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Alkyllysophospholipid ET-18-OCH_3 acts as an activator of protein kinase C in HL-60 cells

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HL-60 cells are very sensitive to the cytotoxic action of ether lipids. Several hypotheses have been proposed to explain this cytotoxicity. We investigated the influence of the alkylphospholipid ET-18-OCH_3 on the activity of protein kinase C. HL-60 cells were incubated with ET-18-OCH_3 at a concentration of 20 μg/ml for 4 h. After the incubation the membrane fraction of the HL-60 cells was isolated and the activity of protein kinase C was determined while it was still associated with the membrane, using the synthetic peptide substrate [Ser^{25}]-protein kinase C (19–31) as a protein kinase C specific substrate. The activity of the membrane-bound protein kinase C was increased in HL-60 cells treated with ET-18-OCH_3 compared to untreated HL-60 cells. The increase in protein kinase C activity was not a consequence of translocation and appeared to be additive to the effect of the phorbol ester 12-myristate 13-acetate. In contrast, solubilized protein kinase C from HL-60 cells could be inhibited or stimulated in vitro by ET-18-OCH_3, dependent on the mode of addition of ET-18-OCH_3 and phospholipids.

Alkyllysophospholipid; HL-60 cell; Protein kinase C

1. INTRODUCTION

The ether lipid ET-18-OCH_3 is a synthetic analog of 2-lysophosphatidylcholine and acts as an strong antitumor agent [1,2]. The mechanisms by which ET-18-OCH_3 exerts its selective antineoplastic activity is a point of great discussion. Various mechanisms have been proposed to be of importance in the cytotoxic action of ether lipids such as disturbance of the phospholipid metabolism of the plasma membrane [3,4], influence on membrane fluidity [5,6] and permeabilization of neoplastic cell membranes [7,8]. Frequently the inhibitory effect of ether lipids on the activity of PK-C have been mentioned as a possible explanation for the antineoplastic action of ether lipids [9–11]. These studies however, were performed in vitro with (partially) purified PK-C and artificial membrane structures composed of phosphatidylserine and diolein. HL-60 cells are highly sensitive to the cytotoxic action of ET-18-OCH_3. To investigate the effect of ET-18-OCH_3 on PK-C in its native environment, we isolated the plasma membrane of HL-60 cells, before and after incubation with ET-18- OCH_3. The activity of PK-C was determined in the plasma membrane fraction when it was still in its native phospholipid environment [12]. The data presented in this paper show that incorporation of ET-18-OCH_3 in the plasma membrane stimulates PK-C activity and that an inhibitory effect of ET-18-OCH_3 on PK-C activity is only achieved under conditions where the ether lipid is present in micelles.

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2. MATERIALS AND METHODS

2.1. *Materials*

All materials used for cell culture were obtained from Gibco (Grand Island, NY). ET-18-OCH_3, phosphatidylserine, diolein, leupeptin, aprotinin and dithiothreitol were from Sigma Chemical Co. (St. Louis, MO). DE-52 (DEAE-cellulose) and P81 filter paper were purchased from Whatman. [γ-^{32}P]ATP (3000 Ci/mmol) was supplied by New England Nuclear (Boston, MA). Calpain inhibitor-I was from Boehringer (Mannheim, Germany) and protein kinase C synthetic peptide substrate [Ser^{25}]-protein kinase C (19–31) was supplied by Bissendorf Biochem. (Hannover, Germany).

2.2. *Cells*

HL-60 cells obtained from the American Type Culture Collection (Rockville, MD) were grown in suspension in RPMI 1640 medium, containing 10% FCS (heat-inactivated), 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C and 5% CO_2. HL-60 cells were grown in log-phase and harvested routinely. Phorbol 12-myristate 13-acetate (PMA) was dissolved in DMSO and added to the cells at a final concentration of 200 nM (and 0.01% DMSO), 20 min before harvesting. ET-18-OCH_3 was dissolved in RPMI 1640 with 20% AB serum and filtered through a 22 μm filter and stored at −20°C. ET-18-OCH_3 at a final concentration of 20 μg/ml was added to the cell suspension 4 h before harvesting.

