

# Protein Kinase C $\delta$ Activation and Translocation to the Nucleus Are Required for Fatty Acid–Induced Apoptosis of Insulin-Secreting Cells

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**Insulin resistance as well as pancreatic  $\beta$ -cell failure can be induced by elevated free fatty acid (FFA) levels. We studied the mechanisms of FFA-induced apoptosis in rat and human  $\beta$ -cells. Chronic treatment with high physiological levels of saturated fatty acids (palmitate and stearate), but not with monounsaturated (palmi-toleate and oleate) or polyunsaturated fatty acids (li-noleate), triggers apoptosis in  $\sim$ 20% of cultured RIN1046-38 cells. Apoptosis restricted to saturated FFAs was also observed in primary cultured human  $\beta$ -cells, suggesting that this mechanism is potentially relevant in vivo in humans. To further analyze FFA-induced signaling pathways leading to apoptosis, we used RIN1046-38 cells. Apoptosis was accompanied by a rapid (within 15 min) nuclear translocation of protein kinase C (PKC)- $\delta$  and subsequent lamin B1 disassembly. This translocation was impaired by the phospholipase C inhibitor U-73122, which also substantially reduced apoptosis. Furthermore, lamin B1 disassembly and apo-ptosis were decreased by cell transfection with a domi-nant-negative mutant form of PKC- $\delta$ . These data suggest that nuclear translocation and kinase activity of PKC- $\delta$  are both necessary for saturated fatty acid-induced apoptosis. *Diabetes* 52:991–997, 2003**

**I**nsulin resistance and abnormal  $\beta$ -cell function are the major characteristics in the pathogenesis of type 2 diabetes. A complex interplay of insulin resistance of skeletal muscle, liver, and fat, as well as secretion abnormalities and declining  $\beta$ -cell function, determines the preclinical development and further progression of the disease. Both genetic and environmental factors seem to contribute through many different molecular and cellular mechanisms to the individual degree of insulin resistance as well as  $\beta$ -cell failure. Lipotoxicity was proposed as an attractive concept to explain parallel developments in the impairment of insulin signaling and  $\beta$ -cell function (1–3).

Before the onset of overt type 2 diabetes, insulin resis-

tance is closely associated with  $\beta$ -cell dysfunction characterized by  $\beta$ -cell hyperplasia and insulin hypersecretion. During further progression of the disease, however, the  $\beta$ -cell is no longer able to compensate for peripheral insulin resistance and declines in its function. From animal models of obesity-linked type 2 diabetes, it is known that both impairment of insulin secretion linked to intra-cellular fat deposition (lipotoxicity) (4) and loss of cell mass by fatty acid–induced apoptotic cell death contribute to the development of relative insulinopenia (5). Recently, it has been shown that exposure to free fatty acids (FFAs) has cytostatic and proapoptotic effects on human pancreatic  $\beta$ -cells (6). Furthermore, human autopsy studies show a relative reduction of  $\beta$ -cell mass in patients with type 2 diabetes compared with weight-matched nondiabetic subjects (7,8). These findings support the idea that in genetically predisposed human subjects, prolonged exposure to elevated FFAs may contribute to  $\beta$ -cell death and development/progression of type 2 diabetes.

The focus of the present study was to further understand the mechanisms involved in fatty acid–induced secretion failure. The intracellular signaling pathway for fatty acid–mediated  $\beta$ -cell apoptosis is incompletely understood. Different mechanisms for lipoapoptosis, such as increased ceramide formation, mitochondrial cytochrome c release, and nitric oxide generation, have been suggested (5,9,10).

Furthermore, it was shown in different cell types that the fatty acid–dependent signaling process might involve activation of different protein kinase C (PKC) isoforms (2,11,12). However, the exact role of PKC isoforms in fatty acid–induced apoptosis has not been defined.

In agreement with other studies in  $\beta$ -cells (13–15), we have reported that the rat insulinoma cell line RIN1046-38 expresses the PKC isoform  $\delta$  and minor levels of other classical, novel, and atypical PKC isoforms other than PKC- $\gamma$  (16). Since PKC- $\delta$  on the one hand can be activated by fatty acids (17) and on the other hand has been described as a protein kinase that mediates apoptosis in response to different cytotoxic and chemotherapeutic agents (14,18–20), we have also investigated whether PKC- $\delta$  is involved in mediating the lipoapoptotic effects of FFAs. Here, we provide new evidence for a proapoptotic effect of PKC- $\delta$  that is specifically induced by the saturated fatty acids palmitate and stearate but not by the mono- or polyunsaturated fatty acids palmitoleate, oleate, and linoleate.

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FFA, free fatty acid; PKC, protein kinase C; PLC, phospholipase C; TUNEL, transferase-mediated dUTP nick-end labeling.

