PKCδ Is Required for Mitochondrial-dependent Apoptosis in Salivary Epithelial Cells*

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We report here that the novel protein kinase C isoform, PKCδ, is required at or prior to the level of the mitochondria for apoptosis induced by a diverse group of cell toxins. We have used adenoviral expression of a kinase-dead (KD) mutant of PKCδ to explore the requirement for PKCδ in the mitochondrial-dependent apoptotic pathway. Expression of PKCδKD, but not PKCαKD, in salivary epithelial cells resulted in a dose-dependent inhibition of apoptosis induced by etoposide, UV-irradiation, brefeldin A, and paclitaxel. DNA fragmentation was blocked up to 71% in parotid C5 cells infected with the PKCδKD adenovirus, whereas caspase-3 activity was inhibited up to 65%. The activation of caspase-9-like proteases by all agents was also inhibited in parotid C5 cells expressing PKCδKD. The ability of PKCδKD to block the loss of mitochondrial membrane potential was similarly determined. Expression of PKCδKD blocked the decrease in mitochondrial membrane potential observed in cells treated with etoposide, UV, brefeldin A, or paclitaxel in a dose-dependent manner. In contrast to the protective function of PKCδKD, expression of PKCδWT resulted in a potent induction of apoptosis, which could be inhibited by co-infection with PKCδKD. These results suggest that PKCδ is a common intermediate in mitochondrial-dependent apoptosis in salivary epithelial cells.

Apoptosis is a genetically regulated form of cell death that plays a critical role in the destruction of unwanted cells such as tumor cells and cells damaged by viral infection, drugs, chemical radiation, and aging. Critical genes in the apoptotic process have been identified and include the Bcl-2 family of proteins (1), as well as a family of cysteine proteases known as caspases, which specifically cleave their substrates at aspartic acid residues (2). Apoptosis is initiated either by ligand binding to cell surface receptors or by cell toxins via a pathway that targets the mitochondria (3). In the receptor-mediated pathway, ligand binding to the tumor necrosis factor (TNF)α/FAS family of receptors results in activation of the initiator caspase, caspase-8. Active caspase-8 then activates downstream "effector" caspases such as 3, 6, and 7, which cleave cell proteins ultimately resulting in DNA fragmentation and cell death.

The mitochondrial-dependent apoptotic pathway is induced by agents such as drugs, chemicals, irradiation, and some types of cell stress. Although the specific cell signals delivered by these agents differ, all appear to converge at the mitochondria resulting in the release of cytchrome c, activation of the initiator caspase, caspase-9, and the subsequent activation of downstream caspases. How many diverse signals initiate this common pathway is not known. One possibility is that specific protein kinase cascades may function to integrate these signals upstream of the mitochondria. In line with this, recent evidence implicates specific serine/threonine protein kinases as regulators of apoptosis. These include the phosphoinositide 3-kinase/AKT pathway (4, 5), members of the mitogen-activated protein kinase/extracellular-regulated kinase family (6–8), and the protein kinase C (PKC)1 pathway (9–12).

The PKC family consists of 12 serine/threonine kinases, of which specific isoforms have been shown to be either pro-apoptotic or anti-apoptotic, depending on the stimulus and cell type (11–15). For instance, PKCα suppresses apoptosis in several cell types, and work from Ruvolo et al. (13) suggests that this occurs through phosphorylation and activation of Bcl-2 (12). In contrast, PKCδ is required for apoptosis induced by genotoxins (11), phorbol ester (16), and Fas ligand (17). Recent reports from two laboratories suggest that PKCδ may function at the mitochondria to facilitate apoptosis (16, 18). Specific isoforms of PKC are cleaved by caspases to release a catalytically active fragment in cells induced to undergo apoptosis. These include PKCθ (19), PKCμ (20), PKCζ (21, 22), and PKCδ (23–25). Expression of the PKCδ or PKCθ catalytic domain induces apoptosis (19, 23). Interestingly, although PKCδ has been shown to contribute to phorbol ester-induced apoptosis, caspase cleavage of PKCδ does not occur under these conditions, suggesting a role for the uncleaved form of PKCδ in apoptosis (9).

