Differential effects of free and liposome-associated 1-*O*-octadecyl-2-*O*-methylglycerophosphocholine on protein kinase C

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Abstract Incorporation of ET-18-OCH_3 into well-characterized liposomes known as ELL-12 has eliminated its gastrointestinal and hemolytic toxicity without loss of growth inhibiting activity. ET-18-OCH_3, but not ELL-12, blunted the increase in membrane protein kinase C (PKC) activity induced by 12-*O*-tetradecanoylphorbol 13-myristate (TPA) and markedly reduced levels of PKCα in NIH 3T3 fibroblasts. Furthermore, prolonged treatment with ELL-12 neither inhibited TPA-induced translocations of PKCα and PKCδ to the particulate fraction nor caused down-regulation, and did not affect the cellular distribution of TPA-insensitive PKCζ. In Jurkat T cells, where ELL-12 markedly induced apoptosis that was blocked by an inhibitor of caspase-3-like activities, it had no effect on PKC activity or translocation induced by TPA. Thus, it seems unlikely that PKC is involved in the therapeutic effects of ELL-12.

Key words: Liposome; Protein kinase C; 1-*O*-Octadecyl-2-*O*-methylglycerophosphocholine; 12-*O*-Tetradecanoylphorbol 13-myristate; Apoptosis

1. Introduction

The ether lipid ET-18-OCH_3 has shown a selective inhibitory effect on the growth of cultured cancer cells in vitro [1], in animal models in vivo [2], and has been used in clinical trials [3]. However, therapeutic utilization of ET-18-OCH_3 has been limited by non-specific membrane detergent effects [4], which cause gastrointestinal and other toxicities, including hemolysis [2]. Incorporation of ET-18-OCH_3 into liposomes, composed of dioleoylphosphatidylcholine:cholesterol:glutaric acid derivatized dipalmitoylphosphatidylethanolamine:ET-18-OCH_3 (4:3:1:2, v/v), known as ELL-12 [5], has eliminated these side effects, reduced toxicity, expanded the dose range, and allowed more targeted delivery into tumor cells [6].

Despite extensive investigation, the mechanism of action of ET-18-OCH_3 is not yet fully elucidated. A growing body of evidence suggests that the antineoplastic effect of this ether lipid may be due to inhibition of several vital signal transduction pathways [7–9], particularly protein kinase C (PKC), a ubiquitous protein kinase that transduces growth signals (for recent reviews see [10,11]). ET-18-OCH_3 inhibits the phosphotransferase activity of PKC in vitro [1,12], acting as a competitor of phosphatidylserine (PS), a known cofactor of PKC [12–15]. ET-18-OCH_3 also effectively inhibits PKC-dependent phosphorylation of endogenous proteins in human breast cancer MCF-7 cells, whose growth is markedly inhibited by ET-18-OCH_3 [16], and inhibits 12-*O*-tetradecanoylphorbol 13-myristate (TPA)-induced protein phosphorylation in intact HL60 cells [17]. Moreover, in HL60 cells, 1-β-D-arabinofuranosylcytosine (ara-C)-induced apoptosis was stimulated by pretreatment with ET-18-OCH_3, which inhibits both ara-C- and TPA-induced translocation of PKCβII [18]. ET-18-OCH_3 also inhibits TPA-stimulated phospholipase D activity and DNA synthesis in NIH 3T3 fibroblasts [19], further supporting the notion that PKC is one of the primary targets of ET-18-OCH_3 [19]. Recently, however, it has been noted that the mode of addition of ET-18-OCH_3 to PKC assays influences its effects: when added from an ethanol solution, ET-18-OCH_3 inhibits PKC activity [12–14], whereas ET-18-OCH_3 stimulates PKC activity when added together with PS and 1,2-diacylglycerol (DAG) as liposomes [20]. Moreover, other studies have shown only a moderate effect on PKC activity in vitro, and, furthermore, similar inhibitory effects were found with active and inactive anomers of an ether lipid [21]. In view of the importance of PKC in signal transduction and cellular proliferation, we examined whether ET-18-OCH_3, when stably associated with liposomes as ELL-12, affects PKC in a similar manner as the free form.

