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PROTEIN KINASE C IS NOT INVOLVED IN THE CYTOTOXIC ACTION OF 1-OCTADECYL-2-*O*-METHYL-*sn*-GLYCEROL-3-PHOSPHOCHOLINE IN HL-60 AND K562 CELLS

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Abstract—We studied the effects of the alkyllysophospholipid 1-octadecyl-2-*O*-methyl-*sn*-glycerol-3-phosphocholine (ET-18-OCH_3) on membrane associated protein kinase C (PK-C) activity in ET-18-OCH_3 sensitive HL-60 cells and in two resistant cell types, Me_2SO differentiated HL-60 cells and K562 cells. HL-60 cells expressed a lower PK-C activity level compared with both resistant cell types. However, membrane-bound PK-C activity in the sensitive HL-60 cells was approximately 3-fold increased in the presence of ET-18-OCH_3, whereas in differentiated HL-60 cells and K562 cells PK-C was not influenced by ET-18-OCH_3. The increase in PK-C activity in HL-60 cells was not due to translocation of cytosolic PK-C or synthesis *de novo*. The effect of ET-18-OCH_3 on kinetic parameters of PK-C in all three cell types was investigated in order to elucidate the nature of the ET-18-OCH_3 effects on PK-C activity in both sensitive and resistant cell types. A functional relationship between PK-C level and effect of ET-18-OCH_3 on PK-C activity in the different cell types could not be found. Moreover, cells depleted of PK-C activity showed similar sensitivity or resistance to ET-18-OCH_3 as cells expressing PK-C activity. These results suggest that a role of PK-C in the cytotoxic action of ET-18-OCH_3 is very unlikely.

Key words: ether lipid; HL-60; K562; protein kinase C; pseudosubstrate; plasma membrane

The alkyllysophospholipid ET-18-OCH_3,† a synthetic analog of 2-lysophosphocholine, exhibits selective antineoplastic activity [1–3]. The mechanisms by which this cytotoxicity is accomplished are still unknown and therefore no explanation for its selectivity towards neoplastic cells is found yet. In recent years it has become clear, however, that the plasma membrane is the main target for the cytotoxic action of ether lipids. Several investigators pointed at a disturbed phospholipid metabolism in plasma membranes after ET-18-OCH_3 incorporation [4, 5]; influences on membrane fluidity [6, 7] and permeabilization are also reported [8, 9]. Recently, Bazill and Dexter reported that endocytosis may play a role in the selective cytotoxic action of ether lipids [10].

The role of protein kinase C (PK-C) in the biological activity of alkyllysophospholipids has been subject to many investigations in the past years [11–14]. PK-C belongs to a large family of closely related proteins with multiple subspecies. The enzyme is present in the cytosol in inactive conformation. After receptor-mediated hydrolysis of phosphatidylinositol bisphosphate, resulting in an increase in intracellular Ca^{2+} and diacylglycerol in the lipid bilayer, PK-C becomes tightly associated with the plasma membrane. Once associated with the plasma membrane PK-C specific substrates interact with the substrate binding site on the catalytic subunit. The kinase–substrate–phospholipid complex possesses a high degree of cooperativity and specificity (for a review see Ref. 15). The enzyme plays a crucial role in several important cellular activities including transmembrane signaling, growth and differentiation. The inhibitory effect of ET-18-OCH_3 on the PK-C activity was suggested as one of the mechanisms by which ET-18-OCH_3 exerts its cytotoxic effects. The majority of the investigations focussed on the effects of ether lipids on (partially) purified PK-C from various sources [11–13]. In quoted reports PK-C activity was measured in a cell-free system with histon as a substrate and an artificial membrane system (liposomes consisting of a mixture of phosphatidylserine and diacylglycerol) as cofactors. As the plasma membrane is known to be the main target for the biological activity of ether lipids and PK-C is activated when associated with the plasma membrane, we decided to investigate the effect of ET-18-OCH_3 on the PK-C activity while the enzyme was still in its native phospholipid environment. As we reported earlier [16], in HL-60 cells there seems to be a major difference in the effect of ET-18-OCH_3 on PK-C activity when PK-C is either (partially) purified or still associated with the plasma membrane. Purified PK-C was inhibited by ET-18-OCH_3 when the latter was added as micelles to the assay mixture, whereas PK-C in the membrane-

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† Abbreviations: Me_2SO, dimethylsulfoxide; ET-18-OCH_3, 1-octadecyl-2-*O*-methyl-*sn*-glycerol-3-phosphocholine; PK-C, protein kinase C.

associated state was activated after prior incubation with ET-18-OCH_3. The ET-18-OCH_3-induced increase in PK-C activity was not due to translocation of PK-C from the cytosol to the plasma membrane nor to synthesis *de novo* [16].

