Positive feedback of protein kinase C proteolytic activation during apoptosis

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In contrast with protein kinase Cα (PKCα) and PKCϵ, which are better known for promoting cell survival, PKCδ is known for its pro-apoptotic function, which is exerted mainly through a caspase-3-dependent proteolytic activation pathway. In the present study, we used the rat GH3B6 pituitary adenoma cell line to show that PKCα and PKCϵ are activated and relocalized together with PKCδ when apoptosis is induced by a genotoxic stress. Proteolytic activation is a crucial step used by the three isoforms since: (1) the catalytic domains of the PKCα, PKCϵ or PKCδ isoforms (CDα, CDϵ and CDδ respectively) accumulated, and this accumulation was dependent on the activity of both calpain and caspase; and (2) transient expression of CDα, CDϵ or CDδ sufficed to induce apoptosis. However, following this initial step

of proteolytic activation, the pathways diverge; cytochrome c release and caspase-3 activation are induced by CDϵ and CDδ, but not by CDα. Another interesting finding of the present study is the proteolysis of PKCδ induced by CDϵ expression that revealed the existence of a cross-talk between PKC isoforms during apoptosis. Hence the PKC family may participate in the apoptotic process of pituitary adenoma cells at two levels: downstream of caspase and calpain, and via retro-activation of caspase-3, resulting in the amplification of its own proteolytic activation.

Key words: calpain, caspase, cysteine protease, pituitary, programmed cell death.

INTRODUCTION

Apoptosis is an evolutionarily conserved mode of cell death [1], characterized by distinct morphological changes that are regulated by a series of biochemical events. These events have been divided into two distinct phases: an initial commitment phase, when cells receive a signal, followed by an execution phase, when all of the characteristic morphological and biochemical features of apoptosis occur [2]. Caspases, a family of aspartate-specific cysteine proteases, play a critical role in the execution phase of apoptosis. 'Initiator' caspases, such as caspases-8 and -9, either directly or indirectly activate 'effector' caspases, such as caspases-3, -6 and -7 [3,4]. These effector caspases then cleave intracellular substrates, including poly(ADP-ribose) polymerase (PARP) and lamins, resulting in the dramatic morphological changes of apoptosis [2,4].

The protein kinase C (PKC) family, mostly isoenzymes belonging to the novel group, is also implicated in the apoptotic processes of many cell types. These kinases are substrates of caspases and calpains, the two cysteine protease families that contribute to the major intracellular modifications that occur in response to various death stimuli [5]. The α, β and γ isoforms of PKC are substrates of μ- and m-calpains [6,7], whereas the δ, θ and ζ isoforms are caspase substrates [8–11]. Since the first study of Emoto et al. [10] demonstrating that the catalytic domain of PKCδ (CDδ) is the kinase responsible for the increased phosphorylation of myelin basic protein in apoptotic U-937 myeloid leukaemia cells, PKCδ proteolysis by caspase-3 has been shown to be involved in apoptosis of other cell types [9,12,13]. More recently, using a microarray approach, limited proteolysis of PKCδ during apoptosis has been shown to be accompanied by

up-regulation of PKCδ mRNA; in line with this observation, overexpression of PKCδ in LNCaP prostate cancer cells was shown to markedly enhance the apoptosis-inducing effect of PMA [14,15]. Moreover, down-regulating PKCδ or overexpressing a kinase-dead PKCδ mutant provides a survival signal that prevents apoptosis induced by serum deprivation [16]. In addition to its proteolytic activation by caspase, PKCδ has been shown to accumulate in mitochondria, where it induces cytochrome c release [17].

Other PKC isoforms have also been implicated in the apoptotic process, i.e. PKCθ [8], PKCβ1 [18] and PKCϵ [19,20]. In most cases, the catalytic domains of PKC, known to be apoptotic in the cases of PKCδ [21] and PKCθ [8], are generated by caspase-3-mediated cleavage. Another piece of evidence in favour of PKC proteolysis triggering apoptosis comes from the observation that overexpression of a PKCζ mutant that is resistant to degradation by caspase-3 protects cells from apoptosis [11]. Hence the caspase-mediated proteolytic activation of some novel and atypical PKC isoforms has been clearly assigned to the apoptotic programme. In contrast, there is only one report concerning the involvement of calpain-dependent proteolysis of the classical PKCα isoform in apoptotic cells [7]. Native PKCα is, however, thought to convey survival signals, since both PKCα antisense oligonucleotides and the PKCα T/A dominant negative mutant can induce apoptosis [22,23]. In contrast, during apoptosis, PKCα is autophosphorylated in HL60 cells treated with 7-hydroxystaurosporine [18], and has also been shown to be a downstream target of tumour necrosis factor α in L929 fibroblasts [24].

In the present study, we investigate the putative involvement of the α, ϵ and δ isoforms of PKC during apoptosis. Rat GH3B6

pituitary adenoma cells were submitted to various apoptotic stimuli (serum deprivation, *cis*-platinum treatment or UV irradiation). We found that: (1) in apoptotic GH3B6 cells, both calpain and caspase are activated, leading to the accumulation of CDα, CDε and CDδ; (2) proteolytic activation of PKC is concomitant with the translocation of all three native isoforms to a particulate compartment; (3) expression of CDε induces proteolytic activation of PKCδ; and (4) CDε and CDδ, but not CDα, induce cytochrome *c* release and caspase-3 activation, suggesting the existence of a feedback loop amplifying the mitochondrial pathway.