## RESEARCH DESIGN AND METHODS

**Cell culture of insulinoma cells.** RIN1046-38 cells were grown in medium 199-Earle's salts (M199) containing 5 mmol/l glucose and 10% FCS under an atmosphere of 93% air and 7% CO<sub>2</sub> at 37°C. At 80% confluence, the medium was exchanged with fatty acid-containing medium. In some experiments, cells were treated with rottlerin (Calbiochem) or U-73122 (Biomol) in the indicated concentrations. Fatty acids (Sigma) were bound to fatty acid-free BSA as previously described (21) with minor modification. Fatty acids (200 mmol/l in ethanol) were diluted 1:25 into Krebs Ringer Hepes buffer containing 20% BSA. The fatty acid mixture was gently agitated at 37°C under nitrogen overnight. Control medium containing ethanol and BSA was prepared similarly. The stock solutions were stored under nitrogen at -20°C. Experiments were performed with a constant fatty acid-to-BSA ratio. For example, at the highest FFA concentration used (1 mmol/l), BSA content was 2.5%. Glucose concentration was 5 mmol/l in all experiments.

**Stable transfection of kinase-negative PKC- $\delta$  in RIN1046-38 cells.** The cDNA for the kinase-negative PKC- $\delta$  (PKC- $\delta$ K376R) was described earlier (22). Kinase-negative PKC- $\delta$  stably overexpressing RIN1046-38 cells was generated by retroviral infection of exponentially growing cells. The retroviruses were obtained by transfection of 293-BOSC cells (23) with the retroviral expression vector pLXSN-PKC- $\delta$ K376R (24). For mock transfection, pLXSN without insert was used. RIN1046-38 cells were incubated with recombinant retroviruses containing supernatants of 293-BOSC cells three times for 8 h at 37°C in the presence of 8  $\mu$ g/ml polybrene. Then, virus-containing medium was removed and cells were grown in normal medium for 24 h before shifting them to medium containing 750  $\mu$ g/ml G418 (Gibco). After several weeks of selection, clones were picked and analyzed for the expression level of the transduced protein.

**Islet isolation and culture of human  $\beta$ -cells.** Islets were isolated from the pancreas of multiorgan donors as previously described (25). Islets were cultured in CMRL 1066 medium supplemented with 5 mmol/l glucose, dithiothreitol, glutamine, Na-pyruvate, Ciprobay, penicillin/streptomycin, HEPES, hydrocortisone, and 10% FCS at 37°C. Two days after plating, when most islets were attached and had begun to spread, cells were trypsinized and seeded on collagen A (Biochrom)-coated glass coverslips. Two days after seeding on coverslips, the medium was exchanged with fatty acid-containing medium.

**Immunofluorescence.** Cells on coverslips were treated as indicated. After fixation in ice-cold (-20°C) methanol for 20 min, cells were washed with PBS and then incubated for 15 min with 0.1% NaBH<sub>4</sub> and 0.1 mol/l glycine in PBS to block autofluorescence. Nonspecific antibody binding was blocked for 45 min with PBG (PBS with 0.045% fish gelatin) containing 5% normal goat serum and 1% BSA. Incubation with the primary antibodies (Sigma) was carried out for 24 h at 4°C after dilution in PBG containing 5% normal goat serum. After four washes with PBG, primary antibody binding was detected with isotype-specific secondary antibodies conjugated with Alexa546 or Alexa488 fluorescent dye. The coverslips were mounted in PermaFluor (Immunotech, Marseille). Localization of the proteins was examined using confocal laser microscopy (Leica, Germany).

Specificity of the PKC- $\delta$  antibody was tested with a PKC- $\delta$ -specific blocking peptide. PKC- $\delta$ -specific immunofluorescence was completely blocked by this peptide, suggesting that the antibody is specific (data not shown).

**Transferase-mediated dUTP nick-end labeling assay.** Islets were grown on collagen A-coated glass coverslips as described above. After treatment as indicated, transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed according to the manufacturer's instructions (in situ cell death detection kit fluorescein; Boehringer Mannheim, Mannheim, Germany). Insulin staining was performed as described above.

**Measurement of DEVD-amc cleaving caspase activity.** Cells were seeded in triplicates in 96-well plates at a density of 10<sup>4</sup> cells/well. After stimulation as indicated, the cells were incubated in lysis buffer (25 mmol/l Tris-HCl, pH 8.0, 60 mmol/l NaCl, 2.5 mmol/l EDTA, and 0.25% NP40) for 10 min. Then, the fluorogenic caspase-3 substrate Ac-DEVD-amc (12  $\mu$ mol/l) was added and the fluorescence determined in 15-min intervals using 360-nm excitation and 480-nm emission wavelengths (CytoFluor 2300).

**Cell-cycle analysis.** Cells were treated as indicated. Detached cells were harvested from the supernatant by centrifugation and added to the non-detached cells harvested by trypsinization. Cells were washed with PBS, fixed in 70% ice-cold ethanol, centrifuged, and washed with PBS. After staining with propidium iodide (50  $\mu$ g/ml) diluted in PBS containing RNase A (100  $\mu$ g/ml), cells were subjected to flow cytometric analysis of DNA content using a Becton Dickinson FACScalibur cytometer. Percentages of cells in the different cell-cycle phases were calculated by CellQuest software (Becton Dickinson).