In the present study we addressed the question of whether PK-C in sensitive HL-60 cells is differently affected by ET-18-OCH_3 compared with two relatively ether lipid-resistant cell types, the (Me_2SO-induced) differentiated HL-60 cells and K562 cells, an erythroleukemic cell line, thereby investigating whether the PK-C activities in both types of cell lines (before and after ET-18-OCH_3 incubation) were functionally related. To examine whether PK-C was differently affected by ET-18-OCH_3 in the sensitive and resistant cell lines, we performed some kinetic experiments involving the affinity of PK-C for the PK-C specific synthetic substrate [Ser^{25}]PK-C(19–31), also to elucidate further the nature of the PK-C activation by ET-18-OCH_3 found in HL-60 cells. The specificity of the substrate was affirmed by using the corresponding pseudosubstrate [Ala^{25}]-PK-C(19–36) as a specific inhibitor and subsequently the various inhibitory constants of the pseudo-substrate were determined in the three cell lines before and after ET-18-OCH_3 incubation. To elucidate the question of whether PK-C plays a role, or no role at all, in the cytotoxic effect of ET-18-OCH_3, PK-C activity in the three cell types was inhibited with staurosporine prior to performing a cytotoxicity assay with increasing amounts of ET-18-OCH_3. The results show clearly that, although ET-18-OCH_3 affects PK-C activity in HL-60 cells, PK-C itself is not essential in the cytotoxic action of ET-18-OCH_3 in the above mentioned cell lines.

MATERIALS AND METHODS

Materials. All materials used for cell culture were obtained from Gibco (Grand Island, NY, U.S.A.). ET-18-OCH_3, phorbol 12-myristate 13-acetate (PMA), Me_2SO, aprotinin and leupeptin were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Phenylmethylsulfonyl fluoride (PMSF) was supplied by Merck (Darmstadt, Germany). Calpain inhibitor-I and staurosporine were from Boehringer (Mannheim, Germany) and protein kinase C synthetic peptide substrate [Ser^{25}]PK-C(19–31) and inhibitor peptide PK-C(19–36) were obtained from Bissendorf Biochem. (Hannover, Germany). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Cell lines. The cell lines HL-60 (promyelocytic cell line) and K562 (erythroleukemic cell line) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and 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° and 5% CO_2. HL-60 and K562 cells were grown in log-phase and harvested routinely. Differentiation of the HL-60 cells was initiated by adding Me_2SO to a final concentration of 1.2%. During the differentiation experiments the cells were recultured after 3 days at a concentration of 3×10^5/mL in fresh media containing all supplements and Me_2SO. The extent of differentiation was assessed by reduction of nitroblue tetrazolium dye (NBT) assayed as described by Collins *et al.* [17].

Assay of protein kinase C activity. Cells (approx. 6×10^7), before and after 4 hr of ET-18-OCH_3 treatment (20 μg/mL, viability of the K562 cells >90%, HL-60 cells >80% after ET-18-OCH_3 treatment), were washed twice with cold phosphate-buffered saline (PBS) and resuspended in 1 mL of a hypotonic extraction solution containing 5 mM $MgCl_2$, 1 mM $NaHCO_3$, 1 mM PMSF, 1 μM calpain inhibitor-I and 50 μg/mL leupeptin, pH 7.5. After a 10 min incubation on ice, the cells were disrupted by ultrasonication at 4° with a Branson sonifier (50 W, 5 sec). The cell lysate was centrifuged at 800 *g* for 5 min to remove nuclei and cell debris and subsequently centrifuged at 48,000 *g* for 30 min at 4°. The 48,000 *g* pellet fraction was resuspended in a small volume of PK-C assay buffer (150 μL) consisting of 30 mM Hepes–KOH, pH 7.5 containing a final concentration of 5 mM magnesium acetate. The assay mixture, with a final volume of 50 μL, consisted of assay buffer (final concentration of 30 mM Hepes and 5 mM magnesium acetate), 25 μM [Ser^{25}]PK-C(19–31) and 10 μL membrane fraction. The reaction was started with 20 μM [^{32}P]ATP (1.1×10^6 cpm) and the reaction mixture was incubated for 3 min at 30°. The reaction was stopped by the addition of 5 μL acetic acid (99.8%). Subsequently the membrane fraction was pelleted by 5 min centrifugation and aliquots of the supernatant were spotted in duplicate on P81 filter papers. Excess radioactivity was washed from the filters in a 75 mM H_3PO_4 solution (five times refreshment). The remaining radioactivity was determined with Optifluor in a liquid scintillation counter. In the kinetic studies identical PK-C assay mixtures were used, except for the synthetic peptide substrate concentration, which was used as indicated in the figures. In the inhibition studies a substrate concentration of 0.5 μM [Ser^{25}]PK-C(19–31) was used with increasing concentrations of inhibitory peptide [Ala^{25}]PK-C(19–36), as indicated in the figures. The kinetic parameters were calculated using the Elsevier BIOSOFT Enzfitter data analysis program.

Protein determination. Triton X-100 was added to the membrane-associated PK-C fraction with a final concentration of 0.5%. The mixture was sonicated for 10 sec with a Branson sonifier and kept on ice for 30 min. Protein concentrations were determined according to Bradford [18] with bovine serum albumin as a standard. The final concentration of Triton X-100 did not influence the protein determination.

