Inhibition of Protein Kinase C δ Protects Rat INS-1 Cells Against Interleukin-1β and Streptozotocin-Induced Apoptosis

Lee Carpenter, Damien Cordery, and Trevor J. Biden

Exposure of pancreatic β-cells to cytokines, such as interleukin-1β (IL-1β), is thought to contribute to the β-cell apoptosis that underlies the onset of type 1 diabetes. One important event triggered by IL-1β is induction of nitric oxide synthase (iNOS), an enzyme that catalyzes intracellular generation of the cytotoxic free radical NO. We recently described a novel requirement for the protein kinase C (PKC) isozyme PKCδ in this process. Our current aim, therefore, was to assess whether PKCδ also plays a role in β-cell apoptosis. As assessed by either annexin V staining or DNA fragmentation, IL-1β caused INS-1 cells to undergo apoptosis. This was completely blocked by adenoviral overexpression of a dominant-negative, kinase-dead (KD) PKCδ mutant. The corresponding PKCα virus was without effect. However, apoptosis caused by the cytotoxic agent streptozotocin (STZ), which acts independent of iNOS, was also inhibited by overexpression of PKCδKD. STZ was additionally shown to activate the proteolytic enzyme caspase-3, a key biochemical effector of end-stage apoptosis. Moreover, STZ caused a caspase-dependent cleavage of PKCδ, thereby releasing a COOH-terminal fragment corresponding to the kinase catalytic domain. Thus, proteolytic activation of PKCδ seems to be important in the distal apoptotic pathway induced by STZ. That IL-1β also activated caspase-3 and promoted PKCδ cleavage suggests that this distal pathway also contributes in the apoptotic response to the cytokine. These data therefore support a dual role for PKCδ in IL-1β–mediated cell death: it is required for efficient NO generation through regulation of iNOS levels but also contributes to apoptotic pathways downstream of caspase activation. *Diabetes* 51:317–324, 2002

Type 1 diabetes is an autoinflammatory disease in which pancreatic β-cells are thought to be destroyed by apoptosis (1–5). Release of proinflammatory cytokines, such as interleukin-1β (IL-1β), from infiltrating monocytic cells is a key feature of this process. In many cell types, proinflammatory signaling pathways are known to involve activation of protein kinase C (PKC) (6–10). The PKC family of serine/threonine protein kinases consists of 11 isoforms in three subgroups, comprising conventional, novel, and atypical members, each with varying cofactor requirements and cellular distribution (11). We showed recently that PKCδ is activated after stimulation of pancreatic β-cells with IL-1β and that this activation is essential for a novel pathway mediating active stabilization of the mRNA for inducible nitric oxide synthase (iNOS) (12). This gene encodes an enzyme that generates the free radical NO, which is implicated in β-cell apoptosis (2,13,14). Taken together, these findings suggest that PKCδ, via its effects on iNOS, might be an important and previously unsuspected regulator of β-cell destruction.

In addition, there is now growing evidence for a role of PKC, particularly PKCδ, in the regulation of apoptosis in other cell types in response to ultraviolet radiation, proinflammatory mediators, and a diverse range of cytotoxic and DNA-damaging agents (15–24). However, in these cases, PKCδ plays a direct catalytic role in mediating some of the terminal, biochemical aspects of apoptosis. In this instance, the underlying mechanism of activation involves proteolytic cleavage of PKCδ by caspase 3 (15,17,19). Caspases are a family of cysteine-dependent, aspartate-directed, proteolytic enzymes (25–28). Although their activation is considered a hallmark of apoptosis, caspase involvement in β-cell destruction is relatively poorly defined. Specifically, caspase-3 is an executioner caspase responsible for cellular features of apoptosis such as DNA fragmentation, nuclear condensation, and plasma membrane remodeling (25,26,28). The caspase-3–dependent cleavage of PKCδ results in release of a 40-kDa constitutively active fragment corresponding to the catalytic domain (PKCδCF) of the native protein kinase (15,17,19). This fragment is thus free to phosphorylate target substrates free of regulatory constraints. Documented substrates under these conditions include the DNA repair enzyme DNA-dependent protein kinase (DNA-PK) (23), which is inactivated by PKCδ, and a phosphatidylserine

From the Garvan Institute of Medical Research, St. Vincents Hospital, Darlinghurst, Sydney, Australia.

Address correspondence and reprint requests to Dr. Trevor Biden, Cell Signalling Group, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst, Sydney 2010, Australia. E-mail t.biden@garvan.unsw.edu.au.

Received for publication 18 December 2000 and accepted in revised form 24 October 2001.

CAD, caspase-activated DNase; DNA-PK, DNA-dependent protein kinase; FACS, fluorescence-activated cell sorting; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; KD, kinase dead; NO, nitric oxide; PBS, phosphate-buffered saline; pfu, plaque-forming units; PI, propidium iodide; PKC, protein kinase C; PKCδCF, PKCδ constitutively active fragment; PS, phosphatidylserine; STZ, streptozotocin; TNF-α, tumor necrosis factor-α; WT, wild-type.

(PS) scramblase (29), whose activation by PKCδ may be involved in the plasma membrane remodeling that underlies elimination of apoptotic cells by the immune system (30).

The major goal of the current study was to determine whether activation of PKCδ plays any role in mediating β-cell apoptosis in response to the physiological stimulus IL-1β. We demonstrate that inhibition of endogenous PKCδ in INS-1 cells, using a recombinant adenovirus for overexpression of a PKCδ kinase-dead (KD) mutant, blocks IL-1β–stimulated apoptosis. A similar protection was also afforded to cells treated with streptozotocin (STZ), a cytotoxic agent that causes DNA and mitochondrial damage but that acts independent of alterations in the level of iNOS. These results, together with the finding that both IL-1β and STZ induced a caspase-dependent cleavage of PKCδ, suggest that activation of PKCδ exerts multiple and complex proapoptotic effects in these cells. There is one potential site in the proximal signaling cascade that links the IL-1β receptor to stabilization of iNOS mRNA and at least one other site in the more distal apoptotic cascade in which PKCδ serves as a substrate for executioner caspases.

