Evidence That Protein Kinase Cε Mediates Phorbol Ester Inhibition of Calphostin C- and Tumor Necrosis Factor-α-induced Apoptosis in U937 Histiocytic Lymphoma Cells*

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Protein kinase C (PKC) activators, such as the tumor-promoting phorbol esters, have been reported to protect several cell lines from apoptosis induced by a variety of agents. Recent evidence suggests that PKCε is involved in protection of cardiac myocytes from hypoxia-induced cell death (Gray, M. O., Karliner, J. S., and Mochly-Rosen, D. (1997) J. Biol. Chem. 272, 30945–30951). We investigated the protective effects of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) on U937 histiocytic lymphoma cells induced to undergo apoptosis by tumor necrosis factor-α (TNF-α) or by the specific PKC inhibitor calphostin C. U937 cells were transiently permeabilized with a peptide (εV1-2) derived from the V1 region of PKCε that has been reported to specifically block translocation of PKCε. The εV1-2 peptide blocked the inhibitory effect of TPA on both TNF-α- and calphostin C-induced apoptosis. A scrambled version of εV1-2 and a peptide reported to inhibit PKCβ translocation (βC2-4) had no effect on the ability of TPA to inhibit apoptosis. These results suggest that PKCε is required for the protective effect of TPA in TNF-α- and calphostin C-induced apoptosis. Furthermore, calphostin C reduced membrane-associated PKCε activity and immunoreactivity, suggesting that PKCε may play an important role in leukemic cell survival.

The phospholipid-dependent protein kinase C (PKC) family of isoforms has a central role in the transduction of extracellular signals and has been implicated in tumor promotion (1). PKCs also appear to be important in the regulation of apoptosis, and several reports have indicated that PKC activation can inhibit apoptosis. For example, the tumor-promoting phorbol ester and potent PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) prevents apoptosis induced by tumor necrosis factor-α (TNF-α), Fas, and microtubule-disrupting drugs in human lymphoma cells (2, 3); by growth factor deprivation in leukemic and normal myeloid cells (4); and by anticancer drug treatment of leukemic cells and a human breast cancer cell line (5, 6). TPA also inhibits apoptotic oligonucleosomal DNA degradation in isolated nuclei from thymocytes (7). Other PKC activators have also been reported to inhibit apoptosis. These include phorbol dibutyrate, which protects mouse spleen blast cells against interleukin-2 withdrawal (8), and mezerein and bryostatin-1, which block apoptosis induced by the sphingolipid ceramide (9). A synthetic analogue of the natural PKC activator diaclylglycerol has also been reported to prevent apoptosis in human leukemic cells induced by ceramide and the isoprenoids farnesol and geranylglycerol (9–11).

It should be noted, however, that there are some reports that demonstrate that PKC activation can induce or increase apoptosis in some cell types (12–14). The effects of PKC activation on apoptosis may therefore be cell type-specific and could be determined by factors such as the rate of PKC down-regulation.

Evidence has recently emerged suggesting that specific PKC isoforms may be involved in the prevention of apoptosis. Mochly-Rosen and co-workers (15) have used peptides that specifically block the interaction of PKC subforms with membrane-anchored binding proteins and have demonstrated that a peptide that inhibited membrane translocation of PKCε blocked the protection of cardiac myocytes from hypoxia-induced cell death. In another study, apoptosis in human leukemic cells induced by TNF-α, anti-Fas antibody, sphingomyelinase, and ceramide was associated with the redistribution of PKCδ and PKCε from the membrane to the cytosol. These redistributions were prevented by concentrations of TPA that blocked apoptosis (16).

