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Differential Effect of Alkyl Chain-Modified Ether Lipids on Protein Kinase C Autophosphorylation and Histone Phosphorylation

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ABSTRACT. Analogues of 1-O-octadecyl-2-O-methyl-*rac*-glycerol-3-phosphocholine (ET-18-OMe), containing a carbonyl group at different positions in the alkyl chain and/or a pentylammonium group in *sn*-3 of glycerol, were evaluated as inhibitors of protein kinase C (PKC; EC 2.7.1.37). The presence of a carbonyl group in the alkyl chain of Et-18-OMe had a dual role in decreasing the inhibitory effect on histone phosphorylation and activating this reaction at low concentrations of compound. The optimal stimulatory effect was observed with the compound having the carbonyl function in C-7 of the alkyl chain. In contrast, all of these compounds were only inhibitors of PKC autophosphorylation, its potency decreasing progressively with the distance between the carbonyl group and the *sn*-1 position of glycerol. Replacement of the phosphocholine group of ET-18-OMe by a pentamethylene trimethylammonium group maintained the inhibitory effect on histone phosphorylation and autophosphorylation of PKC, and the simultaneous introduction of a ketone group in C-7 of the alkyl chain did not decrease any of these effects. The effects of all these analogues on PKC autophosphorylation, but not on histone phosphorylation, correlated quite well with their known antiproliferative activity on human tumor celllines and membranolytic activity. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1843–1847, 1996.

KEY WORDS. protein kinase C; autophosphorylation; histone phosphorylation; alkyl lysophospholipids

Synthetic ether-linked analogues of lysophosphatidylcholine (ALPs)§ possess cytotoxic activity against different types of cancer cells [2]. This biological activity could be due to nonselective effects on the activity of different membrane-located enzymes as a consequence of perturbation of the lipid bilayer by incorporation of the ALP molecules or could be linked to a more specific effect on target enzymes [3, 4].

Several lines of evidence have suggested that PKC (EC 2.7.1.37) may be involved in the antineoplastic effect of different drugs, including ALPs (reviewed in [5]). The activity of PKC is increased in cancer cells [5], and the tumor-promoting activity of phorbol esters may be related to their stimulatory effect on PKC [6]. Furthermore, Et-18-OMe, and ALP in phase 1 clinical evaluation as an antitumor agent, is a potent inhibitor of PKC [3]. This finding prompted the inclusion of the assay of PKC inhibition as one of the criteria in the first screening of new synthetic analogues of phosphatidylcholine as potential antineoplastic agents with improved efficiency and specificity. The activity of PKC on exogenous substrates, mainly histone, has been the parameter most widely used [2, 7–10]. However, no correlation has yet been established between cytotoxic effects and inhibition of PKC [11]. In fact, some lysophosphatidylcholine [7] and distearoylphosphatidylcholine analogues [2] play a dual role in histone phosphorylation in activating PKC at low concentrations while exercising and inhibition effect at rather high concentrations of the compound.

It is worth pointing out that, besides its transphosphorylating activity towards protein substrates, PKC is able to phosphorylate itself, and this autophosphorylation reaction may reflect the intrinsic activity of the enzyme [12]. Interestingly, the autophosphorylation and substrate phosphorylation reactions are differently affected by membrane components because diacylglycerol turns the enzyme on, whereas the phospholipid may affect the specificity towards different substrates [12, 13].

In a previous study [1], a series of ALP analogues containing a carbonyl group at different positions in the C-1

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§ *Abbreviations:* ALP, alkyl lysophospholipid; DMSO, dimethyl sulphoxide; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Et-18-OMe, edelfosine; MNEC, maximal nonerythrolytic concentration; PAF, platelet activating factor; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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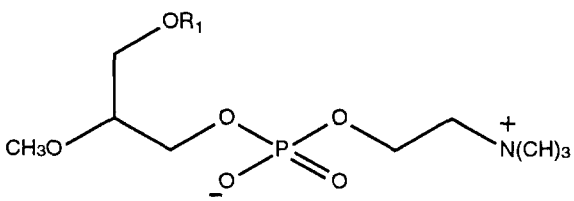
alkyl chain and/or a pentylammonium group in C-3 were shown to possess cytotoxic activity comparable to Et-18-OMe with a lower PAF agonistic effect. The effect of these compounds on the autophosphorylation and transphosphorylation activity of PKC has been evaluated in the present study. An overview of the basic structures of the compounds assayed is presented in Table 1.

MATERIALS AND METHODS

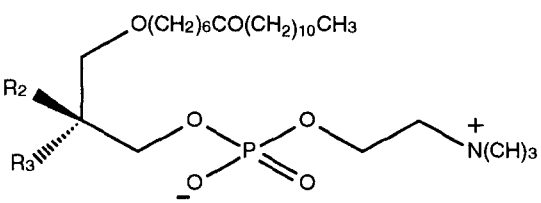
Materials

Phosphatidylserine, diacylglicerol, DMSO and histone (type III-S) were purchased from Sigma Chemical (St. Louis, MO, USA). [γ-^{32}P] ATP was obtained from Amersham (Little Chalfont, Buckinghamshire, UK). Alkyl lysophospholipid analogues were synthesized as previously indicated [1]. The numbering of the compounds has been maintained for the comparison with the data described in this earlier report.

