Protein Kinase C δ Is Essential for Etoposide-induced Apoptosis in Salivary Gland Acinar Cells*

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We have previously shown that parotid C5 salivary acinar cells undergo apoptosis in response to etoposide treatment as indicated by alterations in cell morphology, caspase-3 activation, DNA fragmentation, sustained activation of c-Jun N-terminal kinase, and inactivation of extracellular regulated kinases 1 and 2. Here we report that apoptosis results in the caspase-dependent cleavage of protein kinase C-δ (PKCδ) to a 40-kDa fragment, the appearance of which correlates with a 9-fold increase in PKCδ activity. To understand the function of activated PKCδ in apoptosis, we have used the PKCδ-specific inhibitor, rottlerin. Pretreatment of parotid C5 cells with rottlerin prior to the addition of etoposide blocks the appearance of the apoptotic morphology, the sustained activation of c-Jun N-terminal kinase, and inactivation of extracellular regulated kinases 1 and 2. Inhibition of PKCδ also partially inhibits caspase-3 activation and DNA fragmentation. Immunoblot analysis shows that the PKCδ cleavage product does not accumulate in parotid C5 cells treated with rottlerin and etoposide together, suggesting that the catalytic activity of PKCδ may be required for cleavage. PKCα and PKCδ1 activities also increase during etoposide-induced apoptosis. Inhibition of these two isoforms with G66976 slightly suppresses the apoptotic morphology, caspase-3 activation, and DNA fragmentation, but has no effect on the sustained activation of c-Jun N-terminal kinase or inactivation of extracellular regulated kinase 1 and 2. These data demonstrate that activation of PKCδ is an integral and essential part of the apoptotic program in parotid C5 cells and that specific activated isoforms of PKC may have distinct functions in cell death.

Apoptosis is important for the destruction of tumor cells and cells damaged by viral infection, drugs, chemical radiation, and aging (1–4). An increase or decrease in apoptosis may contribute to the pathology of a wide range of disorders including those associated with development, autoimmune disease, and cancer. In the salivary gland, inappropriate induction of apoptosis via the FAS/FAS ligand pathway has been suggested to lead to the glandular destruction seen in Sjögren’s syndrome (5, 6). In addition, the apoptosis of normal salivary cells in patients treated with head and neck irradiation or chemotherapeutics (7, 8) can result in reduced salivary gland function or xerostomia.

The critical genes in the apoptotic process have been defined genetically in Caenorhabditis elegans and biochemically in other species (9). These include the Bcl-2 family of proteins, a family of related regulatory proteins, which either promote or suppress apoptosis (10), and the caspases, cysteine proteases that are responsible for initiation and execution of the apoptotic signal (11). Other signaling molecules, including members of the mitogen-activated protein kinase family and protein kinase C (PKC) family, have also been shown to be involved in the regulation of apoptosis (12–18).

In this report we have focused on the PKC family of enzymes as potential regulators of apoptosis in salivary acinar cells. The PKC family consists of 11 isoforms, whose expression varies between cell types (19, 20). Individual isoforms exhibit varying substrate specificity, as well as differences in their subcellular localization and response to specific stimuli (20–22), arguing that they have specialized roles in cell signaling. A variety of studies indicate that specific isoforms of PKC may be either pro-apoptotic or anti-apoptotic, depending on the stimulus and cell type (23–25). In support of an anti-apoptotic function, PKC inhibitors are potent inducers of apoptosis in many hematopoietic and neoplastic cells (26–28), and treatment with phorbol 12-myristate 13-acetate to activate PKC antagonizes apoptosis induced by many agents (29–31). Recently PKCα has been shown to phosphorylate Bcl-2 in vitro, and overexpression of PKCα results in increased Bcl-2 phosphorylation and suppression of apoptosis in human pre-B REH cells (32). The atypical PKC isoforms, PKCα and PKCζ, have likewise been shown to protect against apoptosis in many cell types (33–35).

In support of a pro-apoptotic role for PKC, activation of PKC with phorbol 12-myristate 13-acetate, or overexpression of PKCα, can induce apoptosis in prostatic carcinoma cells (36, 37). Likewise, PKCα is activated following induction of apoptosis by genotoxic agents in HL-60 myeloid cells (38). Several laboratories have reported the proteolytic activation of PKCδ to release a catalytically active fragment in cells induced to undergo apoptosis with ionizing radiation and DNA-damaging drugs (39–42). Furthermore, in several cell types expression of the PKCδ catalytic domain induces phenotypic changes indicative of apoptosis (38, 39, 43). A recent report also shows that cleavage and activation of PKCδ by caspase-3 occurs in U937

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† The abbreviations used are: PKC, protein kinase C; JNK, Jun-N-terminal kinase; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinase; Z-VAD.FMK, N-benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-CH₂F; DEVD.FMK, N-benzyloxycarbonyl-Asp-Glu-Val-Asp(O-methyl)-CH₂F; Ac-DEVDFMK-pNa, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide; ara-C, 1-β-D-arabinofuranosylcytosine; DNA-PK, DNA-protein kinase; MEKK1, mitogen-activated protein kinase kinase 1; GST, glutathione S-transferase.