Further reports suggest that PKCε is anti-apoptotic in some cells. For example, in HT58 human lymphoblastic cells, TPA causes growth inhibition and down-regulation of PKC (50). These cells could be induced to undergo apoptosis by the general kinase inhibitor staurosporine, but only when the cells had been pretreated with low concentrations of TPA that down-regulated PKCε, but not PKCε and PKCβ (17). In oncogene-transformed rat embryo fibroblasts, susceptibility to the anticancer drug cisplatin is increased, whereas PKCε expression is decreased compared with nontransformed cells. Stable transfection of these cells with PKCε prevents cisplatin-induced apoptosis and also protects these cells against cisplatin cytotoxicity (17).

Taken together, these reports suggest that PKCε may be of particular significance in the negative regulation of apoptosis, and this possibility has been explored further in this study. We have used U937 histiocytic lymphoma cells, in which apoptosis induced by ceramide, TNF-α, and the PKC inhibitor calphostin C is strongly inhibited by TPA. In this study, we used peptides based on unique sequences within PKC isoforms that specifically block the binding of individual isoforms to anchoring proteins, termed RACK proteins (receptors for activated C-
kinase). Recent work has demonstrated that a peptide corresponding to amino acids 14–21 in the V1 region of PKCe prevents phorbol ester-induced translocation of PKCe and inhibits contraction in cultured cardiac myocytes (18). In a further study, the eV1-2 peptide inhibited hypoxic preconditioning and phorbol ester-mediated protection of cardiac myocytes from hypoxia-induced cell death. In this study, we found that this peptide inhibited the ability of TPA to prevent apoptosis induced by TNF-α and calphostin C (calC). calC also reduced putative PKC activity and displaced PKCe from the membrane to the cytosol.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, benzamidine, calC, Hoechst 33258, phenylmethylsulfonyl fluoride, and TPA were obtained from Sigma. [γ-32P]ATP (10,000 Ci/mmol) was obtained from Brescetae (Adelaide, South Australia, Australia). Leupeptin was from Tokyo Kasai Kogyo Co. Aprotinin was obtained from Boehringer Mannheim. Primary antibodies for PKC isoforms were obtained from Santa Cruz Biotechnology, Inc., and secondary antibodies were from Silenius Laboratories (Hawthorn, Australia). TNF-α was kindly provided by Dr. D. Rathjen (Women’s and Children’s Hospital, Adelaide, South Australia). PKC Selectide™ substrate neurogranin-(28–43) and the PKCe translocation inhibitor peptide (EAVSLKPR7; eV1-2) were from Calbiochem. PKC peptide substrate (epidermal growth factor receptor; H-Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-NH2), PKC pseudosubstrate-(19–31) inhibitor peptide, the scrambled peptide (LSSETKPAV; scrambled eV1-2), and the PKβ translocation inhibitor peptide (SLNPFWNET; β2-4) were obtained from Auspep (Parkville, Australia). HYPERfilm-ECL was obtained from Amersham Pharmacia Biotech. Benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone was obtained from Enzyme Systems Products (Dublin, CA). ECL reagent was obtained from NEN Life Science Products. The TransPort™ transient permeabilization kit was from Life Technologies, Inc.

**Cell Culture**—U937 human myeloid leukemic cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM nonessential amino acids, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were transferred to RPMI 1640 medium supplemented with 2.5% heat-inactivated fetal calf serum for treatment with TNF-α or calC. After addition of calC, cells were illuminated for 10 min with a 40-watt incandescent lamp at a distance of 30 cm.

**Preparation of Cell Extracts—**Cytosolic and solubilized particulate fractions were prepared by sonicating (3 × 10 s) in buffer A (20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 2 mM DTT, 10 mM benzamidine, 0.01% leupeptin, 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 0.1 unit/ml aprotinin, and 0.25 M sucrose). The sonicate was cleared of unlysed cells by centrifugation at 5000 g for 5 min, and the cleared supernatant was centrifuged at 100,000 g for 5 min. The resulting pellet was resuspended in buffer A containing Triton X-100 (as indicated), incubated on ice for 30 min, and centrifuged as described above to obtain a supernatant particulate fraction.