**Immunoblotting.** Cells were stimulated as indicated and lysed in ice-cold lysis buffer (50 mmol/l Hepes, pH 7.2, 150 mmol/l NaCl, 1.5 mmol/l MgCl<sub>2</sub>, 1 mmol/l EGTA, 10% [vol/vol] glycerol, 1% [vol/vol] Triton X-100, 100 mmol/l

TABLE 1

Different effects of FFAs on lipoapoptosis in RIN1046-38 cells

	Sub-G <sub>1</sub> cells
Control	0.5 $\pm$ 0.2
Palmitate	19.7 $\pm$ 3.9
Palmitoleate	0.8 $\pm$ 0.3
Stearate	20.3 $\pm$ 3.6
Oleate	0.6 $\pm$ 0.4
Linoleate	0.4 $\pm$ 0.2

Data are %  $\pm$  SD. Cells were treated with different FFAs (1 mmol/l for 24 h) and subjected to cell-cycle analysis. Data were derived from repeated experiments ( $n \geq 3$ ) and evaluated as described in RESEARCH DESIGN AND METHODS.

NaF, 10 mmol/l Na<sub>2</sub>VO<sub>4</sub>, 100  $\mu$ mol/l Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mmol/l PMSF, and 10  $\mu$ g/ml aprotinin). Cleared crude cell lysates (20 min at 12,000g) were run through a 10% SDS-polyacrylamide gel (50  $\mu$ g protein per lane). Separated proteins were transferred to nitrocellulose membranes by semidry electroblotting (transfer buffer: 48 mmol/l Tris/HCl, pH 7.5, 0.0004% [wt/vol] SDS, 39 mmol/l glycine, and 20% [vol/vol] methanol). After transfer, the membranes were blocked with NET buffer (50 mmol/l Tris/HCl, pH 7.4, 150 mmol/l NaCl, 5 mmol/l EDTA, 0.05% [vol/vol] Triton X-100, and 0.25% [wt/vol] gelatin) for 1 h. Subsequently, filters were incubated with the primary antibody (anti-PKC- $\delta$ , c-20; Santa Cruz) overnight at 4°C. The membranes were washed four times with NET buffer before incubation with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Visualization of immunocomplexes was performed by enhanced chemiluminescence.

## RESULTS

**FFA-induced apoptosis in RIN1046-38 cells.** We have tested whether different fatty acid species show proapoptotic effects on RIN1046-38. Several indicators of apoptosis, such as caspase-3 activity and sub-G<sub>1</sub> DNA formation in flow cytometry, have been studied to quantify and ensure the apoptotic effects of FFAs. Palmitate (16:0) and stearate (18:0) were tested as saturated FFAs. In addition, the mono- and polyunsaturated fatty acids palmitoleate (16:1), oleate (18:1), and linoleate (18:2), with the corresponding carbon chain length, were tested. Quantification of the flow cytometry analysis is given in Table 1. The sub-G<sub>1</sub> fraction was very low under basal conditions (0.5  $\pm$  0.2%) and increased up to ~20% (40-fold increase over basal) with the saturated fatty acids palmitate and stearate, while palmitoleate and oleate as well as linoleate had no effect on the appearance of cells in the sub-G<sub>1</sub> fraction.

**FFA-induced apoptosis in human  $\beta$ -cells.** The concept of lipoapoptosis in  $\beta$ -cells was developed from an animal model of obesity-linked type 2 diabetes (5). Although this animal model shows many similarities to human type 2 diabetes, there is poor direct experimental proof for lipoapoptosis in human  $\beta$ -cells. Therefore, we have investigated whether the results of RIN1046-38 cells can be reproduced in human  $\beta$ -cells. Human islets were obtained from nondiabetic, brain-dead multiorgan donors. Isolated islet cells were incubated with saturated and unsaturated fatty acids for 72 h (1 mmol/l). Apoptotic  $\beta$ -cells were identified by double staining with TUNEL, indicating DNA strand breaks in apoptotic cells, and insulin antibodies. As shown in Fig. 1, only the saturated FFAs palmitate and stearate are able to induce apoptosis of  $\beta$ -cells, as indicated by TUNEL- and insulin-positive cells. In contrast, the unsaturated FFAs did not induce apoptosis in human  $\beta$ -cells (Fig. 1). In summary, this suggests that lipoapoptotic effects occur in normal human  $\beta$ -cells after incubation with saturated FFAs, whereas unsaturated FFAs are

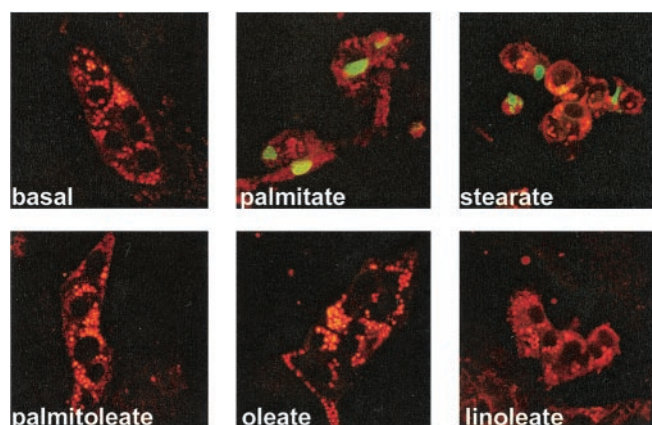


FIG. 1. Different effects of FFAs on lipoapoptosis in human  $\beta$ -cells. Isolated human islet cells were incubated with FFAs (1 mmol/l) for 72 h. Apoptotic  $\beta$ -cells were identified by double staining with TUNEL (green) and insulin antibodies (red). Representative pictures of three independent experiments are shown.

inactive. This is consistent with our results obtained in RIN1046-38 cells.