ET-18-OCH_3 *cytotoxicity assay.* Undifferentiated and differentiated HL-60 cells and K562 cells were incubated with 0, 10, 20 and 50 μg ET-18-OCH_3/mL culture medium for 24 hr at 37° and 5% CO_2 humidified atmosphere in a 24-well multidish (Nunc, Denmark) containing a final volume of 1 mL/well and a final cell concentration of 2×10^6/mL. Viability of the cells was determined by Trypan blue dye exclusion. The proliferative capacity of the cells was analyzed by [^{3}H]thymidine incorporation in a 96-well multidish. After 24 hr incubation with ET-18-

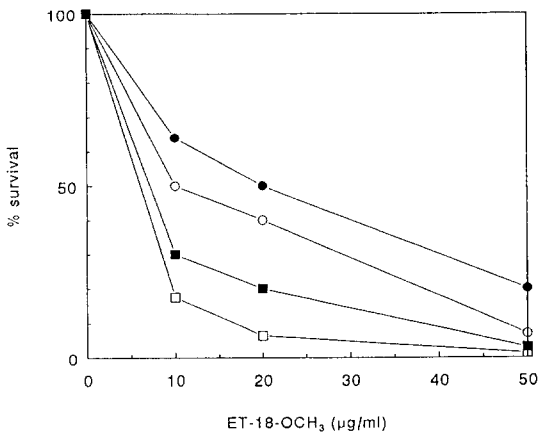

Fig. 1. Cytotoxic effect of ET-18-OCH_3 on HL-60 cells at various stages of differentiation. (□) Undifferentiated HL-60 cells; (■) after 1 day; (○) after 2 days; and (●) after 7 days of exposure to 1.2% Me_2SO. Results are mean values from three separate experiments each determined in triplicate (SE was always <10%). Control values (100% viable cells) represent viability of cells treated with Me_2SO in the absence of ET-18-OCH_3.

OCH_3 in the concentration mentioned above, 1 μCi of [^{3}H]thymidine (Radiochemical Centre, Amersham, U.K.) was added to 10^5 cells/microwell for 1 hr at 37° and 5% CO_2. Cells were harvested with a multisample culture harvester and incorporated radioactivity was determined with a liquid scintillation counter.

RESULTS

PK-C activity in HL-60 cells, differentiated HL-60 cells and K562 cells

We compared the PK-C activity in the ET-18-OCH_3-sensitive HL-60 cells with that of both the (relatively) resistant cell lines K562 and Me_2SO-induced differentiated HL-60 cells. HL-60 cells obtain morphologically and biochemically granulocytic properties after Me_2SO induction [19]. The increase in differentiation is associated with a decline in sensitivity towards the cytotoxic activity of ET-18-OCH_3 according to Vallari *et al.* [20] and our own observations, as shown in Fig. 1. At day 7 of differentiation 90% of the cells were NBT positive. Me_2SO-differentiated cells appear to be significantly less sensitive to the cytotoxic action of the ether lipid. After 24 hr ET-18-OCH_3 incubation period the $T_{1/2}$ kill was obtained at doses increasing from 6 μg/mL for control cells to 8, 12 and 23 μg/mL for cells of day 1, 2 and 7 of differentiation, respectively.

PK-C activity was measured when the enzyme was still associated with its native membrane, in the presence of a specific synthetic peptide substrate, $MgCl_2$ and radiolabeled ATP. The two ET-18-OCH_3 resistant cell lines expressed a higher PK-C activity compared to the undifferentiated HL-60 cells as shown in Fig. 2. Membrane-associated PK-C represented approx. 10–15% of total cellular PK-C activity. Addition of calcium (0.5 mM final concentration) and phorbol ester (1 μM final concentration) to the assay mixture did not result in an increase in PK-C activity in all three cell lines. In the same experiment we analysed the effect of the calcium chelator EGTA on the PK-C activity in the membrane fraction of the three cell types. EGTA in a final concentration of 0.5 mM had no influence on the PK-C activity in the undifferentiated and differentiated HL-60 cells; in K562 cells, however, PK-C activity was markedly reduced by EGTA (Fig. 2).

Effect of ET-18-OCH_3 *on PK-C activity*

Incubation of the three cell lines with ET-18-OCH_3 (20 μg/mL for 4 hr) induced an increase in PK-C activity in the undifferentiated HL-60 cells.

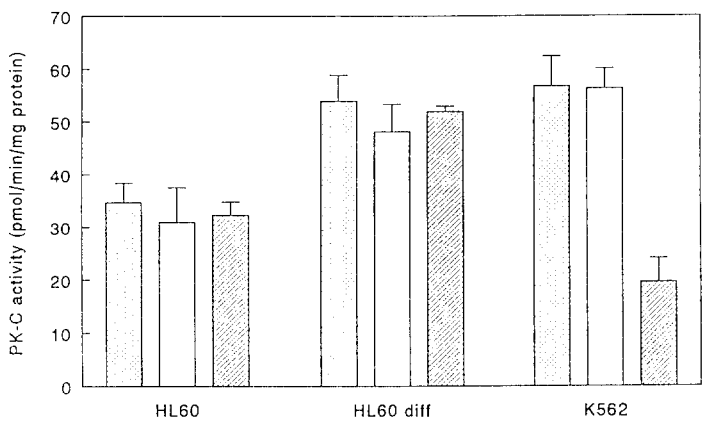

Fig. 2. PK-C activity in the membrane fraction of HL-60 cells, differentiated HL-60 cells and K562 cells. PK-C activity was determined under three different conditions: no extra additions in the PK-C assay mixture (dotted bars), addition of 0.5 mM Ca^{2+} and 1 μM PMA (open bars) and addition of 0.5 mM EGTA to the reaction mixture (hatched bars). PK-C activity is expressed in pmol/min/mg protein. Values represent the means of five independent experiments ±SD.

