxicity seen in primary rat and human islets (Maedler *et al.* 2001, 2003). More specifically, we have investigated whether activation of PKCδ may play a role in mediating the response to palmitate.

Multiple isoforms of PKC are expressed in β-cells (Knutson & Hoenig 1994, Yaney *et al.* 2000) but the major forms are the PMA-sensitive enzymes, PKCα and PKCδ (Knutson & Hoenig 1994, 1996, Carpenter *et al.* 2001). In the present work, a down-regulation protocol was used to manipulate PMA-sensitive PKC levels in BRIN-BD11 cells since Eitel *et al.* (2003) reported that one of these, PKCδ, may be important in mediating the cytotoxic effects of palmitate in β-cells. In this procedure, the cells were exposed to PMA for an initial period of 24 h, leading to the loss of PKC-mediated responses and a diminution in the expression of relevant PKC isoforms. In

Table 1 Summary of the effects of kinase inhibitors on palmitate-induced β-cell death

Inhibitor	Effectiveness as an inhibitor of palmitate-induced death	Kinases reportedly sensitive to inhibition
Rottlerin	+	PKCδ CaM kinases MAPKAP-K2 PRAK GSK3β
KN-62	+	CaM kinase II MAPKAP-K2 PRAK GSK3β
Autocamtide-2-related inhibitory peptide	–	CaM kinase II
SB203580	–	p38 MAPK (regulates MAPKAP-K2 and PRAK)
SB216273	–	GSK3β

confirmation of this, BRIN-BD11 cells that had been exposed to PMA for 24 h no longer responded with an increase in insulin secretion when re-exposed to the drug. Such cells also displayed a dramatic reduction in the expression of PKCδ relative to controls (as judged by Western blotting), confirming that PKCδ had been effectively depleted during the period of culture with PMA. Thus, the fact that palmitate was equally effective as an inducer of apoptosis in β-cells that were either replete with, or depleted of, PKCδ shows that this enzyme cannot be critical for the response.

It is of interest to note that the present data on PKCδ down-regulation in BRIN-BD11 cells stand in contrast to those of Yaney *et al.* (2002) who reported that this isoform was retained in HIT-T15 β-cells during long-term PMA treatment. The reasons for this difference are unclear but may relate to the finding that, unlike other isoforms of PKC, PKCδ requires PMA-induced hyperphosphorylation for down-regulation (Srivastava *et al.* 2002). It is conceivable, therefore, that the enzyme may be less efficiently phosphorylated in HIT-T15 cells, leading to its retention during PMA exposure.

Despite the evidence arising from the PKCδ down-regulation experiments, it was also observed that the PKC inhibitor, rottlerin, markedly attenuated palmitate-induced toxicity. This result is similar to that of Eitel *et al.* (2003) in RIN1046-38 cells and, taken at face value, could point to the involvement of PKCδ. However, it is well known that rottlerin can exert a range of effects in cells, not all of which result from inhibition of PKCδ (Gschwendt *et al.* 1994, Zhao *et al.* 2002, McGovern & Shoichet 2003). Indeed, in one recent study it was concluded that rottlerin does not inhibit PKCδ at all, but is much more effective as an inhibitor of MAPKAP-K2 and PRAK (which form part of the p38 MAP kinase pathway) and GSK3β (Davies *et al.* 2000). Moreover, in the original paper describing the characteristics of rottlerin, it was also reported to inhibit some isoforms of CaM kinase (Gschwendt *et al.* 1994). Thus, the ability of rottlerin to inhibit palmitate-induced apoptosis cannot be taken as unequivocal evidence for the involvement of PKCδ. In support of this, we observed that rottlerin still blocked palmitate-induced β-cell death in cells that had been pre-treated with PMA to deplete the levels of PKCδ. Thus, under these conditions, the effects of rottlerin cannot have been due to inhibition of PKCδ.

In view of these findings, we considered it important to test a further range of compounds that selectively inhibit other enzymes that are also reported to be sensitive to rottlerin (which include

CaM kinases, p38 MAP kinases and GSK3β). Accordingly, we examined the effects of SB203580 and SB216763 (selective inhibitors of the p38 MAP kinase pathway and GSK3β respectively) as well as KN-62 (a widely used inhibitor of CaM kinase II). Neither SB203580 nor SB216763 attenuated the cytotoxic actions of palmitate whereas KN-62 markedly inhibited the response. However, a second, more selective pseudo-substrate inhibitor of CaM kinase II, AIP, failed to reproduce this effect. Thus, we conclude that the inhibitory effects of rottlerin do not reflect its ability to inhibit PKCδ, CaM kinase II, the p38 MAP kinase pathway or GSK3β and we suggest that none of these enzymes plays a critical role in mediating palmitate toxicity in β-cells.

