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Activation of PKC is sufficient to induce an apoptotic program in salivary gland acinar cells

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Abstract

Accumulating evidence suggests that specific isoforms of PKC may function to promote apoptosis. We show here that activation of the conventional and novel isoforms of PKC with 12-O-tetradecanoyl phorbol-13- ester (TPA) induces apoptosis in salivary acinar cells as indicated by DNA fragmentation and activation of caspase-3. TPA-induced DNA fragmentation, caspase-3 activation, and morphologic indicators of apoptosis, can be enhanced by pretreatment of cells with the calpain inhibitor, calpeptin, prior to the addition of TPA. Analysis of PKC isoform expression by immunoblot shows that TPA-induced downregulation of PKCα and PKCδ is delayed in cells pre-treated with calpeptin, and that this correlates with an increase of these isoforms in the membrane fraction of cells. TPA-induced apoptosis is accompanied by biphasic activation of the c-*jun*-N-terminal kinase (JNK) pathway and inactivation of the extracellular regulated kinase (ERK) pathway. Expression of constitutively activated PKCα or PKCδ, but not kinase negative mutants of these isoforms, or constitutively activated PKCε, induces apoptosis in salivary acinar cells, suggesting a role for these isoforms in TPA-induced apoptosis. These studies demonstrate that activation of PKC is sufficient for initiation of an apoptotic program in salivary acinar cells. *Cell Death and Differentiation* (2000) **7**, 1200–1209.

Keywords: protein kinase C; apoptosis; salivary acinar cells

Abbreviations: β-gal, β-galactosidase; ERK, extracellular regulated kinase; JNK, *jun*-N-terminal kinase; PKC, protein kinase C; MAPK, mitogen activated kinase; Rb, retinoblastoma protein; TPA, 12-O-tetradecanoyl phorbol-13-acetate

Introduction

Alterations in apoptosis may contribute to the pathology of a wide range of disorders including those associated with development, autoimmune disease and cancer. Induction of apoptosis via the FAS/FAS ligand pathway has been suggested to lead to the salivary gland destruction seen in the autoimmune disorder known as Sjögren's syndrome.[1] In addition, apoptosis of normal salivary cells in patients treated with head and neck irradiation or chemotherapeutics can result in reduced salivary gland function, or xerostomia.[2,3]

The critical genes in the apoptotic process have been defined genetically in *C. elegans*, and biochemically in other species.[4] These include the Bcl-2 family of proteins, a family of related regulatory proteins which either promote or suppress apoptosis,[5] and the caspases, cysteine proteases that are responsible for initiation and transduction of the apoptotic signal.[6] In addition, signaling molecules, including members of the mitogen activated protein kinase (MAPK) family, as well as protein kinase C (PKC), have been shown to be involved in the regulation of apoptosis.[7–13] Activation of extracellular regulated kinases (ERKs) induces protection against apoptosis,[14,15] and in some cases ERK activity must be inhibited for apoptosis to proceed.[16,17] In contrast, many studies suggest that activation of the c-Jun terminal kinases (JNKs) is critical and sometimes sufficient to induce apoptosis.[15,18] Dominant negative mutants of JNKs have been shown to block apoptosis of HEK 293 cells induced by gamma irradiation and UVC,[11] UV-induced apoptosis of small cell lung carcinoma cells,[10] and cardiomyocyte cell death in response to ischemia.[19]

The PKC family consists of multiple isoforms whose regulation and expression varies between cell types. Activation of the conventional (PKCα, βI, βII, and γ) and novel isoforms of PKC (PKCδ, ε, η, θ and μ), is regulated by diacylglycerol (DAG) in a calcium dependent (conventional isoforms) or calcium-independent (novel isoforms) manner.[20] The tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA), can also activate these isoforms by substituting for endogenous DAG.[20] A variety of studies show that specific isoforms of PKC may be either pro-apoptotic or anti-apoptotic, depending on the stimulus and cell type.[7–9,21] Evidence for an anti-apoptotic role for PKC is the observation that pre-treatment with TPA antagonizes apoptosis induced by many agents.[22–24] Likewise, PKC inhibitors such as staurosporine, calphostin C and chelerythrine induce apoptosis in many hematopoetic and neoplastic cells.[25–27] The atypical PKC isoforms, PKCγ and PKCζ, which are not activated by TPA, also appear to protect against apoptosis.[28–30] In contrast, TPA can induce apoptosis in prostate and breast cancer epithelial cells, and in the human monocytic cell line, U937, suggesting a pro-apoptotic role for PKC.[31–34] Likewise, PKCβ appears to be required for ceramide and TNFα-induced apoptosis in HL-

60 cells.[35] PKCδ is cleaved and activated in a caspase dependent manner in response to a variety of apoptotic stimuli, and expression of activated PKCδ has been shown to induce apoptosis in several cell types.[21,36–38] Recent work from our laboratory demonstrates that inhibition of endogenous PKCδ blocks etoposide induced apoptosis in salivary acinar cells.[39]

In this study we show that activation of conventional and novel isoforms of PKC with TPA is sufficient for induction of apoptosis in salivary acinar cells. Furthermore, TPA-induced apoptosis is enhanced under conditions where the downregulation of activated PKC is inhibited. Expression of constitutively activated, but not wild type or kinase negative, PKCα and PKCδ also induces apoptosis, suggesting that TPA may induce apoptosis via the activation of one or both of these isoforms.