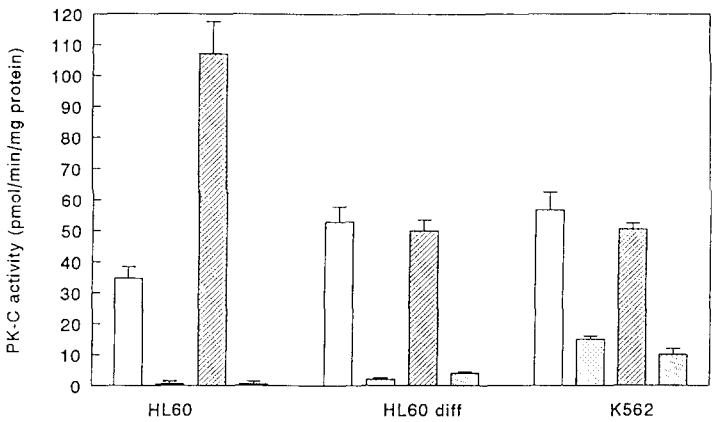

Fig. 3. PK-C activity in the membrane fraction of HL-60, differentiated HL-60 and K562 cells before (open bars) and after (hatched bars) 4 hr 20 μg ET-18-OCH_3/mL incubation, both in the absence (dotted bars) and presence (condensed dotted bars) of 25 μM pseudosubstrate [Ala^{25}]PK-C(19–36). PK-C activity was determined as described and is expressed in pmol/min/mg protein. Values represent the means of five independent experiments ±SD.

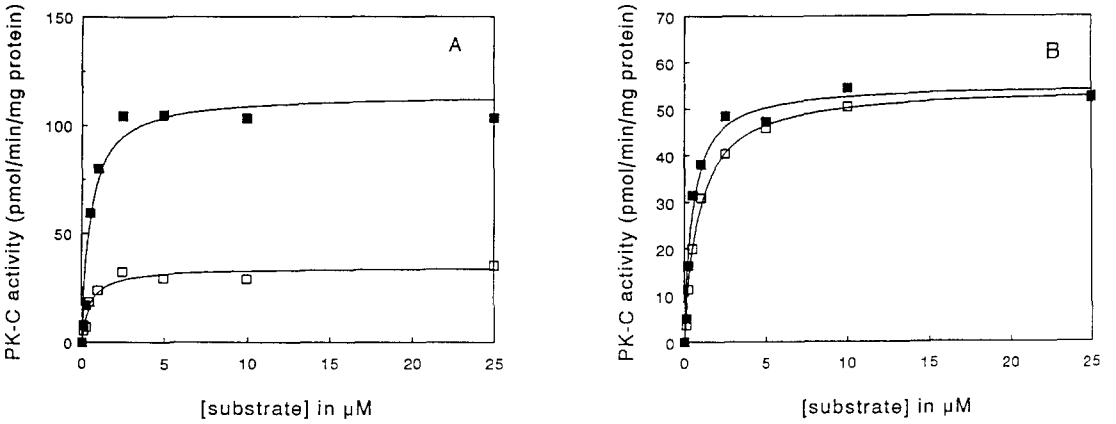

Fig. 4. PK-C activity in the membrane fraction of HL-60 cells (A) and differentiated HL-60 cells (B) as function of the [Ser^{25}]PK-C(19–31) substrate concentration before (open symbols) and after (closed symbols) 4 hr 20 μg ET-18-OCH_3/mL incubation. Figure 4 reflects one representative sample out of three experiments. PK-C activity is expressed in pmol/min/mg protein.

PK-C activity in the membrane fraction of differentiated HL-60 cells and K562 cells remained unchanged after 4 hr of ET-18-OCH_3 treatment (Fig. 3). Similar to ET-18-OCH_3-untreated cells (Fig. 2), addition of calcium and phorbol ester to the PK-C assay mixture of ET-18-OCH_3-treated cells did not result in an increase in PK-C activity. Moreover, EGTA affected PK-C activity of ET-18-OCH_3-treated cells identically to untreated cells; only PK-C of K562 cells was inhibited (data not shown). As the PK-C activity increased 3-fold in HL-60 cells after ET-18-OCH_3 incubation, and as this increase in activity could not be explained by synthesis *de novo* nor translocation of the enzyme [16], we decided to analyse the kinetic behavior of the enzyme towards its substrate before and after ET-18-OCH_3 treatment of the three different cell types. The affinity of PK-C for [Ser^{25}]PK-C(19–31) was tested in the PK-C assay mixture as described above with increasing substrate concentrations as indicated in the figures. The results of the kinetic experiments are shown in Fig. 4 and again an approx. 3-fold increase in PK-C activity after ET-18-OCH_3 incubation is seen in undifferentiated HL-60 cells but not in differentiated HL-60 cells or K562 cells. The level of PK-C activity in the K562 cells is very similar to that of the differentiated HL-60 cells (not shown). The V_{max} and K_m values as calculated with the Elsevier BIOSOFT program are shown in Table 1. The Michaelis–Menten constant of differentiated HL-60 cells is slightly lower in ET-18-OCH_3-treated cells, whereas the constants of undifferentiated HL-

Table 1. Kinetic parameters of PK-C

Cell line	ET-18-OCH_3	V_{max}* pmol/min/mg protein	K_m* μM
HL-60	−	34.5 ± 1.8	0.52 ± 0.12
	+	113 ± 3.4	0.54 ± 0.07
HL-60 diff.	−	54.5 ± 0.7	0.87 ± 0.05
	+	55.1 ± 1.8	0.48 ± 0.07
K562	−	58.8 ± 1.4	1.34 ± 0.12
	+	54.0 ± 1.5	1.34 ± 0.14

* Maximal activity and Michaelis–Menten constant determined as described in Materials and Methods and calculated by the Elsevier BIOSOFT Enzfitter data analysis program.

