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Inhibition of PKCα induces a PKCδ-dependent apoptotic program in salivary epithelial cells

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Abstract

We have used expression of a kinase dead mutant of PKCα (PKCαKD) to explore the role of this isoform in salivary epithelial cell apoptosis. Expression of PKCαKD by adenovirus-mediated transduction results in a dose-dependent induction of apoptosis in salivary epithelial cells as measured by the accumulation of sub-G1 DNA, activation of caspase-3, and cleavage of PKCδ and PKCζ, known caspase substrates. Induction of apoptosis is accompanied by nine-fold activation of c-Jun-N-terminal kinase, and an approximately two to three-fold increase in activated mitogen-activated protein kinase (MAPK) as well as total MAPK protein. Previous studies from our laboratory have shown that PKCδ activity is essential for the apoptotic response of salivary epithelial cells to a variety of cell toxins. To explore the contribution of PKCδ to PKCαKD-induced apoptosis, salivary epithelial cells were cotransduced with PKCαKD and PKCδKD expression vectors. Inhibition of endogenous PKCδ blocked the ability of PKCαKD to induce apoptosis as indicated by cell morphology, DNA fragmentation, and caspase-3 activation, indicating that PKCδ activity is required for the apoptotic program induced under conditions where PKCα is inhibited. These findings indicate that PKCα functions as a survival factor in salivary epithelial cells, while PKCδ functions to regulate entry into the apoptotic pathway.

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Keywords: protein kinase C; apoptosis; salivary epithelial cells

Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; JNK, jun-N-terminal kinase; ERK, extracellular regulated kinase; MAPK, mitogen-activated kinase; Ac-DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline

Introduction

Apoptosis is important for the clearance of damaged or variant cells, and alterations in apoptosis may contribute to the pathogenesis of cancer and other disorders.[1–4] Key regulators of the apoptotic pathway include pro- and antiapoptotic members of the Bcl-2 family, caspase proteases, which cleave cellular proteins, and the family of IAP proteins, which regulate the activity of activated caspases.[5–7] Serine/threonine protein kinases are also known to regulate apoptosis, including the phosphoinositide 3-kinase/AKT pathway,[8,9] members of the mitogen-activated protein kinase family (MAPKs),[10–12] and the protein kinase C (PKC) pathway.[13–17]

The PKC family consists of 11 isoforms, with individual isoforms exhibiting varying substrate specificity, as well as differences in their subcellular localization and response to specific stimuli.[18,19] This argues that specific isoforms of PKC play unique roles in transducing cell signals. In support of this, both pro- and antiapoptotic isoforms of PKC have been described. For example, PKCδ is required for apoptosis induced by genotoxins,[16] phorbol ester,[20] and Fas ligand,[21] while PKCλ and PKCζ protect against apoptosis.[22,23] Accumulated evidence suggests an antiapoptotic/proproliferative function for PKCα. PKCα is overexpressed in a variety of tumor cells and has been suggested to play a role in the proliferation of gliomas,[24] liver,[25] and endometrial tumors.[26] Depletion of PKCα induces apoptosis in glioblastoma multiforme cells[27] and in COS-7 cells.[17] Two studies have explored the molecular mechanism by which PKCα protects against apoptosis. Ruvolo *et al*.[28] have shown that PKCα can phosphorylate Bcl-2 *in vitro*, and that overexpression of PKCα results in increased Bcl-2 phosphorylation and suppression of apoptosis in human pre-B REH cells. Li *et al*.[29] demonstrate that in 32D cells, PKCα overexpression stimulates the prosurvival protein kinase, AKT, and suppresses apoptosis following growth factor withdrawal.

Our studies have focused on understanding the contribution of specific isoforms of PKC to apoptosis in salivary epithelial cells. We have previously shown that activation of PKC by phorbol ester is sufficient to induce an apoptotic program in parotid salivary epithelial cells[15] and that PKCδ is essential for genotoxin-induced cell death in these cells.[13,16] Here, we demonstrate that inhibition of endogenous PKCα induces an apoptotic program in salivary epithelial cells. As we have previously reported for other apoptotic stimuli, induction of apoptosis under these conditions requires PKCδ activity, suggesting that in salivary epithelial cells PKCδ may function to regulate entry into the apoptotic pathway.