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cells in response to agents that induce apoptosis (44).

The present studies were undertaken to ask if specific isoforms of PKC regulate apoptosis in salivary acinar cells in response to genotoxic agents. These studies demonstrate that PKCδ is activated during etoposide-induced apoptosis in a cell line derived from parotid gland acinar cells and that the activity of this isoform is essential for complete apoptosis in these cells. PKCα and PKCβ1 are likewise activated following treatment of parotid acinar cells with etoposide; however, the contribution of these activated isoforms to the apoptotic process appears to be more modest, suggesting that specific isoforms of PKC may have distinct functions in cell death.

MATERIALS AND METHODS

Cells and Cell Culture—The isolation of the immortalized salivary parotid C5 cell line has been described elsewhere (45). Cells were cultured on Primaria 60-mm culture dishes (Falcon Plastics, Franklin Lakes, NJ) in Dulbecco's modified Eagle's medium/F-12 (1:1 mixture) supplemented with 25% fetal calf serum, 1 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 4.1 mM HEPES, 2.5 mM MgCl2, 0.2 mM ATP, and 2.5 mM CaCl2, and resuspended in 20 μM of 125I-iodo-L-thyroxine. Cells were grown to confluence, washed once with phosphate-buffered saline, and resuspended in 1 ml of JNK lysis buffer. The lysate was allowed to sit on ice for 30 min and then clarified by spinning at 12,500 rpm for 5 min in a refrigerated Savant SRF13K microcentrifuge. For the assay a 100-μl volume of a 10% suspension of GST-c-Jun (24) was added to the reaction buffer, boiled, and the reaction products resolved on a 10% SDSPAGE and transferred to a nitrocellulose membrane. The position of GST-c-Jun was determined by staining the gel, and the extent of GST-c-Jun phosphorylation was determined by autoradiography.

Immunoblotting—Adherent and floating cells were scraped into the culture media, collected by centrifugation (3,000 × g for 10 min), washed once with phosphate-buffered saline, and resuspended in 1 ml of JNK lysis buffer. The lysate was allowed to sit on ice for 30 min and then clarified by spinning at 12,500 rpm for 5 min in a refrigerated Savant SRF13K microcentrifuge. For the assay a 100-μl volume of a 10% suspension of GST-c-Jun (24) was added to the reaction buffer, boiled, and the reaction products resolved on a 10% SDS-PAGE gel. The position of GST-c-Jun was determined by staining the gel, and the extent of GST-c-Jun phosphorylation was determined by autoradiography.

RESULTS

Changes in the Expression of Specific PKC Isoforms Occur during Etoposide-induced Apoptosis—Apoptosis occurs in a series of well defined steps that involve specific biochemical changes to the cell. We have previously examined the ability of etoposide to induce apoptosis in the parotid C5 cell salivary acinar cell line.2 These cells were derived from the rat parotid gland and retain many characteristics displayed by parotid acinar cells in vivo (45). Apoptosis was demonstrated by the appearance of cytoplasmic blebbing and nuclear condensation, DNA fragmentation, and caspase-3 activation.3 In addition, etoposide induced activation of JNK and suppressed accumulation of activated ERK1 and ERK2.4 The current studies were undertaken to explore the contribution of the PKC family of enzymes to specific events in the apoptotic process. As an initial approach we asked if treatment of parotid C5 cells with etoposide results in changes in the abundance of specific PKC isoforms. Parotid C5 cells strongly express PKCα, PKCδ, and PKCγ, whereas much weaker expression of PKCε and PKCe is detected. Expression of PKCβ1, PKCγ, PKCe, and PKCα is not detectable in this cell line.4 As shown in Fig. 5B, by 18 h of treatment with 50 μM etoposide, >80% of parotid C5 cells display an apoptotic morphology. Fig. 1 shows an immunoblot of untreated parotid C5 cells or cells treated with 50 μM etoposide for up to 18 h, and probed for expression of PKCα, PKCβ1, PKCδ, PKCe, and PKCγ. As seen here, the expression of PKCα protein (panel A), and PKCβ1 protein (panel B) increases by 4 h and continues to increase up to 18 h after the addition of etoposide. PKCe expression increased about 2–3-fold in 5 similar experiments, whereas PKCε expression is increased about 3–5-fold in 4 similar experiments. In contrast, the abundance of PKCe (panel C) decreases slightly in etoposide-treated cells by about 12 h. Expression of full-length PKCδ protein also decreases during apoptosis, whereas a cleavage product of approximately 40 kDa begins to accumulate by 4 h following the addition of etoposide (panel D). Accumulation of this cleavage product increases about 3-fold after 18 h of etoposide treatment.