We have previously shown that activation of PKC by phorbol ester is sufficient to induce an apoptotic program in parotid salivary acinar cells (10) and that PKCδ is essential for genotoxin-induced cell death (11). Here we demonstrate that expression of a dominant inhibitory form of PKCδ from an adenovirus...
viral vector is sufficient to suppress apoptosis in parotid cells in response to diverse stimuli and that PKCδ is required for loss of mitochondrial membrane potential in apoptotic cells. This argues that PKCδ is important for the integration of diverse death signals at or prior to their convergence at the mitochondria.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The isolation of the salivary parotid C5 cell line has been described elsewhere (26). Cells were cultured on Primaria 60-mm culture dishes (Falcon Plastics, Franklin Lakes, NJ) in a 1:1 mixture of Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented with 2.5% fetal calf serum, 5 μM transferrin, 1.1 μM hydrocortisone, 0.1 μM retinoic acid, 2.0 nM T3, 5 μg/mL insulin, 80 ng/ml epidermal growth factor (Collaborative Biomedical Products, Bedford, MA), 5 μM 1-glutamine, 50 μg/mL gentamicin sulfate, and a trace element mixture (Biofluids, Rockville, MD). Tissue culture reagents were obtained from Life Technologies, Inc. (Manassas, VA) unless otherwise indicated. Etoposide, paclitaxel, and brefeldin A were purchased from Sigma-Aldrich. UVC irradiation was done using a UV cross-linker (Fisher Scientific model UV XL-1000) at a wavelength of 254 nm.

Construction of Adenoviral Vectors and Infection of Parotid C5 Cells—Generation of the wild-type and kinase-dead recombinant parotid PKCδ adenosinase has been previously described (27). The kinase-dead mutant (K376R) has been shown to function as an isoform-specific dominant inhibitory kinase (28). To make the kinase-dead mutant of PKCα (PKCα/KD), mutagenesis was performed using a primer that targeted the conserved lysine in the ATP binding domain (5′AGCCCATCAGGATCTGAAA′) of the mouse PKCα cDNA. The resulting mutant (K368R) cDNA was subcloned into the adenoviral shuttle vector, pX-CMV, and recombinant adenosinase was prepared essentially as described (29). An adenoviral vector expressing the β-galactosidase gene (AdLacZ) was a generous gift from J. Schack, University of Colorado Health Sciences Center (30). Adenoviruses were titered on 293HEK cells using a focus-forming assay that detects expression of the adenoviral protein E2 (31).

Subconfluent parotid C5 cells were infected with AdLacZ, PKCδKD, PKCδWT, or PKCαKD for one hour at different multiplicities of infection (MOI) (12–30) focus-forming units/cell) in a 1:1 mixture of Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented as described above but without the addition of serum. Following the infection period, the virus-containing medium was replaced with medium containing 2.5% fetal bovine serum, and cells were incubated for an additional 24 h prior to the addition of apoptosis-inducing agents. Unless indicated otherwise, doses of the apoptotic agents were as follows: etoposide, 50 μM; UV, 128 J/m²; brefeldin A, 3 μM; taxol, 5 μM.

Immunoblotting—Adherent and floating cells were scraped into the culture medium, collected by centrifugation (3,000 × g for 10 min), washed with phosphate-buffered saline, and resuspended in 1 ml of JNK lysis buffer (25 mM HEPES, pH 7.5, 20 mM sodium orthovanadate, 0.1 mM retinoic acid, 2.0 mM T3, 5 μg/mL insulin, 80 μg/mL epidermal growth factor (Collaborative Biomedical Products, Bedford, MA), 5 mM 1-glutamine, 50 μg/mL gentamicin sulfate, and a trace element mixture (Biofluids, Rockville, MD). Tissue culture reagents were obtained from Life Technologies, Inc. (Manassas, VA) unless otherwise indicated. Etoposide, paclitaxel, and brefeldin A were purchased from Sigma-Aldrich. UVC irradiation was done using a UV cross-linker (Fisher Scientific model UV XL-1000) at a wavelength of 254 nm.