Results

Activation of PKC induces DNA fragmentation and activation of caspase-3 in parotid C5 cells

Treatment of cells with TPA results in activation of the conventional and novel isoforms of PKC and their translocation to cellular membranes. The major PKC isoforms expressed in parotid C5 cells are PKCα, PKCδ, and PKCζ, however low level expression of PKCβ1 and PKCε can also be detected.[39] Of the major isoforms expressed, PKCα and PKCδ are responsive to activation by TPA. Figure 1 shows a dose response and time course of induction of apoptosis in parotid C5 cells by TPA. In the experiment in Figure 1A, subconfluent parotid C5 cells were treated with increasing amounts of TPA for 18 h, after which the cells were harvested and DNA fragmentation was assayed using a biochemical assay which detects apoptosis by quantitation of histone-associated DNA fragments present in the cytoplasm. As seen here, as little as 1 nM TPA induced DNA fragmentation in the parotid C5 cells, while maximal induction was seen at 10 nM TPA. At doses of TPA >10 nM DNA fragmentation appears to decrease slightly in this experiment, and this was a consistent finding in other experiments. Furthermore, TPA appears to be a relatively weak inducer of DNA fragmentation in parotid C5 cells. In other experiments in which we used the assay described above to measure DNA fragmentation, cells treated with 10 μM TPA for 18 h gave an average relative value of 0.36 ± 0.15, compared to an average relative value of 1.86 ± 0.23 for cells treated with 50 μM etoposide for 18 h. Figure 1B shows the time course of apoptosis in parotid C5 cells treated with 1, 2.5 or 10 nM TPA. A small amount of DNA fragmentation is detectable by 4 h, however, fragmented DNA accumulates most rapidly between 8 and 18 h after the addition of TPA. These experiments indicate that activation of a TPA-sensitive isoform of PKC can induce apoptosis in parotid C5 cells. Based on the PKC isoform expression pattern in parotid C5 cells, PKCα and PKCδ are candidate isoforms for this function.

Numerous apoptotic stimuli induce the activation of caspase-3.[6,40] Activation of caspase-3 in response to TPA was examined with a commercial kit in which the cleavage of Ac-DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline) is detected by a colorimetric assay. As seen in Figure 2, caspase-3 activation is detectable 4–6 h after treatment with 10 nM TPA. Caspase-3 activity continues to increase in a linear fashion until 18 h after stimulation with TPA, and at 18 h the level of caspase-3 activity was nearly fourfold higher than the level detected in unstimulated cells.

Blocking degradation of activated PKC enhances TPA-induced apoptosis

Recruitment of PKC to cellular membranes is required for the activation of this family of kinases.[20] However, the membrane

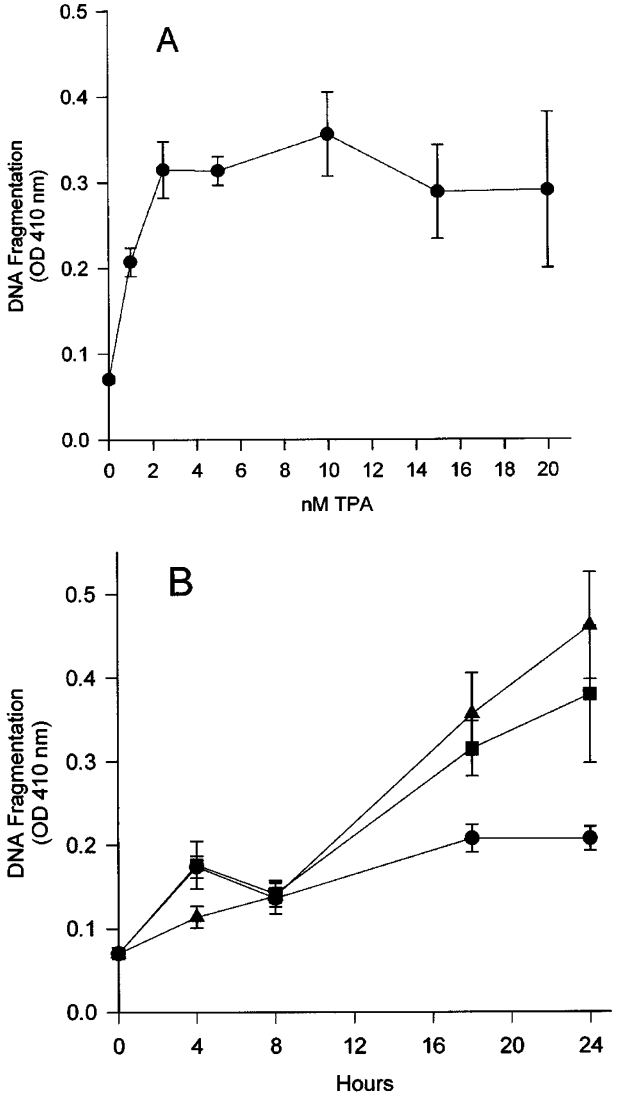


Figure 1 Activation of PKC induces DNA fragmentation in parotid C5 cells. (**A**) Subconfluent parotid C5 cells were treated with increasing doses of TPA for 18 h. (**B**) Parotid C5 cells were treated with 1 (circles), 2.5 (squares) or 10 nM (triangles) TPA for the times indicated. Cells were harvested and DNA fragmentation was assayed using the Cell Death Detection assay kit from Roche Molecular Biochemicals as described in Materials and Methods. Values are expressed as the average of four measurements plus and minus the S.E.M. This experiment was repeated three times with similar results