RESEARCH DESIGN AND METHODS

Cell culture. The rat insulinoma cell line INS-1 was grown in RPMI medium containing 10% heat-inactivated FCS, 500 units/ml penicillin G sodium, 50 μg/ml streptomycin, and 2 mmol/l glutamine (reagents from Gibco BRL, Melbourne, Australia) and passaged by trypsinization into T150 flasks (Becton Dickinson, Franklin Lakes, NY). INS-1 cells (passage 15–30) were routinely seeded at 0.5×10^6/well of a 24-well plate for experiments and used on day 4 at ~80% confluence.

Generation and expression of PKCα and PKCδ recombinant adenovirus. To generate KD mutants, we performed site-directed mutagenesis on mouse PKCα and rat PKCδ cDNA before recombination into adenovirus, as previously described (12). Overexpression of KD PKCs have been well-documented to act in an isozyme-specific, dominant-negative manner (31–34). INS-1 cells were exposed to PKC wild-type (WT) or KD recombinant viruses or MX17 (an E1/E3 deficient adenovirus lacking a transgene) at 10–20 plaque-forming units (pfu)/cell, in 200 μl of media and incubated for 1 h at 37°C, mixing every 15 min. Virus was then removed, and fresh media were applied. At 48 h postinfection, by which time there is a robust expression of the transgene (12), cells were exposed to IL-1β or STZ as described below, and cells or lysates were prepared 9 or 24 h later.

Analysis of caspase 3 activation in response to IL-1β and STZ. INS-1 cells were exposed to 300 pg/ml IL-1β or 10 mg/ml STZ for 9 h. Pilot studies established that this time point was optimal for caspase activation (not shown). Cells were then extracted for a caspase 3 activity determination using the caspase activity assay (Promega, Annandale, New South Wales, Australia) according to the manufacturer's instructions.

Immunoblot analysis. Whole-cell lysate fractions from cells exposed to IL-1β or STZ (for 24 h or 9 h, respectively) were prepared from INS-1 cells by washing monolayers in ice-cold phosphate-buffered saline (PBS) and resuspending in sample buffer containing 1% SDS and 10% mercaptoethanol. Proteins were separated by SDS-PAGE on 10% gels, transferred to nitrocellulose membranes (Trans-blot; Biorad, Regents Park, New South Wales, Australia) by electroblotting, blocked with 5% (wt/vol) milk powder, and probed with rabbit anti–C-term PKCδ antibody (C-17) (Santa Cruz, CA) and donkey anti–rabbit horseradish-peroxidase–conjugated secondary antibody (from Jackson Immuno Research Laboratories, West Grove, PA). The antigen-antibody complexes were visualized by ECL detection (Amersham, Little Chalfont, Bucks, U.K.) and exposure to X-ray film (Fuji, Tokyo, Japan). Signal intensities were determined by densitometric analysis (on a Molecular Dynamics Personal Densitometer SI and software by IPLabGel, Signal Analytics, Fairfax, VA).

Analysis of IL-1β– and STZ-induced apoptosis in INS-1 cells by fluorescence-activated cell sorting. INS-1 cells were infected as above, and expression of the transgene was allowed to proceed for 48 h before exposure to 300 pg/ml IL-1β or 10 mg/ml STZ for 24 h. Cells were stained with both 5 μg/ml propidium iodide (PI; Calbiochem, Alexandria, New South Wales, Australia) and AnnexinV-FLUOS (Roche Diagnostics, Australia, Castle Hill, New South Wales, Australia) according to the manufacturers' instructions. Stained cells were then washed in PBS, harvested by pipetting, and fixed in 90% ice-cold methanol. Cells were analyzed by multiparameter flow cytometry performed on a FACStar bench top flow cytometer (Becton Dickinson) with excitation at 480 nm. Emitted light was split and collected at 530 nm (annexin V) and 585 nm (PI) with the fluorescent signal acquired in log mode. For each flow cytometer run, 10,000 events were acquired in list mode. Increased staining with annexin V (or FL1 fluorescence) alone is indicative of apoptosis, whereas staining with both annexin V and PI is indicative of necrosis. For quantification, cells were restained with PI (postfixation) and reanalyzed by fluorescence-activated cell sorting (FACS). In our hands, this strengthened signal intensity and allowed for better discrimination between the apoptotic and nonapoptotic cell populations.

Analysis of IL-1β– and STZ-induced INS-1 apoptosis by DNA banding. INS-1 cells were infected as above, and expression of the transgene was allowed to proceed for 24 h before exposure to 300 pg/ml IL-1β or 2 and 10 mg/ml STZ for an additional 24 h. Cells were then digested in a buffer containing 100 mmol/l Tris HCl (pH 8), 200 mmol/l EGTA, 2% SDS, and 100 μg/ml proteinase K (Roche) for 16 h at 37°C. DNA was precipitated in 300 mmol/l $NaCl_3$ and 70% isopropyl alcohol, pelleted at 12,000*g* for 15 min, washed with 70% (vol/vol) ethanol, and dried before resuspending in 10 mmol/l Tris-HCl (pH 8.0) and 1 mmol/l EDTA, containing 100 μg/ml RNase (Roche). RNA digestion was carried out for 30 min at 37°C. Total DNA from each well ($\sim 1 \times 10^6$ cells) was run on a 1% agarose gel and visualized after ethidium bromide staining using a GelDoc 1000 illuminator (Biorad) and analyzed using IPLabGel. Banded DNA intensity is expressed relative to its intact genomic band intensity to standardize for loading.

Statistics. All results are presented as mean ± SE. Statistical significance was determined by unpaired Students' *t* test.