2.3. *Assay of protein kinase C activity*

Membrane associated PK-C: HL-60 cells ($6 \cdot 10^6$/assay) were washed twice with phosphate buffered saline (PBS) and lysed in a 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM $NaHCO_3$, 5 mM $MgCl_2$,

1 mM phenylmethylsulphonylfluoride (PMSF), 1 μM calpain inhibitor-I and 50 μg/ml leupeptin. Cells were disrupted by successively 2 min agitation on a vortex mixer, 30 strokes in a potter Elvehjem and 5 s sonication in a Branson sonifier. All procedures were carried out at 4°C. The cell lysate was centrifuged at 800 × g for 10 min to remove nuclei and cell debri and subsequently centrifuged at 48 000 × g for 30 min at 4°C. The 48 000 × g pellet fraction was resuspended in lysis buffer and again centrifuged at 48 000 × g for 30 min at 4°C. The resulting cell pellet was resuspended in a small volume of assay buffer (10 μl/6·10⁶ cells) containing 30 mM HEPES-KOH (pH 7.5) and 5 mM magnesium acetate. The assay mixture, with a final volume of 50 μl, consisted of assay buffer, 25 μM [Ser[25]]-protein kinase C(19–30) and 10 μl membrane fraction. The reaction was started with 20 μM ^{32}P-ATP (1.1 × 10⁶ cpm) and carried out at 30°C for 3 min. The reaction was stopped by spotting aliquots of reaction mixture in duplicate on H_3PO_4 pretreated P81 filter papers. Excess of radioactivity was washed from the filters in a 75 mM H_3PO_4 solution (5 times refreshed). The remaining radioactivity was determined with Optifluor and counted with a liquid scintillation counter. PK-C activity is expressed in pmol ATP/min/10⁵ cells. Partially purified PK-C: 10⁸ HL-60 cells were washed twice with PBS. Cells were lysed in extraction containing: 20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM PMSF, 10 μg/ml leupeptin, 200 nM aprotinin and 1 μM calpain inhibitor-I. Cells were disrupted by ultrasonication, (2 × 5 s with 1 min interval) at 4°C with a Branson sonifier. The extracts were centrifuged for 30 min at 48 000 × g at 4°C. The supernatant was collected and PK-C was partially on DE52 gel [13]. The membrane fraction was subsequently resuspended in extraction buffer to which 0.5% Triton X-100 was added, incubated for 30 min on ice and centrifuged for 30 min 48 000 × g. The supernatant with the solubilized PK-C was collected and subjected to DE-52 [13]. PK-C activity was determined as described by Kikkawa et al. [13] with some modifications. Briefly, the reaction mixture with a final volume of 50 μl contained: 30 mM HEPES-KOH, pH 7.5, 5 mM magnesiumacetate, 0.5 μg phosphatidylserine, 0.1 μg diolein and 0.5 mM $CaCl_2$. Negative controls were performed with 0.5 mM EGTA instead of $CaCl_2$, phosphatidylserine and diolein. The reaction was started with 10 μM [^{32}P]ATP (2.2 × 10⁵ cpm) and performed at 30°C for 3 min. The incorporated radioactivity was determined as described above.

3. RESULTS AND DISCUSSION

In search for answers to the mechanisms by which ALP exerts its antineoplastic action, many investigators focussed on the inhibitory action of ET-18-OCH_3 on PK-C, an enzyme that plays a central role in cellular signal transduction and also serves as an intracellular receptor for tumor-promoting phorbol esters [14]. Purified PK-C, isolated from HL-60 cells, was inhibited by ET-18-OCH_3 with a K_i of 9–15 μM [13,15,16]. The inhibition appeared to be competitive with respect to phosphatidylserine.