Table 2. Inhibition of PK-C activity in HL-60, differentiated HL-60 and K562 cells by the specific inhibitor [Ala^{25}]PK-C(19–36)

Cell line	ET-18-OCH_3	IC_{50}*	% Activity†
HL-60	−	1.1	0.5
	+	0.7	0.5
HL-60 diff.	−	1.2	2
	+	0.8	4
K562	−	1.5	15
	+	1.2	10

*IC_{50} represents the inhibitor concentration (in μM) at which half maximal activity is measured as described in Materials and Methods.

† The percentage PK-C activity determined at a concentration of 25 μM inhibitor, V_{max} (without inhibitor) is taken as 100%.

Table 3. Effect of staurosporine on PK-C activity in HL-60 and K562 cells after 4 hr incubation

Staurosporine	PK-C activity 0	20 nM	50 nM
HL-60	34.7 ± 3.7	5.1	3.4
HL-60 diff.	52.9 ± 4.9	3.5	5.9
K562	56.7 ± 5.7	3.9	2.1

The PK-C activity is expressed in pmol/min/mg protein. Values represent the means ± SD of five experiments (0 nM staurosporine) or the mean of two independent experiments performed in duplicate (20 and 50 nM staurosporine).

60 cells and K562 cells are similar in cells with or without ET-18-OCH_3. The K_m values of the K562 cells are significantly higher than the values of both HL-60 cell types.

Inhibition of PK-C activity with the pseudosubstrate [Ala^{25}]*PK-C*(19–36)

The synthetic peptide substrate used in the PK-C assay system is the pseudosubstrate sequence of PK-C with Ser^{25} substituted for Ala^{25}, as described by House *et al.* [21]. The unmodified pseudosubstrate is a very potent and specific inhibitor of PK-C. We used the pseudosubstrate as a competitive inhibitor in the PK-C assay system. The goal was 2-fold, firstly to investigate the specificity of the assay system, and secondly to determine the IC_{50}, defined as the inhibitor concentration at which 50% inhibition is obtained, of the three cell lines before and after ET-18-OCH_3 treatment. The results are shown in Table 2. At a concentration of 0.5 μM [Ser^{25}]PK-C(19–31) and increasing amounts of inhibitor an almost complete inhibition of PK-C activity was found in all cell types, except for K562 cells in which a small amount of residual PK-C activity was present. The presence of ET-18-OCH_3 in the plasma membrane had no influence on the rate and degree of inhibition.

Inhibition of PK-C by staurosporine and its effect on ET-18-OCH_3 *cytotoxicity*

The dual effect of ET-18-OCH_3 on PK-C activity in the sensitive and resistant cell types and the higher level of PK-C activity in the latter made us question whether there is any involvement of PK-C in the cytotoxic action of ET-18-OCH_3 at all. For that reason we inhibited the PK-C activity in HL-60 cells and K562 cells with staurosporine. After 4 hr of incubation with 50 nM staurosporine the membrane-bound PK-C activity in both cell types was completely inhibited (Table 3). Viability of the cells was determined after 24 hr of staurosporine treatment with trypan blue dye exclusion. As shown in Fig. 5A, the viability of the HL-60 cells was not influenced by the staurosporine whereas that of K562 cells dropped to 70% and 35% at 20 nM and 50 nM, respectively. The [^{3}H]thymidine incorporation was markedly reduced in HL-60 and almost diminished in K562 cells after 24 hr of staurosporine (Fig. 5B). The killing potential of ET-18-OCH_3 in PK-C-inhibited cells was determined with the cytotoxicity assay as described above and compared with that of control HL-60 and K562 cells. Cells were pretreated for 4 hr with 50 nM staurosporine in the culture medium, whereafter increasing amounts of ET-18-OCH_3 were added. After 24 hr viability and [^{3}H]-thymidine incorporation were determined. Strikingly, both PK-C-inhibited cells and control cells exhibited an identical percentage of survival (Fig.