Results

Inhibition of endogenous PKCα activity induces apoptosis

We have previously reported that both the expression and the specific activity of protein kinase C-α (PKCα) is increased in parotid C5 cells induced to undergo apoptosis by treatment with etoposide.[16] In the present studies, we have used an inhibitory form of PKCα to explore the function of endogenous PKCα in the apoptotic pathway. Parotid C5 cells were transduced with increasing amounts of an adenoviral vector, which expresses a kinase inhibitory mutant of PKCα (PKCαKD), or with the control adenovirus, AdlacZ. Experiments were matched for relative levels of expression of PKCαKD, as viral infectivity varied somewhat between experiments. As shown in Figure 1, compared to nontransduced cells (panel 1), parotid C5 cells that express PKCαKD (panels 2–5) are rounded up and detached from the monolayer, characteristic of cells undergoing apoptosis. In contrast, these changes are not seen in cells that express the control adenovirus, AdLacZ (panel 6). Appearance of the apoptotic morphology is evident at 18–24 h after transduction, and maximal by 42 h after transduction, while maximal expression of PKCαKD is observed at 12–18 h and remains stable throughout the course of apoptosis (data not shown). To determine if the morphologic changes in PKCαKD-expressing cells are associated with the accumulation of fragmented DNA, transduced parotid C5 cells were stained with propidium iodide, and the percent sub-G1 DNA was determined by FACS analysis. As shown in Figure 2a, while there is little detectable sub-G1 DNA in untreated cells, inhibition of endogenous PKCα results in a dramatic increase in sub-G1 DNA which increases in a dose-dependent manner with the amount of PKCαKD expressed. Figure 2b shows quantification of sub-G1 DNA in a similar experiment. As seen here, expression of PKCαKD, but not the control adenovirus, AdlacZ, resulted in a dose-dependent accumulation of sub-G1 DNA. At the highest level of PKCαKD expression, nearly 50% of the cellular DNA was in the sub-G1 peak, a value similar to that observed in parotid C5 cells treated with 50 μM etoposide for 18 h. As shown in Figure 6b, expression of PKCαKD in parotid C5 cells likewise resulted in a dose-dependent induction of caspase-3 activity. These data indicate that inhibition of endogenous PKCα induces an apoptotic program in parotid C5 cells.

Activation of specific signal transduction pathways is well documented in cells induced to undergo apoptosis. In particular, members of the MAPK family are activated in response to stimulation with mitogenic or apoptotic agents. In some, but not all, cells, activation of the c-Jun-N-terminal kinase (JNK) pathway has been shown to be required for the apoptotic response to genotoxins and other agents. We have previously demonstrated activation of the JNK pathway, and inactivation of the MAPK pathway in parotid C5 cells induced to undergo apoptosis with etoposide[16] and phorbol ester.[15] Furthermore, inhibition of PKCδ activity blocks activation of JNK and inactivation of ERK in etoposide-treated cells, linking PKC to the regulation of these pathways.[16] To determine if JNK activity is altered in parotid C5 cells induced to undergo apoptosis by expression of PKCαKD, JNK activity was assayed in parotid C5 cells transduced with increasing amounts of PKCαKD. As shown in Figure 3a, transduction with increasing amounts of PKCαDN resulted in increased PKCα protein expression. When JNK activity was assayed in the same experiment, an increase in JNK activity was observed that correlated with increased expression of PKCαKD (Figure 3b). As seen here, JNK is activated up to nine-fold in cells transduced with PKCαKD for 24 h. Analysis of the time course of JNK activation demonstrates an increase in JNK activity by 12 h after transduction and maximal activation by 18–24 h (data not shown). No increase in JNK activity is seen in nontransduced cells, or in cells transduced with the AdLacZ control virus. Since an increase in JNK activity could be explained by increased JNK protein expression, we

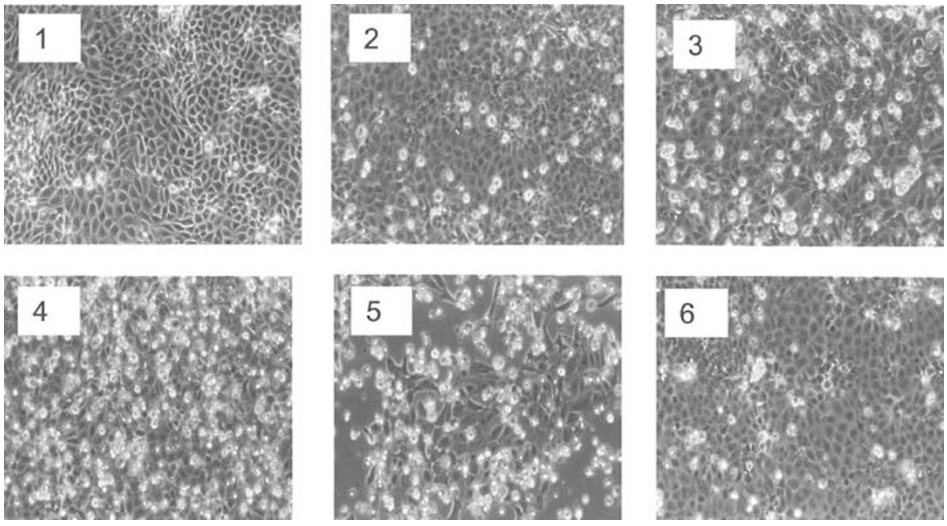

Figure 1 Inhibition of endogenous PKCα induces apoptosis in parotid C5 cells. Subconfluent parotid C5 cells were transduced with PKCαKD or AdlacZ (LacZ) for 42 h. The figure shows nontransduced cells (1), cells transduced with PKCαKD at an MOI of 6, 12, 25, and 50 (2, 3, 4, and 5 respectively), cells transduced with AdlacZ at an MOI of 68 (6). The appearance of the cells was examined microscopically to assess the apoptotic morphology.