EXPERIMENTAL

Materials

cis-Platinum(II) diammine dichloride and monoclonal anti-(goat/sheep IgG)–peroxidase conjugate antibodies were from Sigma (Saint Quentin Fallavier, France). Mouse monoclonal antibody against μ- and m-calpains (NCL-CALP-11B3) was from Novocastra Laboratories (Newcastle, U.K.). Anti-calpastatin was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-PKCδ antibody and calpeptin (benzyl-oxycarbonyl-Leu-Nle-CHO; a calpain inhibitor) were from Calbiochem (Meudon, France). Anti-PKCε rabbit polyclonal IgG and monoclonal anti-CDα were from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). ExGen 500 (linear polyethyl-enimine) was from Euromedex (Souffelweyersheim, France). Purified mouse anti-(human PARP) (7D3-6) and purified mouse anti-cytochrome *c* monoclonal antibodies were from PharMingen (San Diego, CA U.S.A.). Anti-(mouse IgG)–peroxidase Fab fragments and chemiluminescence detection kit were from Roche Molecular Biochemicals. Cy3-conjugated AffiniPure F(ab′)₂ fragment donkey anti-mouse IgG (H+L) was from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Z-VAD-FMK (benzyloxycarbonyl-valylalanyl-D,L-aspartylfluoro-methane) was from Biomol (Plymouth Meeting, PA, U.S.A.). Goat anti-rabbit IgG (H+L)–horseradish peroxidase conjugate was from Pierce (Rockford, IL, U.S.A.). pIRES-EGFP plasmid (where EGFP is enhanced green fluorescent protein) was from Clontech (Palo Alto, CA, U.S.A.). The cDNA clone coding for human PKCα was provided by Dr V. Alvaro and Dr I. B. Weinstein (Columbia Cancer Center, New York, NY, U.S.A.), and the cDNAs coding for rat PKCδ and mouse PKCε were provided by Dr P. Parker (ICRF, London, U.K.).

Cell culture, and time courses of serum deprivation-, *cis*-platinum- and UV-induced apoptosis and inhibitor studies

GH3B6 cells were cultured in Ham's F10 medium supplemented with 2.5% (v/v) fetal bovine serum and 15% (v/v) horse serum, both heat-inactivated at 56 °C for 1 h. Cells were maintained in a 5% CO₂/95% humidified air atmosphere at 37 °C. To induce apoptosis, cells were cultured in a serum-free medium, treated with 10 μM *cis*-platinum(II) diammine dichloride or UV-irradiated at 1 J/m². For the inhibitor studies, cells cultured as described above were pretreated with either 40 μM calpeptin or 40 μM Z-VAD-FMK for 1 h. Cells were collected after 24, 48 or 72 h of serum deprivation, or after 6, 9 or 16 h of *cis*-platinum or UV treatment.

Construction of plasmids encoding CDα, CDδ or CDε, and transient transfection in GH3B6 cells

The coding region of the catalytic domain of human PKCα, rat PKCδ or mouse PKCε, corresponding to the amino acid

sequences of the products generated by cleavage by calpain (for CDα and CDε) or caspase (for CDδ), was introduced into the bi-cistronic vector pIRES-EGFP, which permits the protein of interest and EGFP to be translated separately from a unique transcript containing the two cistrons. The nucleotide sequences of CDα (nt 1011–2019 in the cDNA sequence) and CDδ (nt 981–2021 in the cDNA sequence) with an *Eco*RV site and a start codon at the 5′-end, and an *Eco*RI site and a stop codon at the 3′-end, were produced by PCR. That of CDε (nt 1096–2191 in the cDNA sequence) with an *Eco*RV site and a start codon at the 5′-end, and a *Not*I site and a stop codon at the 3′-end, was also produced by PCR. PCR was performed using human PKCα cDNA subcloned in the pBabe vector, rat PKCδ cDNA subcloned in pCO2 and mouse PKCε cDNA subcloned in pMT2 as template. The sense and antisense primers used to generate the CDα construct were CGGATATCCGATGGACTTCAATTTC-CTCATGG and CGGAATTCCGTCATACTGCACTCTGTA-AG respectively; the sense and antisense primers used to generate the CDδ construct were CGGATATCCGATGAACAACGGG-ACCTATGGC and CGGAATTCCGCTATTCCAGGAATT-GCTCATATTTGGGG respectively; and the sense and antisense primers used to generate the CDε construct were CGGAT-ATCCGATGGCCTTGTCATTTGACAACC and TTGCGG-CCGCAATCAGGGCATCAGGTCTTCACC respectively. The cycle parameters were 94 °C for 40 s, 55 °C (CDα), 62 °C (CDδ) or 60 °C (CDε) for 1 min 30 s, and 72 °C for 1 min 30 s (30 cycles), followed by 72 °C for 5 min. The PCR fragments were gel purified and ligated into pIRES-EGFP. All sequences were checked by DNA sequencing and no mutations were detected. Constructions were transiently transfected in GH3B6 cells with ExGen, as described previously [25]. Cells were seeded in a six-well plate (on 20 mm × 20 mm coverslips for immuno-chemistry) in 2 ml of Ham's F10 medium at a density of 2×10^6 cells/well and grown for 24 h before transfection.

Visualization of internucleosomal DNA fragmentation in apoptotic cells

GH3B6 cells were washed with ice-cold PBS and scraped into lysis buffer [5 mM Tris/HCl, pH 7.5, 20 mM EDTA, 0.5% (v/v) Triton X-100]. Lysates were solubilized for 20 min and centri-fuged for 30 min at 10 000 *g*. Supernatants were collected and proteins were digested with 0.3 mg/ml proteinase K for 1 h at 60 °C, followed by an overnight incubation in a 37 °C water bath. The digested cells were extracted for DNA with 1 vol. of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). The extracted DNA was precipitated with 0.1 vol. of 3 M sodium acetate and 2.5 vol. of absolute ethanol. DNA was pelleted, resuspended in a buffer containing 10 mM Tris/HCl, pH 8, 50 mM NaCl, 1 mM EDTA and 60 μg/ml RNase A, and incubated for 1 h at 37 °C. The recovered DNA (10 μg of each sample) was electrophoresed on 1.7% (w/v) agarose gels at 60 V and incubated with ethidium bromide. The DNA pattern was examined by UV transillumination.