**Western Blot Analysis**—Aliquots of cytosolic and particulate fraction extracts (described above) were added to well loading buffer (20 mM Tris-HCl (pH 6.8), 40% sucrose, 6% SDS, and 10 mM 2-mercaptoethanol) (2 vol extract: 1 vol loading buffer and heated for 5 min at 100 °C. Aliquots (5 μg of protein; determined by a modified method (19) of Lowry et al. [49]) were size-separated by SDS-polyacrylamide electrophoresis using 12% gels in a Bio-Rad minigel system (200 V, 45 min) and then electrophoretically transferred to Schleicher & Schuell nitrocellulose paper (0.2 μm; 100 V, 90 min) as described (20). Membranes were blocked with 5% milk powder and incubated with the primary antibodies for 1 h at 37 °C. To determine specificity, primary antibodies were preincubated for 2 h with 10-fold excess peptide against which the antibody was raised. After incubation with primary antibodies, the membranes were washed, incubated with the horseradish peroxidase-conjugated secondary antibody for 45 min, and washed again. The bound secondary antibody was detected with ECL reagent. Chemiluminescence was photographed with HYPERfilm-ECL.

**Direct Measurement of Membrane-associated PKC Activity—**PKC activity in the particulate fraction was measured using the method of Durkin et al. (21) with modifications. This method does not require a preliminary extraction with subsequent reconstitution and artificial activation of the enzyme with Ca2+ and phosphatidylserine. PKC activity in the particulate fraction was measured using a PKC peptide substrate based on the epidermal growth factor receptor or a highly selective peptide substrate based on neurogranin-(28–43) that allows measurement of PKC in crude extracts, and a PKC inhibitor peptide based on the pseudosubstrate-(19–31) region of PKC.

The assay reaction mixture contained 4–7 μg of protein from the particulate fraction in assay buffer (50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, and 100 μM phenylmethylsulfonyl fluoride) and 50 μM peptide substrate in a final volume of 90 μl. The reaction was started by the addition of 10 μl of 500 μM [γ-32P]ATP (220 cpm/yl in Tris buffer, 0.5 μC/assay) and stopped after 10 min by the addition of 10 μl of 75 mM orthophosphoric acid. The samples were clarified by centrifugation at 16,000 × g for 5 min, and a 90-μl sample of each supernatant was applied to Whatman P-81 paper (2 cm2). The papers were washed twice in 75 mM orthophosphoric acid with gentle agitation for 10 min. The radioactivity bound to the washed papers was determined by liquid scintillation counting.

**Assessment of DNA Fragmentation—**Qualitative assessment of DNA degradation was performed by agarose gel electrophoresis (22). Pelleted cells (5 × 10⁶) were fixed by suspension in 70% ethanol at −20 °C overnight. Cells were then centrifuged at 200 × g for 5 min, and the ethanol was thoroughly removed. Cell pellets were resuspended in 40 μl of phosphate/citrate buffer (192 parts 0.2 M Na2HPO4, 8 parts 0.1 M citric acid (pH 7.8)) at room temperature for 30 min. After centrifugation at 1000 × g for 5 min, the supernatant was transferred to new tubes, dried by vacuum in a SpeedVac Savant Instruments, Inc., Farmingdale, NY), and reconstituted with 15 μl of sterile distilled water. The DNA extract was then incubated with the addition of 0.3 μl of 0.25% Nonidet P-40 and 3 μl of RNase (1 mg/ml) for 30 min at 37 °C. The extract was incubated for a further 30 min with proteinase K (3 μl, 1 mg/ml). After this digestion, 5 μl of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, and 30% glycerol) was added, and the extract was fractionated by electrophoresis on 1.8% agarose at 5 V/cm for 3 h.

**FIG. 1. Effect of TPA on calC-induced apoptosis in U937 cells.** The percentage of cells undergoing apoptosis was measured by morphology and Hoechst chromatin staining as described under “Experimental Procedures.” The results were obtained from four different experiments. A, U937 cells were treated with the indicated concentrations of calC with or without 100 nM TPA for 7 h. B, shown is the time course of apoptosis induced in U937 cells by 1000 units/ml TNF-α and 125 nM calC with or without 100 nM TPA.
DNA was visualized under UV light after staining with 0.5 g/ml ethidium bromide.