Assay for DNA Fragmentation—DNA fragmentation was assayed using a Cell Death Detection Assay kit from Roche Molecular Biochemicals. This assay detects the appearance of histone-associated low molecular weight DNA in the cytoplasm of cells and was performed in accordance with the manufacturer's recommendations.

Assay for Caspase-3 Activity—The activation of caspase-3 was detected with the Caspase-3 Cell Activity Assay Kit PLUS obtained from Biomol (Plymouth Meeting, PA) which uses N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-FMK-pNA) as a substrate. The assays were conducted in accordance with the manufacturer's recommendations. Z-VAD.FMK (Z-VAD-Ala-Asp(O-methyl)-CH2.F) and DevD.FMK (Z-Asp-Glu-Ala-Asp(O-methyl)-CH2.F) were obtained from Enzyme Systems (Livermore, CA).

Kinase Assay for JNK Activity—The GST-c-Jun (1–79) expression vector was kindly provided by Dr. Lynn Heasley (University of Colorado Health Sciences Center, Denver, CO), and the fusion proteins were prepared as described (14). JNK activation was assayed using the GST-Jun kinase assay (47). To collect both adherent and floating cells, cells were scraped into the culture media, collected by centrifugation (3,000 × g for 10 min), washed once with phosphate-buffered saline, and resuspended in 1 ml of JNK lysis buffer. The lysate was allowed to sit on ice for 30 min and then clarified by spinning at 12,500 rpm for 5 min in a refrigerated Savant SRF13K microcentrifuge. For the assay a 100-μl volume of a 10% suspension of GST-c-Jun (1–79) was added to 300 μg of total cellular protein in a final volume of 1 ml and incubated for 2 h at 4 °C. The beads were then washed three times with 20 μl HEPES, pH 7.7, 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% Triton X-100. Forty μl of 50 μM β-glycerophosphate, pH 7.6, 0.1 mM sodium orthovanadate, 10 mM MgCl2, and 20 μM ATP containing 10 μl [γ-32P]ATP (5000 cpm/pmol in the final reaction) was added to the washed beads, and the reaction was incubated at 30 °C for 20 min. The reaction was terminated by the addition of 10 μl of 5% SDS sample buffer, boiled, and the reaction products resolved on a 10% SDS-polyacrylamide gel. The position of GST-Jun was determined by staining the gel, and the extent of GST-Jun phosphorylation was determined by autoradiography.

Assay for Caspase-3 Activity—Caspase-3 activity was assayed using an immunoprecipitation kinase assay as follows. Cytosolic protein (0.25 or 0.5 mg), prepared as described for immunoblotting, was immunoprecipitated for 4 h at 4 °C using 2 μg of anti-PKCα (C-20), anti-PKCβ1 (C-17), or PKCδ (C-17) antibody. The antigen-antibody complexes were collected by incubation with Sepharose-protein A (Sigma) for 1 h at 4 °C, washed 3 times in JNK lysis buffer, 3 times in 2× kinase buffer (40 μl Tris, pH 7.4, 20 mM MgCl2, 20 μM ATP, and 2.5 mM CaCl2), and resuspended in 20 μl of 2× kinase buffer. To prevent contamination with activated PKCδ, immunoprecipitation of PKCα and PKCβ1 was done using cell lysates that were pre-cleared by immunoprecipitation with anti-PKCδ as described above. Twenty μl of reaction buffer (0.4 mg of H1 histone (Sigma), 50 μg/ml phosphatidylserine, 1 μM dioleoylglycerol, and 5 μCi of [γ-32P]ATP (3000 Ci/mM)) was added, and the samples were incubated for 10 min at 30 °C. In some experiments phosphatidylserine and dioleoylglycerol was omitted from the reaction buffer. Reactions were terminated by the addition of 2× SDS sample buffer, boiled, and the reaction products resolved on a 12.5% SDS-polyacrylamide gel. The extent of H1 histone phosphorylation was determined by autoradiography and in some experiments quantified using a PhosphorImager (Molecular Dynamics).
PKC activity was determined by immunoblot analysis as described under “Materials and Methods.” Solid and open arrows indicate migration of the 68- and 43-kDa molecular mass markers, respectively.

