MITOCHONDRIAL TRANSLOCATION OF PROTEIN KINASE C δ IN PHORBOL ESTER-INDUCED CYTOCHROME C RELEASE AND APOPTOSIS

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Abbreviations used are: PKC, protein kinase C; TPA, 12-0-tetradecanoylphorbol-13-
Apoptosis is induced by the release of cytochrome c from mitochondria to the cytoplasm. The present studies demonstrate that the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) induces translocation of protein kinase C (PKC)δ from the cytoplasm to mitochondria. The results also show that translocation of PKCδ results in release of cytochrome c. The functional significance of this event is further supported by the demonstration that PKCδ translocation is required for TPA-induced apoptosis. These findings demonstrate that translocation of PKCδ to mitochondria is responsible at least in part for inducing cytochrome c release and apoptosis.
INTRODUCTION

The protein kinase C (PKC) family of serine/threonine protein kinases is involved in intracellular signals that regulate growth, differentiation and apoptosis. The PKC isoforms have been subdivided into: i) the conventional PKCs (cPKCs; α, β, γ) which are dependent on calcium and activated by diacylglycerol (DAG) or 12-O-tetradecanoylphorbol-13-acetate (TPA); ii) the novel PKCs (nPKCs; δ, ε, θ, η) which are calcium-independent and activated by DAG or TPA; and iii) the atypical PKCs (aPKCs; ζ, λ) which are calcium-independent and not activated by DAG or TPA (1,2). The cPKCs are cleaved in the third variable region by calpains I and II to catalytically active fragments (3). Other studies have demonstrated that the nPKC, PKCδ, is cleaved in the third variable region by caspase 3 in the apoptotic response of cells to DNA damage and engagement of the tumor necrosis factor (TNF) receptor (4-6). The cleaved catalytic fragment of PKCδ is constitutively activated and, when overexpressed in HeLa and other cells, is sufficient to induce apoptosis (6-8). Proteolytic activation of PKCδ has also been implicated in ultraviolet radiation-induced apoptosis of keratinocytes (9). These findings have supported a role for the PKCδ isoform in the apoptotic response of cells to diverse stimuli.

The treatment of human myeloid leukemia cells with TPA is associated with the induction of apoptosis (10). Other studies have demonstrated that TPA induces apoptosis of human MCF-7
breast cancer cells and Jurkat T cells (11,12). These findings have suggested that TPA-induced activation of certain PKC isoforms confers signals that induce apoptosis. However, the identity of the PKC isoform(s) that are activated in TPA-induced apoptosis and the downstream signals that confer the apoptotic response are unknown. The available evidence indicates that at least two pathways induce apoptosis in the cellular response to other stimuli. One pathway is initiated by engagement of the TNF or Fas receptors and thereby activation of caspase 8 (13,14). In turn, caspase 8 cleaves Bid and induces cytochrome c release (15,16). Caspase 8 can also directly activate caspase 3 (17). In the second pathway, other signals, which importantly remain undefined, converge to induce the release of cytochrome c (18,19). Cytosolic cytochrome c binds to Apaf-1, induces the autoprocessing of caspase 9 and thereby the activation of caspase 3 (16,20,21). Neither of these pathways has been linked to TPA-induced apoptosis.

The present studies demonstrate that TPA treatment is associated with the translocation of cytoplasmic PKCδ to mitochondria. The results show that translocation of PKCδ induces the release of cytochrome c and the activation of caspase 3. These findings support a novel mechanism for TPA-induced cytochrome c release and apoptosis.
MATERIALS AND METHODS

Cell culture and reagents. Human U-937 myeloid leukemia cells (ATCC, Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. MCF-7, MCF-7/neo, MCF-7/PKCδRD and 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS. Cells (3 x 10⁶/150 mm culture dish) were plated 24 h before treating with 250 nM TPA (Sigma Chemical Co.), 100 nM bryostatin 1 (ICN, Ohio), 10 µM 1,2-dioctanoyl-sn-glycerol (DOG; Calbiochem) and 0.5 units/ml phospholipase C (PLC; Sigma). Cells were also treated with 10 µM rottlerin (Calbiochem).