RESULTS

Apoptosis measured by annexin V staining is induced by IL-1β and STZ and is dependent on PKCδ activity. We first sought to investigate the potential role of PKCδ in apoptosis in response to IL-1β using FACS analysis. Annexin V-FLUOS binds to PS, which is exposed on the outer leaflet of the (intact) plasma membrane as an early marker of apoptosis. To exclude a possible contribution as a result of necrosis, we also cells treated with PI, which labels the nuclei of nonintact (necrotic) cells. However, as shown in the representative contour blots in Fig. 1*A*, upward shifts in the cell populations (indicative of necrosis) were minimal after experimental treatment as compared with rightward shifts (indicative of apoptosis). In these experiments, INS-1 cells were analyzed after infection with control recombinant adenovirus (MX17) or viruses for overexpression of either PKCδWT or KD. Control cells (upper panels) underwent significant levels of apoptosis when treated with IL-1β, as indicated by the appearance of a population with increased FL1 fluorescence to the right of the dashed line. Overexpression of PKCδWT augmented both basal and IL-1β–stimulated levels of apoptosis (middle panels), and the latter response was shown to be highly significant from nine experiments (see Fig. 1*B*). Conversely, overexpression of the PKCδKD mutant significantly protected INS-1 cells from the IL-1β–stimulated apoptotic shift (lower panels), although basal apoptosis is unaffected. Quantitative data from these and additional studies of cells infected with both PKCα and PKCδ adenoviruses are shown in Fig. 1*B*. Exposure of control INS-1 cells (infected with MX17) to IL-1β increased apoptosis to ~35% of the cell population from a basal level of 10%. Overexpression of PKCαWT virus had a tendency to increase both basal and IL-1β–stimulated apoptosis, but this was not statistically significant. This lack of significance, together with the fact that PKCαKD overexpression did not effect either the basal or stimulated responses, suggests that PKCα has no specific role in IL-1β–induced apoptosis. In marked

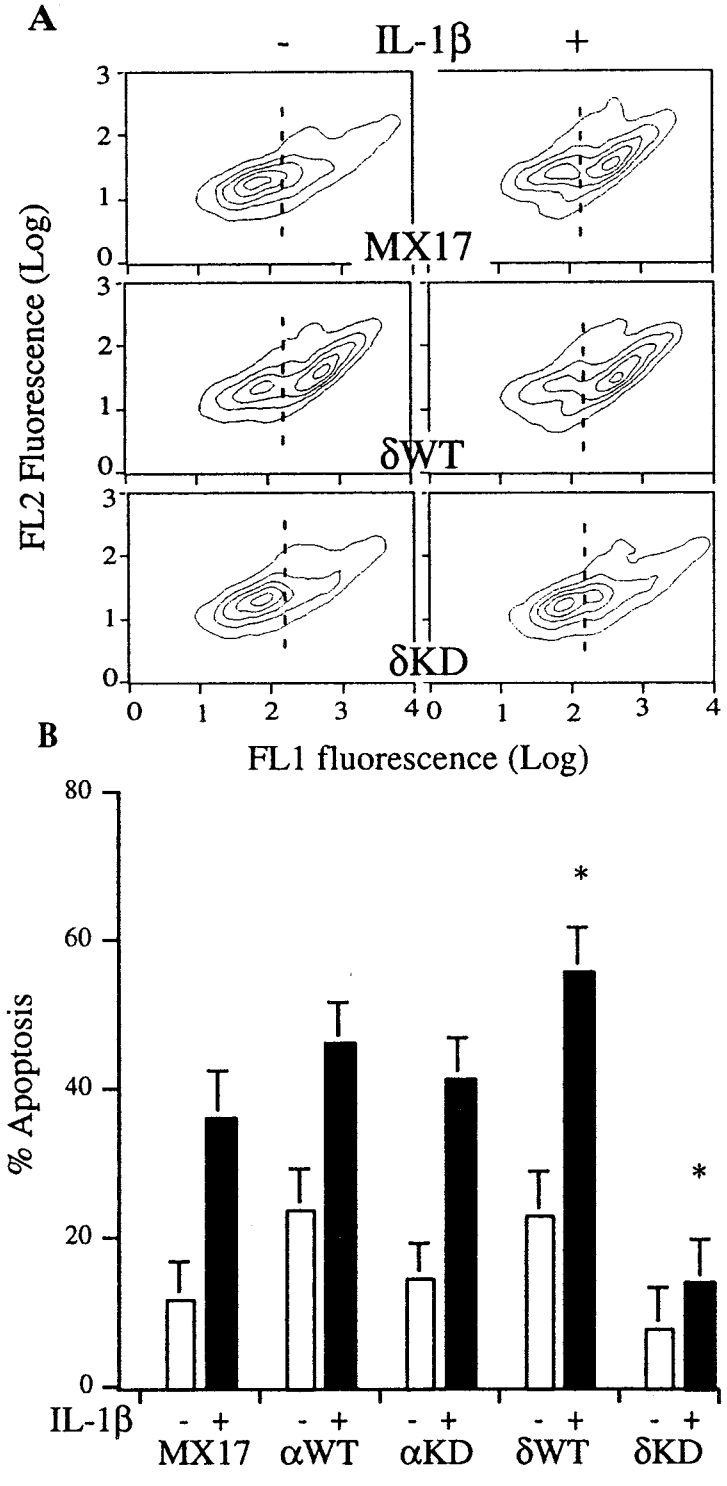

FIG. 1. Effects of PKCδ and PKCα on IL-1β–induced apoptosis in INS-1 cells measured by annexin V staining. *A*: INS-1 cells were infected with MX17 (control virus) or PKC δWT and KD adenovirus constructs as described in RESEARCH DESIGN AND METHODS. At 48 h postinfection, cells were treated where indicated with IL-1β (300 pg/ml) for an additional 24 h. Cells were then stained with annexin V and PI, washed, fixed, and analyzed by FACS. Data are presented as dual-parameter contour plots of FL1 or annexin V fluorescence (*x* axis) versus PI or FL2 fluorescence (*y* axis). The apoptotic population is to the right of the dotted line. *B*: Quantitative data are presented as mean ± SE from nine individual experiments with background apoptosis (routinely ~15% in uninfected cells without IL-1β) subtracted. **P* < 0.05 when compared to the MX17 control with IL-1β.

contrast, the IL-1β response was reciprocally modulated by the overexpression of the two PKCδ viruses. Thus, relative to the MX17 control, stimulated apoptosis was significantly elevated by PKCδWT and, most important, was almost entirely abolished in the presence of PKCδKD.