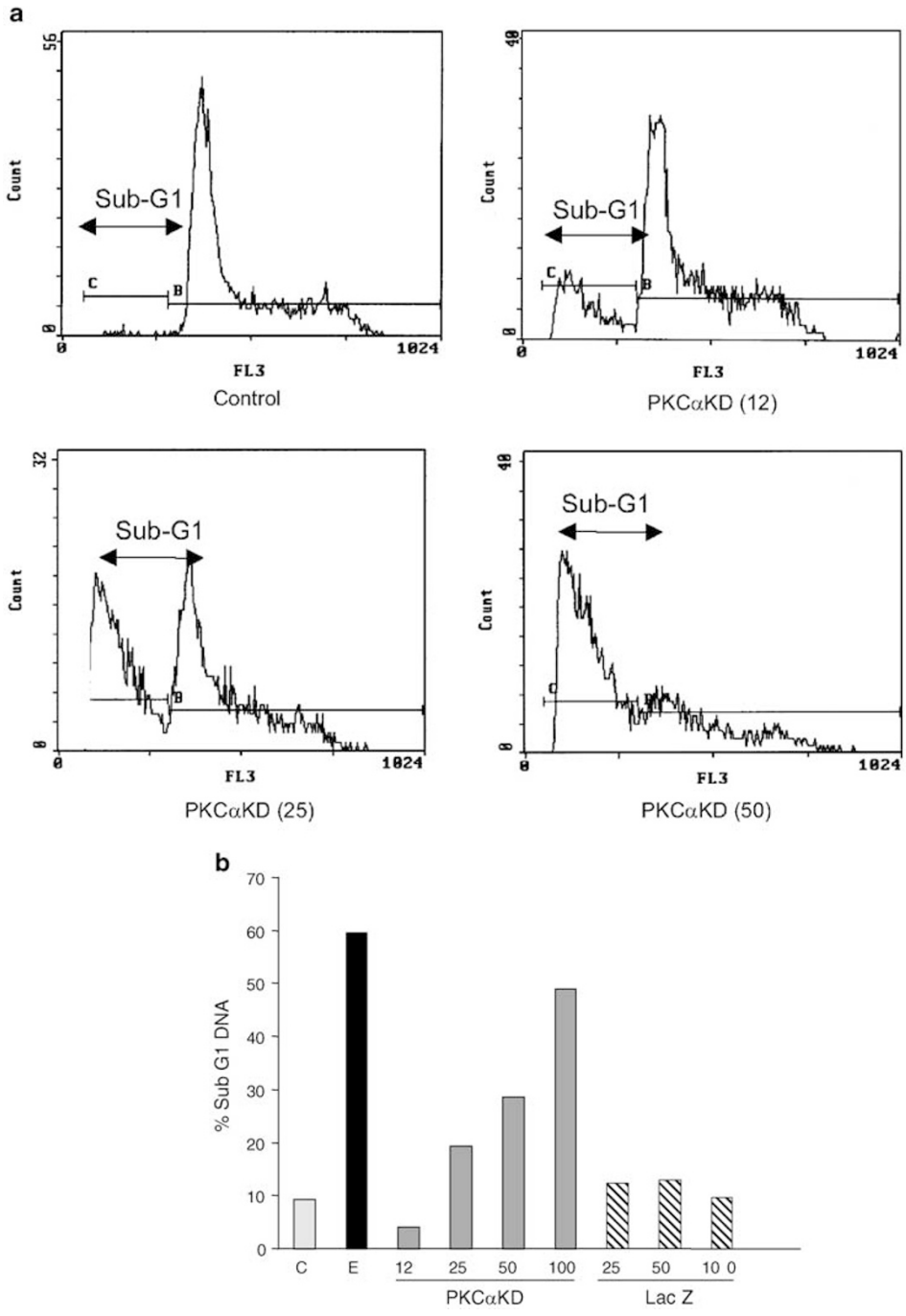

Figure 2 Inhibition of endogenous PKCα induces DNA fragmentation in parotid C5 cells. (**a**) Subconfluent parotid C5 cells were transduced with PKCαKD 42 h at the MOI indicated in parentheses. Cells (adherent and floating) were collected, permeabilized with saponin and stained with propidium iodide as described in Materials and Methods. DNA content was analyzed by FACS. The sub-G1 DNA peak, marked by the double-headed arrow, indicates fragmented DNA. (**b**) Quantification of DNA fragmentation in parotid C5 cells expressing PKCαKD or AdlacZ. Subconfluent parotid C5 cells were transduced with PKCαKD or AdlacZ (LacZ) at the indicated MOI for 42 h. The graph shows quantification of sub-G1 DNA in a single experiment which is representative of three similar experiments. C=untreated cells; E=cells treated with 50 μM etoposide for 18 h.

determined the expression of JNK protein in cells transduced with PKCαDN (Figure 3c). No change in JNK protein expression was observed, indicating that the increase in JNK activity observed is because of activation of the kinase.