Although we have not been able to identify the site of action of rottlerin in the present studies, it is significant that, during a comprehensive analysis of the effects of widely-used kinase inhibitors, both rottlerin and KN-62 were observed to inhibit a similar subset of enzymes (Davies *et al.* 2000). Since we now show that both agents also block palmitate-induced cell death in BRIN-BD11 cells, it seems probable that a critical (but still unidentified) kinase is expressed in β-cells that is sensitive to both rottlerin and KN-62 and is required for palmitate to initiate apoptosis. One caveat to this conclusion, however, is the emergence of new evidence that rottlerin and KN-62 can form aggregates that may also inhibit certain non-kinase enzymes when assayed *in vitro* (McGovern & Shoichet 2003).

In conclusion, the present results provide strong evidence that PKCδ activation is not a pre-requisite for palmitate-induced cell death in BRIN-BD11 cells. As such, they are supported by the conclusions of Kasahara and Kikkawa (1995) and Yaney *et al.* (2000), who demonstrated that, although palmitate can increase the activity of some PKC isoforms, it has little effect on the activity on PKCδ. In addition, over-expression of PKCδ only marginally increased apoptosis in virally transduced β-cells (Carpenter *et al.* 2002). Despite these considerations, it would be premature to conclude that the enzyme plays no role in β-cell apoptosis. Indeed, evidence from other studies suggests the contrary in that certain aspects of the cytotoxic actions of interleukin-1β and streptozotocin may involve the activation of PKCδ (Carpenter *et al.* 2002). However, the present results reveal that it is not required for completion of the apoptotic programme in cells exposed to palmitate.

Acknowledgements

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References

Alcazar O, Qiu-yue Z, Gine E & Tamarit-Rodriguez J 1997 Stimulation of islet protein kinase C translocation by palmitate requires metabolism of the fatty acid. *Diabetes* **46** 1153–1158.

Aucouturier S, Bernard C, Roche C, Philippe J, Chayvialle JA & Cuber JC 1994 Functional coupling between the cyclic adenosine monophosphate pathway and cholecystokinin secretion in RIN cells. *Biochemical and Biophysical Research Communications* **200** 1382–1390.

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA & Butler PC 2003 Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* **52** 102–110.

Carpenter L, Cordery D & Biden TJ 2001 Protein kinase C delta activation by interleukin-1 beta stabilizes inducible nitric-oxide synthase mRNA in pancreatic beta-cells. *Journal of Biological Chemistry* **276** 5368–5374.

Carpenter L, Cordery D & Biden TJ 2002 Inhibition of protein kinase C delta protects rat INS-1 cells against interleukin-1 beta and streptozotocin-induced apoptosis. *Diabetes* **51** 317–324.

Carpentier A, Mittelman SD, Bergman RN, Giacca A & Lewis GF 2000 Prolonged elevation of plasma free fatty acids impairs pancreatic beta-cell function in obese nondiabetic humans but not in individuals with type 2 diabetes. *Diabetes* **49** 399–408.

Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L *et al.* 2000 Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chemical Biology* **7** 793–803.

Cross DA, Culbert AA, Chalmers KA, Facci L, Skaper SD & Reith AD 2001 Selective small-molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurones from death. *Journal of Neurochemistry* **77** 94–102.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR & Lee JC 1995 SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Letters* **8** 229–233.

Davies SP, Reddy H, Caivano M & Cohen P 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal* **1** 95–105.

Efanova IB, Zaitsev SV, Zhivotovsky B, Kohler M, Efendic S, Orrenius S & Berggren PO 1998 Glucose and tolbutamide induce apoptosis in pancreatic beta-cells. A process dependent on intracellular Ca^{2+} concentration. *Journal of Biological Chemistry* **273** 33501–33507.

Eitel K, Staiger H, Rieger J, Mischak H, Brandhorst H, Brendel MD, Bretzel RG, Haring HU & Kellerer M 2003 Protein kinase C delta activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. *Diabetes* **52** 991–997.

Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichselbaum R *et al.* 1995 Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. *EMBO Journal* **15** 6148–6156.

Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, Usellini L, Nano R, Bonini P, Bertuzzi F *et al.* 2001 High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. *Diabetes* **50** 1290–1301.

Gey GO & Gey MK 1936 Maintenance of human normal cells in continuous culture - preliminary report: cultivation of mesoblastic tumors and normal cells and notes on methods of cultivation. *American Journal of Cancer* **27** 45–76.

Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G & Marks F 1994 Rottlerin, a novel protein kinase inhibitor. *Biochemical and Biophysical Research Communications* **28** 93–98.

Ishida A, Kameshita I, Okuno S, Kitani T & Fujisawa H 1995 A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. *Biochemical and Biophysical Research Communications* **26** 806–812.

Kasahara K & Kikkawa U 1995 Distinct effects of saturated fatty acids on protein kinase C subspecies. *Journal of Biochemistry* **117** 648–653.

Kayali AG, Austin DA & Webster NJ 2002 Rottlerin inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes by uncoupling mitochondrial oxidative phosphorylation. *Endocrinology* **143** 3884–3896.

Kikkawa U, Matsuzaki H & Yamamoto T 2002 Protein kinase Cδ: activation mechanisms and functions. *Journal of Biochemistry* **132** 831–839.