Corresponding studies were also undertaken using INS-1 cells treated with 10 mg/ml STZ, a cytotoxin that, unlike IL-1β, acts independent of effects on gene transcription. As shown in Fig. 2, this toxin augmented the number of annexin V–stained cells to nearly 55% of total in the

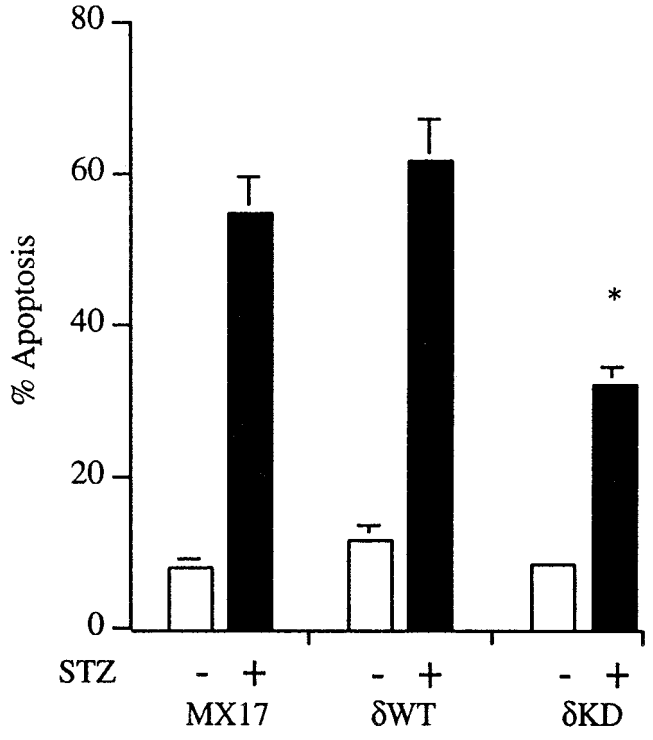

FIG. 2. Effects of PKCδ on STZ-induced apoptosis in INS-1 cells measured by annexin V staining. INS-1 cells were infected with MX17 (control virus) or PKCδ recombinant adenovirus as described in the legend to Fig. 1. At 48 h postinfection, cells were treated where indicated with STZ (10 mg/ml) for 4 h and cultured for an additional 18 h. Cells were stained and analyzed by FACS as described in the legend to Fig. 1. Mean data ± SE from five separate experiments are presented, with background apoptosis (uninfected cells without STZ) subtracted. *$P < 0.05$ when compared to the MX17 control with STZ.

MX17-infected population. Broadly similar results were obtained in cells infected with the PKCδWT virus, but cells that overexpressed PKCδKD were markedly protected against the cytotoxic effects of STZ, with apoptosis reduced to ~35%. This represents a protection of 40% by PKCδKD from STZ-induced apoptosis.

DNA fragmentation is induced by IL-1β and STZ and is dependent on PKCδ activity. We next sought to determine whether PKCδ activation was important in IL-1β– and STZ-induced apoptosis as assessed using the DNA-banding assay. Although less easy to quantify than the annexin V analysis, this technique allows direct visualization of one of the committed steps of apoptosis that distinguishes it from necrosis, namely the cleavage of genomic DNA into integers of ~180 base pairs (14,35). As shown in a representative gel in Fig. 3*A*, both IL-1β and two doses of STZ-induced fragmentation of INS-1 cell in control (MX17)-infected cells. Basal DNA fragmentation was most obviously enhanced by overexpression of PKCδWT, whereas there was a profound protection from DNA fragmentation under all conditions in the cells that overexpressed PKCδKD. Figure 3*B* summarizes the corresponding quantitative data meaned from five separate experiments. MX17-infected cells showed an approximate doubling in DNA fragmentation caused by either IL-1β or 2 mg/ml STZ, with a corresponding larger (fourfold) increase seen at 10 mg/ml STZ. Overexpression of PKCδWT significantly enhanced basal DNA fragmentation but was without significant further effect on the responses of any of the cytotoxic agents. Most important, the ability of all three of these stimuli to induce apoptosis was completely abolished in cells that overexpressed the PKCδKD mutant.

Caspase 3 activity and PKCδ cleavage is induced by IL-1β and STZ. Caspase 3 activity in INS-1 was increased twofold upon exposure to 300 pg/ml IL-1β and 2 mg/ml STZ and fourfold in response to 10 mg/ml STZ (Fig. 4*A*). These same stimuli also enhanced the accumulation of a 45-kDa fragment of PKCδ, with 10 mg/ml STZ having the most pronounced effect (Fig. 4*B*). Generation of this fragment was completely blocked by zVAD, a caspase 3 inhibitor (Fig. 4*C*). These data clearly suggest that, as in other cell types, apoptotic stimuli in β-cells induce a caspase-dependent cleavage of PKCδ into a 45-kDa, constitutively active, COOH-terminal fragment. (15,17,19).

DISCUSSION

The major finding of the current study is that overexpression of a PKCδKD mutant protects INS-1 cells against apoptosis induced by both STZ and IL-1β. The corresponding PKCα mutant was without effect. Identical KD mutants have been widely used in functional studies to inhibit endogenous PKC in an isozyme-specific manner (12,31–34,36). The current results suggest for the first time that PKCδ plays a specific and important role in mediating apoptosis in pancreatic β-cells. Our findings also confirm and extend earlier studies in which pharmacological inhibitors of PKCδ inhibited spontaneous apoptosis in neutrophils (21), as well as the cytotoxic effects of ultraviolet radiation on keratinocytes (19) and of etoposide on salivary cells (20). However, the specificity of the pharmacological agents used in these earlier studies, and especially the use of rottlerin as a PKCδ inhibitor, is now open to doubt (37). Therefore, molecular approaches, such as those used here, are increasingly the methods of choice.