2. Materials and methods

2.1. Materials

Anti-PKC-α, -δ, and -ζ antibodies were purchased from Upstate Biotechnology (Lake Placid, NY), Gibco-BRL (Gaithersburg, MD), and Transduction Laboratories (Lexington, KY), respectively. TPA was from Calbiochem (La Jolla, CA). ET-18-OCH_3 was from Alexis Corp. (San Diego, CA) and used from a concentrated ethanol stock solution. All lipids and other reagents were of the highest purity available. Ac-DEVD-CHO was from Bachem (King of Prussia, PA).

2.2. Liposome preparation and characterization

ET-18-OCH_3 liposome formulation ELL-12 (DOPC/Chol/DOPE-GA/ET-18-OCH_3 (4:3:1:2)), was prepared as previously described [22] and kept at 4°C. In all experiments, ELL-12 concentration refers to the concentration of the of ET-18-OCH_3 component in the liposomes.

2.3. Cell culture

NIH 3T3 fibroblasts and Jurkat T cells were cultured in high glu-

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Abbreviations: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; ara-C, 1-β-D-arabinofuranosylcytosine; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methylglycerophosphocholine; Chol, cholesterol; DOPC, dioleoylphosphatidylcholine; DOPE-GA, dioleoylphosphatidylcholine-glutaric acid; ELL-12, DOPC/Chol/DOPE-GA/ET-18-OCH_3 (4:3:1:2); FBS, fetal bovine serum; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-myristate

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cose (4.5 g/l) Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640, respectively, supplemented with 10% FBS, antibiotics and 2 mM L-glutamine [23].

2.4. Cell fractionation

After washing with cold PBS, NIH 3T3 cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 25 μg/ml leupeptin, and 25 μg/ml aprotinin). All subsequent steps were carried out at 4°C as described earlier [24]. Briefly, the cells were homogenized by passing 10 times through a 25 gauge needle with a syringe and centrifuged at $1000\times g$ for 5 min. Supernatant was centrifuged at $100\,000\times g$ for 1 h to produce a nuclei-free particulate membrane fraction (pellet) and a cytosol fraction (supernatant). Protein concentration was determined by the BCA method (Pierce, Rockford, IL).

2.5. Measurement of PKC activity

Particulate fractions were solubilized in 100 μl lysis buffer containing 0.1% (v/v) Triton X-100 by passage through a 27 gauge needle 10 times. After centrifugation for 20 min at $14\,000\times g$, supernatants were collected and designated membrane fractions. Equal amounts of protein (5–10 μg) from cytosolic and membrane fractions were assayed for PKC activity using a PKC assay kit (Upstate Biotechnology, Lake Placid, NY) as described previously [25]. In some cases, cytosolic and membrane preparations were partially purified on DEAE cellulose columns as previously described before assaying PKC activity [24].

2.6. Western analysis

PKC isozymes were analyzed by Western blotting as previously described [26]. To prevent proteolysis during sample preparation, cell extracts were prepared in a denaturing, hot SDS buffer and then separated by electrophoresis through a 12% polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose membranes and blocked with 3% non-fat milk in PBS containing 0.5% Tween-20 (TPBS) for 30 min. The membranes were then incubated for 2 h at room temperature with anti-PKC-α, -δ, or -ζ antibodies diluted at least 500-fold in milk-TPBS, and subsequently with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA). After washing, immunoreactive bands were visualized by the SuperSignal system (Pierce, Rockford, IL). To compare relative levels of PKC isozymes, the same blots were stripped several times by incubating in 50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% β-mercaptoethanol for 1 h at 60°C and re-probed with another antibody.