**Use of inhibitors to screen for potential signaling pathways: effects of phospholipase C and PKC- $\delta$  inhibitors.** Recent studies have shown that PKC- $\delta$  can be activated during apoptosis (18–20). Since PKC- $\delta$  is obviously expressed in RIN1046-38 cells (16), we have studied whether this PKC isoform is involved in lipoapoptosis of  $\beta$ -cells. PKC- $\delta$  is activated by diacylglycerols, and a rapid rise in diacylglycerols in most cases results from phospholipase C (PLC) activity. Therefore, we have assessed whether the general PLC inhibitor U-73122 (7.5  $\mu$ mol/l) and the inactive control U-73343 are able to decrease fatty acid-induced  $\beta$ -cell apoptosis. Determined by flow cytometry, the PLC inhibitor reduces the effect of palmitate by  $56 \pm 9\%$  ( $n = 3$ ,  $P = 0.002$ ,  $t$  test) and of stearate by  $53 \pm 6\%$  ( $n = 3$ ,  $P = 0.001$ ,  $t$  test). In contrast to U-73122, the inactive control U-73343 did not prevent apoptosis. Even more, coincubation of U-73343 with FFAs increased  $\beta$ -cell apoptosis (data not shown). These findings support the idea that FFAs increase PLC activity by a still unknown mechanism. The following rise in diacylglycerol levels might lead to activation and translocation of PKC- $\delta$  in RIN1046-38 cells. To test this hypothesis, we have used the PKC- $\delta$ -specific inhibitor rottlerin (26). Figure 2 shows that rottlerin itself has proapoptotic activity that caused a

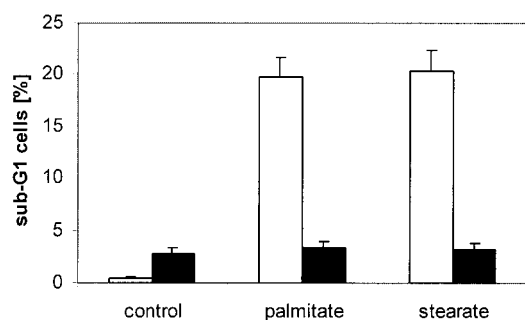


FIG. 2. Rottlerin reduces FFA-induced apoptosis in RIN1046-38 cells. Cells were (■) or were not (□) pretreated with rottlerin (15  $\mu$ mol/l for 30 min) before palmitate or stearate (1 mmol/l) was added for 24 h. Sub-G1 formation was assessed as described in RESEARCH DESIGN AND METHODS. ( $n = 3$ ,  $P < 0.05$ ,  $t$  test).

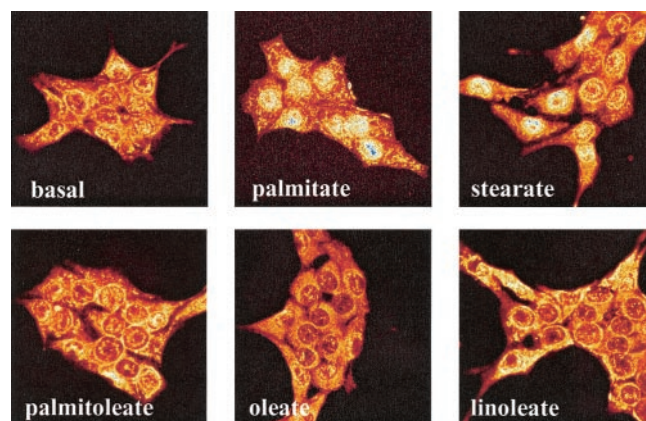


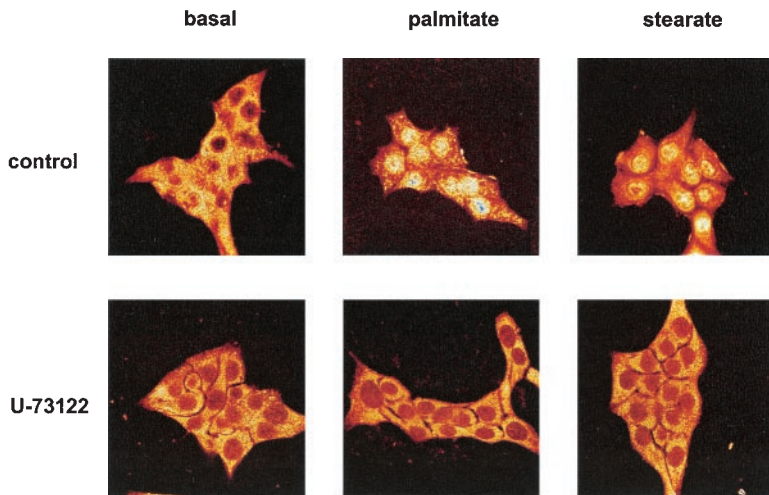
FIG. 3. Different effects of FFAs on PKC- $\delta$  translocation in RIN1046-38. Cells were left untreated or exposed to FFAs (0.4 mmol/l for 15 min). Immunostaining was performed as described in RESEARCH DESIGN AND METHODS. Representative pictures of three independent experiments are shown.