![Figure 1](image1.png)

**Fig. 1.** Changes in the levels of PKC isoforms following etoposide treatment. Subconfluent cultures of parotid C5 cells were treated for the indicated times with 50 μM etoposide. PKC isoform expression was determined by immunoblot analysis as described under “Materials and Methods.” Solid and open arrows indicate migration of the 68- and 43-kDa molecular mass markers, respectively.

product correlates with loss of the full-length PKCδ protein (Fig. 1, panel D, and Fig. 2). Expression of PKCζ (panel E) likewise decreases slightly following treatment with etoposide, and this decrease correlates with the accumulation of a small amount of a PKCζ cleavage product of about 40 kDa. No PKCa, PKCβ1, or PKCe cleavage products were detected (data not shown).

Caspase-dependent cleavage and activation of specific PKC isoforms, as well as other signaling molecules, as been previ-
the increase in PKC 
lysates that had been precleared with anti-PKC 
activity was assayed by an immunoprecipitation kinase assay using cell 
ylates following the addition of etoposide. In the case of PKC 
isms. As seen in Fig. 4 a, PKC 
activity increases 5–6-fold following the addition of etoposide. Quantification of histone phosphoryla-
tion of 3 similar experiments indicates that PKCo activity increases 3–8-fold during etoposide-induced apoptosis. In some experiments the increase in PKCo activity appears to exceed the increase in PKCo protein expression (see Fig. 1A), suggesting that PKCo activity may be regulated by additional mechanisms. As seen in Fig. 4B, PKCβ1 activity increases 5–6-fold following the addition of etoposide. In the case of PKCβ1, however, the increase in kinase activity closely parallels the observed increase in protein abundance (compare Fig. 1 to Fig. 4). Unlike PKCδ, the increased activity of both PKCo and PKCβ1 during apoptosis is dependent on the addition of exog-
ogenous lipid to the kinase reaction (data not shown).

Inhibition of PKC Suppresses the Morphological Changes Associated with Etoposide-induced Apoptosis—The experi-
ments described above demonstrate that specific isoforms of 
PKC are activated during etoposide-induced apoptosis. Al-
though activation of PKC by apoptotic stimuli may be impor-
tant, the fundamental question is whether the activation of 
these molecules is required for apoptosis to occur. To address 
the role of PKCδ, PKCo, and PKCβ1 activation in apoptosis, we 
have examined the effect of isoform-specific inhibitors on bio-
chemical and morphologic markers of apoptosis. To inhibit 
particularly PKCδ in vivo, we have used rottlerin. The IC50 of 
rottlerin for inhibition of PKCδ is reported to be 3–6 μM, whereas 
PKCo, PKCβ, and PKCγ are inhibited at significantly higher 
concentrations (40 μM) (50). To inhibit PKCα and 
PKCβ1 we have used the calcium-dependent PKC isoform in-
hibitor Go6976. The IC50 of Go6976 for inhibition of the PKCo 
and PKCβ1 is reported to be 2–6 nM, whereas no inhibition of 
PKCδ is seen even at μM concentrations (51).

Microscopically, apoptosis can be monitored by the condensa-
tion of the nucleus and cytoplasmic blebbing (52). To deter-
mine the effect of inhibition of PKCδ on appearance of the 
apoptotic morphology, parotid C5 cells were preincubated with 
or without rottlerin for 30 min prior to the addition of 50 μM 
etoposide for 18 h. Compared with untreated cells (Fig. 5A), >80% of parotid C5 cells treated with etoposide demonstrate 
morphologic changes consistent with apoptosis, and in fact 
many are detached from the culture dish by this time (Fig. 5B). 
Pretreatment of parotid C5 cells with 1 μM rottlerin prior to 
the addition of etoposide, however, totally blocks appearance of 
the apoptotic morphology and cell death (Fig. 5D), whereas 
rottlerin alone has no effect on cell morphology (Fig. 5C). 
These results demonstrate that PKCδ activity is essential for initia-
tion of the morphological changes associated with apoptosis in 
response to DNA damage. To determine if rottlerin blocks 
etoposide-induced DNA damage, or the entry of damaged cells 
into apoptosis, parotid C5 cells were treated with etoposide and 
1 μM rottlerin for 24 h, washed to remove the drugs, and then 
placed in drug-free media. Under these conditions the cells 
undergo apoptosis and by 18 h resemble cells treated with 
etoposide alone (Fig. 5E). Thus, inhibition of PKCδ with 
rottlerin appears to block the entry of cells damaged by etopo-
side into the apoptotic pathway.