In our laboratory we were interested in the action of ET-18-OCH_3 on PK-C under more physiological conditions, e.g. when PK-C was still associated with the plasma membrane. In order to investigate this we developed a new model for testing PK-C activity as a modification of the model of Chakravarthy et al. [12]. HL-60 cells were treated 10, 20 and 30 min with the phorbol ester PMA. Afterwards the membrane fraction was isolated and the PK-C activity was measured as described in section 2. An optimum in PK-C activity was achieved after 20 min of PMA incubation (Fig. 1). These results are in accordance to what was found in

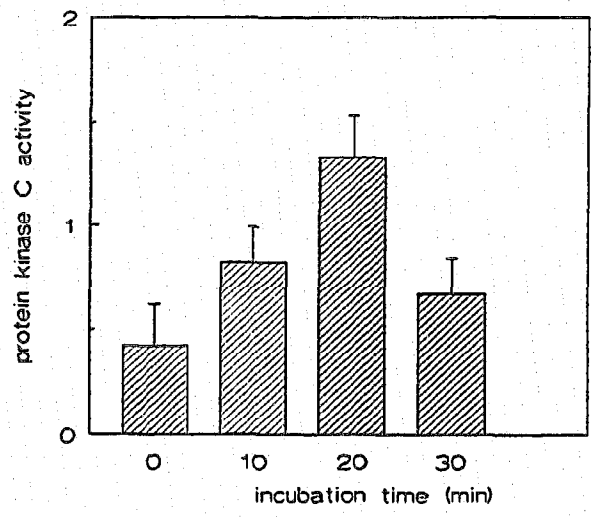

Fig. 1. Time course of PMA incubation. HL-60 cells were incubated with PMA for 10, 20 or 30 min. PK-C activity in the membrane fraction was determined as described in section 2. PMA dissolvent DMSO, in a final concentration of 0.01%, had no influence on the PK-C activity. PK-C activity is expressed in pmol/min/10⁶ cells. Values represent mean of 3 independent experiments ±SD.

HL-60 cells measured with (partially) purified PK-C [17] showing that our model is very useful to investigate the activity of PK-C in its native environment. The action of ET-18-OCH_3 on PK-C in HL-60 cells was investigated at subtoxic concentrations of ET-18-OCH_3, 20 μg/ml for 4 h at 37°C [18]. After 4 h incubation the viability of the cells was approx. 80% as determined with Trypan blue dye exclusion.

Quite unexpectedly, we found a 3-fold increase in PK-C activity in the membranes of ET-18-OCH_3 treat-

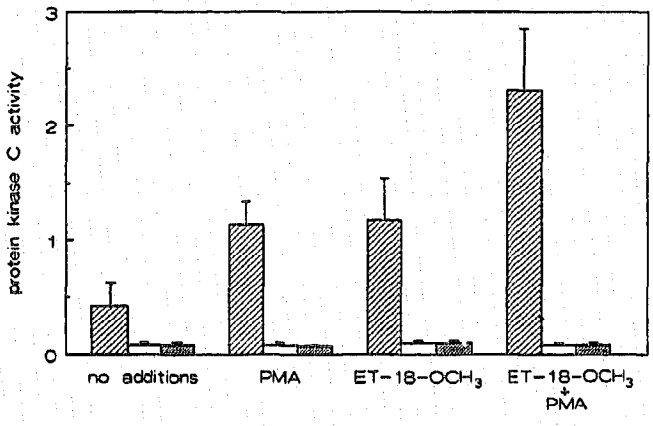

Fig. 2. PK-C activity in the membrane fraction of control HL-60 cells, cells incubated with 200 nM PMA for 20 min, 20 μg/ml ET-18-OCH_3 for 4 h, or with both ET-18-OCH_3 (20 μg/ml for 4 h and 200 nM PMA for 20 min). Hatched bars: PK-C activity in the membrane fraction determined as described in section 2. Open bars: PK-C activity in the membrane fraction measured in the presence of 20 nM staurosporin. Condensed hatched bars: endogenous phosphorylation, phosphorylation without synthetic peptide as substrate in the PK-C assay mixture. PK-C activity is expressed in pmol/min/10⁶ cells. Values represent mean of 6 independent experiments ±SD.