localization of activated PKC is typically transient, since membrane associated PKC is a target for calpain proteases.[41–43] Calpain cleaves activated PKC at the hinge region between the regulatory and catalytic domain and cleaved PKC is subsequently rapidly degraded by other cellular proteases.[44] This process, known as 'downregulation' functions to attenuate the PKC signal, thus preventing the persistent accumulation of activated kinases. Our data indicates that activation of PKC with low doses of TPA results in a modest induction of apoptosis as indicated by DNA fragmentation and caspase-3 activation. Based the low dose of TPA required, as well as the kinetics of DNA fragmentation and caspase activation, induction of apoptosis in response to TPA is likely to be due to activation, and not downregulation of PKC isoforms. To address this directly, we have used the calpain inhibitor, calpeptin, to determine if the magnitude of TPA-induced apoptosis can be increased under conditions where the degradation of activated PKC is inhibited. Parotid C5 cells were pre-treated with calpeptin prior to the addition of TPA to induce apoptosis. As seen in Figure 3A, treatment of parotid C5 cells with low doses of TPA results in the appearance of a faint DNA ladder, indicating fragmentation of DNA into nucleosomal units. However, pre-treatment of cells with calpeptin results in an increase in TPA-induced DNA fragmentation, as indicated by an increase in DNA ladder formation. Pre-treatment with 10 μM calpeptin increased DNA ladder formation slightly at all doses of TPA, while pre-treatment with 50 μM calpeptin resulted in a more robust increase in TPA-induced DNA fragmentation. However, while 10 μM calpeptin alone induce little or no DNA fragmentation, at 50 μM calpeptin DNA fragmentation was evident. Microscopically, apoptosis can be monitored by the condensation of the nucleus and cytoplasmic blebbing.[45] Figure 3B shows the morphology of untreated parotid C5 cells (panel 1), cells treated with 10 nM TPA (panel 2), cells treated with 50 μM calpeptin (panel 3), or cells pre-treated with 50 μM calpeptin proir to the addition of TPA (panel 4). As seen here, 10 nM TPA alone causes only minor rounding and detachment of the cells; changes in cell morphology consistent with the apoptotic phenotype. Pre-treatment with 50 μM calpeptin however dramatically increased the morphologic indicators of apoptosis, consistent with our observation that it enhances the apoptotic response to TPA. Quantification of the cells shown in Figure 3B indicates that $<1\%$ of untreated cells, 13% of TPA treated cells, 4% of calpeptin treated cells, and 73% of cells treated with both calpeptin and TPA, show morphologic indicators of apoptosis.

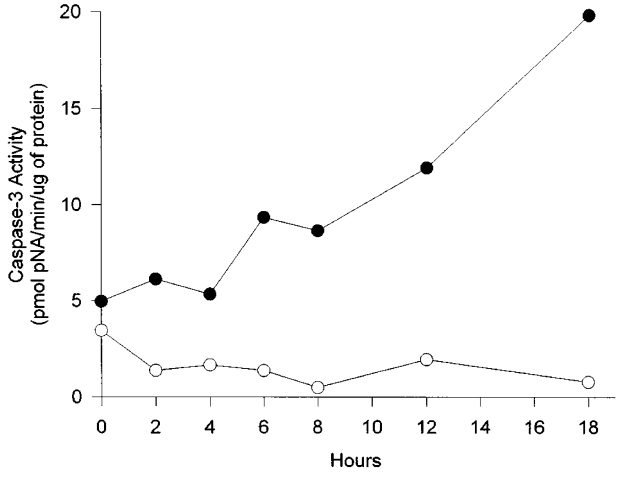
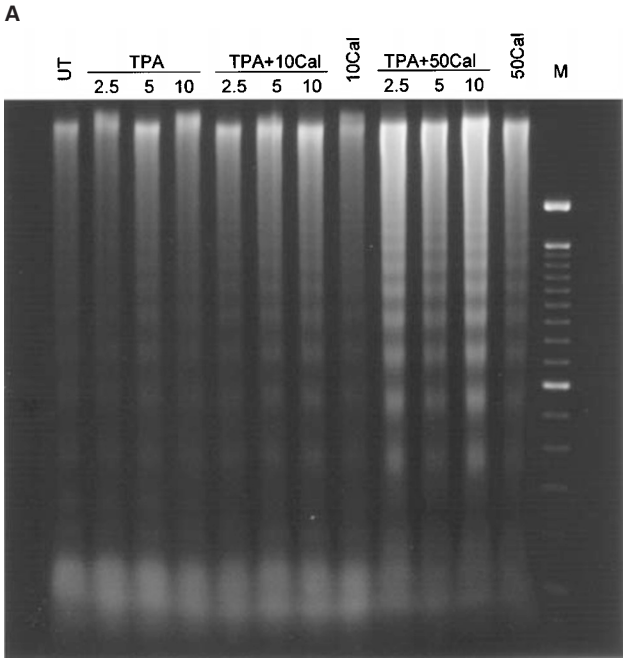


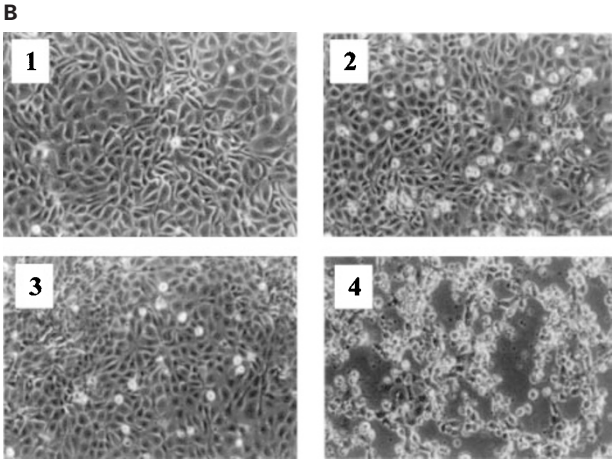
Figure 2 Activation of PKC induces caspase-3 activity in parotid C5 cells. Parotid C5 cells were treated with 10 nM TPA for the times indicated. Caspase-3 activity was assayed using the Caspase-3 Cellular Activity Assay Kit PLUS from BIOMOL Research Laboratories as described in Materials and Methods. This experiment was repeated three times with similar results

To determine if pre-treatment with calpeptin can enhance the activation of caspase-3 by TPA, parotid C5 cells were treated with 10 nM TPA, pre-treated with calpeptin (10 or 50 μM) prior to the addition of TPA, or treated with calpeptin alone for 4 h. As seen in Figure 3C, pre-treatment with 10 μM calpeptin increases TPA induced caspase-3 activity by about twofold. Pre-treatment with 50 μM calpeptin results in a slight, but not significant, further enhancement of caspase activity. This data suggests that one mechanism by which calpeptin enhances TPA induced apoptosis is by increasing caspase-3 activation.