Preparation of total-cell lysate and Western blot analysis

Cells were washed with cold PBS and then scraped into RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 mM NaF, 1 mM benzamidine and 0.5 mM sodium orthovanadate). Proteins were solubilized on ice for 30 min and centrifuged at 12 000 *g* for 30 min at 4 °C. Supernatants were collected and quantification of protein was carried out with a

Bio-Rad protein assay. Equals amounts of protein (20–40 μg) were loaded on an SDS/7.5%-PAGE gel. Proteins were electrophoretically transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubation with TBS (50 mM Tris, 150 mM NaCl, pH 7.4) containing 8% (w/v) powdered milk for 1 h at room temperature. Membranes were then incubated with anti-PKCα (1:2000), anti-PKCδ (1:4000), anti-PKCϵ (1:500), anti-PARP (1:2000), anti-calpain (1:300) or anti-calpastatin (1:500) antibodies overnight at 4 °C. After washing with TBS containing 0.1% Tween, membranes were incubated for 1 h at room temperature with anti-(mouse IgG)–peroxidase antibody (1:4000) for PKCα, PARP and calpain staining, with anti-(rabbit IgG)–peroxidase (1:6000) for the detection of PKCδ and PKCϵ, and with anti-(goat/sheep IgG)–peroxidase (1:6000) for calpastatin detection. Immunoreactive bands were visualized with a chemiluminescence detection kit.

Cell fractionation

Cells were washed with cold PBS, followed by scraping into homogenization buffer (10 mM Tris, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 10 μg/ml leupeptin, 1 mM benzamidine and 0.5 mM sodium orthovanadate). Cells were then homogenized in a glass Dounce homogenizer and centrifuged for 5 min at 500 g. Supernatants were collected and centrifuged for 30 min at 15000 g; supernatants corresponded to soluble fractions. Pellets were resuspended in homogenization buffer supplemented with 1% (v/v) Nonidet P40 and incubated for 30 min on ice; this corresponded to the particulate fraction.

Cytochrome c detection by immunocytochemistry and nuclear labelling

At 1 day after transfection, cells were washed quickly twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4), fixed for 15 min with 4% (v/v) formaldehyde and washed twice. Nuclear labelling and cell permeabilization were performed by a 15 min incubation in PBS supplemented with 0.2% (v/v) Triton X-100 and 2 μg/ml Hoechst reactive. Cells were then washed twice, incubated for 20 min with PBS containing 5% (v/v) normal goat serum and then incubated for 1 h with the antibody against cytochrome c (dilution 1:100 in PBS containing 5% normal goat serum). After being washed, cells were incubated further for 45 min with Cy3-conjugated goat anti-(mouse IgG) (dilution 1:1000). Cells were washed before mounting coverslips in Mowiol and observed by conventional fluorescence microscopy.

RESULTS

Limited proteolysis of PKCα, PKCδ and PKCϵ during apoptosis induced by *cis*-platinum and UV irradiation, but not after serum deprivation

Apoptosis was induced by three different means in the GH3B6 rat pituitary adenoma cell line: serum deprivation, treatment with the anti-tumour drug *cis*-platinum and UV irradiation; the last two are known DNA-damaging agents. As shown in Figure 1(a), internucleosomal DNA fragmentation attested by DNA laddering was induced in all cases, but with different kinetics. As expected, induction of apoptosis was faster with *cis*-platinum or UV irradiation than with serum deprivation. DNA fragmentation was detected after 48 h of serum deprivation and was increased further after 72 h, whereas upon *cis*-platinum treatment or UV irradiation DNA fragmentation appeared after 9 h and was increased further after 16 h. Another criterion of apoptotic cell
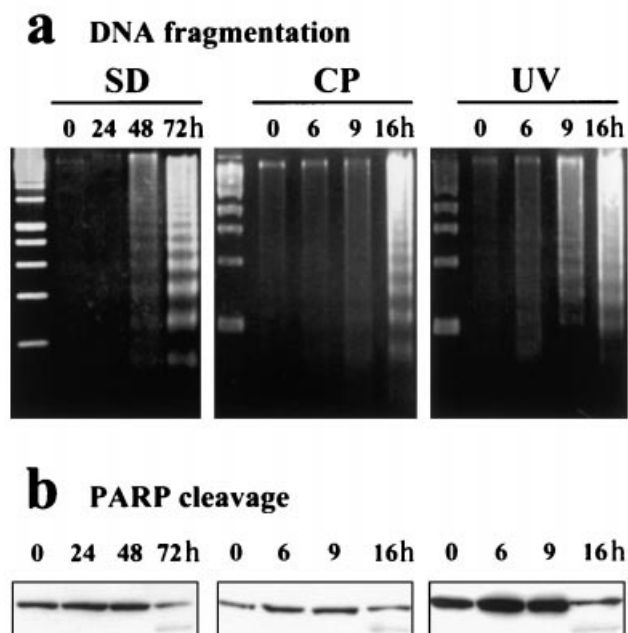

Figure 1   Serum deprivation (SD), *cis*-platinum (CP) treatment and UV irradiation induce DNA fragmentation and PARP cleavage in the GH3B6 pituitary adenoma cell line

Cells were treated in three different ways: serum deprivation for 24, 48 or 72 h; 10 μM *cis*-platinum for 6, 9 or 16 h; or UV irradiation at 1 J/m^2 followed by incubation for 6, 9 or 16 h. After treatment, cells were harvested and extracted for analysis of DNA laddering (**a**) or PARP cleavage (**b**) as detailed in the Experimental section.

death is the activation of caspase-3. We thus examined the fate of PARP, a well-known substrate of caspase-3. Whereas almost all recombinant caspases can cleave PARP *in vitro*, caspase-3 is the effector that yields the characteristic 24 and 89 kDa fragments in cells [26]. In GH3B6 cells, the three pro-apoptotic treatments that we used resulted in the caspase-3-dependent cleavage of PARP to its characteristic 89 kDa fragment (Figure 1b). Interestingly, proteolysis of PARP occurring after 72 h of serum deprivation, or 16 h after *cis*-platinum treatment or UV irradiation, was preceded by an increase in PARP accumulation.