To determine the contribution of PKCo and PKCβ1 to the 
morphologic changes seen during apoptosis, parotid C5 cells 
were pretreated with Go6976 for 30 min prior to the addition of
etoposide. As seen in Fig. 5G, preincubation with 100 nM G06976 inhibits the apoptotic morphology induced by etoposide, although less dramatically than pretreatment with rottlerin (compare Fig. 5, D–G). Treatment with G06976 alone appears to have no effect on cell morphology (Fig. 5F). Since 100 nM G06976 is about 20-fold above the reported IC₅₀ for PKCa and PKCβ1 in vitro, it is possible that other isoforms of PKC, or other kinases, may be inhibited at this dose and may account for the observed phenotype. Alternatively, G06976 may be a less effective inhibitor of PKCa and PKCβ1 in intact cells, and thus a higher dose may be required to see inhibition of PKCa and PKCβ1-dependent events. As discussed above, PKCδ is not inhibited by G06976 even at micromolar concentrations (51) and therefore is unlikely to account for suppression of the apoptotic morphology.

Inhibition of PKC Suppresses DNA Fragmentation in Etoposide-Treated Parotid C5 Cells—The breakdown of chromosomal DNA into 200-base pair nucleosomal fragments is characteristic of cells undergoing apoptosis. To determine if inhibition of PKCδ and/or PKCα/β1 suppresses DNA fragmentation, parotid C5 cells were treated with 50 μM etoposide, with or without the addition of rottlerin or G06976. DNA fragmentation was assayed using an enzyme-linked immunosorbent assay that quantitates cytoplasmic low molecular weight histone-associated DNA. As seen in Fig. 6, pretreatment with rottlerin suppresses etoposide-induced DNA fragmentation by about 30% at 1 μM and by almost 50% at 10 μM. Pretreatment of parotid C5 cells with G06976 prior to the addition of etoposide likewise inhibits DNA fragmentation (Fig. 6), although less effectively than pretreatment with rottlerin. At 10 nM G06976, etoposide-induced DNA fragmentation is inhibited by about 10%, whereas at 100 nM G06976 it is inhibited by about 40%. These data indicate that PKCδ and PKCα/β1 activity are required for maximal fragmentation of DNA in etoposide-treated parotid C5 cells.

Inhibition of PKC Suppresses Caspase-3 Activation in Etoposide-Treated Parotid Cells—We have previously shown that pretreatment with caspase inhibitor, Z-VAD.FMK, prior to the addition of etoposide inhibits biochemical and morphological indicators of apoptosis in parotid C5 cells. Cleavage and activation of PKCδ is likewise inhibited by Z-VAD.FMK (Figs. 2 and 3B), indicating that activation of at least some caspases must lie upstream of PKCδ in the apoptotic pathway. To determine if activation of caspases also occurs downstream of PKCδ, we have asked if pretreatment with rottlerin can suppress caspase-3 activity in etoposide-treated parotid C5 cells. In the experiment shown in Fig. 7, parotid C5 cells were treated with etoposide for 18 h, and activation of caspase-3 under these conditions was set at 100%. Pretreatment with 1 μM rottlerin prior to the addition of etoposide suppressed caspase-3 activity by >60%, whereas pretreatment with 2.5 μM rottlerin suppressed activity by about 80%. No further suppression of caspase-3 activity was seen at higher concentrations of rottlerin. These results indicate that PKCδ activity is required for maximal activation of caspase-3 following an apoptotic signal. Since cleavage of PKCδ itself is inhibited by Z-VAD.FMK, caspase activation may occur both upstream and downstream of PKCδ activation in the apoptotic pathway. Pretreatment of cells with the PKCα and PKCβ1 inhibitor, G06976, also suppressed etoposide-induced caspase-3 activity, although less effectively than pretreatment with rottlerin (Fig. 7). Ten nM G06976 suppressed caspase-3 activity by 10%, whereas caspase-3 activity was inhibited by 35% at 100 nM G06976. A small amount of caspase activation reproducibly occurred in cells treated with 100 nM G06976 alone, suggesting that this dose may be a weak inducer of apoptosis. Thus, while caspase-3 activation can occur under conditions where PKCδ, or PKCα and PKCβ1 activity is inhibited, maximal caspase-3 activation appears to require these activated isoforms.