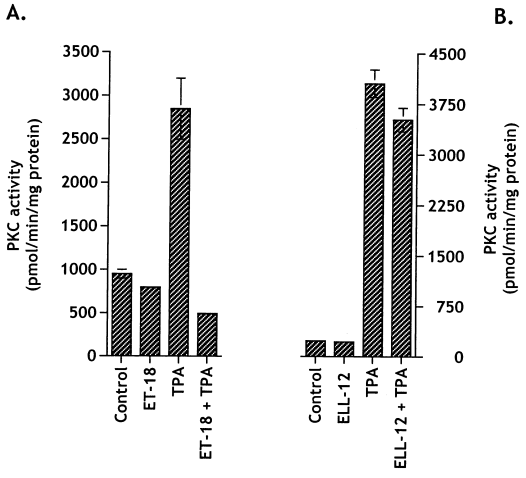

Fig. 1. Effects of acute treatment with ET-18-OCH_3 and ELL-12 on activation of PKC. NIH 3T3 fibroblasts were incubated with vehicle, ET-18-OCH_3 (A, 7.5 μM), or ELL-12 (B, 7.5 μM) for 30 min and then treated without or with 200 nM TPA for 15 min. ELL-12 concentration refers to the concentration of the of ET-18-OCH_3 component in the liposomes. Cells were lysed and PKC in the particulate fractions was assayed as described in Section 2.5. A and B are from two independent experiments. Data are means ± S.D. from a representative experiment carried out in triplicate.

2.7. Cell viability assay

Cell viability was measured with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) proliferation/viability kit essentially as described in the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). The MTT dye reduction assay measures mitochondrial respiratory function and can detect the onset of cell death earlier than dye exclusion based methods. NIH 3T3 fibroblasts were seeded in 96 well flat bottom plates and grown to confluence. Solutions of ET-18-OCH_3 and ELL-12 in culture medium were added and after 24 h, MTT was added and incubations continued for an additional 4 h. After removing the medium and washing the cells, reduced dye was solubilized with 20% SDS in 50% dimethylformamide (pH 4.7) and cell viability assessed from absorbance measurements at 570 and 630 nm.

2.8. [^{3}H]Thymidine incorporation

NIH 3T3 cells were seeded in 24-well plates at 3×10^4 cells per well, cultured for 48 h in medium containing 10% serum, and then the culture medium was replaced with medium containing 2% serum. Cells were treated with the indicated concentrations of ELL-12 for 18 h and then pulsed with 1 μCi of [^{3}H]thymidine for 6 h. Incorporation of radioactivity into trichloroacetic acid-insoluble material was measured as described [27].

2.9. DNA fragmentation assay and staining of apoptotic nuclei

DNA fragmentation measurement and staining of apoptotic nuclei were carried out as described [28].

3. Results and discussion

3.1. Effect of acute ET-18-OCH_3 and ELL-12 treatment on PKC activity

Previously, many investigations have been focused on the inhibitory actions of antineoplastic ether lipids on PKC [12–14,16,29,30]. ET-18-OCH_3 was proposed to accumulate at the cell surface with non-saturation kinetics [31] and to compete with PS at the regulatory domain of PKC isozymes [32]. Thus, it was suggested that ET-18-OCH_3 might decrease the levels of PKC isozymes by proteolytic down-regulation in a similar manner as its activators, PS, DAG, and TPA [33]. Due to the enhanced therapeutic effects of liposome-associated ET-18-OCH_3, it was of interest to compare the effects of ET-18-OCH_3 and ELL-12 on PKC. We have circumvented the shortcomings of previous in vitro approaches by examining the effects of ELL-12 on NIH 3T3 fibroblasts, which express only a limited and well-defined set of PKC isozymes [24], and have previously been shown to be sensitive to ether lipids [9,19]. Moreover, ET-18-OCH_3 markedly inhibits PKC activity in these cells [19,34]. Thus, it was of interest to examine whether ELL-12 alters the effects of TPA on PKC activity and/or translocation in these cells. As expected, TPA increased membrane-bound PKC activity by 6-8-fold within 15 min (Fig. 1). In agreement with previous studies [19,34], treatment of NIH 3T3 fibroblasts with ET-18-OCH_3 for 30 min prior to stimulation with TPA prevented the TPA-induced increase in membrane PKC activity (Fig. 1). In contrast, pre-incubation with ELL-12 had no effect on PKC activity nor did it affect TPA-induced activation of PKC (Fig. 1).