Inhibition of ERK activity and the reciprocal activation of JNK has been shown to correlate with the initiation of apoptosis in calphostin C-induced cell death in glioma cells,[30] growth factor withdrawn PC-12 cells,[31] Fas-induced Jurkat cells,[12] and UV-irradiated fibroblasts.[23] To determine if ERK activity is altered in parotid C5 cells transduced with PKCαKD, active ERK1 and ERK2 were assayed using an antiactive ERK antibody that specifically recognizes the phosphorylated

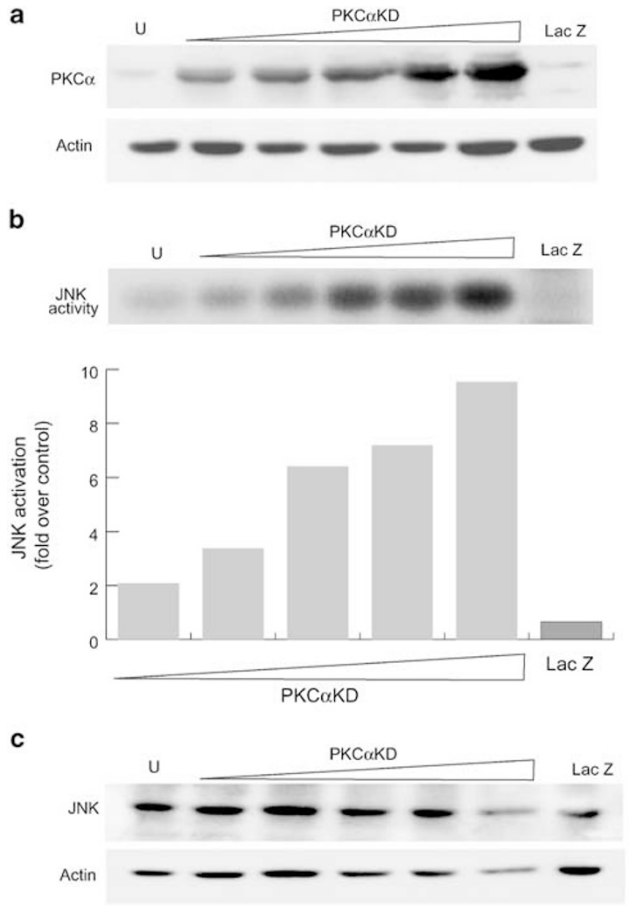

Figure 3 Inhibition of endogenous PKCα results in the activation of c-terminal Jun kinase. (**a**) Parotid C5 cells were transduced with increasing amounts of PKCαKD or AdLacZ for 24 h. U=untreated. Top: Cell lysates were prepared and assayed for PKCα expression by immunoblot. Bottom: The blot was stripped and reprobed for actin to show equal protein loading. (**b**) JNK activity was assayed using the GST-Jun kinase assay as described in Materials and Methods. The reaction products were displayed on a 10% SDS-polyacrylamide gel. An autoradiogram of the dried gel is shown (top) with a graph that shows quantification of the assay (bottom). The data are expressed as fold activation over untreated cells and is representative of three similar experiments. (**c**) Cell lysates from the above experiment were analyzed for total JNK protein by immunoblot. The blot was stripped and reprobed for actin.

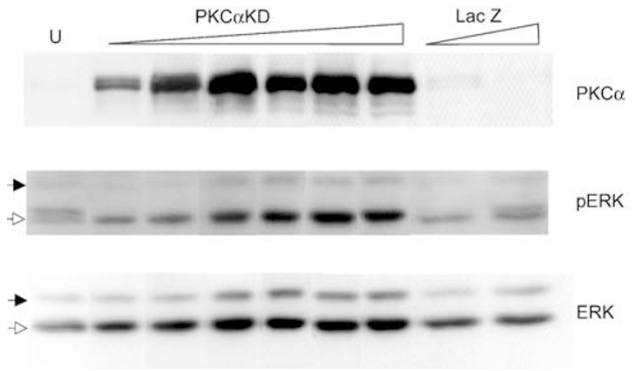

Figure 4 Inhibition of endogenous PKCα results in an increase in ERK expression and activity. Parotid C5 cells were transduced with increasing amounts of PKCαKD or AdLacZ for 24 h. Cell lysates were resolved on a 10% SDS-polyacrylamide gel and immunoblotted with anti-PKCα (top panel) or anti-active ERK2 which cross-reacts with both phosphorylated ERK1 and ERK2 (middle panel). The immunoblot was stripped and reprobed with an anti-ERK antibody that recognizes total ERK1 and ERK2 (bottom panel). The positions of both ERK1 (solid arrow) and ERK2 (open arrow) are noted on the left side of each panel. These experiments were repeated three or more times with similar results.