Table I

Distribution of Protein kinase C activity in HL-60 control cells of PMA/ET-18-OCH_3 treated cells

Fraction	PMA	ET-18-OCH_3	PK-C activity (nmol/min/mg protein)
Cytosol	–	–	2.1 ± 0.5
	+	–	0.5 ± 0.2
	–	+	2.0 ± 0.5
Membrane	–	–	0.7 ± 0.3
	+	–	1.8 ± 0.5
	–	+	0.9 ± 0.2

HL-60 cells were incubated with 20 μg ET-18-OCH_3 for 4 h or 200 nM PMA for 20 min. PK-C was partially purified and assayed as described in section 2. The results are the mean of at least 3 independent experiments ±SD.

ed cells compared to untreated HL-60 cells (Fig. 2). The phosphorylation reaction could be inhibited to a level of 0.13±0.01 pmol ATP/min/10^6 cells in the presence of 20 nM staurosporin, an inhibitor of PK-C [19] (Fig. 2). Endogenous phosphorylation, without the synthetic peptide in the assay mixture, resulted in a similar level of phosphorylation (Fig. 2). The question remained whether the increase in PK-C activity was the result of a PK-C redistribution from the cytosolic fraction to the membrane fraction (similar to the PMA

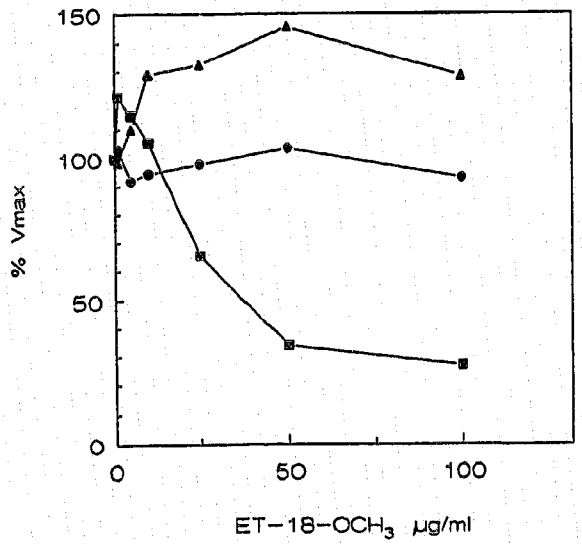

Fig. 3. Effect of ET-18-OCH_3 on partially purified PK-C. ET-18-OCH_3 was added to the reaction mixture under 3 different conditions as described in section 3, respectively methods (i), (ii) and (iii). (■) ET-18-OCH_3 dissolved in ethanol as described in method (i). Ethanol, with a final concentration of 1% had no influence on the PK-C activity. (▲) ET-18-OCH_3 added to the reaction mixture as mixed liposomes (with phosphatidylserine and diolein), as described in method (ii). (●) ET-18-OCH_3 added to the reaction mixture as separate liposomes, as described in method (iii). V_{max} represents the PK-C activity measured under standard conditions (without ET-18-OCH_3 in the assay mixture) as described in section 2. 100% V_{max} activity of PK-C is 2.1±0.5 nmol/min/mg protein. Values represent mean of 3 independent experiments with SD always under 10%.