A second important aspect of the current work was the demonstration that STZ and IL-1β both increased caspase-3 activity and generated a 45-kDa fragment by cleavage of full-length PKCδ. This is consistent with the knowledge that PKCδ contains a specific caspase 3 cleavage site and also with results obtained using a variety of other cell types demonstrating the generation of a constitutively active PKCδ fragment in response to apoptotic stimuli (15,17,19). Furthermore, overexpression of PKCδCF alone was sufficient to induce chromatin condensation, nuclear fragmentation and cytotoxicity in fibroblasts (17), and apoptosis in leukemia cells (23). Taken together, these and our current findings suggest that caspase 3–mediated cleavage of PKCδ also plays an important role in the terminal events of apoptosis in pancreatic β-cells.

The events underlying the execution phase of apoptosis in β-cells are poorly understood, and only very recently has the role of caspase activation been addressed. This is surprising because there is growing evidence that apoptosis is the key means by which β-cells are destroyed during the onset of type 1 diabetes (3–5). In any event, it is clear that pancreatic β-cells exposed to cytotoxic stimuli in vitro undergo biochemical and morphological changes consistent with apoptosis (2,4). For example, their DNA is fragmented into 180 base-pair lengths (14), a response that, in other cell types, is known to be due to caspase-activated DNases (CADs) (35,38,39). In INS-1 cells, over-

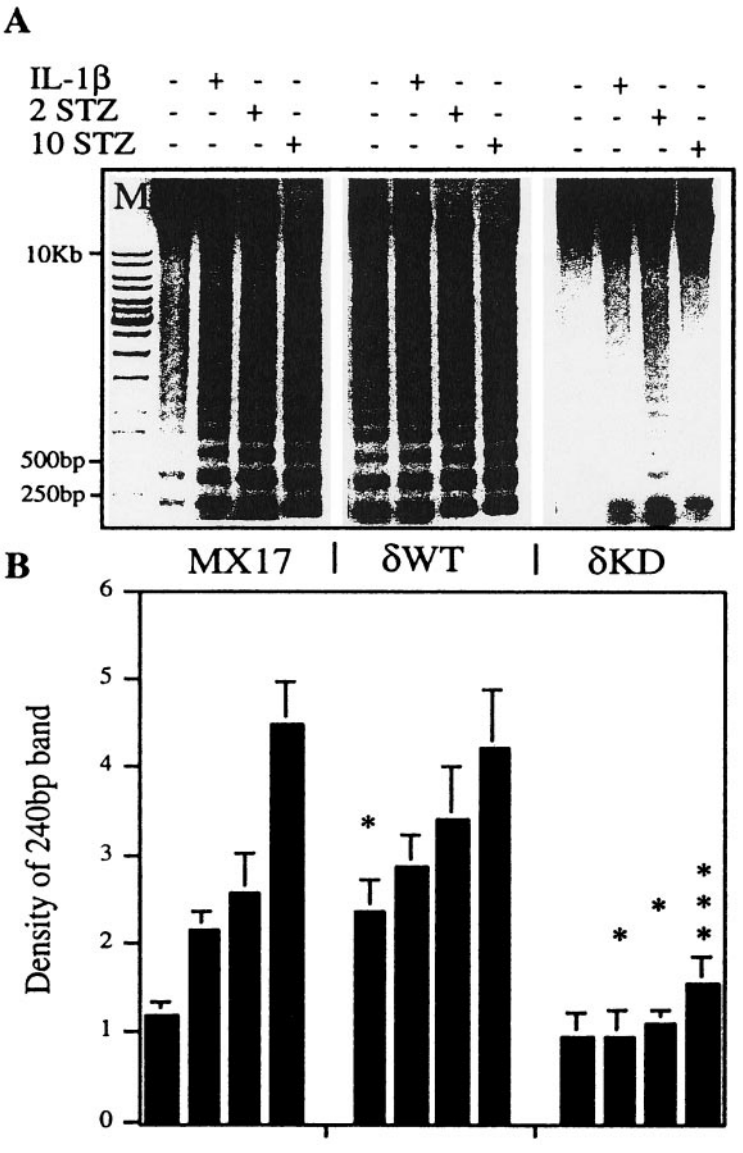

FIG. 3. Effects of PKCδ on IL-1β– and STZ-induced apoptosis in INS-1 cells measured by DNA banding. INS-1 cells were infected with MX17 (control virus) or PKCδ recombinant adenovirus as described in the legend to Fig. 1. At 48 h postinfection, cells were treated with IL-1β (300 pg/ml) for a total of 24 h or with STZ (2 or 10 mg/ml) for 4 h followed by additional culture for 20 h. Cells were harvested, DNA-purified, quantified, and separated by electrophoresis on a 1% agarose gel. Band density was quantified by densitometry, and results are presented relative to the genomic band density in the same sample. For further details, see RESEARCH DESIGN AND METHODS. *A*: A representative gel is shown where M = markers. *B*: Mean data ± SE from five separate experiments is shown, where **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 when compared with the corresponding treatment conditions in control-infected cells. Within the MX17 group alone, treatment with IL-1β and 2 and 10 mg/ml STZ induces significant increases in DNA banding (*P* values of <0.05, <0.05, and <0.001, respectively) when compared with the control MX17 group.

expression of PKCδWT alone was sufficient to induce DNA fragmentation, although there was little further effect on IL-1β– and STZ-induced apoptosis. This may be due to a lack of sensitivity of the assay in discriminating between intermediate to high levels of apoptosis, or it might indicate that PKCδ and the experimental stimuli induce apoptosis through a common and therefore nonadditive mechanism. In any event, results using the PKCδKD mutant clearly demonstrated that activation of PKCδ was absolutely required for DNA fragmentation in response to IL-1β and STZ. It is not yet known whether CADs are regulated directly by PKCδ, but it is noteworthy that there seems to be a requirement by CAD for histone phosphorylation during DNA fragmentation (40), and histone is a known substrate for PKCδ (41). Alternatively—or in addition—the DNA repair enzyme DNA-PK has also been identified as a direct substrate for PKCδ (23), and inhibition of its activity by phosphorylation may be sufficient to promote CAD-mediated DNA fragmentation.