Inhibition of PKCδ, but Not PKCα/β1, Prevents Sustained Activation of JNK and Suppression of ERK1/2 Activation—Different members of the mitogen-activated protein kinase family have been demonstrated to be activated in response to stimulation with mitogenic or apoptotic agents. We have previously shown that etoposide induces activation of JNK in parotid C5 cells. To ask if activation of JNK occurs upstream or downstream of PKCδ activation, parotid C5 cells were pretreated with rottlerin, and JNK activation was assayed at various times after the addition of etoposide using the GST-Jun kinase assay (47). As seen in Fig. 8, lanes 1–7, in cells treated with etoposide alone, activation of JNK is evident by 2 h following the addition of etoposide and is sustained for at least 12 h. In cells pretreated with 5 μM rottlerin prior to the addition of etoposide, however, some activation of JNK is apparent at 2 and 4 h (see Fig. 8, lanes 8 and 9), although the sustained activation of JNK appears to be nearly totally blocked (Fig. 8, lanes 10–13). No activation of JNK is seen in cells treated with rottlerin alone (Fig. 8, lanes 14–19). These data suggest that whereas early activation of JNK may occur independent of PKCδ, sustained activation of JNK is likely to be PKCδ-dependent. To ask if JNK activation requires PKCα and/or PKCβ1 activity, the same experiment was repeated in cells pretreated for 30 min with 100 nM G06976. As seen in Fig. 8, lanes 27–32, in contrast to inhibition of PKCδ, pretreatment of
cells with an inhibitor of PKCα and PKCβ1 does not block the activation of JNK by etoposide. As seen in Fig. 8, lane 32, a small amount of JNK activation is seen in parotid C5 cells treated with Gö6976 for 12 h, again suggesting that at this dose Gö6976 may be a weak inducer of apoptosis. These results suggest that, in contrast to PKCδ, sustained activation of the JNK pathway does not require PKCs or PKCβ1 activity.

We have previously shown that in addition to activating JNK, stimulation of parotid C5 cells with etoposide results in a decrease in the amount of activated ERK1 and ERK2, suggesting that these pathways are reciprocally regulated in apoptotic cells. To ask if this decrease in activated ERK1 and ERK2 is blocked in etoposide-treated cells which are pretreated with rottlerin or Gö6976, activated ERK1 and ERK2 were assayed by immunoblotting using an anti-active ERK antibody which specifically recognizes the phosphorylated (active) forms of these kinases. As seen in Fig. 9A, activated ERK1 and ERK2 can be detected in untreated parotid C5 cells. Although the stimulus responsible for the activation of ERK1 and ERK2 in these cells is not clear, the tissue culture media that the cells are maintained in contains epidermal growth factor that is capable of activating ERKs. As seen in Fig. 9A, following the addition of etoposide, ERK1 and ERK2 activity is initially stimulated and then decreases by 8 h to a level at, or below, that seen in untreated cells. This decrease in activated ERK1 and ERK2 is coincident with the increase in JNK activity shown in Fig. 8, suggesting that these pathways are coordinately regulated. In parotid C5 cells pretreated with rottlerin before the addition of etoposide, however, the decrease in ERK1 and ERK2 activity at 6–8 h appears to be blocked, with no decline in ERK1 or ERK2 activity apparent even after 12 h of etoposide treatment. Thus PKCδ appears to be required for the decrease in ERK1 and ERK2 activity seen in etoposide-treated cells. In contrast, pretreatment of parotid C5 cells with Gö6976...
had no effect on the decrease in ERK1 and ERK2 activity seen in etoposide-treated cells (Fig. 9C). This is consistent with the inability of Go6976 to suppress JNK activation in etoposide-treated cells (Fig. 8). Interestingly, treatment of parotid cells with Go6976 alone resulted in the initial activation and subsequent inactivation of ERK2, again suggesting that Go6976 is a weak inducer of apoptosis in these cells (Fig. 9C). Uniform loading of the gels was demonstrated by reprobing the blots with an anti-ERK antibody that recognizes both ERK1 and ERK2 (Fig. 9, B and D). There was no change in the amount of either ERK1 or ERK2 over the period examined indicating that the changes in the amount of activated ERK2 did not result from a decrease in the amount of the ERK2 protein. These results suggest that, in contrast to PKCδ which appears to be required for this event, activation of PKCα and/or PKCβ1 most likely occurs parallel to, or downstream of, the suppression of ERK1 activity.