Thus the programmed cell death of GH3B6 pituitary cells is accompanied by caspase activation. Since PKCα, δ and ϵ are either calpain or caspase substrates, and since the three isoforms are expressed at high levels in GH3B6 cells, we analysed their accumulation and limited proteolysis during apoptosis. Whatever the duration of serum deprivation, the levels of native PKCα, δ and ϵ remained constant, and their catalytic domains were not detected by the technique of Western blotting (Figure 2a). In contrast, the levels of the three native PKC isoforms decreased progressively upon *cis*-platinum treatment or UV irradiation (Figure 2a). This decrease began as early as 6 h after the beginning of the treatment. Moreover, 16 h after *cis*-platinum treatment or UV irradiation, at a time when a large proportion of cells was apoptotic, Western blot analysis revealed the accumulation of lower-molecular-mass products (approx. 50 kDa) corresponding to CDα, CDϵ and CDδ. The progressive decrease in the levels of native enzymes suggests that proteolysis starts as soon as 6 h, even though the catalytic domains were not detectable until 16 h after the onset of apoptosis-inducing treatment.
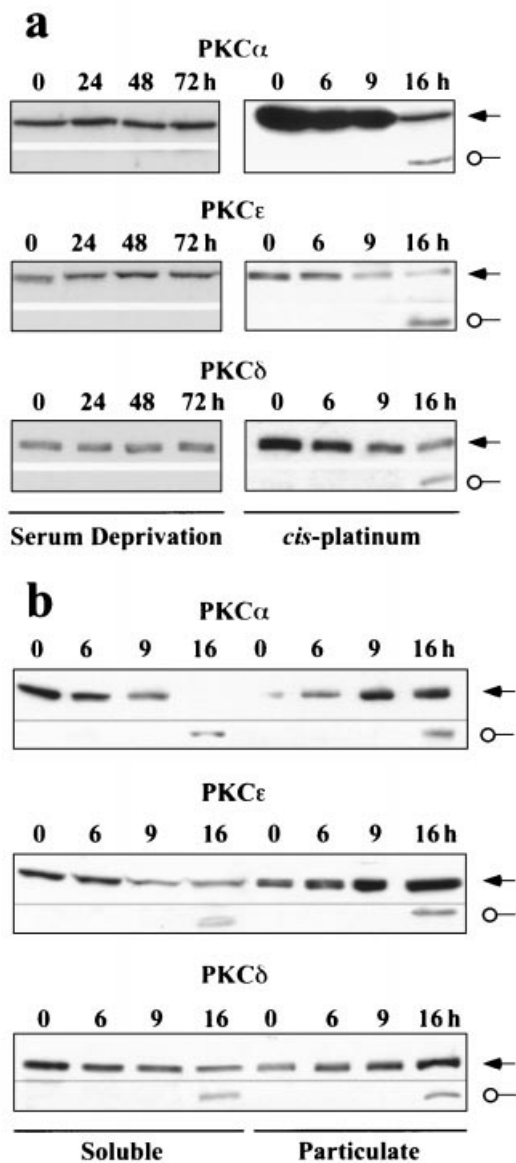
Figure 2 PKCα, PKCδ and PKCε are proteolysed and relocalized from the soluble to the particulate fraction during *cis*-platinum- or UV irradiation-induced apoptosis

(**a**) GH3B6 cells were treated as described in the legend to Figure 1; total-cell lysates were analysed by Western blot for immunodetection of PKCα (←, native form of 82 kDa; ○–, catalytic domain of 46 kDa), PKCε (←, native form of 96 kDa; ○–, catalytic domain of 48 kDa) or PKCδ (←, native form of 78 kDa; ○–, catalytic domain of 42 kDa). Catalytic domains appeared after 16 h of *cis*-platinum treatment. (**b**) After various periods (from 0 to 16 h) of *cis*-platinum treatment, GH3B6 cell homogenates were fractionated into soluble and particulate fractions. Both fractions were analysed by Western blot and probed with antibodies against PKCα, PKCδ or PKCε. Native isoforms (←) were translocated from the soluble fraction to the particulate one, and catalytic domains (○–) were present in both fractions after 16 h of treatment. Both proteolysis and relocalization of the three PKC isoforms were observed with the same kinetics after UV treatment (results not shown).

Subcellular relocalization of PKCα, PKCδ and PKCε during *cis*-platinum- or UV-induced apoptosis

Since, at least for the conventional PKCs, proteolysis is concomitant with activation [27], the cleavage of PKCα, δ and ε occurring in apoptotic GH3B6 cells suggested that an activation
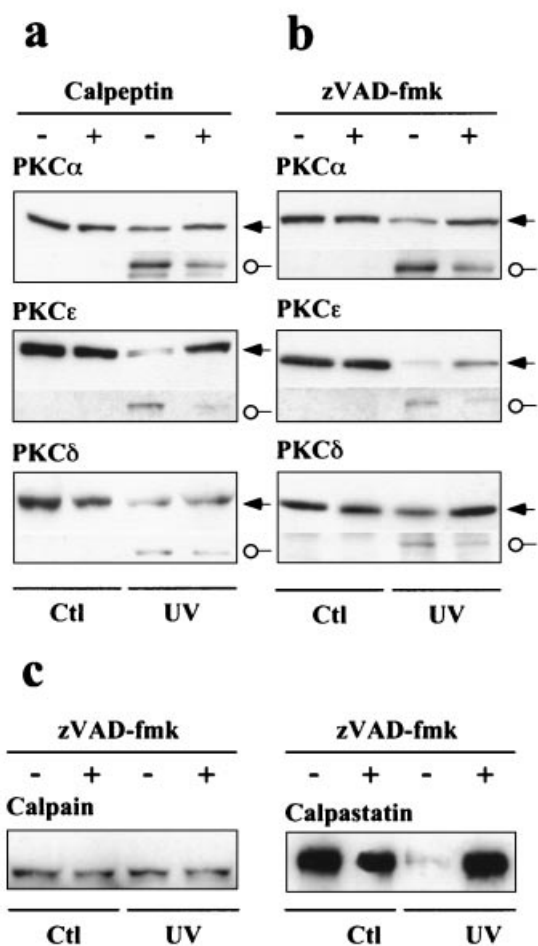

Figure 3 Effects of calpain and caspase inhibitors on the proteolysis of PKCα, PKCε and PKCδ

At 16 h after UV treatment, with or without pretreatment with 40 μM calpeptin (calpain inhibitor) (**a**) or 40 μM Z-VAD-FMK (pan-caspase inhibitor) (**b** and **c**), GH3B6 cells were extracted for protein analysis. (**a**, **b**) Total-cell lysates were analysed by Western blot for immunodetection of PKCα, PKCδ and PKCε. Degradation of native PKC isoforms (←), that occurred after UV treatment, was partially or not reversed by calpeptin or Z-VAD-FMK, as attested by changes in the accumulation of catalytic domains (○–). (**c**) Total-cell lysates were also analysed for their content of calpain and calpastatin (the endogenous calpain inhibitor). Calpain accumulation remained constant, whereas proteolysis of calpastatin after UV treatment was totally reversed by caspase inhibition. Similar results (not shown) were obtained after 16 h of treatment with 10 μM *cis*-platinum.