The studies described above suggest that the stabilization of activated PKC by pre-treatment with calpeptin can enhance the apoptotic response to TPA. To determine if enhancement of TPA-induced apoptosis correlates with an increase in membrane associated PKC, PKC isoform expression was analyzed by immunoblot. As seen in Figure 4A, treatment of parotid C5 cells with TPA results in the selective downregulation of the conventional isoforms, PKCα and PKCδ. PKCα protein abundance in whole cell lysates is decreased dramatically at 4 h, and is nearly completely absent by 8 h. However, pre-treatment of parotid C5 cells with calpeptin prior to the addition of TPA results in the stabilization of PKCα. Under these conditions only a slight decrease in PKCα protein was observed at 4 h, although by 8 h PKCα protein abundance was significantly reduced. Similar results were observed for PKCδ, although PKCδ downregulation in response to TPA was somewhat slower than that observed for PKCα (Figure 4A). Pre-treatment of parotid C5 cells with calpeptin results in the stabilization of PKCδ as seen in Figure 4A. In TPA treated cells PKCδ protein is downregulated significantly at 4 h, however in calpeptin pre-treated cells, no decrease in PKCδ protein abundance was observed until 8 h after the addition of TPA, when it decreased by about 50%. The atypical isoform, PKCζ, which is not responsive to TPA, was not downregulated, and calpeptin alone had no effect on the total cellular level of PKCα, PKCδ or PKCζ.

Figure 4B and C show the subcellular distribution of PKCα and PKCδ, respectively in parotid C5 cells treated with TPA, with or without pre-treatment with calpeptin. In untreated cells, the majority of PKCα is found in the cytosol, however, upon the addition of TPA, PKCα protein in the cytosol decreases dramatically (Figure 4B). The rate of loss of PKCα from the cytosol appears to be similar in cells treated with TPA alone, and cells pre-treated with calpeptin prior to the addition of TPA. Loss of PKCα from the cytosol is associated with a transient increase in membrane-





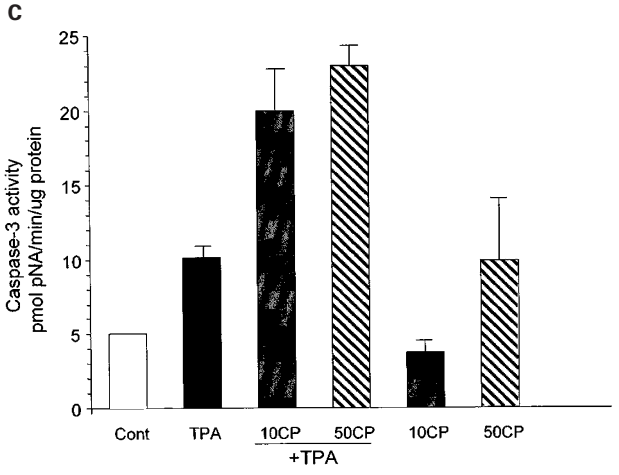


Figure 3 Calpeptin enhances TPA-induced apoptosis. (**A**) DNA fragmentation was assayed using a DNA ladder assay.[46] Parotid C5 cells were treated with 2.5, 5 or 10 nM TPA, pretreated with 10 or 50 μM calpeptin for 30 min prior to the addition of TPA, or treated with calpeptin alone for 24 h. UT=untreated, Cal=calpeptin, M=molecular weight markers (100 base pair DNA ladder). (**B**) Parotid C5 cells were treated with 10 nM TPA (panel 2), 50 μM calpeptin (panel 3), or pre-treated with 50 μM calpeptin for 30 min prior to the addition of TPA (panel 4). Total time of treatment was 24 h. Photomicrographs of the treated cells are shown; untreated cells are shown in panel 1. (**C**) Parotid C5 cells were treated with 10 nM TPA, pre-treated with calpeptin (CP; 10 or 50 μM) prior to the addition of TPA, or treated with calpeptin alone for 4 h. Caspase-3 activity was assayed using the Caspase-3 Cellular Activity Assay Kit PLUS from BIOMOL Research Laboratories as described in Materials and Methods

associated PKCα in TPA treated cells, and pre-treatment with calpeptin results in a more sustained increase in membrane-associated PKCα. In the absence of calpeptin, PKCα in the membrane fraction increases at 2 h after TPA treatment, but then decreases dramatically after 4 h, and is non-detectable after 8 h of TPA. In cells pre-treated with calpeptin, there is an increase in membrane associated PKCα at 2 h, which is maintained for up to 8 h following TPA treatment. Interestingly, although PKCα is downregulated at 18 h in cells pre-treated with calpeptin, in cells treated with TPA alone, PKCα (and PKCδ, see Figure 4C) re-accumulates in the cytosol at 18 h. This suggests that downregulation triggers an increase in PKC protein expression which results in replenishment of the basal level in the cell.

In contrast to PKCα, PKCδ appears to be evenly distributed between the cytosol and membrane in untreated parotid C5 cells (Figure 4C). Stimulation with TPA results in loss of PKCδ from the cytosol by 2 h, however, there is little or no increase in membrane-associated PKCδ protein at 2 h. The most likely explanation is that translocated PKCδ is rapidly downregulated, and thus does not accumulate at this time point. However, this data is also consistent with degradation of PKCδ in the cytosol of TPA treated parotid C5 cells. In parotid C5 cells pre-treated with calpeptin prior to the addition of TPA, PKCδ is depleted from the cytosol at a rate similar to that seen in cells treated with TPA alone, however, the membrane association of PKCδ is dramatically stabilized. Under these conditions membrane-associated PKCδ is not downregulated until 18 h, while in cells treated with TPA alone it is downregulated by 4 h. Taken together, these results suggest that association of activated PKC with the membrane fraction induces an apoptotic program in parotid C5 cells, and that apoptosis is enhanced under conditions where this association is stabilized.