Knutson K & Hoenig M 1994 Identification and subcellular characterization of protein kinase-C isoforms in insulinoma beta-cells and whole islets. *Endocrinology* **135** 881–886.

Knutson KL & Hoenig M 1996 Arachidonic acid-induced down-regulation of protein kinase C delta in beta-cells. *Journal of Cellular Biochemistry* **62** 543–552.

Lu X, Yang XY, Howard RL & Walsh JP 2000 Fatty acids modulate protein kinase C activation in porcine vascular smooth muscle cells independently of their effect on *de novo* diacylglycerol synthesis. *Diabetologia* **43** 1136–1144.

Lupi R, Dotta F, Marselli L, Del Guerra S, Masini M, Santangelo C, Patane G, Boggi U, Piro S & Anello M 2002 Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* **51** 1437–1442.

McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FP, Yoon TW, Swanston-Flatt SK & Flatt PR 1996 Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. *Diabetes* **45** 1132–1140.

McGarry JD & Dobbins RL 1999 Fatty acids, lipotoxicity and insulin secretion. *Diabetologia* **42** 128–138.

McGovern SL & Shoichet BK 2003 Kinase inhibitors: not just for kinases any more. *Journal of Medical Chemistry* **10** 1478–1483.

Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N & Donarth M 2001 Distinct effects of saturated and monounsaturated fatty acids on β-cell turnover and function. *Diabetes* **50** 69–76.

Maedler K, Oberholzer J, Bucher P, Spinas GA & Donath MY 2003 Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes* **52** 726–733.

Mandrup-Poulsen T 2001 Beta-cell apoptosis: stimuli and signaling. *Diabetes* **50** S58–S63.

Mizuno K, Noda K, Araki T, Imaoka T, Kobayashi Y, Akita Y, Shimonaka M, Kishi S & Ohno S 1997 The proteolytic cleavage of protein kinase C isotypes, which generates kinase and regulatory fragments, correlates with Fas-mediated and 12-*O*-tetradecanoyl-phorbol-13-acetate-induced apoptosis. *European Journal of Biochemistry* **15** 7–18.

New L, Jiang Y, Zhao M, Liu K, Zhu W, Flood LJ, Kato Y, Parry GC & Han J 1998 PRAK, a novel protein kinase regulated by the p38 MAP kinase. *EMBO Journal* **17** 3372–3384.

Niwa K, Inanami O, Yamamori T, Ohta T, Hamasu T, Karino T & Kuwabara M 2002 Roles of protein kinase C delta in the accumulation of P53 and the induction of apoptosis in H_2O_2-treated bovine endothelial cells. *Free Radical Research* **36** 1147–1153.

Poitout V & Robertson RP 2002 Minireview: Secondary beta-cell failure in type 2 diabetes - a convergence of glucotoxicity and lipotoxicity. *Endocrinology* **143** 339–342.

Purrello F & Rabuazzo AM 2000 Metabolic factors that affect beta-cell function and survival. *Diabetes, Nutrition and Metabolism* **13** 84–89.

Roche E, Buteau J, Aniento I, Reig JA, Soria B & Prentki M 1999 Palmitate and oleate induce the immediate-early response genes c-fos and nur-77 in the pancreatic beta-cell line, INS-1. *Diabetes* **48** 2007–2014.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T & Nebreda AR 1994 A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **23** 1027–1037.

Soltoff SP 2001 Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase C delta tyrosine phosphorylation. *Journal of Biological Chemistry* **12** 37986–37992.

Srivastava J, Procyk KJ, Iturrioz X & Parker PJ 2002 Phosphorylation is required for PMA- and cell-cycle-induced degradation of protein kinase C delta. *Biochemical Journal* **15** 349–355.

Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M & Hidaka H 1990 KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. *Journal of Biological Chemistry* **15** 4315–4320.

Yaney GC, Korchak HM & Corkey BE 2000 Long-chain acyl CoA regulation of protein kinase C and fatty acid potentiation of glucose-stimulated insulin secretion in clonal beta-cells. *Endocrinology* **141** 1989–1998.

Yaney GC, Fairbanks JM, Deeney JT, Korchak HM, Tornheim K & Corkey BE 2002 Potentiation of insulin secretion by phorbol esters is mediated by PKC-alpha and nPKC isoforms. *American Journal of Physiology, Endocrinology and Metabolism* **283** E880–E888.

Yu HY, Inoguchi T, Kakimoto M, Nakashima N, Imamura M, Hashimoto T, Umeda F & Nawata H 2001 Saturated non-esterified fatty acids stimulate *de novo* diacylglycerol synthesis and protein kinase C activity in cultured aortic smooth muscle cells. *Diabetologia* **44** 614–620.

Zhao H, Tian W & Cohen DM 2002 Rottlerin inhibits tonicity-dependent expression and action of TonEBP in a PKCdelta-independent fashion. *American Journal of Physiology and Renal Physiology* **282** F710–F717.

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