3.2. Effect of ET-18-OCH_3 and ELL-12 on the level and membrane translocation of PKC isozymes in NIH 3T3 fibroblasts

In agreement with previous studies [19,34], prolonged treatment of NIH 3T3 fibroblasts with ET-18-OCH_3 resulted in marked reduction in levels of PKCα (Fig. 2A) and PKCδ holo

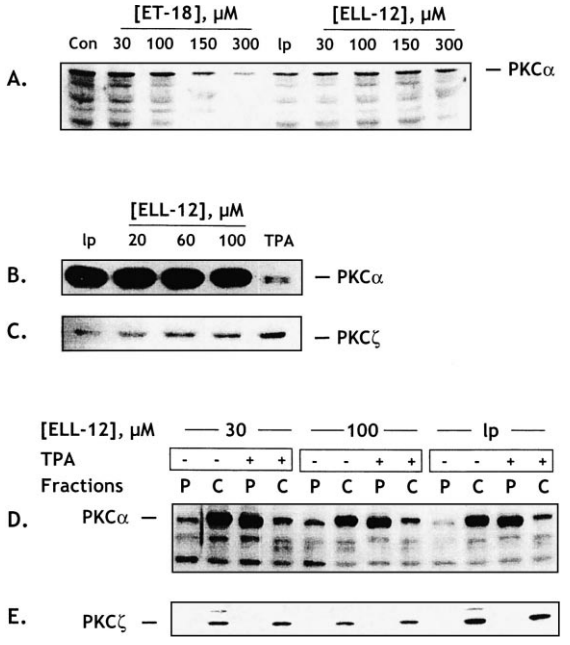

Fig. 2. Effects of ET-18-OCH_3 and ELL-12 on levels of PKC isoenzymes. Confluent monolayers of NIH 3T3 fibroblasts were treated for 20 h with vehicle (Con), with empty liposomes (ET-18-OCH_3-free) at a concentration equivalent to the highest dose of ELL-12 (lp), or with the indicated concentrations of ET18-O-CH_3 (ET-18), ELL-12, or TPA (1 μM) alone. Cells were then lysed and lysates (30 μg) were resolved on SDS-PAGE gels and analyzed by Western blotting with the corresponding anti-PKC isozyme antibodies, anti-PKCα (A, B) and anti-PKCζ (C). Results are from separate experiments with different exposure times. D, E: Effect of long-term ELL-12 pretreatment on TPA-induced translocation of PKC isozymes from the cytosol to the particulate fraction. NIH 3T3 fibroblasts were incubated for 20 h with the indicated concentrations of ELL-12, or control liposome preparation without ET-18-OCH_3 (lp), followed by 15 min stimulation with vehicle (−) or with (+) TPA (200 nM). Cells were harvested and extracted in cell lysis buffer and fractionated into cytosol (C) and nuclei-free, particulate (P) fractions, prepared as described in Section 2.5. Proteins (30 μg) were resolved on 8% SDS-PAGE, transferred to nitrocellulose membranes, probed with specific monoclonal anti-PKC antibodies, and visualized by enhanced chemiluminescence detection.

enzymes (data not shown) in a dose-dependent manner. However, in contrast to downregulation of PKC with TPA, no production of proteolytic fragments of PKCα could be detected. In contrast, ELL-12 did not have any significant effects on the levels of PKCα and PKCδ, even at concentrations as high as 300 μM. As expected, long term incubation with 1 μM TPA caused downregulation of cPKCα (Fig. 2B) and PKCδ (data not shown), but not PKCζ (Fig. 2C).