Preparation of Cells for Fluorescence-activated Cell Sorting—The medium containing floating cells was removed and saved. Cells were removed from a P60 dish by the addition of 3 ml of cell dispersion solution (0.25% trypsin-EDTA, 68 μM EGTA, pH 7.4, 100 μg/mL Pro) and incubated at 37 °C for 15 min. An equal volume of trypsin inhibitor mixture (86 mM NaCl, 30 mM KCl, 1 mM NaH2PO4, 3 mM MgSO4, 0.5 mM CaCl2, 15 mM glucose, 18 mM NaCO3, 0.5 mM adenosine, 20 mM taurine, 2 mM γL-carnitine, 0.5% bovine serum albumin, and 80 μg/ml trypsin inhibitor (Sigma)) was added and a single cell suspension was made by pushing cells 4 times through a 20-gauge needle, 2 times through a 23-gauge needle, and 2 times through a 26-gauge needle. Suspended cells were combined with the medium containing floating cells and centrifuged at 1000 × g for 3 min. The pellet was washed once with phosphate-buffered saline, and the cells were then resuspended in the desired reagent.

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Analysis of DNA Content—Cells were prepared for fluorescence-activated cell sorting (FACS) as described above and the cell pellet was resuspended in 0.5 ml of saponin/propiolamide isoleucine solution (0.3 mg/ml saponin, 25 μg/ml propidium iodide, 10 mM EDTA, and 5 μg/ml RNase). Cells were stained for 5–24 h at 4 °C in the dark prior to FACS analysis using a Beckman Coulter XL.

Expression of PKCδKD Inhibits Apoptosis in Response to Diverse Agents—We have previously demonstrated that etopside-induced apoptosis in parotid C5 cells is accompanied by specific changes in PKC signaling pathways, which include caspase-dependent cleavage and activation of PKCδ (11). The studies reported here utilize expression of a dominant inhibitory form of PKCδ from an adenoviral vector (PKCδKD) to explore the contribution of PKCδ to specific events in the apoptotic pathway. In Fig. 1, expression of PKCδ protein was analyzed by immunoblotting in parotid C5 cells infected with increasing amounts of PKCδKD. As seen here, the abundance of PKCδ protein increased with increasing MOI and was 15–20-fold over endogenous levels at an MOI of 150 as determined by densitometry analysis of immunoblots. The abundance of the other major isoforms of PKC expressed in parotid C5 cells, PKCα and PKCζ (11), was unchanged (Fig. 1). Control studies in parotid C5 cells infected with AdLacZ and stained for β-ga-
lactosidase expression demonstrated that >98% of cells were infected at an MOI of 100 (data not shown).

We hypothesize that PKCδ functions as an integrator of cell toxins that converge at the mitochondria to induce apoptosis. To address this directly, we have analyzed the effect of PKCδKD expression on apoptosis induced by etoposide, as well as three additional cell toxins that have distinct mechanisms of action. Etoposide inhibits topoisomerase type II, resulting in the accumulation of double-stranded breaks (33). UV irradiation results in direct DNA damage as well as free radical-mediated oxidative damage (34, 35). Brefeldin A is a potent inhibitor of vesicular transport and protein secretion and has been proposed to function by inhibiting the membrane binding of COP coat proteins (36, 37). Taxol inhibits microtubule disassociation and blocks cells in G2/M (38). In the experiment shown in Fig. 2, parotid C5 cells were infected with PKCδKD or the control virus, AdLacZ, for 24 h and then challenged with etoposide, brefeldin A, or UV irradiation. Apoptosis in response to these agents is indicated morphologically by rounding, blebbing, and detachment of cells from the monolayer (Fig. 2, top row). Infection with PKCδKD prior to the addition of the stimuli significantly inhibited appearance of the apoptotic morphology (Fig. 2, middle row). However, infection with the control vector, AdLacZ, had no effect on appearance of the apoptotic morphology (Fig. 2, bottom row). PKCδKD expression similarly protected against apoptosis induced by taxol (data not shown).