**Rottlerin Blocks Activation of PKCδ in Etoposide-treated Parotid Cells—**To determine if the ability of rottlerin to block apoptosis is due to a decrease in the generation and/or activity of the PKCδ 40-kDa cleavage fragment, the expression of PKCδ was assayed by immunoblot in etoposide-treated cells pretreated with rottlerin. As seen in Fig. 10A, pretreatment of parotid cells with rottlerin prior to the addition of etoposide inhibits accumulation of the 40-kDa PKCδ cleavage product. In cells pretreated with 1 μM rottlerin prior to the addition of etoposide, accumulation of the cleavage product is blocked significantly, whereas in cells pretreated with 10 μM rottlerin the cleavage product is not detectable. In contrast, pretreatment of parotid C5 cells with Go6976 only slightly suppresses accumulation of the PKCδ cleavage product (Fig. 10C), an effect which may be secondary to its inhibition of caspase activity. The mechanism by which rottlerin blocks accumulation of the PKCδ cleavage product is not clear. Since PKCδ activity is required for caspase-3 activation (see Fig. 7), a decrease in the abundance of the cleavage fragment may be secondary to inhibition of caspase activation. Alternatively, since treatment of parotid cells with 1 or 10 μM rottlerin alone results in a decrease in the abundance of the full-length PKCδ protein (see Fig. 10A), inhibition of PKCδ may decrease the stability of the PKCδ protein. Finally, if the kinase activity of PKCδ is required for its cleavage and activation, rottlerin would be expected to inhibit cleavage directly. To determine if rottlerin inhibits the lipid-independent activation of PKCδ by etoposide, PKCδ activity was assayed in parotid C5 cells pretreated with rottlerin prior to the addition of etoposide. As seen in Fig. 10, panel B, pretreatment of parotid C5 cells with rottlerin blocks the increase in PKCδ activity seen in cells treated with etoposide alone.

**DISCUSSION**

The activation of specific signaling molecules, including some isoforms of PKC, has been demonstrated in apoptotic cells, suggesting that these activated molecules may function to regulate the apoptotic pathway (12, 13, 39, 44). In this report we show that PKCδ is activated in a caspase-dependent manner in parotid salivary acinar cells induced to undergo apoptosis by chemotherapeutic drugs. Inhibition of PKCδ activity partially or totally blocks all parameters of apoptosis examined including appearance of the apoptotic morphology, caspase-3 activation, and DNA fragmentation. PKCα and PKCβ1 activities also increase during etoposide-induced apoptosis; however, inhibition of these isoforms results in a much more modest suppression of apoptosis. These data argues that activation of PKCδ is an integral and essential part of the apoptotic program in parotid C5 cells and that specific activated isoforms of PKC may have distinct functions in cell death.

Proteolytic activation of PKCδ, in which the catalytic domain of the protein is cleaved from the regulatory domain, has been demonstrated in cells induced to undergo apoptosis with the topoisomerase inhibitors etoposide and camptothecin (38), ionizing radiation (41), ara-C and mitomycin C (42), and FAS ligand (43). We show that both cleavage of PKCδ as well as its lipid-independent activation can be blocked by caspase inhibitors, which also block apoptosis. Furthermore, expression of the PKCδ catalytic domain in several cell types induces phenotypic changes indicative of apoptosis (38, 39, 43). Likewise, data from our laboratory show that expression of the catalytic domain of PKCδ, but not a kinase-dead catalytic domain, can induce apoptosis in parotid C5 cells.3 Although these results demonstrate that activation of PKCδ by cleavage can effectively initiate the apoptotic program, the question of whether cleavage of PKCδ is required for apoptosis is still unanswered. In fact, since in our studies PKCδ activity is required for caspase activation (see Fig. 7), at least some functions of PKCδ in the apoptotic pathway may be independent of its cleavage by caspase.

To determine the functional consequence of PKCδ activation in parotid C5 cells we have utilized the PKCδ specific inhibitor,

![Figure 10](https://example.com/fig10.png)
rrottlerin. Pretreatment of parotid C5 cells with rottlerin prior to the addition of etoposide effectively blocks most parameters of apoptosis. However, upon removal of both drugs apoptosis occurs, indicating that the cells have sustained DNA damage but are unable to carry out the apoptotic program. Although rottlerin is thought to inhibit PKCδ at least in part by competing for ATP binding (50), our data suggest that, in addition to inhibiting activated PKCδ, rottlerin also prevents accumulation of the active PKCδ cleavage product (Fig. 10). Although this may be due to an effect of rottlerin on protein stability, an alternative explanation is that the kinase activity of PKCδ is required for its cleavage. Cleavage and activation of MEK kinase 1 (MEKK1) has also been demonstrated during apoptosis, and in this case the kinase activity of MEKK1 has been shown to be required for its cleavage (12).

