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 Ca²⁺/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.


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 (Efamova 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;
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% CO₂ and were grown and maintained in 75-cm² 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 × 10⁵ 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 × 10⁴ 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 CaCl₂ 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-cm² 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 sulphonylic 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-PKCα 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 \( P<0.05 \).

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 Cα 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 PKCα 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).
Taken at face value, these results suggest that PKCα 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 PKCα; 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. [1996]).
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...
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
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 PKC\(\varepsilon\) relative to controls (as judged by Western blotting), confirming that PKC\(\varepsilon\) 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 \(\beta\)-cells that were either replete with, or depleted of, PKC\(\varepsilon\) shows that this enzyme cannot be critical for the response.

It is of interest to note that the present data on PKC\(\varepsilon\) 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 \(\beta\)-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, PKC\(\varepsilon\) 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 PKC\(\varepsilon\) 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 PKC\(\varepsilon\). However, it is well known that rottlerin can exert a range of effects in cells, not all of which result from inhibition of PKC\(\varepsilon\) (Gschwendt et al. 1994, Zhao et al. 2002, McGovern & Shoichet 2003). Indeed, in one recent study it was concluded that rottlerin does not inhibit PKC\(\varepsilon\) 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 GSK3\(\beta\) (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 PKC\(\varepsilon\).

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 Table 1 Summary of the effects of kinase inhibitors on palmitate-induced \(\beta\)-cell death

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Effectiveness as an inhibitor of palmitate-induced death</th>
<th>Kinases reportedly sensitive to inhibition</th>
</tr>
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<tbody>
<tr>
<td>Rottlerin</td>
<td>+</td>
<td>PKC(\varepsilon), CaM kinases, MAPKAP-K2, PRAK, GSK3(\beta)</td>
</tr>
<tr>
<td>KN-62</td>
<td>+</td>
<td>CaM kinase II, MAPKAP-K2, PRAK, GSK3(\beta)</td>
</tr>
<tr>
<td>Autocamtide-2-related inhibitory peptide</td>
<td>–</td>
<td>CaM kinase II</td>
</tr>
<tr>
<td>SB203580</td>
<td>–</td>
<td>p38 MAPK (regulates MAPKAP-K2 and PRAK)</td>
</tr>
<tr>
<td>SB216273</td>
<td>–</td>
<td>GSK3(\beta)</td>
</tr>
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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 prerequisite for palmitate-induced cell death in BRIN-BD11 cells. As such, they are supported by the conclusions of Kasahara and Kikkawa (1995) and Yancey 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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