effect) or whether we are dealing with another phenomenon induced by ET-18-OCH_3. Translocation of PK-C was investigated with two methods. First we incubated HL-60 cells with both ET-18-OCH_3 and PMA (the latter was added the last 20 min of ET-18-OCH_3 treatment). As shown in Fig. 2 an additive efect of ET-18-OCH_3 and PMA on PK-C in the membrane fraction was seen. Obviously, the PMA was still capable of promoting a redistribution of cytosolic PK-C to the membrane fraction after ALP treatment. Secondly, we performed a partial purification of PK-C in both the cytosolic and membrane fraction of cells treated with PMA or ET-18-OCH_3. As shown in Table I, a 75% decrease was observed in the cytosol fraction of PMA treated cells whereas the PK-C activity in the cytosol fraction of ET-18-OCH_3 treated cells remained the same. The PMA treatment resulted in an increase in PK-C activity in the membrane fraction. The PK-C activity in the membrane fraction of HL-60 cells treated with ET-18-OCH_3 was similar to that in control HL-60 cells due to the solubilization procedure with 0.5% Triton X-100. These observations indicate that ET-18-OCH_3 does not promote a redistribution of PK-C from the cytosol to the membrane fraction. Incubation of HL-60 cells with cycloheximide (30 μg/ml), an inhibitor of protein synthesis, together with ET-18-OCH_3 did not influence the increase in PK-C activity, concluding that de novo synthesis of PK-C is not responsible for the increase in activity.

To unravel this striking effect of ET-18-OCH_3 on PK-C when measured in this model and the complete contradictory observations reported by other investigators, we also studied the effect of ET-18-OCH_3 in the conventional conditions as described by Kikkawa et al. [13]. Cytosolic PK-C from HL-60 cells was isolated and partially purified with DE-52 chromatography. ET-18-OCH_3 was added to the assay mixture under three conditions. (i) ET-18-OCH_3 was dissolved in 100% ethanol, subsequently diluted in assay buffer to the desired concentration with a final concentration of 1% ethanol in the assay. PS and diolein were added as a liposome structure as described by Kikkawa et al. [13]. (ii) ET-18-OCH_3 was dissolved in chloroform, each concentration of ET-18-OCH_3 was separately evaporated under a stream of nitrogen together with PS and diolein, and the mixture was resuspended in assay buffer by ultrasonication. By this procedure mixed liposomes are formed containing PS, diolein and an increasing amount of ET-18-OCH_3. (iii) ALP was dissolved in chloroform and each concentration was separately evaporated under N_2 and resuspended in assay buffer by ultrasonication. Liposomes of PS and diolein are made as described under (i) and added to the assay mixture separately from the ET-18-OCH_3 liposomes.

The results of this comparative study are shown in Fig. 3. PK-C activity is inhibited by ET-18-OCH_3 when added under the conditions as described by method (i).

This result is in accordance with those presented by Helmann et al. [9] and Shoji et al. [11]. The inhibition was described as competitively with respect to PS and to the activation by diacylglyerol or TPA. The lack of inhibitory action of ET-18-OCH_3 on PK-C activity when added as separate liposomes (method (iii)) is remarkable and might explain the findings of Van Blitterswijk et al. [20] who found no inhibition of PK-C by the ether lipid when added as separate liposomes. Even more striking is the effect of ET-18-OCH_3 when incorporated in the PS and diolein liposomes (method (ii)). The action of the ether lipid resembles that of lysophosphatidylcholine as described by Oishi et al. [21].

Linking the results obtained in the different experiments we can conclude that ET-18-OCH_3 is an activator of PK-C when incorporated in the membrane of HL-60 cells or in membrane structures as shown in Fig. 3. Inhibition of PK-C by ET-18-OCH_3 can only be achieved under conditions where the ether lipid is present in micelles. The physiological consequences of this finding are unclear. In contrast to the general assumption, ET-18-OCH_3 obviously does not act as an inhibitor of PK-C in HL-60 cells when incorporated in the plasma membrane. In this respect it is of interest that naturally occurring ether-diglycerides are recently described as activators of PK-C [22]. Further investigations on the mechanisms of action of ET-18-OCH_3 on PK-C activity, and additionally the effects of ether lipids on PK-C from resistant cell lines are now ongoing in order to further elucidate or reject a possible role of PK-C in the antineoplastic action of ET-18-OCH_3.

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