Another commonly used assay to demonstrate in vivo activation of PKC is the intracellular redistribution of this enzyme. Upon activation, PKC translocates from the cytosol to the plasma membrane [35]. To investigate what effect, if any, ELL-12 has on the translocation of selected PKC species present in NIH 3T3 fibroblasts, cells were pre-incubated with ELL-12 and then stimulated with TPA for short periods (0–15 min), followed by rapid washing and separation of membrane fractions from the cytosol. The association of cPKCα, nPKCδ and atypical PKCζ isoenzymes with membranes was assessed by Western blot analysis. A liposome preparation without ET-18-OCH_3 served as a control to eliminate any non-specific effects of the lipid carrier. As expected, TPA treatment induced rapid translocation of PKCα to the membrane fraction (Fig. 2D). In contrast, treatment of the cells with ELL-12 did not affect the intracellular distribution of cPKCα (Fig. 2D), or nPKCδ (data not shown). It is well known that atypical PKCζ neither binds TPA, nor is capable of translocation from the cytosol to the plasma membrane in response to DAG and tumor promoter phorbol esters, such as TPA. Incubation with increasing concentrations of ELL-12 also had no significant effect on distribution of atypical PKCζ (Fig. 2E). Moreover, prolonged treatment with ELL-12 had no significant effects on TPA-induced translocation of any of the PKC isoforms.

3.3. *Effect of ELL-12 treatment on growth and apoptosis of NIH 3T3 fibroblasts*

Several studies indicate that NIH 3T3 fibroblasts are moderately sensitive to the cytotoxic actions of ET-18-OCH_3 [9,19]. In agreement, we found that prolonged incubation in serum-free medium with 5, 10, 20, and 100 μM ET-18-OCH_3 for 20 h, reduced viability by 0, 20, 45 and 100%, respectively. We also found that in the presence of serum, the inhibitory effects of ET-18-OCH_3 were much weaker and the IC_{50} was shifted to about a 3-fold higher concentration. ELL-12 was 2-fold less cytotoxic than ET-18-OCH_3, in concordance with previous results showing that associating ET-18-OCH_3 with liposomes abrogated non-specific cell membrane-associated lytic effects [6,36]. However, ELL-12 still has a growth inhibitory effect in media containing 2% serum as measured by [^{3}H]thymidine incorporation (Fig. 3A). A significant effect was observed at concentrations as low as 10 μM with a maximum inhibition at 50 μM.

A previous study demonstrated that ELL-12, similar to ET-18-OCH_3, can induce the formation of DNA laddering, a characteristic of apoptosis, in murine leukemia cells [36]. Thus, it was of interest to examine whether ELL-12 causes apoptosis of NIH 3T3 fibroblasts. Serum deprivation induced apoptosis of NIH 3T3 fibroblasts, where shrinking, blebbing, and condensation of nuclei were clearly evident after 24 h.