Induction of apoptosis correlates with bi-phasic activation of JNK and transient activation of ERK

Members of the mitogen activated kinase (MAPK) family are activated in response to stimulation with mitogenic or apoptotic agents. We have previously demonstrated activation of the c-Jun-N-terminal protein kinase (JNK) pathway, and inactivation of the extracellular regulated kinase (ERK) pathway, in parotid C5 cells induced to undergo apoptosis with etoposide.[46] 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.[39] To determine if JNK activity is altered in parotid

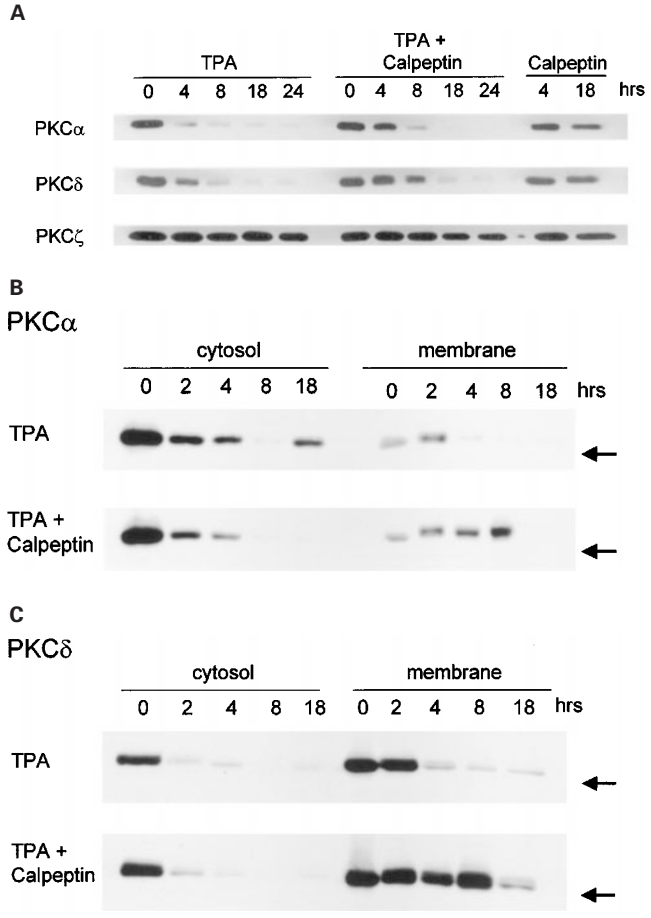


Figure 4 Calpeptin delays the degradation of activated PKC and results in an increase in membrane-associated PKC in TPA treated cells. Parotid C5 cells were treated with 10 nM TPA, pretreated with 50 μM calpeptin for 30 min prior to the addition 10 nM TPA, or treated with calpeptin alone as indicated. Time of treatment in hours is shown at the top of each lane. (**A**) Cells were harvested and expression of PKCα, PKCδ and PKCζ in whole cell lysates was determined by immunoblot analysis as described in Materials and Methods. (**B** and **C**) Cells were harvested and expression of PKCα (B) or PKCδ (**C**) in membrane and cytosol fractions was determined by immunoblot analysis. This experiment is representative of three similar experiments