process had been initiated. One of the earliest steps of PKC activation is translocation from one subcellular compartment to another. Thus we asked whether PKC translocation also occurs during genotoxic-stress-induced apoptosis. Cell homogenates were separated into soluble and particulate (membranes, mitochondria and other insoluble organelles) fractions. During *cis*-platinum- or UV-induced apoptosis, the three native PKC isoforms were progressively relocalized from the soluble to the particulate fraction (Figure 2b). Translocation was evident 6 h after the apoptosis-inducing treatment, and the kinetics for the translocation and degradation of the native isoforms were similar. This indicates that translocation to the particulate compartment and proteolysis are concomitant events. However, after 16 h, the catalytic domains were detected in both the soluble and the
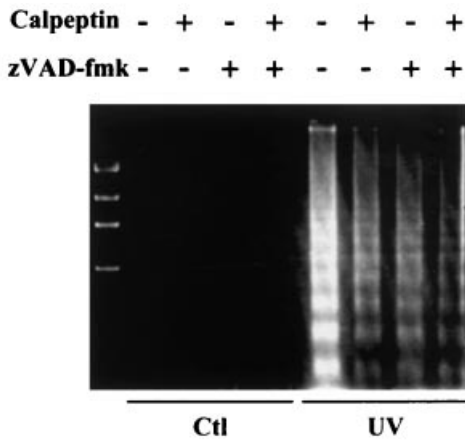
Figure 4   DNA fragmentation is reversed by inhibition of caspase and calpain

As described in the legend to Figure 3, GH3B6 cells were pretreated or not with 40 μM calpeptin and/or 40 μM Z-VAD-FMK. At 16 h after UV treatment, cells were extracted for DNA ladder analysis. UV-induced DNA internucleosomal fragmentation was partially reversed by calpeptin or Z-VAD-FMK pretreatment. When cells were treated with both inhibitors together, the effects were additive. Identical results (not shown) were obtained after 16 h of *cis*-platinum treatment.

particulate fractions. This indicates that proteolysis might occur in both compartments.

Interestingly, PKC relocalization did not occur after serum deprivation (results not shown), which did not induce the proteolysis of PKC. Together with the lack of PKC proteolysis, this further suggests that the apoptotic process induced by serum deprivation is PKC-independent.

Calpain- and caspase-dependent proteolysis of PKCα, PKCε and PKCδ

Whereas PKCα is a known substrate of μ- and m-calpains, and PKCδ a known substrate of caspase-3, PKCε is a potential calpain substrate, since its down-regulation induced by thyrotropin-releasing hormone is calpain-dependent [28]. Hence the accumulation of CDα, CDδ, and CDε that we observed in apoptotic GH3B6 cells suggested that both calpain and caspase may be activated during apoptosis induced by a genotoxic stress. To test this hypothesis, the cells were pretreated with the calpain inhibitor calpeptin or with the pan-caspase inhibitor Z-VAD-FMK. As shown in Figure 3(a), calpeptin inhibited the proteolysis of both PKCα and PKCε by more than 50 % (mean of quantification of four independent experiments), and displayed a weaker (15 %) inhibitory effect on PKCδ proteolysis. In addition, the proteolysis of PKCδ, PKCα and PKCε was partially reversed by treatment with Z-VAD-FMK (Figure 3b).

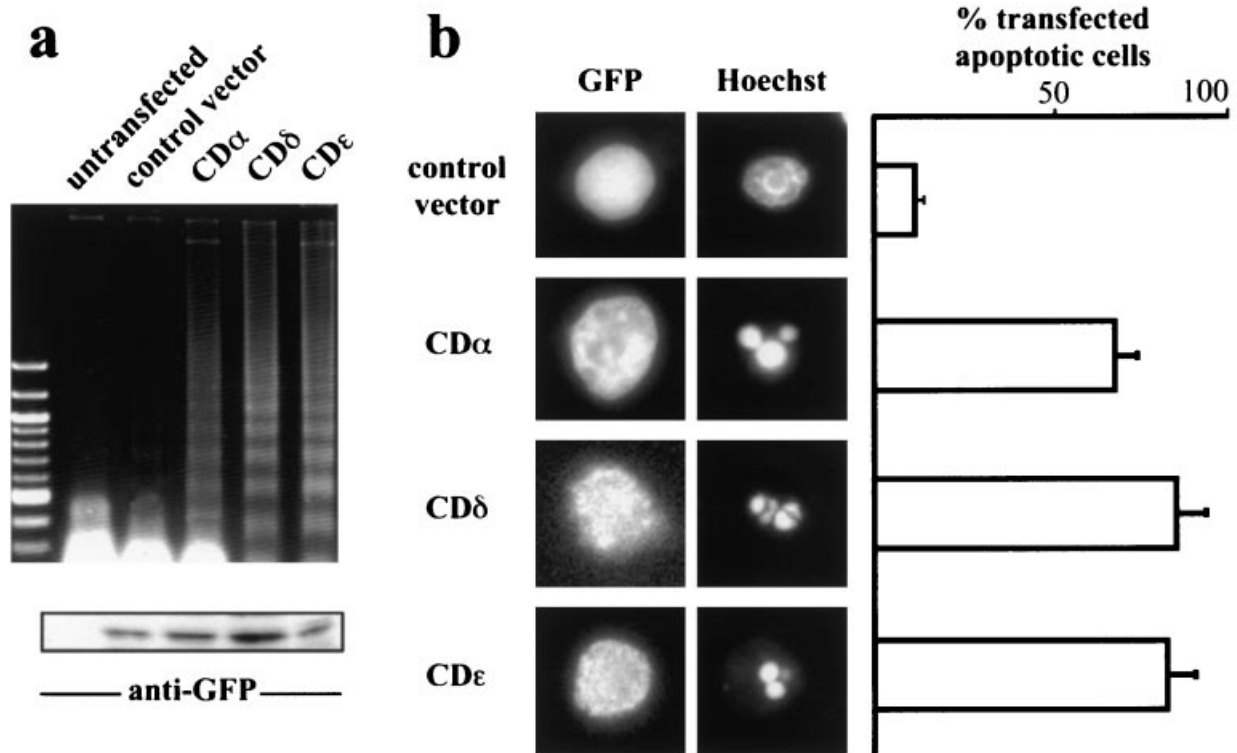**Figure 5   The catalytic domains of PKCα, PKCδ and PKCε are apoptotic**