Another characteristic of apoptosis is the enrichment of PS in the outer leaflet of the plasma membrane, which underlies the recognition of apoptotic cells and their removal by macrophages (30). The enzymes responsible for PS rearrangement are a family of scramblases, a member of which has recently been suggested as a substrate for PKCδ (29). Both IL-1β and STZ exposure increased annexin V binding on INS-1 cells, which is indicative of PS exposure on the external cell surface and is thus a readily quantifiable measure of apoptosis. Stimulated annexin V binding was inhibited by overexpression of PKCδKD. In the case of STZ, however, this inhibition was only partial, which also contrasts with the results

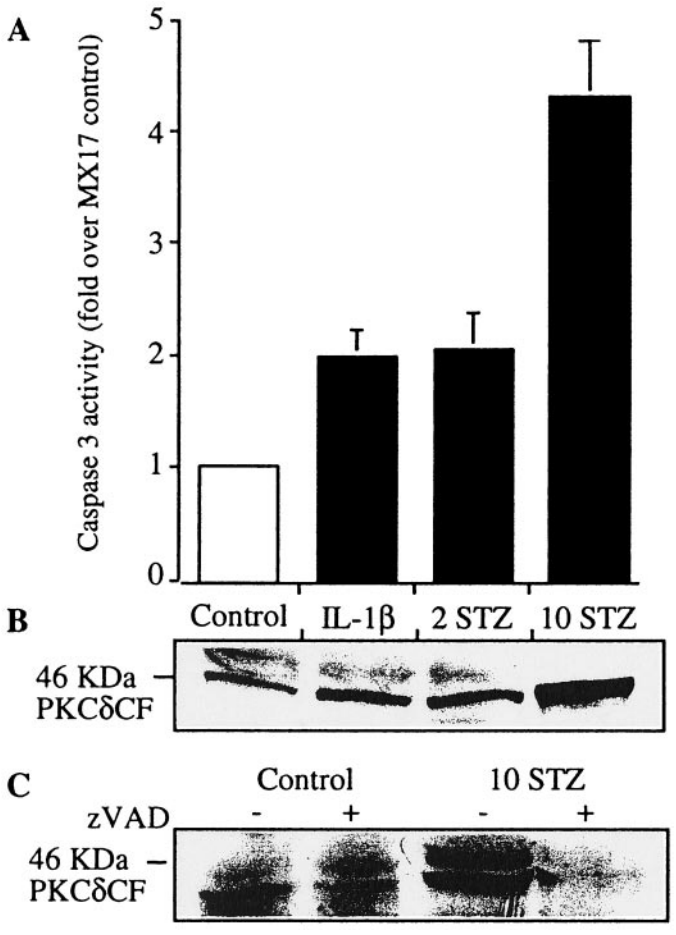

FIG. 4. Effect of IL-1β and STZ on caspase 3 activity (*A*) and caspase 3–mediated PKCδ cleavage (*B* and *C*) in INS-1 cells. Cells were incubated for 9 h with 300 pg/ml IL-1β, with 2 or 10 mg/ml STZ (*A* and *B*), or with 10 mg/ml STZ in the presence or absence of 50 μmol/l zVAD (*C*). Caspase activity (*A*) was measured in cell lysates as described in RESEARCH DESIGN AND METHODS, and data are presented as mean ± SE from four separate experiments. Lysates were also separated by SDS-PAGE and immunoblotted using a PKCδ COOH-terminal specific antibody (*B* and *C*), and representative blots are shown.

using DNA fragmentation that was almost completely abolished under these conditions. This might suggest that the constitutively active PKCδ fragment plays a more crucial role in the pathways that lead to nuclear rather than plasma membrane responses. Alternatively, inhibition of PKCδ might have more pronounced effects on a process such as DNA fragmentation, which is irreversible (and therefore represents a cumulative end point assay), than on PS translocation, which is likely to be in a dynamic equilibrium, albeit one favoring external presentation of PS in apoptotic cells. That inhibition of PKCδ completely abolished IL-1β–stimulated apoptosis, as measured by either assay, is potentially explained by PKCδ acting at multiple sites in the IL-1β signaling cascade, as opposed to a single site in response to STZ.

The potential for multiple sites of action is a consequence of our recent discovery that PKCδ plays an essential role in stabilizing iNOS mRNA in IL-1β–treated β-cells. Activation of PKCδ in this instance is very rapid and therefore probably mediated by lipid signaling molecules rather than caspase activation. Because toxic effects of NO generation on β-cells have been well-documented (2,13,14), stabilization of iNOS mRNA by PKCδ would probably also contribute to cellular destruction. Therefore, a second role for PKCδ in the terminal events of apoptosis, downstream of NO production, would have been difficult to document solely on the basis of results obtained with IL-1β. For that reason, we also used STZ, which induces a robust and rapid apoptotic response that is independent of proinflammatory gene expression (STZ does not induce iNOS (42 and data not shown). Its cytotoxicity has been variously explained by its chemical conversion to NO (14,42,43) and its ability to damage DNA by direct methylation (44). Irrespective of its precise mode of action, use of STZ has allowed us to identify a second potential site of action for PKCδ in the signal transduction pathway leading to β-cell apoptosis.

Surprisingly, little is known about caspase function in pancreatic β-cells. In a single study using RINm5F cells, both IL-1β and NO donors were shown to stimulate caspase 3 activity (45). There have also been reports of cytokines leading to cleavage of known caspase 3 substrates in β-cell lines (46), thereby implicating caspases in tumor necrosis factor-α (TNF-α) (47) and γ-interferon–mediated β-cell destruction (46). We have confirmed these results for IL-1β and extended them to demonstrate cleavage of PKCδ. That IL-1β reproduced the effects of STZ on the distal apoptotic pathway, which supports its physiological relevance, suggests that both sites of action of PKCδ contribute to the apoptotic effects of this cytokine. We do not, however, exclude the possibility of additional mechanisms. Recent findings suggest that PKCδ can also disrupt mitochondrial function in certain cell types (36,48), which, according to current beliefs, should lead to caspase activation (49). This is unlikely to be relevant to INS-1 cells, where modulation of PKCδ expression did not alter caspase activity assays (L.C., D.C., T.J.B., unpublished observations). However, the potential complexity of these multiple facets of PKCδ function meant that a quantitative assessment of their individual contribution to IL-1β–stimulated apoptosis was beyond the scope of the present study. These issues would probably be best addressed by comparing the effects of caspase-resistant mutants of PKCδ with those of PKCδWT on β-cell apoptosis. In any event, both sites of action of PKCδ are almost certainly important, especially in a physiological setting in which β-cells are exposed to mixtures of cytokines, including TNF-α. Caspase activation in response to the latter stimulus is known to be closely linked to receptor occupation (47).