significant increase of sub-G1 cells. This proapoptotic effect of rottlerin might be due to the recently described mitochondrial action of rottlerin (27). Despite the proapoptotic action, rottlerin was still able to significantly reduce palmitate- and stearate-induced sub-G1 cell numbers (Fig. 2). We took this as an indication that PKC- $\delta$  might be necessary for FFA-induced apoptosis.

**Different effects of FFA on PKC- $\delta$  translocation.** Next, we tested whether the presumably proapoptotic PKC isoform  $\delta$  is translocated by different FFAs in RIN1046-38 cells. PKC- $\delta$  translocation was visualized by confocal laser scanning. Cells were exposed for 15 min to FFAs at a concentration of 0.4 mmol/l. This concentration corresponds to high physiological levels of FFAs from obese and insulin-resistant individuals in the fasting state. PKC- $\delta$  in RIN1046-38 cells was identified with an isoform-specific antibody. Under basal conditions, PKC- $\delta$  is mainly found in the cytoplasmic region. The saturated FFAs palmitate and stearate induce PKC- $\delta$  translocation into the nucleus after short-term incubation (Fig. 3). In contrast to these saturated FFAs, the monounsaturated FFAs palmitoleate and oleate, as well as the polyunsaturated FFA linoleate, were not able to induce nuclear translocation and accumulation of PKC- $\delta$  (Fig. 3). This nuclear PKC- $\delta$  translocation could be prevented by the PLC inhibitor U-73122 (7.5  $\mu$ mol/l), which was also a potent inhibitor of apoptosis in RIN1046-38 cells (Fig. 4).

In summary, this suggests that only palmitate and stearate, which translocate PKC- $\delta$  into the nucleus, are able to induce apoptosis in RIN1046-38 cells. As the PKC- $\delta$  inhibitor rottlerin could partially prevent the effect of saturated FFAs on  $\beta$ -cell death, it seems possible that PKC- $\delta$ -dependent phosphorylation of specific substrates is required.

**Lipoapoptosis in RIN1046-38 cells stably overexpressing kinase-negative PKC- $\delta$ .** The data obtained with rottlerin suggest that not only PKC translocation, but also PKC activation, is required for lipoapoptosis. Rottlerin was originally described as a specific inhibitor for PKC- $\delta$  (26). Since we have experienced proapoptotic effects of rottlerin itself, and since rottlerin was recently described as a mitochondrial uncoupler (27), we decided to create stable transfectants of RIN1046-38 cells overex-



**FIG. 4.** Inhibition of PLC blocks FFA-induced PKC- $\delta$  translocation. Cells were left untreated or exposed to the PLC inhibitor U-73122 (7.5  $\mu$ mol/l for 30 min) before palmitate or stearate was added (0.4 mmol/l for 15 min). Immunostaining was performed as described in RESEARCH DESIGN AND METHODS. Representative pictures of three independent experiments are shown.

pressing a transdominant kinase-negative mutant of PKC- $\delta$  or PKC- $\delta$ -wt to specifically determine the role of PKC- $\delta$  kinase activity in lipoapoptosis. In contrast to kinase-negative PKC- $\delta$ , we were not able to propagate any PKC- $\delta$ -wt-overexpressing clones, which might indirectly suggest that PKC- $\delta$ -wt overexpression is toxic to RIN1046-38 cells. Expression of kinase-negative PKC- $\delta$  was ensured by RT-PCR using primers directed against the mutated region of PKC- $\delta$  (data not shown). In addition, the expression level of PKC- $\delta$  was tested by Western blotting. An overexpression of kinase-negative PKC- $\delta$  of about three- to fivefold over the endogenous PKC- $\delta$  level was achieved (data not shown). Three different clones overexpressing kinase-negative PKC- $\delta$  were incubated with FFAs (1 mmol/l for 24 h) and compared with vector controls. Apoptosis was determined by flow cytometry, and representative results of three clones are shown in Fig. 5A. The transfection process itself or the specific culture conditions after transfection of cells lead to an increase in basal and FFA-induced apoptotic activity. Despite this increase in apoptotic activity, the kinase-negative PKC- $\delta$ -overexpressing cells could maximally reach 50% of the apoptotic level with palmitate and stearate, as compared with vector controls (Fig. 5A). These results strongly suggest that activation of PKC- $\delta$  is required to transmit palmitate- and stearate-induced apoptosis.