Isolation of mitochondria. Cells were washed twice with phosphate buffer saline (PBS), homogenized in buffer A (210 mM manitol, 70 mM sucrose, 5 mM HEPES, 1mM EGTA) and 110 ug/ul digitonin in a glass homogenizer (Pyrex no. 7727-07) and centrifuged at 5000g for 20 min. Pellets were resuspended in buffer A, homogenized in a small glass homogenizer (Pyrex no. 7726) and centrifuged at 2000g for 5 min. Supernatant (S1) was collected and the pellet again homogenized in of buffer A. Supernatant (S2) was collected after centrifugation at 2000g for 5 min. Supernatants S1 and S2 were mixed and centrifuged at 11000g for 10 min. Mitochondrial pellets were disrupted in lysis buffer at 4°C for 30 min and then centrifuged at 15000g for 20 min. The concentration of mitochondrial proteins in the
supernatant was determined using Bio-Rad protein estimation kit.

**Isolation of the cytosolic fraction.** Cells were washed twice with PBS and the pellet was suspended in 5 ml of ice-cold buffer B containing 250 mM sucrose. The cells were homogenized by disrupting three times in a Dounce homogenizer in buffer B (20 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 10 µg/ml leupeptin and aprotinin). After centrifugation for 5 min at 4°C, the supernatants then were centrifuged at 105,000 x g for 30 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction.

**Immunoprecipitation and immunoblot analysis.** Total, cytoplasmic or mitochondrial lysates were subjected to immunoprecipitation with anti-GFP, anti-PKCγ (Santa Cruz Biotechnology, CA), anti-PKCμ (Santa Cruz), anti-PKCζ (UBI), anti-PKCθ (Santa Cruz), anti-PKCη (Santa Cruz), anti-PKCE (Santa Cruz) or anti-PKCδ (Santa Cruz) antibodies. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The residual binding sites were blocked by incubating the filters with 5% nonfat dry milk in PBST (PBS/0.05% Tween 20). The filters were incubated with anti-PKCδ, anti-cytochrome c (22), anti-Hsp-60 (Stressgen, Canada), anti-Actin (Sigma), anti-PKCγ, anti-PKCμ, anti-PKCζ or anti-GFP (Clontech, Palo Alto, CA). After washing twice with PBST, the filters were incubated with anti-rabbit or anti-mouse IgG peroxidase conjugate and developed
by ECL (Amersham).

**Plasmids.** pEGFP-PKCδ and PKCδ-RD were prepared as described (23). The pEGFP-PKCδ(K378R) was generated by site-directed mutagenesis (24).

**Transient transfections.** 293T cells were transiently transfected with empty vector (pEGFP-C1), GFP-PKCδ or pEGFPCy-PKCδ (K378R) using SuperFect (Qiagen). At 24 h after transfection, cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 µg/ml leupeptin and aprotinin) and subjected to immunoblotting with anti-PKCδ and anti-GFP. Signal intensities were determined by densitometric analysis.

**Immunofluorescence microscopy.** Cells immobilized on slides were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, incubated with 20 ng anti-PKCδ/slide and then Texas Red-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Inc). Mitochondria were stained with 0.006 ng/slide of Mitotracker Green FM (Molecular Probes). The slides were analyzed using a Zeiss Auxiphot fluorescence microscope coupled to a CCD camera and a power Macintosh 8100. Image analysis was performed using the IPLab Spectrum 3.1 software (Signal Analytics).

**PKCδ activity assays.** 293T cells were transiently transfected with GFP-PKCδ or GFP-PKCδ(K-R). Total cell lysates were subjected to immunoprecipitation with anti-PKCδ, anti-PKCθ, anti-
PKCε, anti-PKCη, anti-PKCµ or anti-PKCζ. The immune complex kinase assays were performed using H1 histone as a substrate as described (25).

**Quantitation of apoptosis by flow cytometric analysis.** Cells were harvested, washed twice with PBS and fixed with 80% ethanol. Cells (10⁶ cells/ml) were washed and incubated with propidium iodide (2.5 µg/ml) and RNase (50 µg/ml). FACScan (Becton Dickinson) was used to assess cells with sub-G1 DNA content.
RESULTS AND DISCUSSION

PKC isoforms regulate diverse cellular processes, but are not known as effectors of mitochondria (1). To determine whether PKC regulates mitochondrial function, human U-937 cells were treated with TPA to activate PKC. PKC translocation was assessed by subjecting cytoplasmic and mitochondrial fractions to immunoblotting with anti-PKC antibodies. The results demonstrate that TPA treatment is associated with decreases in cytoplasmic PKCδ and concomitant increases in mitochondrial PKCδ (Fig. 1A). As controls, the cytoplasmic and mitochondrial fractions were also subjected to immunoblotting with anti-actin and anti-Hsp60 to ensure purity of the preparations (Fig. 1A). By contrast to translocation of PKCδ, TPA had no detectable effect on cytoplasmic or mitochondrial levels of PKCγ and PKCζ (Fig. 1B and data not shown). The immunoblots were scanned to calculate percent PKCδ translocation to mitochondria. The results demonstrate that approximately 40% of PKCδ translocates to mitochondria in response to TPA.