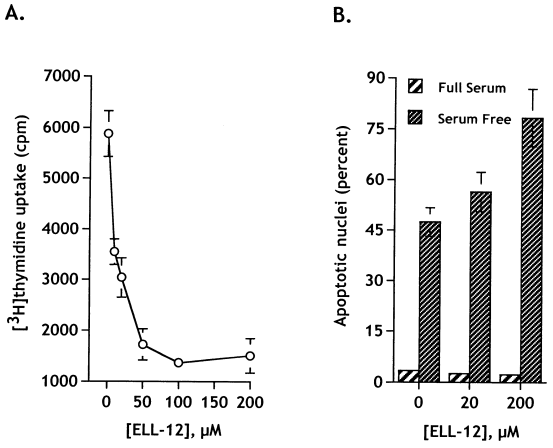

Fig. 3. Effects of ELL-12 on proliferation and apoptosis of NIH 3T3 fibroblasts. A: NIH 3T3 fibroblasts were incubated in media containing 2% fetal calf serum for 20 h in the presence of the indicated concentrations of ELL-12 and [^{3}H]thymidine incorporation was measured. B: NIH 3T3 fibroblasts were cultured in serum-free medium in the presence of the indicated concentrations of ELL-12 and percent apoptotic cells was evaluated with the DNA-specific fluorochrome bisbenzimide. Data are means ± S.D. from a representative experiment carried out in triplicate.

Treatment of NIH 3T3 fibroblasts with 20 μM ELL-12 slightly enhanced the appearance of apoptotic nuclei induced by serum starvation and a significant effect was observed at a concentration of 200 μM (Fig. 3B). In contrast, the presence of serum totally prevented apoptosis even in the presence of 200 μM ELL-12. These observations are similar to those of a previous report demonstrating that untransformed NIH 3T3 fibroblasts cultured in the presence of serum are resistant to the apoptotic effects of ET-18-OCH_3 [37].

3.4. ELL-12 markedly induces apoptosis of Jurkat T cells without affecting PKC activity

Because human leukemic cell lines are highly sensitive to ET-18-OCH_3 and readily undergo apoptosis after exposure to this ether lipid, we examined the effect of ELL-12 on apoptosis and PKC activity in Jurkat T cells. In agreement with previous studies [18,37,38], ET-18-OCH_3 markedly induced apoptosis of Jurkat T cells as measured by a quantitative DNA fragmentation assay at a concentration as low as 2 μM (Fig. 4A). ELL-12 also caused extensive cell death in these cells in a dose-dependent manner (Fig. 4B). In contrast, treatment of cells with liposomes devoid of ET-18-OCH_3 did not have any significant effects (Fig. 4B). Blebbing of cell membranes was observed in almost all cells and cells were fragmented into characteristic condensed nuclei and apoptotic bodies, whereas untreated cells did not show any morphological changes (data not shown). It should be pointed out that, similar to the effects of ET-18-OCH_3, ELL-12 induced apoptosis very rapidly and apoptosis was clearly evident after only 5 h of treatment (Fig. 4B).

Activation of the executionary caspases, especially caspase-3, -6, and -7, represent a converging downstream point in the apoptotic pathway leading to proteolysis of nuclear targets, such as poly(ADP-ribose) polymerase (PARP) and lamins, which are involved in the morphological nuclear changes associated with apoptosis (reviewed in [39]). To examine their role in ELL-12-induced apoptosis, we utilized an irreversible inhibitor, Ac-DEVD-CHO, a tetrapeptide aldehyde corresponding to the motif that is cleaved in PARP, which inhibits a wide spectrum of caspase-3-like caspases [39]. As shown in

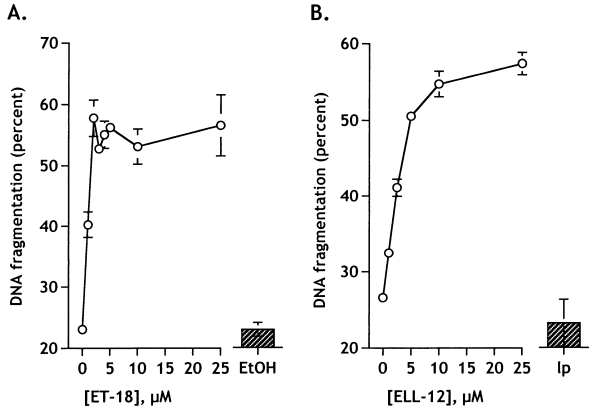

Fig. 4. ELL-12 markedly induces apoptosis of Jurkat T cells. A, B: Jurkat T cells, pre-labeled with [*methyl*-^{3}H]thymidine for 16 h, were treated in serum-free medium for 5 h with the indicated doses of (A) ET-18-OCH_3 or vehicle or (B) ELL-12 or empty liposomes equivalent to the highest dose of ELL-12 (lp) and DNA fragmentation was determined. Data are means ± S.D. Similar results were obtained in four independent experiments.