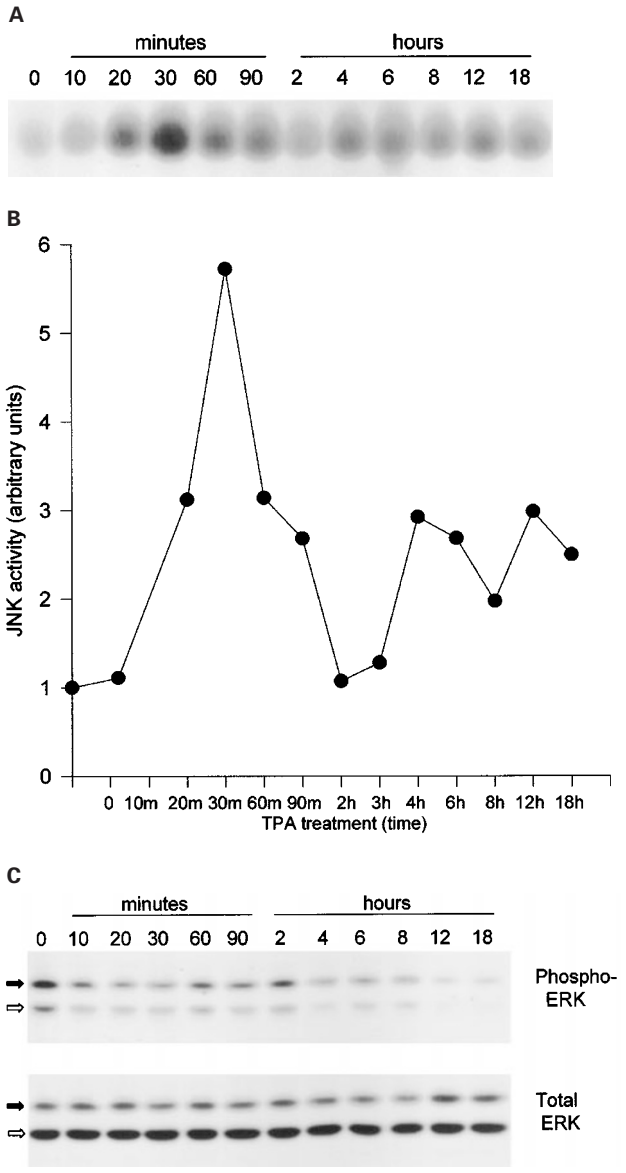


Figure 5 Induction of apoptosis correlates with bi-phasic activation of JNK and inactivation of ERK. Parotid C5 cells were untreated, or treated with 10 nM TPA. Time of stimulation is shown at the top of each lane. (**A**) Cell lysates were prepared and assayed for JNK activity using the GST-jun kinase 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. (**B**) Quantitation of changes in JNK activity upon treatment with TPA. The graph represents single values averaged from two experiments. (**C**) Cell lysates (25 μg) were resolved on an 10% polyacrylamide gel and immunoblotted with anti-active ERK2 which cross-reacts with both phosphorylated ERK1 and ERK2 (top). The immunoblot was stripped and reprobed with an anti-ERK antibody that recognizes both ERK1 and ERK2 (bottom). 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

C5 cells induced to undergo apoptosis with TPA, JNK activity was assayed in parotid C5 cells treated with 10 nM TPA for 10 min to 18 h. Figure 5A shows an autoradiograph of a GST-Jun assay for JNK activity, while Figure 5B shows the data from two such experiments quantified by Phosphoimager analysis. As seen here, activation of PKC with TPA results in the bi-phasic activation of JNK. The first peak of JNK activation is detectable by 10 min, is maximal at 30 min, and returns to a near basal level by 2 h. The maximal activation in this experiment is 10-fold, although this varied from 3–10-fold in five experiments due to differences in the basal level of JNK activity. This initial peak is followed by a second, smaller peak which appears by 4 h, and sustained for at least 12–18 h following the addition of TPA. The maximal fold-increase in JNK activity in this second peak was threefold in this experiment, and ranged from 2–5-fold in five separate experiments.

We have previously shown that in addition to activating JNK, stimulation of parotid C5 cells with etoposide results in transient activation of ERK1 and ERK2, suggesting that these pathways are reciprocally regulated in apoptotic

cells.[46] To determine the status of ERK in TPA treated parotid C5 cells, ERK1 and ERK2 activity was assayed in the same experiment shown in Figure 5A, using an anti-active ERK antibody that specifically recognizes the phosphorylated (active) forms of these kinases (Figure 5C). As seen in Figure 5C, top, ERK1 and ERK2 activity decreases by 10 min after the addition of TPA, increases slightly between 60 min and 2 h, and then decreases to nearly undetectable levels by 18 h. Uniform loading of the gels was demonstrated by reprobing the blots with an anti-ERK antibody that recognizes total ERK1 and ERK2 (Figure 5C, bottom). A comparison of Figure 5A and B with C, clearly shows that inactivation of ERK1 and ERK2 in TPA treated cells is coincident with the biphasic activation of JNK. In fact, the slight increase in ERK activity between 60 min and 2 h corresponds with the valley between the two peaks of JNK activation. This data supports our previous observation that the JNK and ERK signaling pathways are reciprocally regulated in parotid C5 cells undergoing apoptosis.

Transient expression of constitutively activated PKC isoforms induces apoptosis in parotid C5 cells

Our data indicate that activation of a conventional or novel isoform of PKC by TPA induces apoptosis in parotid C5 cells. To ask if expression of activated conventional or novel isoforms of PKC is sufficient to induce apoptosis in parotid C5 cells, we have utilized a transient transfection cell death assay. In this assay cells are transfected with the expression vector of interest, together with a reporter gene, β-galactosidase (β-gal) at a ratio of 10:1. Loss of β-gal expressing cells in the transfected population is indicative of apoptosis. This assay has been previously used to demonstrate regulation of apoptosis by ICE-like proteases,[47] PKCδ[36] and PKCζ.[30] As seen in Figure 6, expression of activated PKCα decreases the number of β-gal expressing cells by about 30% compared to the vector alone (control). Expression of activated PKCδ results in a more dramatic induction of apoptosis as indicated by a 50% decrease in β-gal expressing cells. In contrast, expression of activated PKCε did not induce apoptosis, although increased PKCε protein expression could be detected by immunoblot (data not shown). This supports our conclusions that activation of specific isoforms of PKC by TPA is sufficient to induce apoptosis, and that PKCα and PKCδ contribute to the apoptotic signal in parotid C5 cells.

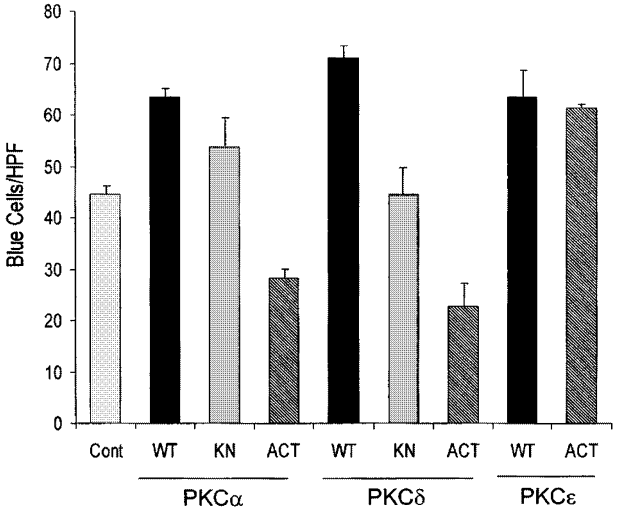


Figure 6 Transfection of activated PKCα or PKCδ induces apoptosis in salivary acinar cells. Parotid C5 cells were transiently transfected with 1 ug of pCMVβ-galactosidase together with 9 ug of the effector plasmid: pSRD empty vector (Cont), pSRDPKCα wild type (WT), pSRDPKCα kinase negative (KN), pSRDPKCα active (ACT), pSRDPKCδ wild type, pSRDPKCδ kinase negative, pSRDPKCδ active, pSRDPKCε wild type or pSRDPKCε active. After 48 h cell viability was determined by staining for β-gal expression. The data is reported as the average number of β-gal expressing cells per high power field (HPF). A minimum of 1000 cells were counted for each determination. This experiment was repeated four times with similar results