To determine whether the suppression of the morphologic characteristics of apoptosis is associated with suppression of biochemical indicators of apoptosis, the ability of PKCδKD to inhibit DNA fragmentation was determined. Parotid C5 cells were infected with PKCδKD or AdLacZ, treated with etoposide, stained with propidium iodide, and the percent sub-G1 DNA determined by FACS analysis. As seen in Fig. 3A, in untreated cells (panel A) or cells infected with PKCδKD alone (panel D), there is little detectable sub-G1 DNA; however, nearly all the DNA is in etoposide-treated cells is found in the sub-G1 fraction (panel B). Infection of cells with PKCδKD prior to the addition of etoposide resulted in a dose-dependent decrease in sub-G1 DNA and an increase in 2N to 4N DNA (panels C, E, F). Fig. 3B shows the quantification of sub-G1 DNA in a similar experiment. PKCδKD suppressed etoposide-induced DNA fragmentation, as indicated by sub-G1 DNA, by 64% when cells were infected at an MOI of 150 and 72% at an MOI of 300. Expression of PKCδKD at an MOI of 75 and 150 also slightly suppressed basal DNA fragmentation in infected but untreated cells, an observation that was consistent in multiple experiments. In contrast, infection with AdLacZ offered no protection against etoposide-induced DNA fragmentation. As a control, parotid C5 cells were infected with an adenovirus which expresses a kinase-dead PKCα protein (PKCαKD). As seen in Fig. 3C, in contrast to PKCδKD, expression of PKCαKD offered no protection against etoposide-induced apoptosis. Fig. 4 shows a series of similar experiments using UV irradiation, brefeldin A, or taxol to induce apoptosis. As seen here, expression of PKCδKD resulted in a dose-dependent decrease in sub-G1 DNA in cells treated with UV (panel A), brefeldin A (panel B), or taxol (panel C). The analysis of multiple experiments using these agents is shown in Table I and shows that suppression of DNA fragmentation by PKCδKD was 67–71% for cells treated with brefeldin A or UV and 54–57% for cells treated with etoposide or taxol. Suppression of brefeldin A and UV-induced apoptosis was slightly but significantly greater than suppression of etoposide-induced apoptosis (p = 0.04), and suppression of UV-induced DNA fragmentation was slightly but significantly greater than suppression of taxol-induced apoptosis (p = 0.05).

PKCδ Functions Prior to the Activation of Caspase-3 and Caspase-9-like Proteases—To address where in the apoptotic pathway PKCδ functions, we have sequentially analyzed specific events for their dependence on PKCδ. Activation of effector caspses such as caspase-3 is required for dismantling of structural proteins, the activation or inactivation of protein kinases, and DNA fragmentation. In the experiment shown in Fig. 5, parotid C5 cells were infected with PKCδKD and Ac-DEVD-pNA cleavage, an indicator of caspase-3 activity, was assayed after the addition of etoposide, brefeldin A, taxol, or treatment with UV. Although the extent of caspase-3 activation varied between agents, Ac-DEVD-pNA cleavage was suppressed in all cases by expression of PKCδKD, indicating that PKCδ is required for, or at a point prior to, activation of caspase-3 protease. Interestingly, expression of PKCδKD did not suppress caspase-3 cleavage entirely, and about the same residual level of caspase-3 activity was seen regardless of the apoptotic agent. This suggests that a PKCδ-independent pathway leading to caspase-3 activation is also induced by this group of agents. The suppression of Ac-DEVD-pNA cleavage by PKCδKD in multiple experiments is shown in Table I. Inhibition of caspase-3 cleavage is similar for all agents; however, PKCδKD appears to be a slightly, although not significantly, less potent inhibitor of etoposide-induced Ac-DEVD-pNA cleavage than of cleavage induced by brefeldin A or UV irradiation.