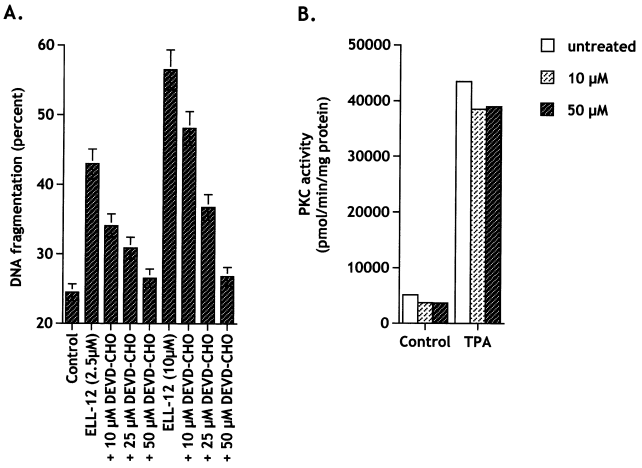

Fig. 5. ELL-12 induces caspase-dependent apoptosis in Jurkat T cells without affecting activity or translocation of PKC. A: Jurkat T cells, pre-labeled with [*methyl*-^{3}H]thymidine for 16 h, were pre-incubated in serum-free medium in the absence or presence of the indicated concentrations of Ac-DEVD-CHO for 1 h, then treated for an additional 5 h with the indicated concentrations of ELL-12 and DNA fragmentation was determined. Data are means ± S.D. Similar results were obtained in three independent experiments. B: Jurkat T cells were incubated with vehicle or the indicated concentrations of ELL-12 for 30 min and then treated without or with 200 nM TPA for 15 min. Cells were lysed and PKC in the particulate fractions was assayed. Data are means from a representative experiment carried out in duplicate.

Fig. 5A, Ac-DEVD-CHO abolished DNA fragmentation induced by ELL-12 in a dose-dependent manner. Significant inhibition was observed at a concentration of Ac-DEVD-CHO as low as 10 μM and complete reversal was observed at 50 μM (Fig. 5A). Similarly, Ac-DEVD-CHO abolished the morphological features associated with apoptosis, including condensation of nuclei and formation of apoptotic bodies (data not shown). Although ET-18-OCH_3 markedly inhibits PKC in human leukemic cell lines [17,18], treatment with 10 and 50 μM ELL-12 had no significant effects on PKC activity or on translocation induced by TPA (Fig. 5B). These results are similar to those obtained with NIH 3T3 fibroblasts (Fig. 1).

In summary, our studies with ELL-12 reinforce previous findings that inhibition of PKC by ET-18-OCH_3 may be important for its anti-proliferative activities [1,12–19]. However, the possibility cannot be excluded that a non-specific 'detergent effect' of ET-18-OCH_3 influences PKC activity. The much less cytotoxic, but very potent antineoplastic ELL-12 form does not alter PKC activity nor induces down-regulation of PKCα, PKCδ, or PKCζ, suggesting that targeted delivery of ET-18-OCH_3 into cells eliminated non-specific membrane perturbations. Moreover, ELL-12 has no effect on TPA-induced redistribution of PKC isoenzymes. Even in the highly sensitive human leukemic Jurkat T cell line, where ELL-12 markedly induced apoptosis, it had no effect on PKC activity or translocation induced by TPA. Our results indicate that the potent antitumor effect of the liposome formulation of ET-18-OCH_3, ELL-12, must result from actions on other intracellular targets in vivo.

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