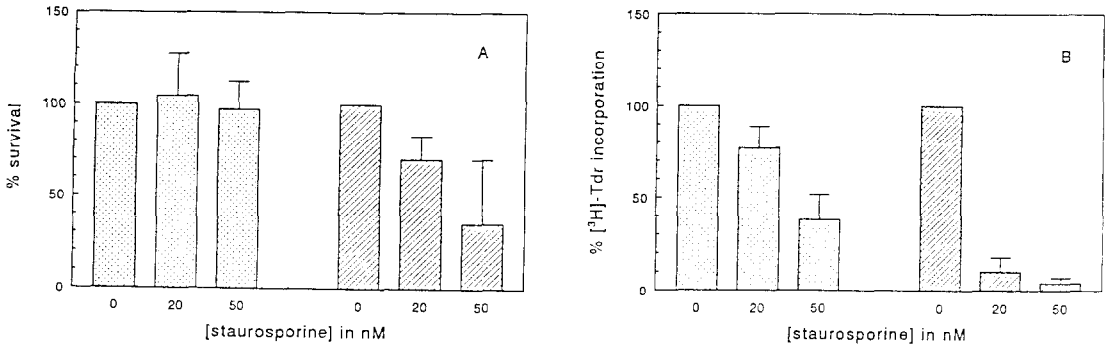

Fig. 5. (A) Percentage survival of HL-60 cells (dotted bars) and K562 cells (hatched bars) after 24 hr incubation with 20 and 50 nM staurosporine, determined by trypan blue dye exclusion. (B) Effect of staurosporine on [^{3}H]thymidine incorporation in HL-60 cells (dotted bars) and K562 cells (hatched bars). Values are expressed as percentage cell survival and [^{3}H]Tdr incorporation of control values (in the absence of staurosporine) and represent the mean of four independent experiments ±SD.

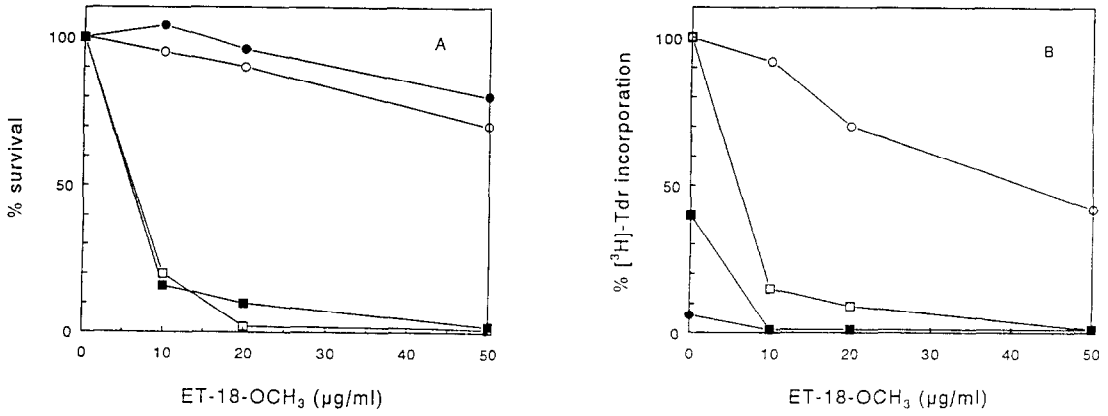

Fig. 6. (A) The effect of ET-18-OCH_3 on the viability of HL-60 (squares) and K562 cells (circles) without staurosporine (open symbols) or with staurosporine (50 nM for 4 hr, closed symbols) in the culture medium. Values are expressed as percentage of control values (in the absence of ET-18-OCH_3 and staurosporine). Values represent means of at least four independent experiments with SD always <15%. (B) [^{3}H]Thymidine incorporation of HL-60 cells and K562 cells (symbols as described in Fig. 6A). Values are expressed as percentage of control values (in the absence of ET-18-OCH_3 and staurosporine). Values represent means of at least four independent experiments with SD always <15%.

6A). The [^{3}H]thymidine incorporation in PK-C-inhibited HL-60 cells was approx. 40% compared to control cells; however, treatment with both staurosporine and ET-18-OCH_3 reduced the incorporation to below 1% of the control value. The incorporation of [^{3}H]thymidine in PK-C-inhibited K562 cells was very low (approx. 5%), and was also reduced to less than 1% after combined incubation with ET-18-OCH_3 (Fig. 6B).

DISCUSSION

HL-60 cells are very sensitive to induction with Me_2SO and they follow the myeloid pathway of hematopoietic differentiation. The increase in differentiation is associated with a decline in sensitivity towards the cytotoxic activity of ET-18-OCH_3 (Fig. 1) in agreement with the findings of Vallari *et al.* [20]. The difference in resistance to ET-18-OCH_3 is most pronounced at a concentration of 20 μg/mL. At this concentration 95% of the undifferentiated cells are killed, in contrast to only approx. 43% of the more mature cells (after 7 days of Me_2SO incubation).

The activity of membrane-bound PK-C in the ether lipid-resistant cell lines, K562 and differentiated HL-60, is approx. 2-fold higher compared to PK-C in the sensitive HL-60 cells (Fig. 2 and Table 1). From the literature it is known that differentiated HL-60 cells (after both PMA- and Me_2SO-induced differentiation) express a higher PK-C activity in the cytosol and membrane fraction [22, 23]. In that case,

however, PK-C activity was measured in a solubilized membrane preparation with histon as (a relatively aspecific) substrate, and an artificial membrane structure composed of phosphatidylserine and diacylglycerol. In our PK-C assay system, which is a modification of the method of Chakravarthy *et al.* [24], we can measure PK-C activity still associated with the plasma membrane, in the presence of its native phospholipid cofactors using a specific peptide substrate. This method enabled us to investigate the ET-18-OCH_3 effect on PK-C activity while the enzyme was still associated with the plasma membrane, mimicking the *in vivo* situation far more than the conventional PK-C assay systems.