(active) forms of these kinases. As shown in Figure 4, expression of PKCαKD (top), but not AdLacZ, increased the abundance of phosphorylated ERK1 and ERK2 (middle), indicating an increase in the active forms of these kinases. When these blots were reprobed with an anti-ERK antibody that recognizes total ERK, an increase in both ERK 1 and ERK2 protein abundance was observed (bottom), indicating that PKCαKD also increases ERK protein expression. These studies suggest that, in contrast to parotid C5 cells induced to undergo apoptosis with genotoxins,[16] the JNK and ERK pathways are not inversely regulated when apoptosis is induced by inhibition of endogenous PKCα.

The activation of caspase-3 and cleavage of cellular proteins is a hallmark of apoptosis induced by most stimuli.[32,33] Among the numerous caspase-3 substrates described in apoptotic cells are the PKC isoforms, PKCδ ,[34] and PKCζ .[35] Caspase cleavage of PKCδ results in the generation of a constitutively active catalytic fragment that is sufficient to induce apoptosis when expressed in a variety of cell types. Caspase cleavage of PKCζ likewise results in the generation of a fragment with catalytic activity,[35] although the function of this fragment in apoptotic cells has not been addressed. To determine if these PKC isoforms are cleaved in parotid C5 cells undergoing apoptosis in response to inhibition of endogenous PKCα, PKCδ and PKCζ protein expression was analyzed by immunoblot. As shown in Figure 5, increased expression of PKCαKD (top) results in cleavage of endogenous PKCδ into a 40 kDa fragment identical to that seen in etoposide-treated cells (middle). Likewise, PKCζ is also cleaved into two fragments in cells expressing PKCαKD (bottom), which appear to be identical in molecular weight to the caspase cleavage fragments produced in response to etoposide.

Apoptosis induced by the inhibition of endogenous PKCα requires PKCδ

PKCδ has been implicated as an intermediate in apoptosis induced by a variety of agents with distinct mechanisms of action. These include phorbol ester,[14,20] Fas/CD95,[21] and etoposide.[16] To ask if apoptosis induced by inhibition of PKCα requires PKCδ activity, parotid C5 cells were transduced with PKCαKD or PKCδKD alone, or cotransduced with both adenoviruses. As shown in Figure 6a, transduction of PKCδKD together with PKCαKD (panel 1) greatly inhibits the ability of PKCαKD (panel 2) to induce apoptosis as assayed by changes in cell morphology. To determine if this block in apoptosis correlates with suppression of DNA fragmentation, parotid C5 cells were transduced with either virus, both viruses, or the control virus, AdlacZ, for 42 h and the percent of sub-G1 DNA was assayed. As shown in Figure 6b, coexpression of PKCδKD suppresses PKCαKD-induced

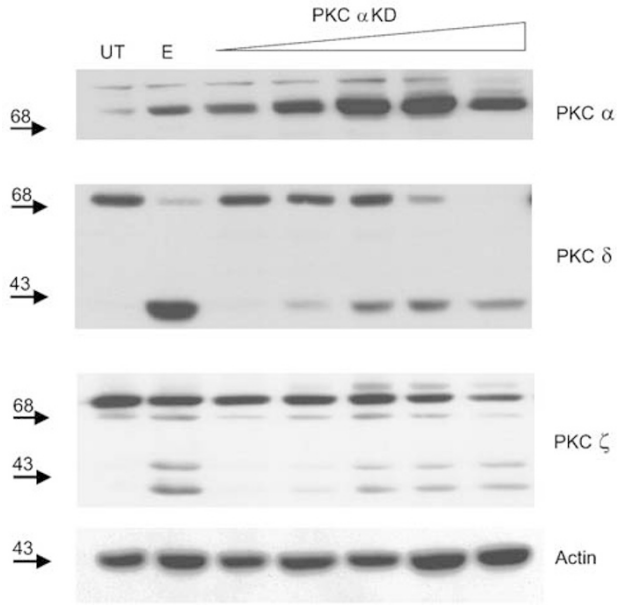

Figure 5 Inhibition of endogenous PKCα results in cleavage of PKCδ and PKCζ. Subconfluent parotid C5 cells were left untreated (UT), treated with 50 μM etoposide for 18 h (E), or transduced with PKCαKD at the indicated MOI for 42 h. Cells were harvested and PKC isoform expression was analyzed by immunoblot as described in Materials and Methods. The blot was stripped and reprobed for actin to show equal protein loading. Arrows indicate position of the molecular weight markers. This experiment was repeated three times with similar results.