The demonstration that PKCδ also translocates to mitochondria in TPA-treated MCF-7 cells indicates that the finding is not restricted to certain cell types (Fig. 2A). In addition, to confirm the subcellular redistribution of PKCδ in TPA-treated cells, we visualized intracellular fluorescence with a CCD camera and image analyzer. Examination of fluorescence markers in control cells showed distinct patterns for anti-PKCδ (red signal) and a mitochondrion-selective dye (Mitotracker;
green signal) (Fig. 2B). The demonstration that TPA induces a marked change in fluorescence signals (red and green—he yellow/orange) supported translocation of PKCδ to mitochondria (Fig. 2B). These findings obtained by immunofluorescence microscopy thus confirm the results of PKCδ redistribution found by subcellular fractionation.

To determine whether the natural product bryostatin, which activates PKC (26), also induces the translocation of PKCδ, mitochondrial lysates from U-937 cells treated with 100 nM bryostatin were subjected to immunoblot analysis with anti-PKCδ. As a control, mitochondrial lysates were also subjected to immunoblot analysis with anti-PKCζ. The results demonstrate that, in contrast to PKCζ, treatment with bryostatin was associated with translocation of PKCδ to mitochondria (Fig. 3A).

Phospholipase C (PLC) is activated by cell membrane-initiated signaling pathways (27,28) and by conferring the hydrolysis of phosphatidylinositol or phosphatidylcholine, results in the formation of DAG (29). To determine whether PLC induces the translocation of PKCδ, mitochondrial lysates from U-937 cells treated with 0.5 units/ml PLC were subjected to immunoblot analysis with anti-PKCδ. The results demonstrate that, treatment with PLC is associated with translocation of PKCδ to mitochondria (Fig. 3B). To confirm the involvement of DAG in mitochondrial translocation of PKCδ, cells were treated with a cell permeable DAG (DOG) (30). Immunoblot analysis of DOG-treated cell lysates demonstrated that DOG induced the translocation of PKCδ to
mitochondria (Fig. 3C). These findings indicate that, like TPA, treatment with byostatin, PLC and DOG is associated with redistribution of cytosolic PKCδ to mitochondria.

To determine whether activation of PKCδ is required for translocation to mitochondria, we transfected cells with a vector expressing green fluorescence protein (GFP)-tagged PKCδ. Immunoblot analysis with anti-GFP demonstrated no detectable PKCδ in the mitochondrial fraction from cells transfected with an empty GFP vector (Fig. 4A). By contrast, transfection of kinase-active GFP-PKCδ was associated with PKCδ expression in mitochondria (Fig. 4A). Moreover, treatment of the GFP-PKCδ-transfected cells with TPA resulted in further increases in levels of mitochondrial PKCδ (Fig. 4A). Significantly, transfection of kinase-inactive GFP-PKCδ(K-R) had no effect on expression of mitochondrial PKCδ (Fig. 4A). In addition, overexpression of GFP-PKCδ(K-R) blocked the TPA-induced translocation of PKCδ to mitochondria (Fig. 4A). To demonstrate that PKCδ(K-R) specifically blocks endogenous PKCδ activity, and not that other isoforms of PKC, 293T cells were transiently transfected with GFP-PKCδ or GFP-PKCδ(K-R). Following transfection, cell lysates were subjected to immunoprecipitation with anti-PKCδ, anti-PKCμ, anti-PKCζ, anti-PKCθ, anti-PKCη or anti-PKCε. The precipitates were assayed in in vitro kinase assays using H1 histone as substrate. The results demonstrate that, in contrast to PKCμ, PKCζ, PKCθ or PKCη, overexpression of PKCδ(K-R) specifically inhibits the activity of endogenous PKCδ.
Of note, the results also indicate that overexpression of PKCδ(K-R) is associated with slight inhibition of the phosphorylated and active PKCε (Fig. 4B). PKCδ consists of an N-terminal regulatory domain (RD) and a C-terminal catalytically active fragment (CF) (6). MCF-7 cells stably transfected to express the 35 kDa RD exhibit attenuation of TPA-induced PKCδ activity (unpublished data). Translocation of PKCδ to mitochondria was also attenuated in TPA-treated MCF-7/PKCδRD cells as compared to that in MCF-7 cells expressing the empty neo vector (Fig. 5A). Other studies were performed with rottlerin, a selective inhibitor of PKCδ activation (31). Treatment of U-937 cells with rottlerin abrogated TPA-induced localization of PKCδ to mitochondria (Fig. 5B). These findings collectively demonstrate that PKCδ activation is necessary for its translocation to mitochondria.