As addition of phorbol ester and Ca^{2+} had no increasing effect on the PK-C activity as shown in Fig. 2, we can conclude that the membrane-bound PK-C fraction in all three cell types is already in fully active conformation. This is in agreement with the findings of Chakravarthy *et al.* [24]. Also, addition of phosphatidylserine to the PK-C assay mixture did not increase the enzyme activity (data not shown). The addition of the calcium chelator EGTA to the reaction mixture dramatically decreased the PK-C activity in K562 cells, but not in both undifferentiated and differentiated HL-60 cells. This suggests a major difference in affinity for Ca^{2+}, a cofactor of PK-C. A possible difference in PK-C subtype expression in K562 cells might explain this phenomenon.

In answer to our question of whether PK-C activity in ether lipid-resistant cell lines K562 and differentiated HL-60 cells is influenced by ET-18-OCH_3 as reported for the ether lipid-sensitive HL-60 cells [16], we can conclude the following: the presence of the ether lipid in the membrane fraction of differentiated HL-60 cells or K562 cells is not of influence on the membrane-bound PK-C activity (Fig. 3). As reported earlier, the increase in PK-C activity in the membrane fraction of HL-60 cells was not due to translocation from the cytosol nor synthesis *de novo* [16]. From Fig. 4 and Table 1 it is clear that this increase in activity is also not explained by a change in the affinity of PK-C for its specific substrate after ET-18-OCH_3 treatment. The question remains open as to whether the affinity of the enzyme for its phospholipid cofactors is changed by the presence of ET-18-OCH_3; unfortunately, extracting PK-C from the plasma membrane will also disintegrate ET-18-OCH_3 from the membrane and therefore its effect on PK-C. Although we performed kinetic experiments with an enzyme preparation which is still incorporated in the membrane structure and under influence of (unknown) phospholipid cofactors instead of a purified enzyme preparation, which is more commonly used in kinetic analysis, the enzyme was obeying Michaelis–Menten kinetics (Fig. 4). The higher K_m value found in the K562 cells might point again at a difference in PK-C subtype compared with the HL-60 cells.

The specificity of the PK-C substrate was confirmed using the Ala^{25} pseudosubstrate of PK-C as a competitive inhibitor. The IC_{50} as shown in Table 2, was nearly identical before and after ET-18-OCH_3 treatment in all three cell types. This is in agreement with the results of the kinetic experiments in which the K_m values for ET-18-OCH_3 of untreated and treated cells were identical, except possibly for the differentiated HL-60 cells which expressed a slightly higher affinity for the peptide substrate in the presence of ET-18-OCH_3.

These results made us question whether the increase in PK-C activity in HL-60 cells after ET-18-OCH_3 treatment and the nonresponsiveness of PK-C in both resistant cell types (also expressing a higher level of PK-C activity) to the ether lipid were functionally related. As the above mentioned data are rather conflicting, and as it is generally assumed that PK-C is inhibited by ET-18-OCH_3, we decided to determine the cytotoxic effect of ET-18-OCH_3 in HL-60 and K562 cells depleted of PK-C activity. PK-C activity in the membrane fraction was completely inhibited after 4 hr of 50 nM staurosporine in the culture medium (Table 3). The 35% survival of the K562 cells after 24 hr 50 nM staurosporine (Fig. 5a), implies a role for PK-C in the basic metabolism of K562 cells in contrast to HL-60 cells which seem to be far less dependent on PK-C for their survival. From the [^{3}H]thymidine incorporation experiments (Fig. 5b), it can be concluded that, in both cell lines, activity of PK-C is essential for the proliferative capacity.

Strikingly, the cytotoxic effect of ET-18-OCH_3 on HL-60 and K562 cells pretreated with staurosporine (50 nM for 4 hr), was identical to the control cells (Fig. 6a). These results suggest that the presence of PK-C activity in the membrane fraction of HL-60 and K562 cells is essential for the proliferative capacity of the cells, but more importantly in the present context not essential for the cytotoxic action of ET-18-OCH_3.

Taken together, a functional relationship between the cytotoxic action of ET-18-OCH_3 and its effect on PK-C in the above-mentioned cell lines is very unlikely. As ET-18-OCH_3 is also capable of inducing differentiation of HL-60 cells [25], the increase in PK-C activity in HL-60 cells after ET-18-OCH_3 treatment might be explained as an early event in the process of differentiation. On the other hand, it is very well possible that differences in the PK-C subtype composition in undifferentiated HL-60 cells and differentiated HL-60 cells, as reported by Hashimoto [26], and K562 cells are partly responsible for the results as presented in this paper. The mechanism(s) responsible for the cytotoxic action of the ether lipids are still unknown and a possible role for PK-C, however, seems highly questionable.