DNA fragmentation by about 40%. To determine if PKCδ is required for PKCαKD-induced caspase-3 activity, caspase-3 activity was assayed in cells transduced with either virus alone or both viruses. As shown in Figure 6c, expression of PKCαKD results in a dose-dependent induction of caspase-3 activity, while no increase in caspase activity was seen in cells transduced with AdlacZ. Cotransduction with PKCδKD together with PKCαKD however greatly suppressed the induction of caspase-3 activity by PKCαKD. These data indicate that apoptosis induced by the inhibition of endogenous PKCαKD proceeds through a PKCδ-dependent pathway.

Discussion

The role of PKC in apoptosis is controversial, with data supporting both pro- and antiapoptotic functions. In the current studies, we have examined the role of PKCα, a PKC isoform associated with proliferation in many cell types.[24, 36–39] Our studies suggest that PKCα is essential for the survival of salivary epithelial cells, and that in the absence of PKCα activity, apoptosis is initiated. Apoptosis can be inhibited by expression of an inhibitory form of PKCδ, indicating that apoptosis induced under these conditions requires PKCδ activity. This is in agreement with previous studies from our laboratory that have demonstrated an essential role for PKCδ in apoptosis induced by a wide variety of agents.[13,16] Thus, PKCα and PKCδ appear to have reciprocal functions in salivary epithelial cells, with PKCα functioning as a survival factor, while PKCδ functions to regulate entry into the apoptotic pathway.

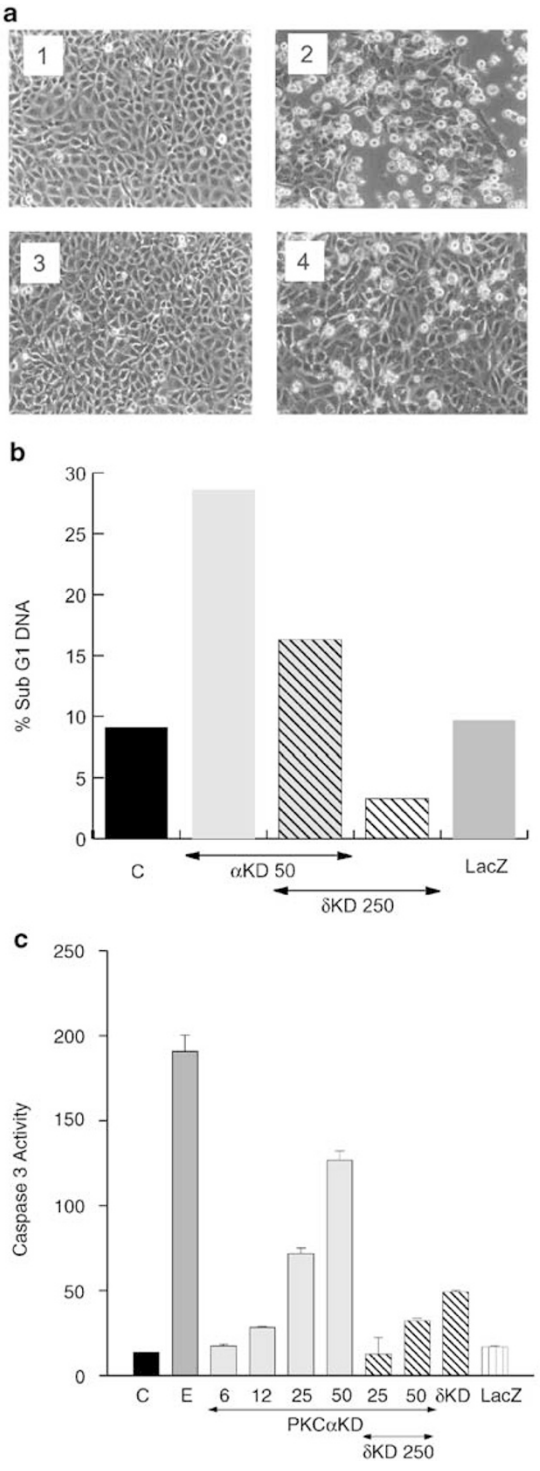

Figure 6 Apoptosis induced by inhibition of PKCα requires PKCδ activity. (**a**) Subconfluent parotid C5 cells were left untreated (1), transduced with PKCαKD at an MOI of 50 (2), transduced with PKCδKD at an MOI of 250 (3), or transduced with PKCαKD (MOI=50) together with PKCδKD (MOI=250) (4) for 42 h. The appearance of the cells was examined microscopically to assess the apoptotic morphology. (**b** and **c**) Subconfluent parotid C5 cells were transduced with PKCαKD, PKCδKD, or both at the indicated MOI. Sub-G1 DNA (**b**) and caspase-3 activity (**c**) were assayed as described in Materials and Methods. These experiments were repeated three or more times with similar results.