**Quantitation of Apoptosis**—U937 cells (5 × 10⁵/ml) were treated with test compounds in RPMI 1640 medium containing 2.5% fetal calf serum. Chromatin was visualized by fluorescent microscopy after a 30-min incubation with the cell-permeable fluorochrome Hoechst 33258 (10 g/ml). The proportion of cells undergoing apoptosis at a given time was determined by counting cells exhibiting two or more membrane blebs and brightly stained condensed and fragmented chromatin. At least 200 cells were counted for each sample. The proportion of cells undergoing apoptosis was also determined using flow cytometry (23). Flow cytometry results are the percentage of cells (10,000) containing subdiploid DNA.

**Permeabilization**—In initial experiments, U937 cells were transiently permeabilized using the TransPort™ kit according to the manufacturer’s instructions. In subsequent experiments, cells were permeabilized by electroporation. U937 cells were suspended at 10 × 10⁶ cells/ml in serum-free RPMI 1640 medium, and 800-μl aliquots were electroporated with or without the indicated peptides at 330 microfarads and 200 V at room temperature. The electroporated cells were kept at room temperature for 10 min and then transferred to RPMI 1640 medium containing 2.5% fetal calf serum to a final concentration of 5 × 10⁵ cells/ml for treatment.

**RESULTS**

**Characterization of Calphostin C-induced Apoptosis in U937 Cells**—In the presence of light, calC is a highly specific PKC inhibitor that interacts with the regulatory domain of the enzyme and inhibits phorbol ester binding at high concentrations (24). calC was chosen for this study because, unlike TNF-α or ceramide, calC induced apoptosis in a large proportion of cells (Fig. 1), thus making biochemical studies more interpretable. Apoptosis induced by low concentrations of calC was acutely
blocked by TPA (Figs. 1 and 2). The protective effect of TPA was less after prolonged incubations (Fig. 1), possibly because of down-regulation of PKC. calC induced apoptosis in U937 cells in a dose-dependent manner and generated characteristic oligonucleosomal DNA fragmentation (Fig. 3), membrane blebbing (data not shown), and chromatin condensation (Fig. 2). Using time-lapse microcinematography, it was observed that calC-treated U937 cells underwent fragmentation into clusters of apoptotic bodies in a similar way to cells treated with TNF-α (data not shown).

PKC Isozymes and Distribution in U937 Cells—The main intracellular receptors for TPA are the PKC family of isozymes. Because U937 cells have been reported to contain different profiles of PKC isozymes, we screened our U937 cells using immunoblotting. U937 cells have been reported to express PKCα, PKCβI, PKCβII, PKCε, and PKCζ (25); PKCα, PKCβI, PKCε, and PKCζ (26, 27); or PKCβI, PKCβII, PKCε, and PKCζ (28). The U937 cells used in this study expressed PKCβI, PKCβII, PKCε, and PKCζ and very low levels of PKCδ, but did not contain detectable levels of PKCa or PKCθ (Fig. 4). PKCβI and PKCβII were predominantly located in the cytosol, whereas PKCε and PKCζ were distributed between the cytosolic and particulate fractions (Fig. 4).

TPA Protection against calC- and TNF-α-induced Apoptosis Is Inhibited by eV1-2—To determine whether PKCe translocation is required for the protective effect of TPA, we used a peptide derived from the V1 region of PKCe (eV1-2) that specifically inhibits the TPA-induced membrane translocation of this isof orm of PKC. The eV1-2 peptide blocks the interaction between PKCe and anchoring proteins that have been termed RACK proteins. Transient permeabilization with this peptide has been demonstrated to block TPA-induced contraction and protection against hypoxia-induced cell death in cardiac myocytes (15, 18). Furthermore, expression of the V1 fragment of PKCe from which eV1-2 was derived blocked TPA enhancement of neurite outgrowth in PC-12 rat neuroblastoma cells (29). Similar peptides have been identified with specificity for PKCd (29) and PKCβ (βC2-4) (30). Clearly, such peptides provide a powerful tool to evaluate the biological roles of individual PKC subforms.