GH3B6 cells were transiently transfected (or not) with control vector or with a vector containing CDα, CDδ or CDε cDNA. (**a**) DNA extractions were performed for each condition. Expression of CDα, CDδ or CDε induced DNA fragmentation. DNA fragmentation was reproducibly greater in cells expressing CDδ and CDε than in cells expressing CDα. (**b**) Left: Hoechst staining of transfected cells. CDα-, CDδ- or CDε-expressing cells (EGFP-positive cells) exhibited chromatin condensation. 1 cm = 7 μm. Right: 200 EGFP-positive cells were counted in three independent experiments. The percentage of transfected cells that were apoptotic was then calculated. Results are given as means ± S.D.
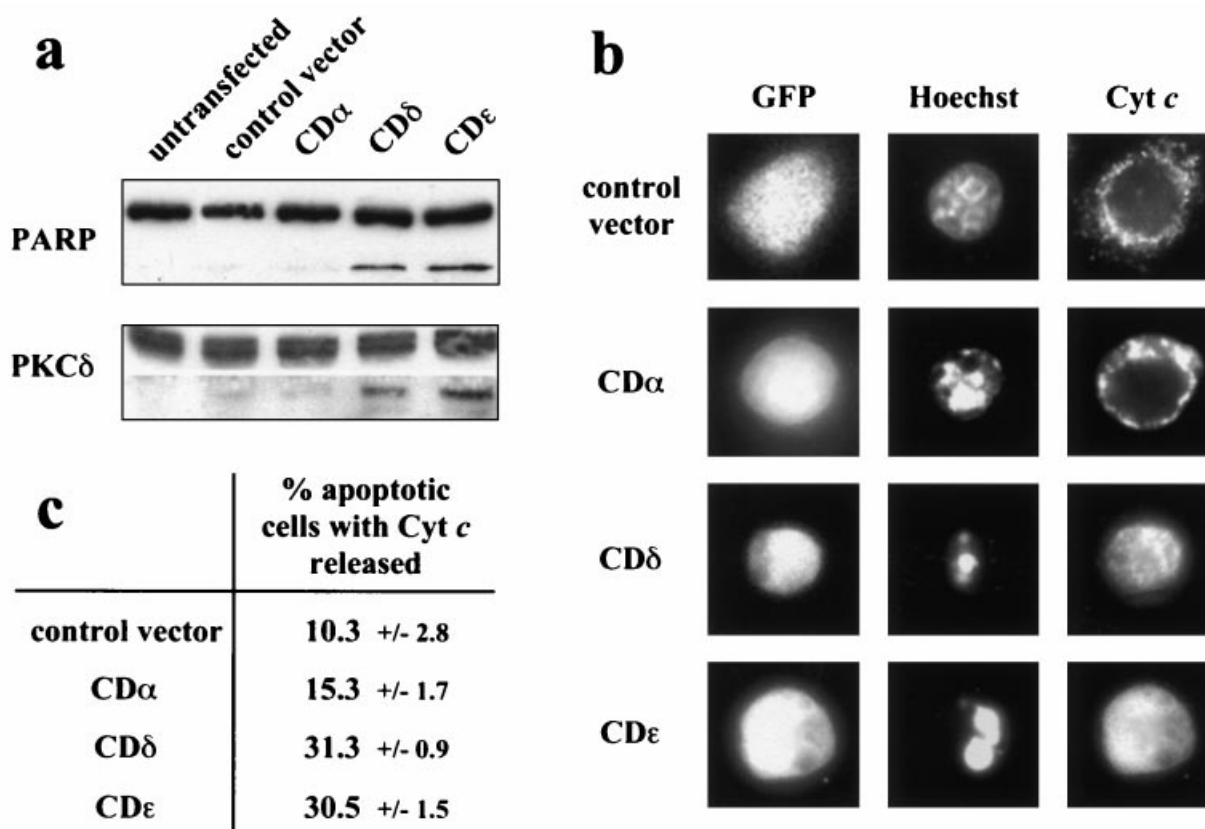
Figure 6   CDδ and CDε induce caspase-3 and cytochrome c release from mitochondria

As described in the legend to Figure 5, GH3B6 cells were transfected (or not) with control vector or with a vector containing CDα, CDδ or CDε cDNA. (**a**) Total-cell lysates were analysed for their PARP, native PKCδ and CDδ contents. (**b**) Cytochrome c (Cyt c) localization was analysed by immunochemistry together with Hoechst nuclear staining. When CDδ or CDε was expressed, EGFP-positive cells showed chromatin condensation and cytochrome c was released from the mitochondria into the cytoplasm. 1 cm = 7 μm. (**c**) For each condition, the number of apoptotic transfected cells in which cytochrome c was released from mitochondria was counted (140 EGFP-positive apoptotic cells were counted in three independent experiments). Results are given as means ± S.D.

These results demonstrate that both calpain and caspase are activated in apoptotic GH3B6 pituitary adenoma cells. Furthermore, they indicate that, in GH3B6 cells, calpain activation is closely linked to caspase activity. Indeed, despite the absence of consensus caspase cleavage sites in PKCα and PKCε, the limited proteolysis of these isoforms is reduced in the presence of the pan-caspase inhibitor Z-VAD-FMK. Figure 3(c) shows that, in apoptotic GH3B6 cells, the level of active calpains (μ- and/or m-calpain, as the monoclonal antibody that we use does not distinguish between these calpains) remained constant for at least 16 h after UV irradiation, whereas calpastatin was degraded. Pretreating the cells with Z-VAD-FMK prevented calpastatin degradation (Figure 3c). This suggests that the effect of Z-VAD-FMK on the proteolysis of PKCα and PKCε may be mediated indirectly via its effect on calpastatin degradation. Indeed, calpain activation is dependent, at least partially, upon caspase activation.