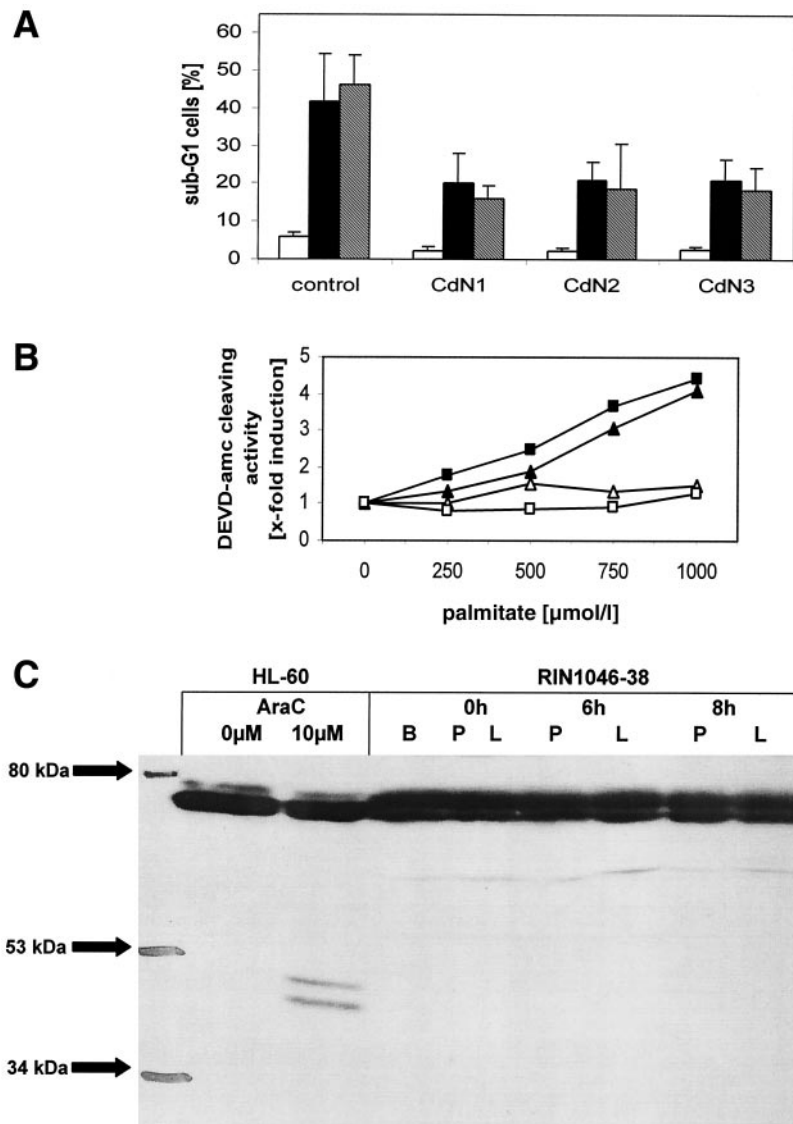
Activation of caspases is specific for apoptosis and distinguishes it from necrosis (28). We have therefore determined caspase-3 activity as an indicator for fatty acid-induced apoptosis in RIN1046-38 cells. Palmitate was able to stimulate caspase-3 activity in a dose-dependent manner from 0.25 to 1 mmol/l, with a maximal increase of about fourfold over basal activity, while linoleate, as well as different BSA concentrations (not shown), had no effect on caspase-3 activity. Overexpression of kinase-negative PKC- $\delta$  prevents caspase-3 activation (Fig. 5B), suggesting that PKC- $\delta$  is required for palmitate-induced caspase-3 activation.

Furthermore, we have tested whether PKC- $\delta$  serves as a substrate of caspase-3. In contrast to some earlier studies (14,19), we could not detect PKC- $\delta$  cleavage after incubation with FFAs (Fig. 5C). This suggests that PKC- $\delta$  acts upstream of caspase-3 and that PKC- $\delta$  fragmentation is not necessary for FFA-induced apoptosis in our cells.

**Lamin B1 disassembly.** To obtain further evidence that PKC- $\delta$ -dependent phosphorylation of a specific substrate is required, we studied the effect on lamin B1. Lamin B1 disassembly represents one of the various features of apoptotic cells. Recently, PKC- $\delta$  has been described as a proapoptotic lamin B kinase in HL60 cells (19). Since PKC- $\delta$  is specifically translocated to the nucleus upon stimulation with saturated fatty acids, we have determined whether PKC- $\delta$  translocation is associated with lamin B1 disassembly. We have detected lamin B1 proteins in the nuclear lamina with a specific antibody by confocal laser microscopy. It can be seen in Fig. 6 that under basal conditions, lamin B1 is stained along the nuclear lamina. After incubation of RIN1046-38 cells with 1 mmol/l palmitate or stearate for 16 h, lamin B1 is disintegrated from the nuclear lamina and diffusely distributed in the nucleus. This palmitate- or stearate-induced lamin B1 disintegration can be completely prevented in kinase-negative PKC- $\delta$ -expressing RIN1046-38 cells (Fig. 6). This is consistent with the idea that the kinase activity of PKC- $\delta$  is important for lamin B1 disintegration.