In conclusion, we demonstrated that activation of PKCδ plays a key role in mediating apoptosis in INS-1 cells in response to both IL-1β and STZ. Our results further suggest that this represents a dual role of PKCδ, with one site in the execution phase of apoptosis as defined here with STZ and the second site in regulating iNOS as demonstrated in our earlier study. An appreciation of this dual physiological role now suggests that inhibition of PKCδ might be beneficial therapeutically. Indeed, our current finding that overexpression of the PKCδKD mutant completely protects INS-1 cells from IL-1β–induced apoptosis supports this contention. However, a fuller substantiation of these in vitro findings would require additional experiments, preferably in vivo and possibly involving

PKCδ null mice, to determine whether these are protected against experimentally induced diabetes.

ACKNOWLEDGMENTS

This work was supported by a grant for the Juvenile Diabetes Foundation (1999-555), a Postdoctoral Research Fellowship from the Faculty of Medicine, University of New South Wales, and a generous donation from Sandra Gross.

We thank Dr. E. Musgrove for assistance with FACS analysis and Dr. Greg Cooney, Dr. Kieren Scott, and Professor Peter Scholfield for critical review of the manuscript.

REFERENCES

1. Mandrup-Poulsen T: The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* 39:1005–1029, 1996
2. McDaniel ML, Kwon G, Hill JR, Marshall CA, Corbett JA: Cytokines and nitric oxide in islet inflammation and diabetes. *Proc Soc Exp Biol Med* 211:24–32, 1996
3. Rabinovitch A, Suarez-Pinzon WL: Cytokines and their roles in pancreatic islet β-cell destruction and insulin-dependent diabetes mellitus. *Biochem Pharmacol* 55:1139–1149, 1998
4. Mauricio D, Mandrup-Poulsen T: Apoptosis and the pathogenesis of IDDM: a question of life and death. *Diabetes* 47:1537–1543, 1998
5. Thomas HE, Kay TW: Beta cell destruction in the development of autoimmune diabetes in the non-obese diabetic (NOD) mouse. *Diabetes Metab Res Rev* 16:251–261, 2000
6. Hirano M, Hirai S, Mizuno K, Osada S, Hosaka M, Ohno S: A protein kinase C isozyme, nPKCϵ, is involved in the activation of NF-kappa B by 12-O-tetradecanoylphorbol-13-acetate (TPA) in rat 3Y1 fibroblasts. *Biochem Biophys Res Commun* 206:429–436, 1995
7. St-Denis A, Chano F, Tremblay P, St-Pierre Y, Descoteaux A: Protein kinase C-α modulates lipopolysaccharide-induced functions in a murine macrophage cell line. *J Biol Chem* 273:32787–32792, 1998
8. Chen CC, Wang JK, Lin SB: Antisense oligonucleotides targeting protein kinase C-α, -β I, or -δ but not -ϵ inhibit lipopolysaccharide-induced nitric oxide synthase expression in RAW 264.7 macrophages: involvement of a nuclear factor kappa B-dependent mechanism. *J Immunol* 161:6206–6214, 1998
9. Lallena MJ, Diaz-Meco MT, Bren G, Paya CV, Moscat J: Activation of I kappa B kinase β by protein kinase C isoforms. *Mol Cell Biol* 19:2180–2188, 1999
10. Lin CH, Sheu SY, Lee HM, Ho YS, Lee WS, Ko WC, Sheu JR: Involvement of protein kinase C-γ in IL-1β-induced cyclooxygenase-2 expression in human pulmonary epithelial cells. *Mol Pharmacol* 57:36–43, 2000
11. Mellor H, Parker PJ: The extended protein kinase C superfamily. *Biochem J* 332:281–292, 1998
12. Carpenter L, Cordery D, Biden TJ: Protein kinase Cδ activation by interleukin-1β stabilizes inducible nitric-oxide synthase mRNA in pancreatic β-cells. *J Biol Chem* 276: 5368–5374, 2001
13. Ankarcrona M, Dypbukt JM, Brune B, Nicotera P: Interleukin-1 β-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. *Exp Cell Res* 213:172–177, 1994
14. Kaneto H, Fujii J, Seo HG, Suzuki K, Matsuoka T, Nakamura M, Tatsumi H, Yamasaki Y, Kamada T, Taniguchi N: Apoptotic cell death triggered by nitric oxide in pancreatic β-cells. *Diabetes* 44:733–738, 1995
15. Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichselbaum R, et al: Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. *EMBO J* 14:6148–6156, 1995
16. Emoto Y, Kisaki H, Manome Y, Kharbanda S, Kufe D: Activation of protein kinase CΔ in human myeloid leukemia cells treated with 1-β-D-arabinofuranosylcytosine. *Blood* 87:1990–1996, 1996
17. Ghayur T, Hugunin M, Talanian RV, Ratnofsky S, Quinlan C, Emoto Y, Pandey P, Datta R, Huang Y, Kharbanda S, Allen H, Kamen R, Wong W, Kufe D: Proteolytic activation of protein kinase C δ by an ICE/CED 3-like protease induces characteristics of apoptosis. *J Exp Med* 184:2399–2404, 1996
18. Shao RG, Cao CX, Pommier Y: Activation of pkc-alpha downstream from caspases during apoptosis induced by 7-hydroxystaurosporine or the topoisomerase inhibitors, camptothecin and etoposide, in human myeloid leukemia hl60 cells. *J Biol Chem* 272:31321–31325, 1997
19. Denning MF, Wang Y, Nickoloff BJ, Wrone-Smith T: Protein kinase Cδ is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes. *J Biol Chem* 273:29995–30002, 1998
20. Reyland M, Anderson S, Matassa A, Barzen K, Quissel D: Protein kinase C δ is essential for etoposide-induced apoptosis in salivary gland acinar cells. *J Biol Chem* 274:19115–19123, 1999