Our current studies indicate that inhibition of PKCα by expression of a kinase negative mutant is sufficient to induce apoptosis, suggesting that PKCα functions to suppress apoptosis in parotid C5 cells. We have previously reported that the expression and activity of both PKCα and PKCβ1 increase following treatment of parotid C5 cells with etoposide.[16] An increase in PKCα protein abundance in etoposide-treated cells can also be shown in Figure 5. Shao *et al.*[40] also show that PKCα is activated following induction of apoptosis by genotoxic agents in HL-60 myeloid cells. In the light of our current findings, this increase in PKCα in response to genotoxins may represent a survival signal. Previous studies from our laboratory have shown that transfection of parotid C5 cells with a plasmid that expresses a kinase dead mutant of PKCα did not induce apoptosis.[15] The apparent discrepancy between these studies and our current observation may be owing to the higher level of expression achieved by adenoviral-facilitated expression of PKCαKD (Matassa and Reyland, unpublished data). Importantly, our current findings are in line with studies from other investigators including Whelan and Parker,[17] who showed that loss of PKC function induced apoptosis in COS-1 and U937 cells. In addition, specific targeting of PKCα by antisense oligonucleotides or ribozymes blocks the growth of gliomas and increases apoptosis,[38,39] and inhibits the growth of lymphoma cells.[41] Several studies suggest that the prosurvival function of PKCα may result from direct regulation of specific components of the apoptotic machinery. Ruvolo *et al.*[25] show that activation of mitochondrial PKCα results in phosphorylation of the anti-apoptotic protein, Bcl-2. In these studies, overexpression of PKCα correlated with increased resistance to etoposide-induced apoptosis and increased phosphorylation of Bcl-2.[28] Li *et al.*[29] report that overexpression of PKCα activates the prosurvival function of serine–threonine kinase, Akt, and suppresses apoptosis induced by growth factor withdrawal.

Activation of the c-Jun terminal kinases (JNKs) has been shown to promote either apoptosis or survival signaling depending on the cellular context.[42] In particular, the sustained or persistent activation of JNK has been associated with apoptosis in a number of cell types.[43–45] We have previously reported that in salivary epithelial cells, apoptosis induced by etoposide or TPA correlates with sustained activation of JNK.[15,46] Treatment of parotid C5 cells with etoposide resulted in the activation of JNK in 4–6 h, which was maintained throughout the time course of apoptosis,[46] while treatment of parotid cells with TPA resulted in a biphasic activation of JNKs with the later phase commencing by 4 h.[15] In the current studies, we extend these observations to show that sustained activation of JNK also occurs in parotid C5 cells induced to undergo apoptosis by inhibition of PKCα. Activation of JNK occurred as early as 12 h after transduction and was maximal at 18–24 h (data not shown). These studies suggest that activation of JNK is an early event in the response of salivary epithelial cells to apoptotic stimuli. This is supported by studies in JNK null MEF cells in which a marked deficiency in mitochondrial-dependent apoptosis is observed including a block in cytochrome *c* release, an early event in the apoptotic pathway.[47]

In contrast to the JNK pathway, most studies indicate that activation of extracellular regulated kinases (ERKs) protects against apoptosis,[31,48,49] while more limited data suggest a role for ERK activation in promoting apoptosis.[50–52] Indeed, we have previously shown that ERK is inactivated in parotid C5 cells induced to undergo apoptosis by treatment with etoposide[46] or TPA.[15] However, the data presented in the current manuscript show that apoptosis associated with PKCα inhibition correlates with an increase in the expression of ERK1 and ERK2 protein as well as an increase in the abundance of the phosphorylated or activated forms of these kinases. While this may indicate that PKCα normally functions to suppress ERK expression and activity in parotid C5 cells, this seems unlikely given a variety of reports which demonstrate that PKCα, as well as other PKC isoforms, activate ERK.[53–55] A more likely explanation is that activation of ERK1/2 and its increased expression is a component of the apoptotic pathway induced by the loss of PKCα activity, and does not reflect loss of a normal physiologic function of PKCα. Interestingly, PKCδ has also been shown to be a positive regulator of ERK,[50,55,56] and expression of a dominant negative PKCδ construct blocked the activation of ERK in cells induced to undergo apoptosis by treatment with UV light.[50] We show that PKCδ is activated during apoptosis in parotid C5 cells which express PKCαKD (Figure 5) and that PKCαKD-induced apoptosis requires PKCδ activity (Figure 6); thus, activation of PKCδ may account for the activation of ERK under these conditions.

Our current studies clearly define distinct and opposing functions for PKCα and PKCδ in salivary epithelial cells. Studies by Mandil *et al.*[24] suggest that these two isoforms play similar opposing roles in glioma cells. Here, we link these isoforms in the apoptotic pathway by demonstrating that apoptosis induced by PKCα inhibition is dependent upon PKCδ activity. Similar to these other apoptotic agents, inhibition of PKCδ suppresses caspase activation as well as DNA fragmentation, indicating that PKCδ functions at an early point in the apoptotic pathway. However, expression of PKCδ KD does not totally block PKCαKD-induced apoptosis, suggesting that apoptosis induced by inhibition of PKCα may also involve an additional pathway that is independent of PKCδ. The requirement of PKCδ for apoptosis in response to diverse signals, including inhibition of endogenous PKCα, suggests that PKCδ may function to regulate entry into the apoptotic pathway in epithelial and perhaps other cell types.