Discussion

The activation of specific isoforms of PKC occurs in response to a variety of apoptotic stimuli, suggesting that this family of protein kinases may contribute to regulation of the apoptotic pathway. The role of PKC in apoptosis is controversial however, with data supporting both pro- and anti-apoptotic functions.[22–24,31–34,48] Here we show that direct activation of the novel and conventional forms of PKC by TPA is sufficient to induce DNA fragmentation and activation of caspase-3 in salivary acinar cells. Our studies in salivary acinar cells are in agreement with data from other epithelial cell models, including breast, prostate and thyroid cells, that demonstrate that activation of PKC with TPA induces apoptosis,[32–34,48–50] and with data from keratinocytes showing that over expression of PKCδ induces apoptosis.[21]

Whelan and Parker[51] have recently reported that the loss of PKC function is sufficient to induce an apoptotic response in U937 cells and COS-1 cells. Since TPA causes both activation and downregulation of PKC, results obtained using this agent could conceivably reflect either of these processes. Our data argue that activation of PKC is responsible for the apoptotic response. First, only very low doses of TPA induce apoptosis; above 10 nM TPA the apoptotic response is typically reduced. Although the reason for this decrease is not clear, it may reflect the amount of time activated PKC is membrane associated, since at higher doses of TPA depletion of PKC from the membrane occurs more rapidly. Second, TPA-induced apoptosis can be enhanced by prior treatment of cells with a calpain inhibitor. Pre-treatment with calpeptin prolongs the association of PKCα and PKCδ with the membrane, which presumably prolongs the activated state of these isoforms. Third, expression of constitutively activated PKCα and PKCδ is sufficient to induce apoptosis in parotid C5 cells. Our studies therefore suggest that the translocation of activated PKC to the membrane signals an event (or events) that initiates the apoptotic pathway.

In breast and thyroid derived-epithelial cells, as well as in the monocytic cell line, U937, TPA-induced apoptosis appears to be p53-independent, since these cells either lack p53 or have a mutant form of p53.[31,33,50] Likewise,

PKCδ-induced cell death in HeLa cells and HPV-transformed keratinocytes is presumably p53-independent.[21] In the LNCaP prostate epithelial cell line however, TPA-induced apoptosis was preceded by induction of the cdk inhibitor, p21, and dephosphorylation of the retinoblastoma protein (Rb).[52] An essential role for Rb was confirmed by the demonstration that DU145 prostate epithelial cells, which do not express functional Rb, or LNCaP cells transfected with the Rb inhibitor, E1a, were resistant to TPA-induced apoptosis.[52] Since parotid C5 cells were derived from salivary gland acinar cells transfected with an SV40 T-antigen encoding plasmid, and still express SV40 T-antigen, the function of both retinoblastoma (Rb) protein and p53 is likely to be abrogated in these cells.[53] This is supported by our unpublished data which indicates that both wild type and mutant p53 can be detected in parotid C5 cells (Reyland and Matassa, unpublished data). Thus, TPA-induced apoptosis in this cell line is most likely mediated via a p53-independent pathway.

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,[54] growth factor withdrawn PC-12 cells,[15] Fas-induced Jurkat cell[55] and UV-irradiated fibroblasts.[30] In addition, we have previously reported that etoposide-induced apoptosis in parotid C5 cells correlates with activation of JNK and inactivation of ERK, and that regulation of these pathways in response to etoposide requires PKCδ activity.[46] In the current studies we extend this observation to demonstrate that reciprocal regulation of the ERK and JNK signaling pathways also occurs in parotid C5 cells undergoing TPA-induced apoptosis. Treatment of the parotid C5 cell line with TPA resulted in the bi-phasic activation of JNKs and in a decrease in the level of activated ERK. The first peak of JNK activation occurs at 20–60 min after the addition of TPA, while the second peak occurs at 4–12 h. In etoposide treated parotid C5 cells activation of JNK occured at 6–12 h, and this corresponded to the onset of apoptosis. Likewise, in TPA treated parotid C5 cells the onset of the second peak of JNK activation, the induction of DNA fragmentation, and the activation of caspase-3 were comparable. This suggests that the early peak of JNK activity in TPA treated cells is dissociated from apoptosis, whereas the second delayed peak, coupled with a decrease in the level of activated ERK, may contribute to the induction of TPA-induced apoptosis in parotid C5 cells. In this regard, Chen *et al*[11,56] have reported that early transient activation of JNK is associated with proliferation in Jurkat cells, while sustained activation of JNK is associated with apoptosis. Likewise, delayed, persistent activation of JNK is also seen in human KB-3 carcinoma cells undergoing apoptosis in response to chemotheraputic drugs,[13] and in human glioma cells undergoing calphostin C-induced apoptosis.[54]