## DISCUSSION

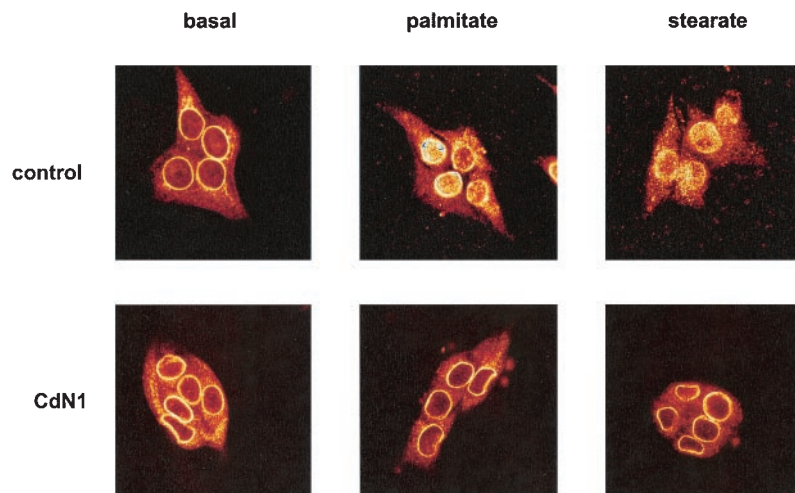
Evidence that  $\beta$ -cell apoptosis might contribute to the development of type 2 diabetes was obtained in studies with obese and insulin-resistant Zucker rats, as well as *Psammomys obesus*, which develop obesity-linked diabetes mainly due to increased apoptosis of  $\beta$ -cells (5,29,30). Increased FFA levels have been proposed to be one of the major triggers for apoptotic  $\beta$ -cell death (5). The results of our study provide evidence that lipoapoptosis specifically occurs with saturated fatty acids, whereas mono- and polyunsaturated fatty acids had no effect. Since only those FFAs that cause  $\beta$ -cell death were able to induce PKC- $\delta$  translocation into the nucleus, we supposed that PKC- $\delta$  might be an important mediator of fatty acid-induced  $\beta$ -cell apoptosis. This hypothesis was further supported by the use of the PKC- $\delta$  inhibitor rottlerin and more specifically by demonstrating that RIN1046-38 cells expressing a kinase-negative PKC- $\delta$  mutant are resistant to FFA-induced apoptosis. Several conditions may explain why only partial, i.e., 50%, inhibition of FFA-induced apoptosis was observed. First, this might indicate that the overexpres-



**FIG. 5.** PKC- $\delta$  activation but not fragmentation is involved in FFA-induced apoptosis in RIN1046-38 cells. **A:** Three different clones of RIN1046-38 cells (CdN1-3) stably overexpressing kinase-negative PKC- $\delta$  were not treated ( $\square$ ) or were treated with palmitate ( $\blacksquare$ ) or stearate ( $\boxtimes$ ) (1 mmol/l for 24 h) and subjected to cell-cycle analysis. Sub-G1 formation was assessed as described in RESEARCH DESIGN AND METHODS ( $n = 3$ ,  $P < 0.05$ ,  $t$  test). **B:** Two different clones of RIN1046-38 cells stably overexpressing kinase-negative PKC- $\delta$  (open symbols) or vector control cells (filled symbols) were exposed to increasing concentrations of palmitate for 6 h. DEVD-amc cleaving activity was measured as described in RESEARCH DESIGN AND METHODS ( $n = 2$ ). **C:** RIN1046-38 cells were treated with 1 mmol/l palmitate (P) or linoleate (L) for 6 or 8 h. As control, HL-60 cells treated with 10  $\mu$ mol/l ara-C for 6 h were used (19). Immunoblotting was performed as described in RESEARCH DESIGN AND METHODS.

sion level of PKC- $\delta$  in RIN1046-38 cells (approximately threefold) is not sufficient to completely suppress endogenous PKC- $\delta$  kinase activity. Second, this might also point to PKC- $\delta$ -independent signaling pathways involved in FFA-induced  $\beta$ -cell apoptosis.

The present knowledge about the FFA-induced signaling pathways is very fragmentary, and results are inconclusive. However, nitric oxide generation and de novo ceramide synthesis have been described as mediators of  $\beta$ -cell lipoapoptosis (9,10,31,32). Although it is well ac-



**FIG. 6.** A kinase-negative mutant of PKC- $\delta$  prevents FFA-induced disintegration of lamin B1 in RIN1046-38 cells. RIN1046-38 cells stably overexpressing kinase-negative PKC- $\delta$  (CdN1) or control cells were exposed to palmitate (1 mmol/l for 16 h). Immunostaining for lamin B1 was performed as described in RESEARCH DESIGN AND METHODS. Representative pictures of three independent experiments are shown.