Of the parameters examined, the morphologic changes associated with apoptosis appear to be the most sensitive to inhibition of PKCδ. The apoptotic morphology is essentially blocked at 1 μM rottlerin, a concentration slightly below the reported IC₅₀ for inhibition of PKCδ activity (Fig. 5) (50). As depicted in Fig. 11, this suggests that activation of PKCδ occurs early in the apoptotic pathway and upstream of events that result in the morphologic changes associated with apoptosis. In fact, accumulation of the PKCδ cleavage fragment and increased PKCδ activity can be detected by 4 h following the addition of etoposide (Figs. 1 and 3), whereas the apoptotic morphology is not apparent until about 6 h.² The activation of caspase-3 in etoposide-treated cells also appears to be quite sensitive to rottlerin, with a maximum inhibition of 80% at 2.5 μM rottlerin (Fig. 7). Although some of the structural changes seen during apoptosis, including cleavage of PARP and FAK, are thought to be caspase-3-dependent (48), our data suggest that caspase-3 activation alone is not sufficient to initiate these morphologic changes in parotid C5 cells.

DNA fragmentation is likewise suppressed under conditions where PKCδ activity is inhibited (Fig. 6). Recently Bharti et al. (53) have reported that activated forms of PKCδ are able to inactivate DNA protein kinase (DNA-PK), an enzyme that is essential for the repair of double-stranded DNA breaks. They suggest that activation of PKCδ during apoptosis inhibits the ability of DNA-PK to repair DNA damage and thus promotes DNA fragmentation. This hypothesis is supported by our observation that inhibition of PKCδ with rottlerin partially suppresses DNA fragmentation. Alternatively, since caspase-3 may be important for the inactivation of factors that suppress DNA fragmentation (54, 55), inhibition of DNA fragmentation may be secondary to inhibition of caspase-3 activity.

Our studies indicate that both the expression and activity of PKCα and PKCβ1 increase following treatment of parotid C5 cells with etoposide. To explore the contribution of this activation to the apoptotic response, we have used the Ca²⁺-dependent PKC isomorph inhibitor Gö6976. Although caspase-3 activity and DNA fragmentation are each suppressed by about 40% at 100 nM Gö6976, cellular rounding and blebbing are only slightly inhibited. Thus, although activation of PKCα and/or PKCβ1 may contribute to DNA fragmentation and caspase-3 activation, these isoforms probably are not required for the morphologic changes associated with apoptosis. One possibility is that activation of these isoforms amplifies specific events in the apoptotic pathway thus ensuring efficient cell demise.

Recent studies indicate that sustained activation of the JNK pathway correlates with the induction of apoptosis by a variety of agents including tumor necrosis factor-α (56), isothiocyanates (57), and TRAIL/apo2 (58). We have previously shown that treatment of parotid C5 cells with etoposide results in the caspase-dependent, sustained activation of JNK, as well as a decrease in the level of activated ERK.² Our current results demonstrate that sustained activation of the JNK pathway, as well as inactivation the ERK pathway, requires PKCδ activity, as rottlerin is able to block both events in etoposide-treated cells. In contrast, inhibition of PKCα and PKCβ1 activity does not suppress the activation of JNK or inactivation of ERK. These findings support our previous observation that the JNK and ERK pathways are reciprocally regulated in parotid C5 cells during apoptosis. Taken together these results suggest that PKCδ activation lies upstream of events that regulate JNK activation/ERK inactivation in apoptotic parotid C5 cells (see Fig. 11), whereas activation of PKCα and/or PKCβ1 occurs downstream, or independently, of the changes in these pathways.

The family of intracellular signaling molecules whose activity is regulated during apoptosis is increasing rapidly and includes a variety of protein kinases (48). Potential roles for these activated kinases include modulating the apoptotic responsiveness of the cell, as well as amplifying the apoptotic program. Our data demonstrate that one direct or indirect target of activated PKCδ is caspase-3, since maximal activation of caspase-3 requires PKCδ activity. This predicts the existence of a positive feedback loop whereby caspase activation of PKCδ results in the activation of more caspase activity, which in turn contributes to the further activation of PKCδ. Preliminary evidence from our laboratory shows that expression of the PKCδ catalytic fragment in parotid C5 cells is sufficient to induce caspase-3 activity.³ In addition, since PKCδ is essential for the apoptotic morphology, and contributes to DNA fragmentation, it is likely to regulate apoptosis through the activation or inactivation of additional effector molecules. DNA-PK has recently been identified as a substrate for activated PKCδ in vitro (53). A more thorough understanding of the role of activated PKCδ in apoptosis awaits the identification of additional substrates for this kinase in the apoptotic pathway.

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