Materials and Methods

Cells and cell culture

The isolation of the salivary parotid C5 cell line has been described elsewhere.[57] Cells were cultured on Primaria 60 mm culture dishes (Falcon Plastics, Franklin Lakes, NJ, USA) in a 1 : 1 mixture of Dulbeccos modified Eagle media/Nutrient mixture F-12 (DMEM/F12) supplemented with 2.5% fetal calf serum, 5 μg/ml 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, USA), 5 mM L-glutamine, 50 μg/ml gentamicin sulfate, and a trace element mixture (Biofluids, Rockville, MD, USA). Tissue culture reagents were obtained from GIBCO/BRL (Gaithersburg, MD, USA) unless otherwise indicated. Etoposide was purchased from Sigma-Aldrich.

Construction of adenoviral vectors and transduction of parotid C5 cells

Generation of the wild-type and kinase dead recombinant rat PKCδ adenoviruses has been previously described.[58] The kinase dead mutant (K376R) has been shown to function as an isoform-specific dominant inhibitory kinase.[59] To make the kinase dead mutant of PKCα (PKCαKD), mutagenesis was performed using a primer that targeted the conserved lysine in the ATP-binding domain (TACGCCATCAGGATCCTGAAG) of the mouse PKCα cDNA. The resulting mutant (K368R) cDNA was subcloned into the adenoviral shuttle vector, pXCMV, and recombinant adenovirus was prepared essentially as described.[60] An adenoviral vector expressing the β-galactosidase gene (AdlacZ) was a generous gift of J Schaack, University of Colorado Health Sciences Center.[61] Adenoviruses were titered on 293 HEK cells using a focus forming assay, which detects expression of the adenoviral protein E2.[62]

Subconfluent parotid C5 cells were transduced with AdlacZ or PKCαKD for 1 h at different multiplicites of transduction (MOIs) (6–100 focus forming units (ffu)/cell) in DMEM/F12 supplemented as described above but without the addition of serum. Following the transduction period, the virus-containing media were replaced with supplemented DMEM/F12 containing 2.5% FBS and cells were incubated for an additional 42 h.

Immunoblotting

Adherent and floating cells were collected as previously described[16] and resuspended in 1 ml of JNK lysis buffer (25 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10 mM NaF, and 4 μg/ml each aprotinin and leupeptin). 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 SRF13 K microfuge. Protein concentration was determined using a Bradford assay kit purchased from Biorad. Cell lysates (25–50 μg) were resolved by 10% SDS-PAGE, transferred to an Immobilon membrane (Millipore), and immunoblotted with the desired antibody as described previously.[16] Enhanced chemiluminescence (ECL; Amersham) followed by autoradiography was used to detect the signal. Antibodies to JNK, actin, and PKC were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-PKC antibodies recognize an epitope in the carboxy-terminal portion of the protein. For immunoblots from cells transduced with PKCδKD, the PKCα and PKCζ antibodies were preabsorbed with four times excess of PKCδ blocking peptide (Santa Cruz) by shaking for 1 h at room temperature. The antiactive ERK2 antibody, which cross-reacts with both phosphorylated ERK1 and ERK2, was obtained from Promega Biotechnology (Madison, WI, USA). An anti-MAP kinase antibody, which cross-reacts with both ERK1 and ERK2, was obtained from Upstate Biotechnology (Lake Placid, NY, USA).

Measurement of caspase activity

N-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD-pNA) (caspase-3) cleavage was assayed as previously described.[46]

Preparation of cells for fluorescence-activated cell sorting (FACS)

The medium, containing floating cells, was removed and saved. Cells were removed from a P60 dish by the addition of 3 ml cell dispersion solution (0.25% trypsin-EDTA, 68 μM EGTA, pH 7.4, 100 μg/ml pronase) and incubation at 37°C for 15 min. An equal volume of trypsin inhibitor cocktail (86 mM NaCl, 30 mM KCl, 1 mM NaH_2PO_4, 3 mM $MgSO_4$, 0.5 mM CaCl, 15 mM glucose, 18 mM $NaCO_3$, 0.5 mM adenosine, 20 mM taurine, 2 mM DL-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 × through a 20 gauge needle, 2 × through a 23 gauge needle, and 2 × 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 PBS and the cells were then resuspended in the desired reagent.

Analysis of DNA content

Cells were prepared for FACS as described above and the cell pellet was resuspended in 0.5 ml of saponin–propidium iodide 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 the FACS analysis.

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, USA), and the fusion proteins were prepared as described.[63] JNK activation was assayed using the GST-Jun kinase assay[64] as previously described.[16] The reaction products were 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.

Acknowledgments

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