cepted that the saturated fatty acids palmitate and stearate can act as precursors for ceramide synthesis, we did not obtain any evidence that de novo synthesis of ceramide (tested by the inhibitor Fumonisin B1) or formation of ceramide by cleavage of sphingomyelin (tested by sphingomyelinase activation) was necessary for FFA-induced apoptosis in RIN1046-38 cells (data not shown). While several investigators found that the ceramide pathway mediates drug- or FFA-induced apoptosis, others argued against a role of ceramides (9,10,31–33). Moreover, a recent study in human  $\beta$ -cells showed that the proapoptotic effects of FFAs were only partially dependent on the ceramide pathway (6), suggesting the involvement of other signaling pathways in lipoapoptosis of  $\beta$ -cells. Our data are in agreement with these reports and suggest that the ceramide pathway is not necessary for FFA-induced apoptosis. PKC- $\delta$  has also been implicated in anti-cancer drug-induced apoptosis (18–20). In this case, it appears that ceramide formation induces PKC- $\delta$  translocation to the mitochondria, which then amplifies ceramide action in the mitochondrial apoptotic pathway (34). In our studies, we could not detect major mitochondrial translocation of PKC- $\delta$ . However, the sensitivity of immunofluorescence and confocal microscopy does not allow to exclude that PKC- $\delta$  also transiently translocates to the mitochondrial membrane.

Although this may be an option, mitochondria are definitely not the only target of PKC- $\delta$ , since another important intracellular apoptotic signaling route exists that is characterized by the movement of PKC- $\delta$  toward the nucleus and by the disassembly of nuclear lamina. Since the PLC inhibitor could prevent lamin B disassembly and reduce the lipoapoptotic effects, we suggest that this pathway is dependent upon PLC activity, which leads to an increase of intracellular diacylglycerol and thereby mediates activation and translocation of PKC- $\delta$  to the nucleus. Lamin B1 was recently described as a substrate for PKC- $\delta$  kinase (19). It has been hypothesized that phosphorylation of lamin B induces conformational changes of the protein, enabling caspases to reach their specific cleavage sites and induce apoptosis. It can be assumed that PKC- $\delta$  induces lamin B disassembly directly by lamin B phosphorylation, since the PKC- $\delta$  kinase-negative mutants could completely prevent lamin B disassembly. Another relevant mechanism for the proapoptotic effect of PKC- $\delta$  may be inactivation of DNA-dependent protein kinase in the nucleus, which plays an essential role in DNA repair mechanisms (35).

Furthermore, we have shown that caspase-3 is involved in the FFA-induced apoptotic signaling pathway. In contrast to other reports (14,19), we did not find cleavage of PKC- $\delta$  by caspase-3. Therefore, PKC- $\delta$  does not seem to serve as a substrate of caspase-3 in the FFA-induced signaling pathway. This and the time course of PKC translocation (<15 min) and caspase-3 activation (not earlier than 6 h) suggest that PKC- $\delta$  acts upstream of caspase-3. This hypothesis is further confirmed by the fact that stable overexpression of kinase-negative PKC- $\delta$  prevents caspase-3 activation. Other authors described cleavage of PKC- $\delta$  in IL-1 $\beta$ - and streptozotocin-induced apoptosis, suggesting that PKC is activated downstream of caspase-3 in these studies (14,19). Since we could not find

evidence for such a signaling sequence, it seems that FFAs induce different upstream mechanisms than cytotoxic stimuli in apoptosis.

Although the evidence of FFA-induced lipoapoptosis from obese insulin-resistant animal models is solid, it cannot necessarily be assumed that this is a relevant mechanism for human  $\beta$ -cells. Studies examining the  $\beta$ -cell mass in humans are very limited in number. Furthermore, metabolic characterization and phenotyping of the individuals was poor, which might be the most likely reason for conflicting results in this field (8,36–38).

We have tested whether normal human  $\beta$ -cells behave identical to RIN1046-38 cells and respond differentially upon exposure to saturated and unsaturated fatty acids. Like the rat insulinoma cell line, normal human  $\beta$ -cells respond only upon saturated fatty acids with apoptosis. Compared with RIN1046-38 cells and in agreement with the results of Lupi et al. (6), it appears that human  $\beta$ -cells are more resistant to apoptosis. This is suggested by the necessity of longer incubation periods (72 instead of 24 h) to induce apoptosis in human cells. Since we have maintained human  $\beta$ -cells in a coculture with other endocrine islet cells, it might be possible that these cells prevent  $\beta$ -cell apoptosis by unknown paracrine mechanisms and therefore longer FFA incubation periods are needed to induce cell death by FFAs. Nevertheless, these data show for the first time that normal human  $\beta$ -cells respond differentially upon saturated and unsaturated fatty acids, which is in accordance with results obtained from rodent  $\beta$ -cells (10). The work of Cnop et al. (39) also suggests a different role for the FFAs oleate and palmitate, although these different effects seem to partially equalize after long-term exposure. Furthermore, our data support the idea that insulin resistance and overnutrition with saturated fatty acids may promote obesity and  $\beta$ -cell death in human individuals. To our knowledge, there are no studies that have specifically addressed this question; however, large cohort studies demonstrating that high saturated fatty acid intake increases the incidence of type 2 diabetes (40,41) might indirectly indicate that such a lipoapoptotic effect truly exists in humans.

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