Because caspase-3 is cleaved and activated by caspase-9 in the mitochondrial-dependent pathway, the ability of PKCδKD to inhibit cleavage of Ac-LEHD-pNA, a caspase-9 substrate, was also assayed. As seen in Fig. 6, etoposide (panel A), UV irradiation (panel B), brefeldin A (panel C), and taxol (panel D)
all induced Ac-LEHD-pNA cleavage, which can be inhibited in a dose-dependent manner by expression of PKC\(\text{KD}\). In contrast, expression of AdLacZ had no effect on Ac-LEHD-pNA cleavage. Inhibition of Ac-LEHD-pNA cleavage by PKC\(\text{KD}\) was about 80% for brefeldin A and UV about 45% for etoposide and about 65% for taxol at an MOI of 200, reflecting the relative potencies observed previously for inhibition of sub-G\(_1\) DNA and caspase-3 activity.

**PKC\(\text{o}\) Is Required for Loss of Mitochondrial Membrane Potential in Response to Agents That Induce Apoptosis**—Loss of the transmembrane potential of mitochondria (MMP) has been shown to occur prior to nuclear condensation and caspase activation and is linked to cytochrome \(c\) release in many but not all apoptotic cells (39–41). To determine whether loss of MMP in response to cell toxins requires PKC\(\text{o}\), we assayed MMP during apoptosis in parotid C5 cells infected with PKC\(\text{o}\)KD or the control vector, AdLacZ. Intracellular fluorescence was assayed by FACS after loading cells with an intramitochondrial dye. High fluorescence at 575 nm corresponds to the aggregated form of the dye and is proportional to an intact MMP, whereas loss of MMP leads to a loss of 575 fluorescence and an increase in fluorescence at 525 nm (monomeric form of the dye).
This experiment has been repeated 35 times with similar results.

As seen in Fig. 7, untreated cells or cells infected with PKCΔKD alone exhibit high 575 fluorescence, indicating normal MMP. Treatment of cells with etoposide, brefeldin A, or UV results in loss of 575 fluorescence and an increase in fluorescence at 525 indicating a loss of MMP. However, in cells infected with PKCΔKD, loss of MMP in response to etoposide, brefeldin A, or UV is significantly inhibited. This is quantified in Fig. 8 in which the MMP is expressed as the ratio of 575 (aggregate) to 525 (monomer) fluorescence. As seen here, loss of MMP in response to each of these agents is inhibited in a dose-dependent manner by expression of PKCΔKD. With the exception of etoposide, MMP is nearly restored to that seen in cells infected with PKCΔKD alone. Expression of AdLacZ did not inhibit loss of MMP (data not shown).

The data presented above show that PKCΔ activity is required for the loss of MMP in apoptotic cells. In most cases, loss of MMP had been shown to occur prior to, and independent of, caspase activation (42, 43); however, recently it has been reported that caspase activity may be required for changes in MMP (42). To determine whether loss of MMP in response to cell toxins requires activated caspase, parotid C5 cells were pretreated with the broad spectrum caspase inhibitor, ZVAD.FMK, prior to induction of apoptosis. As seen in Table II, pretreatment with ZVAD.FMK did not block the loss of MMP seen in cells treated with etoposide, brefeldin A, UV, or taxol, indicating that caspase activation is not required for loss of MMP. ZVAD.FMK however was a potent inhibitor of caspase-3 activity in parotid C5 cells. Taken together, these data indicate that loss of MMP occurs independent of activation of caspase-3. Moreover, whereas PKCΔ activity is required for loss of MMP, activation of PKCΔ by caspase-directed cleavage per se is not required.
Expression of PKCδ WT Is a Potent Inducer of Apoptosis in Parotid C5 Cells—The studies shown above demonstrate an essential role for PKCδ in apoptosis induced by cell toxins and DNA damage. To determine whether overexpression of PKCδ WT alone is sufficient to induce apoptosis, parotid C5 cells were infected with an adenoviral vector, which drives expression of PKCδ WT. As seen in Fig. 9A, expression of PKCδ WT resulted in a robust induction of apoptosis as indicated by DNA ladder formation. Similar results were seen when DNA fragmentation was measured by assaying sub-G1 DNA accumulation (data not shown). DNA fragmentation was accompanied by a dose-dependent activation of caspase-3 as seen in Fig. 9B. Induction of apoptosis by PKCδ WT did not require the addition of a PKC activator such as phorbol ester, indicating that at least some of the overexpressed PKCδ is activated intracellularly. To verify that adenovirus-expressed PKCδ KD blocks apoptosis via inhibition of PKCδ WT, parotid C5 cells were infected with PKCδ WT or co-infected with PKCδ WT together with PKCδ KD. As seen in Fig. 9C, co-infection with PKCδ KD results in inhibition of the caspase-3 activation induced by PKCδ WT. DNA fragmentation induced by infection with PKCδ WT was also inhibited by co-infection of PKCδ KD (data not shown).