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REFERENCES

1. Glasser L, Somberg LB and Vogler WR, Purging murine leukemic marrow with alkyllysophospholipids. *Blood* **64**: 1288–1291, 1984.
2. Andreesen R, Modolell M, Weltzien HV, Eibl H, Common HH, Löhr GW and Munder PG, Selective destruction of human leukemic cells by alkyllysophospholipids. *Cancer Res* **38**: 3894–3899, 1978.
3. Verdonck LF, Witteveen EO, van Heugten HG, Rozemuller E and Rijksen G, Selective killing of

malignant cells from leukemic patients by alkyl-lysophospholipid. *Cancer Res* **50**: 4020–4025, 1990.
4. Modolell M, Andreesen R, Pahlke W, Brugger U and Munder PG, Disturbance of phospholipid metabolism during the selective destruction of tumor cells induced by alkyl-lysophospholipids. *Cancer Res* **39**: 4681–4686, 1979.
5. Herrmann DBJ and Neumann HA, Cytotoxic ether phospholipids. Different affinities to lysophosphocholine acyltransferases in sensitive and resistant cells. *J Biol Chem* **46**: 5803–5809, 1986.
6. Noseda A, Godwin PL and Modest EJ, Effects of antineoplastic ether lipids on model and biological membranes. *Biochim Biophys Acta* **945**: 92–100, 1988.
7. van Blitterswijk WJ, Hilkmann H and Storme GA, Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids* **22**: 820–823, 1987.
8. Lazenby CM, Thompson MG and Hickman JA, Elevation of leukemic cell intracellular calcium by the ether lipid SRI 62-834. *Cancer Res* **50**: 3327–3330, 1990.
9. Dive C, Thompson MG, Watson JV and Workman P, Multiparametric flow cytometry of the modulation of tumor cell membrane permeability by development antitumor ether lipid SRI 62-834 in EMT6 mouse mammary tumor and HL-60 human promyelocytic leukemia cells. *Cancer Res* **51**: 799–806, 1991.
10. Bazill GW and Dexter TM, Role of endocytosis in the action of ether lipids on WEHI-3B, HL-60, and FDCP-mix A4 cells. *Cancer Res* **50**: 7505–7512, 1990.
11. Helfman DM, Barnes KC, Kinkade JM, Vogler WR, Shoji M and Kuo JF, Phospholipid-sensitive Ca^{2+}-dependent protein phosphorylation system in various types of leukemic cells from human patients and in human leukemic cell lines HL60 and K562, and its inhibition by alkyl-lysophospholipid. *Cancer Res* **43**: 2955–2961, 1983.
12. Daniel LW, Small GW and Schmitt JD, Alkyl-linked diglycerides inhibit protein kinase C activation by diacylglycerols. *Biochem Biophys Res Commun* **151**: 291–297, 1988.
13. Shoji M, Raynor RL, Berdel WE, Vogler WR and Kuo JF, Effects of thioether phospholipid BM 41.440 on protein kinase C and phorbol ester-induced differentiation of human leukemia HL60 and KG-1 cells. *Cancer Res* **48**: 6669–6673, 1988.
14. Kiss Z, Deli E, Vogler WR and Kuo JF, Antileukemic agent alkyllysophospholipid regulates phosphorylation of distinct proteins in HL-60 and K562 cells and differentiation of HL-60 cells promoted by phorbol ester. *Biochem Biophys Res Commun* **142**: 661–666, 1987.
15. Parker PJ, Kour G, Marais RM, Mitchell F, Pears C, Schaap D, Stabel S and Webster C, Protein kinase C—a family affair. *Mol Cell Endocrinol* **65**: 1–11, 1989.
16. Heesbeen EC, Verdonck LF, Hermans SWG, van Heugten HG, Staal GEJ and Rijksen G, Alkyl-lysophospholipid ET-18-OCH_3 acts as an activator of protein kinase C in HL-60 cells. *FEBS Lett* **290**: 231–234, 1991.
17. Collins SJ, Ruscetti FW, Gallagher RE and Gallo RC, Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. *J Exp Med* **149**: 969–974, 1979.
18. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
19. Collins SJ, Ruscetti F, Gallagher RE and Gallo RC, Terminal differentiation of human promyelocytic leukemia cells induced by dimethylsulfoxide and other polar compounds. *Proc Natl Acad Sci USA* **75**: 2458–2462, 1978.
20. Vallari DS, Smith ZL and Snyder F, HL-60 cells become resistant towards antitumor ether-linked phospholipids following differentiation into a granulocytic form. *Biochem Biophys Res Commun* **156**: 1–8, 1988.
21. House C and Kemp BE, Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. *Science* **238**: 1726–1728, 1987.
22. Zylber-Katz E and Glazer RI, Phospholipid- and Ca^{2+}-dependent protein kinase activity and protein phosphorylation patterns in the differentiation of human promyelocytic leukemia cell line HL-60. *Cancer Res* **45**: 5159–5164, 1985.
23. Makowske M, Ballester R, Cayre Y and Rosen OM, Immunochemical evidence that three protein kinase C isozymes increase in abundance during HL-60 differentiation induced by dimethyl sulfoxide and retinoic acid. *J Biol Chem* **263**: 3402–3410, 1988.
24. Chakravarthy BR, Franks DJ, Whitfield JF and Durkin JP, A novel method for measuring protein kinase C activity in a native membrane-associated state. *Biochem Biophys Res Commun* **160**: 340–345, 1989.
25. Honma Y, Kasukabe T, Hozumi M, Tsushima S and Nomura H, Induction of differentiation of cultured human and mouse myeloid leukemia cells by alkyl-lysophospholipids. *Cancer Res* **41**: 3211–3216, 1981.
26. Hashimoto K, Kishimoto A, Aihara H, Yasuda I, Mikawa K and Nishizuka Y, Protein kinase C during differentiation of human promyelocytic leukemia cell line, HL-60. *FEBS Lett* **263**: 31–34, 1990.