21. Pongracz J, Webb P, Wang KQ, Deacon E, Lunn OJ, Lord JM: Spontaneous neutrophil apoptosis involves caspase 3-mediated activation of protein kinase C-δ. *J Biol Chem* 274:37329–37334, 1999
22. Khwaja A, Tatton L: Caspase-mediated proteolysis and activation of protein kinase Cδ plays a central role in neutrophil apoptosis. *Blood* 94:291–301, 1999
23. Bharti A, Kraeft SK, Gounder M, Pandey P, Jin S, Yuan ZM, Lees-Miller SP, Weichselbaum R, Weaver D, Chen LB, Kufe D, Kharbanda S: Inactivation of DNA-dependent protein kinase by protein kinase Cδ: implications for apoptosis. *Mol Cell Biol* 18:6719–6728, 1998
24. Frutos S, Moscat J, Diaz-Meco MT: Cleavage of ζPKC but not λιPKC by caspase-3 during UV-induced apoptosis. *J Biol Chem* 274:10765–10770, 1999
25. Thornberry NA, Lazebnik Y: Caspases: enemies within. *Science* 281:1312–1316, 1998
26. Earnshaw WC, Martins LM, Kaufmann SH: Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 68:383–424, 1999
27. Wolf BB, Green DR: Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem* 274:20049–20052, 1999
28. Hengartner MO: The biochemistry of apoptosis. *Nature* 407:770–776, 2000
29. Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA, Bratton DL: Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cδ. *J Biol Chem* 275:23065–23073, 2000
30. Ren Y, Savill J: Apoptosis: the importance of being eaten. *Cell Death Differ* 5:563–568, 1998
31. Ohno S, Konno Y, Akita Y, Yano A, Suzuki K: A point mutation at the putative ATP-binding site of protein kinase C α abolishes the kinase activity and renders it down-regulation-insensitive. A molecular link between autophosphorylation and down-regulation. *J Biol Chem* 265:6296–6300, 1990
32. Hirai S, Izumi Y, Higa K, Kaibuchi K, Mizuno K, Osada S, Suzuki K, Ohno S: Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKC δ. *EMBO J* 13:2331–2340, 1994
33. Baier-Bitterlich G, Uberall F, Bauer B, Fresser F, Wachter H, Grunicke H, Utermann G, Altman A, Baier G: Protein kinase C-τ isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Mol Cell Biol* 16:1842–1850, 1996
34. Li W, Yu JC, Shin DY, Pierce JH: Characterization of a protein kinase C-δ (PKC-δ) ATP binding mutant. An inactive enzyme that competitively inhibits wild type PKC-δ enzymatic activity. *J Biol Chem* 270:8311–8318, 1995
35. McIlroy D, Sakahira H, Talanian RV, Nagata S: Involvement of caspase 3-activated DNase in internucleosomal DNA cleavage induced by diverse apoptotic stimuli. *Oncogene* 18:4401–4408, 1999
36. Matassa AA, Carpenter L, Biden TJ, Humphries MJ, Reyland ME: PKCδ is required for mitochondrial-dependent apoptosis in salivary epithelial cells. *J Biol Chem* 276:29719–29728, 2001
37. Davies SP, Reddy H, Caivano M, Cohen P: Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105, 2000
38. Mukae N, Enari M, Sakahira H, Fukuda Y, Inazawa J, Toh H, Nagata S: Molecular cloning and characterization of human caspase-activated DNase. *Proc Natl Acad Sci U S A* 95:9123–9128, 1998
39. Wolf BB, Schuler M, Echeverri F, Green DR, McIlroy D, Sakahira H, Talanian RV, Nagata S: Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. *J Biol Chem* 274:30651–30656, 1999
40. Rogakou EP, Nieves-Neira W, Boon C, Pommier Y, Bonner WM: Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. *J Biol Chem* 275:9390–9395, 2000
41. McGlynn E, Liebetanz J, Reutener S, Wood J, Lydon NB, Hofstetter H, Vanek M, Meyer T, Fabbro D: Expression and partial characterization of rat protein kinase C-delta and protein kinase C-ζ in insect cells using recombinant baculovirus. *J Cell Biochem* 49:239–250, 1992
42. Kroncke KD, Fehsel K, Sommer A, Rodriguez ML, Kolb-Bachofen V: Nitric oxide generation during cellular metabolization of the diabetogenic N-

methyl-N-nitroso-urea streptozotozin contributes to islet cell DNA damage. *Biol Chem Hoppe Seyler* 376:179–185, 1995
43. Turk J, Corbett JA, Ramanadham S, Bohrer A, McDaniel ML: Biochemical evidence for nitric oxide formation from streptozotocin in isolated pancreatic islets. *Biochem Biophys Res Commun* 197:1458–1464, 1993
44. Murata M, Takahashi A, Saito I, Kawanishi S: Site-specific DNA methylation and apoptosis: induction by diabetogenic streptozotocin. *Biochem Pharmacol* 57:881–887, 1999
45. Tejedo J, Bernabe JC, Ramirez R, Sobrino F, Bedoya FJ: NO induces a cGMP-independent release of cytochrome c from mitochondria which precedes caspase 3 activation in insulin producing RINm5F cells. *FEBS Lett* 459:238–243, 1999
46. Saldeen J: Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. *Endocrinology* 141:2003–2010, 2000
47. Stephens LA, Thomas HE, Ming L, Grell M, Darwiche R, Volodin L, Kay T: Tumor necrosis factor-α-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic β cells. *Endocrinology* 140:3219–3227, 1999
48. Li L, Lorenzo PS, Bogi K, Blumberg PM, Yuspa SH: Protein kinase Cδ targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. *Mol Cell Biol* 19: 8547–8558, 1999
49. Green DR, Reed JC: Mitochondria and apoptosis. *Science* 281:1309–1312, 1998