PKCδ is cleaved by caspase-3 to generate a 40-kDa carboxy-terminal fragment in most but not all cells induced to undergo apoptosis by genotoxins and other cell toxins (11, 24, 25). Our studies indicate that treatment of parotid C5 cells with etoposide, UV, brefeldin A, and taxol all result in the caspase-dependent cleavage of endogenous PKCδ to generate a 40-kDa fragment (data not shown). To determine whether induction of apoptosis by PKCδ correlates with generation of the 40-kDa catalytic fragment, parotid C5 cells were infected with PKCδ WT or PKCδ KD, and PKCδ expression was analyzed by immunoblot. As seen in Fig. 9D, induction of apoptosis by PKCδ WT is accompanied by cleavage of PKCδ to generate a 40-kDa fragment. In contrast, the 40-kDa PKCδ fragment is not seen in etoposide-treated cells, which express PKCδ KD. Because activation of caspase-3 is required for PKCδ cleavage (23), we conclude that PKCδ regulates an event in the apoptotic pathway upstream of caspase-3.

**DISCUSSION**

Many chemicals and drugs induce apoptosis through a mitochondrial-dependent mechanism. However, how these diverse signals are integrated is not known. The data presented here indicate that PKCδ is a required intermediate in the apoptotic pathway induced by agents that cause DNA damage (etoposide; UV) as well as toxins that target the Golgi (brefeldin A) and microtubule network (taxol). Although all agents required PKCδ for a maximal apoptotic response, the dependence on PKCδ for apoptosis induced by UV and brefeldin A was slightly greater than for taxol and etoposide for all apoptotic parameters assayed. This suggests that whereas PKCδ functions as a common integrator of these apoptotic signals, alternative pathways also exist to integrate apoptotic signals initiated by specific types of cell damage. In this regard, etoposide has been reported to induce two distinct pathways of apoptosis: a caspase-dependent pathway at low doses (10 μM) and a caspase-independent pathway at higher doses (>25 μM) (42). The less efficient inhibition of etoposide-induced apoptosis by PKCδ KD may indicate that PKCδ is required for one but not both of these pathways. For all agents except etoposide, upstream events in the apoptotic pathway, such as loss of MMP and activation of caspase-9-like protease, were inhibited to a greater extent than downstream events such as caspase-3 activation and DNA fragmentation. This suggests that PKCδ is required for regulation of a key early component(s) in the
apoptotic pathway and functions at, or prior to, the mitochondria.