The potential role of PKCδ translocation was explored by assessing mitochondrial release of cytochrome c. Whereas diverse apoptotic signals induce cytochrome c release (32-34), phorbol ester treatment of cells has not been associated with this event. Immunoblot analysis of cytoplasmic fractions with anti-cytochrome c demonstrated that TPA treatment of U-937 cells is associated with cytochrome c release (Fig. 6A). Similar results were obtained when U-937 cells were treated with PLC or DOG (Figs. 6B and C). To determine whether PKCδ functions in inducing cytochrome c release, we pretreated U-937 cells with rottlerin before adding TPA. Of note, treatment of cells with rottlerin
alone is associated with cytotoxic effects that contribute to a detectable release of cytochrome c (Fig. 7A). By contrast, analysis of cytoplasmic lysates demonstrated that rottlerin significantly blocks TPA-induced cytochrome c release (Fig. 7A). As these findings indicate that the PKCδ kinase function is required for TPA-induced release of cytochrome c, 293T cells were transfected to express GFP, GFP-PKCδ or GFP-PKCδ(K-R) and then treated with TPA. Immunoblotting of the cytoplasmic fraction from GFP positive cells demonstrated abrogation of TPA-induced cytochrome c release in cells expressing PKCδ(K-R) compared to that in cells transfected with the GFP-PKCδ vector (Fig. 7B). Taken together, these results and those obtained for PKCδ translocation support a role for PKCδ, in the mitochondrial release of cytochrome c.

The release of cytochrome c from mitochondria triggers activation of caspases and induction of apoptosis (35). To determine whether TPA-induced PKCδ translocation and thereby cytochrome c release contributes to apoptosis, U-937 cells treated with rottlerin and TPA were assayed for sub-G1 DNA content. The results demonstrate that treatment with rottlerin alone induces a low level of apoptosis (Fig. 8A). By contrast, the apoptotic response of U-937 cells to TPA was significantly attenuated by inhibition of PKCδ with rottlerin (Fig. 8A). Moreover, treatment of MCF-7/neo cells with TPA was also associated with the induction of apoptosis (Fig. 8B). By contrast, the apoptotic response to TPA was significantly attenuated in MCF-7/PKCδRD cells (Fig. 8B). Taken together with our other findings, these
results support a role for TPA-induced localization of PKCδ to mitochondria and in the induction of apoptosis.

Previous work has demonstrated that TPA treatment is associated with translocation of PKCδ to the cell membrane (36). The present studies demonstrate that TPA treatment of diverse cell types is associated with translocation of PKCδ to mitochondria. These findings have been confirmed by cell fractionation and immunofluorescence studies. The results further demonstrate that the PKCδ kinase function is necessary for TPA-induced mitochondrial localization. The functional significance of PKCδ translocation to mitochondria is supported by the finding that this event is linked to mitochondrial release of cytochrome c. Moreover, the results demonstrate that abrogation of PKCδ translocation to mitochondria significantly inhibits TPA-induced apoptosis. These findings thus support a model in which TPA induces the release of cytochrome c and thereby apoptosis by a PKCδ-dependent mechanism.
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**FIGURE LEGENDS**

**Figure 1.** Translocation of PKCδ to mitochondria in response to TPA treatment. A. U-937 cells were treated with 250 nM TPA for the indicated times. The cells were harvested and separated into cytosolic (cyto) and mitochondrial (mito) fractions. Proteins were subjected to 10% SDS-PAGE and immunoblot analysis with anti-PKCδ. As controls, lysates were also analyzed by immunoblotting with anti-Actin or anti-Hsp60. B. U-937 cells were treated with 250 nM TPA for the indicated times. Cytosolic and mitochondrial fractions were analyzed by immunoblotting with anti-PKCγ or anti-PKCζ.

**Figure 2.** Translocation of PKCδ to mitochondria in response to TPA treatment. A. MCF-7 cells were treated with 250 nM TPA for 1 h. Cytosolic (Cyto) and mitochondrial (Mito) fractions were analyzed by immunoblotting with anti-PKCδ. B. U-937 cells were treated with TPA for 1 h. After washing, the cells were immobilized on slides, fixed and incubated with anti-PKCδ followed by Texas Red-conjugated goat anti-rabbit IgG. Mitochondria were stained with the mitochondria-selective permeant dye Mitotracker Green FM.