Fig. 5. Effect of eV1-2 on prevention of calC-induced apoptosis by TPA. U937 cells were electroporated with the indicated peptides (10 nM (A and B) and as indicated (C)) as described under “Experimental Procedures.” A, U937 cells were treated with 125 nM calC with either 10 nM TPA or 0.1% Me2SO for 3 h. The data indicate the percent protection against calC-induced apoptosis by 10 nM TPA. B, U937 cells were treated with 1000 units/ml TNF-α with or without 10 nM TPA for 2.5 h. C, U937 cells were electroporated with the indicated concentrations of eV1-2 peptide and then treated with 125 nM calC with either 10 nM TPA or 0.1% Me2SO for 3 h. For A–C, cells that displayed two or more blebs and condensed chromatin were scored as apoptotic. 200 cells were counted per treatment. The results for A–C are the mean ± S.E. of three different experiments. D, U937 cells were treated with either 0.1% Me2SO or 125 nM calC with or without 10 nM TPA for 3 h. Cells (10,000) were analyzed by fluorescence-activated cell sorter as described under “Experimental Procedures.” Numbers within each panel indicate the percentage of cells with subdiploid DNA. Percent protection is the relative proportion of cells that are protected against apoptosis by TPA. Results are representative of two different experiments.
a peptide that blocks PKCβI and PKCβII binding to RACK proteins (βC2-4 (Figs. 5 and 6). These data strongly support a role for PKCe in mediating the protective effect of TPA against calC- and TNF-α-induced apoptosis. It should be noted that a low concentration of TPA (10 nM) was used in these experiments, a concentration that gave only partial protection against apoptosis (Fig. 5). The eV1-2 peptide was less effective at a higher concentration of TPA (100 nM) (data not shown), as reported previously in cardiac myocytes (18).

A prediction from these data is that the eV1-2 peptide should specifically inhibit the TPA-induced translocation of PKCe in U937 cells. Such inhibition has been reported in cardiac myocytes (15, 18). However, in a number of experiments including a time course (0–60 min) and several concentrations of Triton X-100, the eV1-2 peptide did not significantly inhibit 10 nM TPA-induced translocation of PKCe by Western blotting (data not shown). A similar lack of effect of the eV1-2 peptide was found in preliminary experiments with immunofluorescence localization (data not shown). We suggest two reasons for the inability to demonstrate an inhibitory effect on PKCe translocation.

First, U937 cells have a high basal level of particulate PKCe (Figs. 4 and 7) (26), which generates a high background level of immunoreactivity. Second, the peptide blocks only the binding of PKCe to RACK proteins. Interaction of PKCe with other binding proteins will not be inhibited. For example, PKCe, PKCβ, and PKCζ have been reported to bind to caveolin (31). PKC also binds to AKAP79 (32) and to PICK1 (33). Consequently, any effects of eV1-2 on PKCe translocation will depend on the relative proportions of RACK and other binding proteins in a particular cell type. We are currently investigating possible effects of the peptide on PKCe intracellular localization in the presence of TPA using confocal microscopy.