Our data demonstrates that TPA, a direct activator of the conventional and novel PKC isoforms can induce apoptosis in parotid C5 cells. TPA however appears to be much weaker stimulus for apoptosis than etoposide; the level of DNA fragmentation and caspase-3 activation in response to TPA is only about 20% of that seen in parotid C5 treated with etoposide.[46] Likewise, TPA only weakly induces an apoptotic morphology (Figure 3B), and cleavage of PKCδ, which we have previously demonstrated in parotid C5 cells in response to other agents which induce apoptosis[39], is not detected in TPA treated cells (Reyland and Matassa, unpublished data). These differences may simply reflect the modest level of apoptosis induced by TPA, or alternatively they may indicate that direct activation of PKC by TPA replicates only part of the total apoptotic program. Previous work from our laboratory demonstrates that PKCα and PKCδ are activated during etoposide induced apoptosis, and that PKCδ activity is essential for etoposide induced apoptosis in parotid C5 cells.[39] These previous studies support a pro-apoptotic function for both full-length and caspase-3 cleaved PKCδ, since the PKCδ inhibitor, rottlerin, blocks caspase-3 activation and PKCδ cleavage in etoposide treated cells.[39] Although it is not known if these forms of PKCδ have specific substrates, it is possible that in the absence of PKCδ cleavage, as in the case of TPA-induced apoptosis, only a partial apoptotic program is induced. Alternatively, since activation of PKCδ by etoposide is due to cleavage and release of the catalytic domain,[39] this may be a more efficient way to activate PKCδ, resulting in higher levels of activated PKCδ, and more pronounced apoptosis in these cells.

Our previous data demonstrate that PKCα and PKCδ are activated during etoposide-induced apoptosis, and that inhibition of PKCδ activity suppresses apoptosis.[39] Here we show that enhancement of apoptosis correlates with the prolonged activation of these isoforms, and that expression of activated PKCα and PKCδ can induce apoptosis in parotid C5 cells. Although we have used a salivary acinar cell line derived from the parotid gland for the studies presented here, TPA, and expression of activated PKCα and PKCδ can also induce apoptosis induce apoptosis in a salivary acinar cell line derived from the submandibular gland (Reyland and Quissell, unpublished data). Taken together, these studies suggest that activation of PKC is an essential part of the apoptotic program in salivary acinar cells, and that PKCα and PKCδ transduce a pro-apoptotic signal in these cells.

Materials and Methods

Cell culture

The isolation of the immortalized salivary parotid C5 cell line has been described elsewhere.[53] Cells were cultured on Primaria 60 mm culture dishes (Falcon Plastics, Franklin Lakes, NJ, USA) in DMEM/F12 (1 : 1 mixture) 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 1-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.

Cell death transfection assay

Parotid C5 cells were transfected using Per-fect 6 (Invitrogen). After 48 h cell viability was determined by staining for β-gal expression. The

induction of apoptosis is associated with the selective loss of β-gal staining cells. The assay is quantitated by counting the average number of stained cells per high power field. All constructs were in the vector pSRD.[57] Wild type PKCα, δ and ε, and the constitutively activated and kinase-negative forms of PKCα and δ, were a generous gift of Dr. S. Ohno, Yokohama University.

Subcellular fractionation

Cells in 100 mm dishes were washed with phosphate buffered saline, extracted in one ml Buffer A (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 25 ug/ul each aprotinin and leupeptin), and homogenized with 25 strokes of a Dounce homogenizer. The homogenate was transferred to a microcentrifuge tube and centrifuged in a microcentrifuge at 4°C, at 10 K for 2 min to clarify. The clarified homogenate was centrifuged at 4°C in a TL100 ultracentrifuge (TL100.3 rotor) at 45 K for 30 min. The supernatant was collected as the cytosol fraction and Triton X-100 was added to a final concentration of 0.5% (vol/vol). The pellet was carefully washed twice with Buffer A, and resuspended in 500 ul Buffer A containing 0.5% Triton X-100. The pellet solution was vortexed vigorously, incubated on ice for 30 min, and centrifuged in a microcentrifuge at 4°C, at 10 K for 2 min. The supernatant was collected as the membrane fraction.

Immunoblot analysis

Adherent and floating cells were scraped into the culture media, collected by centrifugation (3000 $\times$ *g* for 10 min), washed once with phosphate buffered saline, 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 r.p.m. for 5 min in a refrigerated Savant SRF13K microfuge. The preparation of particulate and cytosol preparations has been previously described.[58] Protein concentration was determined using a Bradford assay kit purchased from Biorad. Cell lysates (25–50 μg) were resolved on a 10% gel, transferred to an Immobilon membrane (Millipore), and immunoblotted with the desired antibody as described previously.[59] Enhanced chemiluminescence (ECL, Amersham) followed by autoradiography was used to detect the signal. Antibodies to PKC isoforms were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All anti-PKC antibodies recognize epitopes in the carboxy-terminal portion of the protein. The anti-active 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).

Assay for DNA fragmentation

DNA fragmentation was assayed using the 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. In some experiments DNA fragmentation was assayed using the DNA ladder assay as previously described.[46]

Assay for Caspase-3 activity

The activation of caspase-3 was detected with the Caspase-3 Cellular Activity Assay Kit PLUS obtained from BIOMOL Research laboratories (Plymouth Meeting, PA, USA) which uses N-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD FMK-pNA) as a substrate. The assays were conducted in accordance with the manufacturer's recommendations.

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.[10] JNK activation was assayed using the GST-Jun kinase assay.[60] To collect both adherent and floating cells, cells were scraped into the culture media, collected by centrifugation (3000 $\times$ *g* for 10 mins), washed once with phosphate buffered saline, and resuspended in one ml of JNK lysis buffer. The lysate was allowed to sit on ice for 30 min and then clarified by spinning at 12 500 r.p.m. for 5 min in a refrigerated Savant SRF13K microfuge. For the assay a 100 μl volume of a 10% suspension of GST-c-*jun* (1–79) was added to 300 μg 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 mM HEPES, pH 7.7, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100. Forty μl of 50 mM β-glycerophosphate, pH 7.6, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$ and 20 μM ATP containing 10 mCi γ-^{32}P-ATP (5000 c.p.m./pmol in the final reaction) was added to the washed beads and the reaction was incubated at 30°C for 20 min. The reactions were terminated by the addition of 10 μl 5 $\times$ 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.

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