Previous studies showing a requirement for PKCδ for apoptosis have relied heavily, but not exclusively, on the use of rottlerin to inhibit PKCδ. A recent publication by Davies et al. (44) however indicates that rottlerin does not inhibit PKCδ in vitro, bringing into question reports by Gschwendt et al. (45) and Keenan et al. (46), which demonstrate specific inhibition of PKCδ by rottlerin. Thus alternative inhibitory strategies are required to verify the role of PKCδ in apoptosis. Our current studies, which use a kinase-dead mutant of PKCδ to inhibit endogenous PKCδ activity, provide independent verification of the importance of PKCδ as a regulator of apoptosis. This approach has been used extensively to inhibit the activity of

FIG. 7. PKCδ is required for mitochondrial membrane depolarization in response to apoptotic agents. Parotid C5 cells were untreated or infected with PKCδKD at the indicated MOI for 24 h prior to the addition of the apoptotic agent for an additional 18 h. MMP was assayed by measuring fluorescence at 575 and 525 nm as described under "Experimental Procedures." Healthy cells have high 575 fluorescence, indicating a normal MMP. In apoptotic cells however the dye is excluded from the mitochondria leading to loss of 575 fluorescence (aggregate) and an increase in 525 fluorescence (monomer). Data were converted to density plots using System 2 software.
PKCδ and Regulation of Apoptosis

FIG. 8. Inhibition of mitochondrial membrane depolarization by PKCδKD is dose dependent. Parotid C5 cells were untreated or infected with PKCδKD at the indicated MOI for 24 h prior to the addition of the apoptotic agent. MMP is expressed as the ratio of 575 (aggregate) to 525 (monomer) fluorescence. The striped bars indicate the aggregate/monomer ratio in untreated cells or in cells treated with the apoptotic agent alone. Cells infected with PKCδKD but not treated with an apoptotic agent are shown in black, whereas the gray bars show cells infected with PKCδKD and then induced to undergo apoptosis with the indicated agent. A high ratio indicates healthy cells; a decrease in the ratio indicates apoptosis. This experiment has been repeated three times with similar results.

TABLE II
Mitochondrial membrane depolarization does not require caspase activation

Cells were pretreated with 50 μM ZVAD FMK for 30 min prior to the addition of the apoptotic agent. MMP and caspase-3 activity were assayed as described under "Experimental Procedures." This experiment was repeated four times with similar results. A representative experiment is shown.

| Treatment     | MMP fluorescence | Caspase-3 activity (pmol/min/μg protein) |
|---------------|------------------|----------------------------------------|
| Untreated     | 0.99             | 15.4                                   |
| ZVAD          | 0.96             | >0.1                                   |
| Etoposide     | 0.18             | 78.2                                   |
| Etop + ZVAD   | 0.25             | >0.1                                   |
| Brefeldin A   | 0.16             | 111.9                                  |
| BFA + ZVAD    | 0.11             | >0.1                                   |
| UV            | 0.48             | 106.1                                  |
| UV + ZVAD     | 0.29             | >0.1                                   |
| Taxol         | 0.25             | 59.4                                   |
| Taxol + ZVAD  | 0.13             | >0.1                                   |

First, PKCδ is required for mitochondrial membrane depolarization, a process that we show here to be caspase-independent in parotid C5 cells. Second, expression of PKCδKD inhibits activation of both initiator (caspase-9) and effector (caspase-3) caspases. Third, whereas induction of apoptosis by PKCδWT is accompanied by cleavage of PKCδ in cells which express PKCδKD no cleavage of PKCδ is seen. This suggests that the kinase function of PKCδ is required for activation of a pathway, which subsequently mediates caspase activation. However, expression of the caspase-cleavage fragment of PKCδ is sufficient to induce apoptosis in many cells, including parotid C5 cells, suggesting that both full-length and caspase-cleaved PKCδ contribute to the apoptotic program. One explanation is that the function of the activated full-length and caspase-cleaved forms of PKCδ are the same, with caspase cleavage simply being a more efficient method of activating the kinase. In this paradigm, caspase cleavage would serve to amplify the PKCδ signal in apoptotic cells. In support of this is the demonstration from our laboratory and Fujii et al. that caspase cleavage of PKCδ is not required for phorbol ester-induced apoptosis, and that phorbol ester is a much weaker inducer of apoptosis than agents that induce PKCδ cleavage (9, 11). Alternatively, it is tempting to speculate that either changes in conformation or subcellular localization may result in the regulation of targets specific for the caspase-cleaved form of PKCδ. Thus the functions of full-length and caspase-cleaved PKCδ may be distinct, with full-length PKCδ regulating an early event(s) and the caspase-cleaved form of PKCδ regulating a later event(s) in the apoptotic pathway. Consistent with this possibility is the observation that PKCδ translocates to specific sites following induction of apoptosis (16, 18, 47).