**Figure 3.** Translocation of PKCδ to mitochondria in response to bryostatin, PLC or DOG treatment. A. U-937 cells were treated with bryostatin (Bryo) for the indicated times. Proteins from the mitochondrial (Mito) fraction were subjected to immunoblot
analysis with anti-PKCδ or anti-PKCζ. As control, mitochondrial lysates were also analyzed by immunoblotting with anti-Hsp60. B and C. U-937 cells were treated with 0.5 units/ml PLC (B) or 10 µm DOG (C) and harvested after indicated times. Proteins from the mitochondrial fraction were subjected to immunoblot analysis with anti-PKCδ. As control, mitochondrial lysates were also analyzed by immunoblotting with anti-Hsp60.

Figure 4. PKCδ kinase function is necessary for TPA-induced translocation to mitochondria. A. 293T cells were transiently transfected to express empty vector, PKCδ or PKCδ(K-R). After 24 h, the cells were treated with TPA for 1 h. Lysates prepared from mitochondria (upper panel) or intact cells (lower panel) were subjected to 10% SDS-PAGE and immunoblot analysis with anti-GFP. B. 293T cells were transfected with GFP-PKCδ (wt) or GFP-PKCδ (K-R). Following transfection, total cell lysates were subjected to immunoprecipitation with anti-PKCδ, anti-PKCµ, anti-PKCζ, anti-PKCθ, anti-PKCη or anti-PKCε. The precipitates were assayed for phosphorylation of H1 histone (upper panels). Anti-PKCδ, anti-PKCµ, anti-PKCζ, anti-PKCθ or anti-PKCε immunoprecipitates were analyzed by immunoblotting with anti-PKCδ, anti-PKCµ, anti-PKCζ, anti-PKCθ or anti-PKCε respectively (middle panels). Total Lysates were also analyzed by immunoblotting with anti-GFP (lower panels).
Figure 5. A. MCF-7/neo and MCF-7/PKCδRD cells were treated with TPA for the indicated times. Mitochondrial lysates were subjected to immunoblot analysis with anti-PKCδ. B. U-937 cells were treated with TPA for 1 h or 10 µM rottlerin (ROT) for 1.5 h. Cells were also treated with rottlerin for 0.5 h before adding TPA for an additional 1 h (ROT/TPA). Mitochondrial lysates were subjected to immunoblot analysis with anti-PKCδ.

Figure 6. Regulation of TPA-induced cytochrome c release by PKCδ. A. U-937 cells were treated with TPA for the indicated times. Cytosolic lysates were subjected to immunoblot analysis with anti-cytochrome c (cyt c). B and C. U-937 cells were treated with 0.5 units/ml PLC (B) or 10 µM DOG (C) for the indicated times. Cytosolic lysates were subjected to immunoblot analysis with anti-cytochrome c. As control, cytoplasmic lysates were also analyzed by immunoblotting with anti-Actin.

Figure 7. A. U-937 cells were treated with TPA for 6 h or rottlerin (ROT) for 6.5 h. Cells were also treated with rottlerin for 0.5 h before adding TPA for an additional 6 h (ROT/TPA). Total cell lysates were analyzed by immunoblotting with anti-cytochrome c (upper panel). Signal intensities from the anti-cytochrome c immunoblotting experiments described in the upper panel were analyzed by densitometric scanning. The signal intensities are expressed in arbitrary value as the mean ± S.D of three independent experiments (lower panel). B. 293T cells were
transiently transfected to express empty vector, PKCδ or PKCδ(K-R). After 24 h, the cells were treated with TPA for 3 h. Cytosolic lysates were analyzed by immunoblotting with anti-cytochrome c.

Figure 8. Regulation of TPA-induced apoptosis by PKCδ. A. U-937 cells were treated with TPA for 12 h or rottlerin for 12.5 h. Other cells were treated with rottlerin (ROT) for 0.5 h before adding TPA for an additional 12 h (ROT/TPA). Cells were fixed in 80% ethanol, stained with propidium iodide and analyzed by FACScan. B. MCF-7/neo and MCF-7/PKCδRD cells were treated with TPA for 12 h. Untreated (solid bars) and TPA-treated (hatched bars) cells were fixed in ethanol, stained with propidium iodide and analyzed by FACScan. The results are expressed as mean ± SD percentage apoptosis determined from two independent experiments each performed in duplicate.
### A.

| kDa | MCF-7 | TPA | MCF-7 | TPA |
|-----|-------|-----|-------|-----|
| 89  |       |     |       |     |
| 65  |       |     |       |     |

IB: anti-PKCδ

### B.

- U-937
- TPA
Mitochondrial translocation of protein kinase C delta in phorbolester-induced cytochrome C release and apoptosis
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