The importance of calpain- and caspase-mediated proteolysis during the apoptosis of GH3B6 cells was further suggested by the antagonistic effects of calpeptin and/or Z-VAD-FMK on DNA fragmentation (Figure 4). Treatment with calpeptin or Z-VAD-FMK only partially prevented DNA fragmentation, but their effects were additive. Addition of calpeptin and Z-VAD-FMK to the culture medium was associated with a marked decrease in the number of floating cells, and the attached cells were morpho-

logically normal, suggesting that the decrease in DNA fragmentation following calpain or caspase inhibition was associated with an increase in cell viability.

The catalytic domains of PKCα, PKCδ and PKCε promote apoptosis

The results described above suggest that CDα, CDε and CDδ generated by calpain or caspase could play a major role in the apoptotic process. To test this hypothesis, cDNAs for CDα, CDδ and CDε were subcloned in pIRES-EGFP, a bi-cistronic vector that allows the independent translation of any given catalytic domain and EGFP. This strategy was chosen in order to easily detect transfected cells while eliminating potential problems that may arise from fusion with EGFP. The immunodetection of EGFP in whole-cell extracts (transfected or not) was used as an estimation of transfection efficiency (Figure 5a, lower panel). Figure 5(a) shows that, when transiently transfected into GH3B6 cells, the three catalytic domains induced DNA fragmentation, whereas the control vector had no effect. CDδ and CDε induced reproducibly greater DNA fragmentation than CDα. To directly correlate catalytic domain expression with apoptosis, we performed Hoechst staining to visualize the condensation state of the chromatin in EGFP-positive transfected cells (Figure 5b). The majority of EGFP-positive cells expressing CDα, CDδ or

CDϵ exhibited condensed chromatin and morphological features characteristic of apoptotic nuclei, compared with only 10 % of control EGFP-positive cells (control vector). The number of apoptotic cells was slightly higher in cells transfected with CDδ or CDϵ compared with CDα (80 % and 66 % respectively), in agreement with the corresponding differences in the levels of DNA fragmentation illustrated in Figure 5(a). Therefore, among the various substrates that are proteolysed by calpain and caspase, the α, δ and ϵ isoforms of PKC, through the generation of their catalytic domains, may represent efficient executioners capable of promoting apoptosis in GH3B6 pituitary adenoma cells.

Expression of the catalytic domains of PKCδ and PKCε, but not that of PKCα, induces caspase-3 activation and cytochrome *c* release

To further characterize the pathway involved in the apoptosis induced by the PKC catalytic domains, we examined the caspase-3-dependent cleavage of PARP and cytochrome *c* localization (Figure 6). Surprisingly, when CDδ or CDϵ was overexpressed, PARP was proteolysed, as attested by the accumulation of the 89 kDa degradation fragment, indicating that caspase-3 is activated. However, PARP was not cleaved in control or CDα-transfected cells. We tested the possibility that activation of caspase-3 upon CDδ or CDϵ expression could affect the proteolysis of PKC. This was indeed the case, as shown in the lower panel of Figure 6(a): the accumulation of CDδ was induced by overexpression of CDϵ. It is of note that the accumulation of CDδ observed in CDδ-transfected cells might result from both the expression vector and proteolysis of endogenous PKCδ.

Caspase-3 activation in CDδ- or CDϵ-expressing cells could have several consequences. We tested the possibility that the mitochondrial pathway could be involved via the release of cytochrome *c* into the cytoplasm, which in turn would activate caspase-9 and caspase-3. Figures 6(b) and 6(c) show that, indeed, this pathway might contribute to caspase activation in CDδ- or CDϵ-expressing cells. The localization of cytochrome *c* was clearly cytoplasmic in approx. 30 % of CDδ- and CDϵ-expressing cells. This was not the case for control or CDα-expressing cells, in accordance with the fact that no PARP cleavage was observed in these cells (Figure 6a). All CDδ- or CDϵ-expressing cells in which cytochrome *c* was released into the cytoplasm had condensed chromatin. However, it should be noted that cytochrome *c* apparently remained mitochondrial in some CDδ- or CDϵ-expressing cells exhibiting condensed chromatin. In the latter case, the level of cytoplasmic cytochrome *c* may be too low to be detectable by immunocytochemistry.

Thus CDδ- and CDϵ-induced apoptosis involves cytochrome *c* release, which probably participates in caspase-3 activation. Our results also highlight the fact that the pathway of apoptosis induction following expression of CDδ or CDϵ is different from that involved following expression of CDα.

DISCUSSION

The present findings demonstrate that the PKC family play an important role in the programmed cell death of pituitary adenoma cells. Indeed, we present strong evidence that three PKC iso-enzymes may contribute to the apoptotic process in GH3B6 cells. They not only act downstream of caspase and calpain activation, but also act by retro-amplifying caspase-3, and one consequence of this action is the retro-amplification of their own proteolytic activation. However, the engagement of PKC in apoptosis is dependent upon the mode of induction of apoptosis. In contrast

with genotoxic stresses, and despite the activation of caspase-3 attested by PARP cleavage, serum deprivation neither affected the accumulation nor induced the proteolytic activation of PKCα, PKCϵ and PKCδ.

Calpain and caspase are both activated upon death induction in pituitary adenoma cells

Caspase-mediated proteolysis is a hallmark of apoptosis, whereas calpain-mediated proteolysis has been shown to contribute primarily to necrotic neuronal death following ischaemic and excitotoxic types of injury [5]. However, various calpain inhibitors have been found to provide protection against apoptosis in cerebellar granule neurons, neutrophils and thymocytes [29–31] and, in the GH3B6 model, cell death has the morphological and biochemical characteristics of apoptosis, but not necrosis. The pituitary originates from the anterior ridge of the neural plate [32,33], and under specific conditions the GH3B6 cells can differentiate into neuronal-like cells, with neurite-like processes expressing the neurofilament protein [34]. Therefore the involvement of calpain in pituitary cell death might be related to the neuronal-like features of this cell type, rather than to the type of cell death.

Calpain or caspase: which PKC is cleaved by which protease?