It should be noted that expression of PKCδKD did not totally inhibit apoptosis in response to any agent tested. Because PKCδKD is thought to function as a competitive inhibitor of endogenous PKCδ, it is possible that we cannot achieve the level of expression of the inhibitory protein required to completely suppress the function of the endogenous kinase (28). In addition it is likely that other regulatory signals contribute to the induction and/or amplification of apoptosis by these agents. In particular, members of the mitogen-activated protein kinase/extracellular-regulated kinase family have been shown to regulate apoptosis (6–8). In addition, the caspase cleavage and constitutive activation of other protein kinases such as MEKK-1, MST1, PAK-2, FAK have also been shown to contribute to apoptosis as does caspase cleavage and inactivation of enzymes required for DNA repair (48, 49).

Our studies suggest that PKCδ functions at, or prior to, the mitochondria in apoptotic cells, and that overexpression of protein kinases; however, pitfalls include a potential lack of specificity, as well as the possibility that the subcellular localization of the kinase may be altered because of the mutation and/or overexpression. To address the issue of specificity, we have shown that inhibition is specific for PKCδ, as expression of PKCαKD does not inhibit etoposide-induced apoptosis. Likewise, preliminary experiments using PKCδKD also show no inhibition of apoptosis.3 Furthermore, as expected, expression of PKCδWT has the opposite effect of PKCδKD, i.e. it is an inducer of apoptosis. Expression of PKCδKD can block apoptosis induced by overexpression of PKCδWT, indicating that PKCδKD is a target of PKCδKD. Inhibition of apoptosis with PKCδKD increased in a dose-dependent manner when the kinase-dead mutant was expressed in the range of 5–30-fold over endogenous PKCδ (MOI of 25–250). Whereas it is not clear why such high ratios of kinase-dead to endogenous PKCδ are required, one possibility, not addressed in the current manuscript, is that some/much of the overexpressed protein does not localize to the correct subcellular localization, and hence is not available to suppress the biological effect being assessed.

PKCδ is proteolytically activated by caspase-3 in response to many agents that induce mitochondrial-dependent apoptosis, including the agents utilized in these studies. Whereas caspase cleavage and activation of PKCδ clearly contributes to apoptosis in many cells, our data are also consistent with a role for PKCδ in apoptosis prior to caspase cleavage and activation.

3 R. Bell and M. E. Reyland, unpublished data.
PKCδWT is sufficient to induce apoptosis. This is consistent with a recent report from Li et al. (18) in which phorbol ester treatment of keratinocytes which overexpress PKCδWT induced apoptosis. In this study apoptosis correlated with translocation of PKCδWT, but not PKCδKD to the mitochondria, suggesting that mitochondrial localization of PKCδ facilitates apoptosis (16, 18). This conclusion is supported by data from Majumder et al. (16), which demonstrates that in phorbol ester-treated cells, translocation of PKCδ to the mitochondria facilitates cytochrome c release. Whereas our studies are consistent with the hypothesis that PKCδ regulates mitochondrial events in apoptosis, they do not address whether this is a direct effect at the level of the mitochondria or an indirect effect perhaps via regulation of another component of the apoptotic pathway. Notably, the nuclear proteins DNA-PK (50) and lamin B (51) are reported to be substrates for PKCδ in apoptotic cells and Scheel-Toellner et al. (47) show translocation of PKCδ to the nucleus in T cells treated with IFN-β to induce apoptosis (47). Future studies directed at identifying substrates of PKCδ in apoptotic cells will greatly enhance our understanding of the function of this essential signaling pathway.

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