Membrane-associated PKCe Is Active in Untreated U937 Cells and Is Greatly Reduced during Calphostin C-induced Apoptosis—The above results suggest that the binding of PKCe to RACK proteins mediates the protective effect of TPA against both calC- and TNF-α-induced apoptosis in U937 cells. These results further suggest that the anti-apoptotic effect of TPA requires the phosphorylation of target proteins by PKCe, as PKC only binds to RACK proteins in the presence of activating cofactors (34). Recent reports also suggest that PKCe plays a role in the survival of cells that have not been stimulated with agents that directly activate PKC. Expression of PKCe, but not of PKCζ, extended the survival of interleukin-3 (IL-3)-dependent cells in the absence of the cytokine (35), and stable expression of PKCe protected transformed rat embryo fibroblasts from cisplatin-induced apoptosis (17). However, we found that the eV1-2 peptide had no effect on the levels of apoptosis induced by calC and TNF-α or in untreated cells, suggesting that association of PKCe with RACK proteins is not involved in U937 cell survival. PKCe does, however, appear to be inhibited in TNF-α-, Fas- and ceramide-induced apoptosis, where PKCe is displaced from the particulate fraction to the cytosol (16). We therefore determined if calC also displaced PKCe from the membrane and whether membrane-associated PKCe was active in U937 cells.

PKCe immunoreactivity was divided between the particulate and cytosolic fractions of proliferating U937 cells, as has been reported by others (26). Particulate PKCe immunoreactivity was reduced at 10 min by calC and TNF-α (Fig. 7) and ceramide (data not shown) and was associated with increased immunoreactivity in the cytosol (Fig. 7). In calC-treated cells, the re-
PKC\(\epsilon\) Is Required for TPA Inhibition of Apoptosis

U937 cells (\(10^6\)) were treated with 125 nm calC and/or 100 nm TPA for 3 h. Particulate fractions were preincubated with either a PKC pseudosubstrate inhibitor or Tris buffer for 30 min and then assayed for kinase activity using a peptide substrate derived from the epidermal growth factor receptor as described under “Experimental Procedures.” Inhibitor-sensitive PKC activity was calculated by subtracting counts of inhibitor-preincubated particulate fractions from Tris-preincubated particulate fractions. The data are expressed as mean ± S.E. of quadruplicate determinations and are representative of three separate experiments.

| Treatment       | Kinase activity \(\pm\) S.E. | Inhibitor-sensitive PKC activity |
|-----------------|------------------------------|---------------------------------|
| \(\text{Me}_2\text{SO}\) |                             |                                |
| + Inhibitor     | 121 ± 11                     | 55                              |
| calC            | 121 ± 5                      | 65                              |
| + Inhibitor     | 66 ± 4                       | 269                             |
| TPA             | 371 ± 21                     | 163                             |
| + Inhibitor     | 103 ± 7                      |                                 |
| calC + TPA      | 265 ± 12                     |                                 |
| + Inhibitor     | 101 ± 8                      |                                 |

DISCUSSION

Much evidence has accumulated that suggests a role for PKC\(\epsilon\) in the inhibition of apoptosis. The calcium-independent isomorph PKC\(\epsilon\) has recently been implicated in the protection of cardiac myocytes from hypoxia-induced cell death (15). The studies presented here suggest that PKC\(\epsilon\) is also required for the inhibition of apoptosis by the tumor-promoting phorbol ester TPA. The major evidence for this is that the eV1-2 peptide derived from the V1 region of PKC\(\epsilon\) blocked TPA protection against both calC- and TNF-\(\alpha\)-induced apoptosis. A peptide made from scrambled amino acids present in the eV1-2 peptide and a peptide specific for PKC\(\epsilon\) had no effect on TPA protection. We were unable, however, to consistently demonstrate an effect of eV1-2 on TPA-induced translocation of PKC\(\epsilon\). This result may be partly due to the high level of particulate PKC\(\epsilon\) in U937 cells (26), which generates a high background level of immunoreactivity. Furthermore, the eV1-2 peptide will not block the interaction of PKC\(\epsilon\) with other binding proteins such as caveolin (31), AKAP79 (32), and PICK1 (33). RACK proteins, however, apparently bind a large proportion of translocated PKC\(\epsilon\) (34), so it is also possible that the peptides that inhibit binding to RACK proteins allow PKC to associate with proteins in the particulate fraction that are not bound under normal conditions. This phenomenon may be cell type-specific, and we are currently investigating this using immunolocalization and confocal microscopy. Studies in other cells, however, have established the specificity of eV1-2 for inhibiting PKC\(\epsilon\) translocation. In neonatal rat cardiomyocytes, the eV1-2 peptide inhibited TPA-induced translocation of PKC\(\epsilon\) measured by confocal microscopy and hypoxia-induced PKC\(\epsilon\) translocation measured by immunoblotting (15, 18). It is therefore likely that TPA-induced binding of PKC\(\epsilon\) to RACK proteins is required for the suppression of apoptosis.