The involvement of calpain in the proteolytic activation of PKC is poorly documented in the literature. Only one study refers to the calpain-dependent proteolytic activation of PKCα during apoptosis [7], and such activation has never been documented for PKCϵ. PKCα and PKCϵ contain calpain cleavage sites that have been clearly identified for PKCα [6]. They are potentially present in PKCϵ at positions 398–399 and 404–405 of the V3 region. The latter possibility is further supported by the fact that the down-regulation of PKCϵ induced by thyrotropin-releasing hormone in GH4C1 pituitary cells is calpain-dependent [28]. Despite the fact that PKCα and PKCϵ do not contain caspase cleavage sites in their V3 regions, their proteolysis is partially reversed by the pan-caspase inhibitor Z-VAD-FMK. A similar observation has been made for PKCβ$_I$, a calpain substrate. The proteolysis of PKCβ$_I$ has been shown to be totally reversed by caspase inhibitors in UCN-01-treated HL60 cells. In addition, we have shown that the degradation of calpastatin that occurs during apoptosis of GH3B6 cells is caspase-dependent. Likewise, the indirect activation of calpain by caspase, via the degradation of calpastatin, could be one mechanism used by GH3B6 cells during apoptosis, as shown previously [35–38]. However, here we show for the first time that CDϵ can retro-activate caspase-3, providing a possible additional link between calpain and caspase. Does this link function upon a genotoxic stress? If so, calpeptin should inhibit the proteolysis of PKCδ. We observed only a weak effect of calpeptin on PKCδ proteolysis. However, this could be expected, as the calpain-generated product (CDϵ) probably only amplifies caspase activation, but does not initiate it. This may not be the case for calpain activation, which could be the sole consequence of caspase-mediated calpastatin degradation.

Concomitant proteolysis and relocalization of PKCα, PKCε and PKCδ

Only a limited number of studies have addressed the question of the relocalization of PKC isoenzymes during apoptosis. Proteolysis and activation/translocation are linked events, since the sensitivity of PKCα to calpain depends upon the release of the pseudosubstrate from the catalytic site [27]. In our present study,

we show that proteolysis and relocalization are concomitant events for PKCα, PKCε and PKCδ. In contrast with the study of Chen et al. [39], which showed translocation of PKCδ and PKCε within 5 min of irradiation in mouse epidermal JB6 cells, translocation is not a rapid event in apoptotic GH3B6 cells, since it occurs several hours after the induction of apoptosis. Is the proteolysis of PKC dependent upon translocation or not? In the case of PKCδ, nuclear translocation in cytokine-deprived T cells has been shown to be necessary for proteolytic activation by caspase-3, and rescue by interferon-β caused a rapid exit of PKCδ from the nucleus [40]. In the present study, we observed the accumulation of PKC catalytic domains in both soluble and particulate compartments, suggesting that proteolysis might take place not only after activation-induced translocation, but also in the pool of enzyme that remains in the cytoplasm. Upon PMA stimulation, calpain is known to co-migrate with PKCα to the plasma membrane [41]. This could well be the case in apoptotic GH3B6 cells, and explain the presence of the catalytic domain in the particulate fraction, which contains (in addition to mito-chondria) Golgi and plasma membranes. Upon the induction of apoptosis by PMA, intact PKCα and PKCδ have been detected in mitochondria [17,42]. A detailed analysis of the subcellular localization of native PKC isoforms and their catalytic fragments in apoptotic GH3B6 pituitary cells will certainly be of interest. Upon the induction of apoptosis, PKC is possibly involved in two ways: (1) generation of the constitutively active catalytic fragment, and (2) accumulation or sequestration of the native active enzyme. The catalytic fragments and the native enzymes possibly have different locations and/or different substrate specificity, and therefore different roles in the execution of the apoptotic programme. The fact that catalytic fragments alone can induce apoptosis suggests, nevertheless, that they are the major PKC executioners of apoptosis.

PKC catalytic domains: efficient executioners of apoptosis and inducers of caspase-3 activity

Upon transient transfection, the catalytic domains of the three PKC isoforms, i.e. CDα, CDε and CDδ, were each sufficient to induce apoptosis. This has already been described for CDδ, but is a novel observation for CDα and CDε. Thus calpain and caspase can target the PKC family with the same net effect: accumulation of the catalytic fragments that will take on their message to ensure the execution of apoptosis. However, we have observed that CDε and CDδ were reproducibly more efficient in inducing apoptosis than CDα. Further analysis indicated that the three PKC isoforms used different pathways: the expression of CDε and CDδ, but not that of CDα, was associated with PARP cleavage and cytochrome c release from the mitochondria, both suggesting caspase-3 activation. This was substantiated by the fact that the catalytic domain of endogenous PKCδ accumu-lated in cells overexpressing CDε, but not in cells overexpressing CDα. This indicates that not only are PKC isoforms calpain and caspase targets during apoptosis, but that some of their pro-teolytic products can retro-activate caspase-3. There are only a few studies in the literature suggesting that PKCδ might retro-activate caspase. Inhibition of PKCδ by Rottlerin inhibited etoposide- or cis-platinum-induced caspase-3 activity [9,43], and expression of a kinase-dead PKCδ mutant blocked caspase-9 and -3 activation [44]. Interestingly, with regard to the mitogen-activated protein kinase (MAPK) pathway [45], MEKK-1 (MAPK/ERK kinase kinase, where ERK is extracellular-signal-regulated kinase) has been shown to be proteolytically activated by caspase and, as in our study, the catalytic fragment could retro-activate caspase. An additional, interesting but contro-

versial point is the dependence on kinase activity of the pro-apoptotic function of the catalytic domains. For example, in human keratinocytes, expression of an active CDδ was sufficient to induce apoptosis, whereas expression of a kinase-inactive CDδ was not [46]. In contrast, Goerke et al. [47] recently showed that the induction of apoptosis by PKCδ is independent of kinase activity, as a kinase-negative mutant induced apoptosis to the same extent as the wild-type enzyme.

Conclusion

The genotoxic stress-induced apoptosis of the GH3B6 pituitary adenoma cell involves the PKC family. Activation of both calpain and caspase is responsible for the proteolytic activation of all three isoforms analysed, and this proteolytic activation may retro-activate the mitochondrial pathway via the PKC catalytic domains themselves.

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