The binding of PKC to RACK proteins requires the presence of PKC activating cofactors (34). This suggests that suppression of apoptosis by TPA requires that PKC\(\epsilon\) not only bind to RACK proteins, but that PKC\(\epsilon\) also phosphorylate target proteins. One potential target for PKC\(\epsilon\) in the TPA-induced suppression of apoptosis is the Bcl-2 protein, which requires phosphorylation on putative PKC phosphorylation sites for its apoptosis-suppressive function. Serine-to-alanine mutations at putative PKC phosphorylation sites in Bcl-2 prevented the PKC activator bryostatin-1 from blocking apoptosis induced by IL-3 withdrawal (37). Expression of Bcl-2 may also be regulated by PKC. In IL-3-dependent B cells, phorbol ester treatment increased Bcl-2 expression via a cyclic adenosine monophosphate response element in the bcl-2 promoter. This element is bound by cAMP response element-binding protein, which is phosphorylated by PKC after phorbol ester stimulation on the same serine residue that is phosphorylated when IL-3 is pres-
ent (38, 39). A role for PKC in regulating the levels of Bcl-2 protein is further supported by a report showing that PKC inhibition resulted in apoptosis and a reduction in Bcl-2 in glioma cells (40). Furthermore, overexpression of PKCe, but not of PKCeβ, protected IL-3-dependent TF-1 cells from apoptosis and increased Bcl-2 levels, but did not abrogate IL-3 dependence (35). Overexpression of Bcl-2 in IL-3-dependent hematopoietic cells also did not cause IL-3 independence, suggesting that the anti-apoptotic signal transduction cascade from IL-3 may operate via PKCe and Bcl-2 (41). Overexpression of PKCe has also been shown to protect transformed rat embryo fibroblasts against cisplatin-induced apoptosis (17).

The overexpression data suggest that PKCe may have a role in cell survival where PKCe has not been activated by phorbol ester, and it is therefore intriguing that diverse apoptotic stimuli displace PKCe from the membrane (16). We stress, however, that there is as yet no direct evidence that this displacement is causally linked to apoptosis. Although we provided evidence that particulate PKCe had catalytic activity, the εV1-2 peptide did not induce apoptosis or increase the sensitivity of the cells to TNF-α or calC. These results suggest that if PKCe does protect cells from apoptosis in the absence of TPA, then this does not involve a PKCe-RACK interaction. The effect of calC on displacement of particulate PKCe could be a direct result of calC binding to the enzyme. This may explain the lack of an effect on PKCe, which is not inhibited by calC (16). Alternatively, the calC effect could be indirect. For example, calC has been reported to elevate ceramide levels in WEHI-231 cells (42), and exogenous ceramide has been reported to displace particulate PKCe (16).

Although cell-permeable ceramides induce apoptosis in U937 cells (2, 43) and other cells (44–46), it has not been demonstrated that ceramide production is required for TNF-α or calC. These results suggest that if PKCe does protect cells from apoptosis in the absence of TPA, then this does not involve a PKCe-RACK interaction. The effect of calC on displacement of particulate PKCe could be a direct result of calC binding to the enzyme. This may explain the lack of an effect on PKCe, which is not inhibited by calC (16). Alternatively, the calC effect could be indirect. For example, calC has been reported to elevate ceramide levels in WEHI-231 cells (42), and exogenous ceramide has been reported to displace particulate PKCe (16).

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