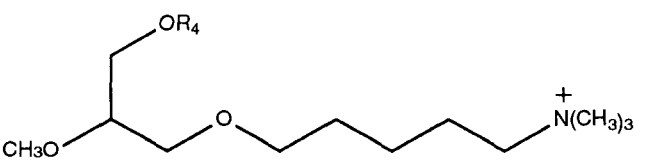
TABLE 1. Chemical Structures of ALP Analogues



Compound	R_1
ET-18-OMe	$-(CH_2)_{17}CH_3$
17	$-CH_2CO(CH_2)_{15}CH_3$
1	$-(CH_2)_3CO(CH_2)_{13}CH_3$
2	$-(CH_2)_6CO(CH_2)_{10}CH_3$
3	$-(CH_2)_{11}CO(CH_2)_5CH_3$



Compound	R_2	R_3
2-S	$-OCH_3$	-H
2-R	-H	$-OCH_3$



Compound	R_4
19	$-(CH_2)_{17}CH_3$
6	$-(CH_2)_6CO(CH_2)_{10}CH_3$

PKC Purification

PKC was purified from rat brain essentially as described up to the hydrophobic chromatography step [14] but by using phenyl-Sepharose instead of Phenyl-5PW and conventional liquid chromatography systems. At this step, the enzyme preparation was nearly homogeneous and showed a single 86-kDa autophosphorylated band, and its histone kinase activity was stimulated 6- to 8-fold by the standard phospholipid mixture.

Lipid Preparations

Phosphatidylserine and diacylglicerol were mixed in chloroform at the desired concentrations, dried under an N_2 stream, suspended in 20 mM Tris buffer (pH 7.5) and subjected to sonication for 45 sec. After this procedure, the mixture of lipids was used immediately. The alkyl lysophospholipid analogues were stored dry at −20°C and were dissolved in ethanol/0.5% DMSO at the moment of use.

Assay of PKC Activity

Assays were done in a total volume of 75 μL and all tubes contained 10 mM $MgCl_2$, 0.2 mg/mL of histone, 10 μM ATP (including 2 μCi of [γ-^{32}P] ATP) and 20 mM Tris (pH 7.5). The assays in the presence of lipids and calcium contained 5 μg/mL phosphatidylserine, 0.4 μg/mL diacylglicerol and 100 μM $CaCl_2$. In the assays without activators, 1 mM EGTA was used instead of calcium and lipids. Enzyme activity was determined as the incorporation of ^{32}P from [γ-^{32}P] ATP into histone. Reactions were initiated by the addition of the enzyme preparation and stopped after 5 min of incubation at 30°C by spotting 30 μL of the reaction mixture on P81 Whatman paper squares (2 × 2 cm). The papers were submerged into a cold solution of 75 mM phosphoric acid and washed for 20 min. After three washes in phosphoric acid, the P81 papers were dehydrated in ethanol and then in acetone. The papers were then dried and their radioactivity determined by scintillation counting.

Determination of PKC Autophosphorylation

The conditions for the autophosphorylation assays were the same as those in the determination of the PKC activity but without histone. After incubation for 5 min at 30°C, the autophosphorylation reaction (80 μL) was stopped by the addition of 20 μL of a sample buffer that contained 0.5 M sucrose, 10% SDS, 312.5 mM Tris, 10 mM EDTA, 250 mM DTT and 0.05% bromophenol blue, and boiled for 2 min. The samples were then analyzed by SDS-PAGE in 10% polyacrylamide gels, according to Laemmli [15]. The gels were dried and analyzed by autoradiography on Amersham Hyperfilm-MP and densitometry of the bands.

RESULTS AND DISCUSSION

The influence of a carbonyl group in the C-1 chain of alkyl ether phospholipids on the inhibition of PKC was tested by

using phosphocholine analogues 1, 2, 2-R, 2-S, 3 and 17 and compared with that obtained with the model compound ET-18-OMe. Under the standard assay conditions described in Materials and Methods, histone phosphorylation by PKC was inhibited by ET-18-OMe with an IC_{50} of approximately 16 μM (Fig. 1A), which is comparable to the values reported in the literature [2, 8, 9]. The presence of a carbonyl group in the C-1 chain renders the compound less inhibitory (Fig. 1). In fact, the ketone analogues started to show a dual role by becoming activators of PKC at low concentrations of compound. Both the loss of inhibition and the compound's potency as activator increased progressively with the distance between the carbonyl group and the *sn*-1 position of glycerol. However, the extent of activation caused by the analogue with the carbonyl group in C-12 was less than that of the compound with the ketone group in C-7, suggesting that the optimal position for the carbonyl function is at, or around, C-7. This notion would be in agreement with a previous report on the effects of branched-chain analogues of diasteroylphosphatidylcholine, where the optimal stimulatory effects were detected with compounds containing butyl groups at the C-8 position of the stearic acid bound to the *sn*-1 and *sn*-2 positions of glycerol [2].

The extent of activation caused by the enantiomers 2-R and 2-S was very similar (Fig. 1). This result indicates that the effect on PKC was not stereospecific.

Stimulation of PKC by these ketone analogues was also observed in the assays in the absence of other phospholipids, which suggested that they could substitute for phosphatidylserine in the activation of PKC. To explore this possibility, the effect of ketone 2 on the activation of PKC by phosphatidylserine was assayed. Low concentrations of this analogue potentiated the stimulatory effect of phosphatidylserine and decreased the apparent Ka values from 2.3 μg/mL to 0.9 μg/mL (Fig. 2A). In contrast, ET-18-OMe behaved as a competitive inhibitor (Fig. 2B), as would be expected from the literature [3]. These data indicate that the distortion of the alkyl chain caused by the presence of the ketone group makes the diacylglycerol/phosphatidylserine/ALP complexes more suitable to direct protein kinase activity toward histone, probably through interaction of the complexes with this substrate [13].

The effect of the ketone analogues on PKC autophosphorylation was also studied (Fig. 3). These compounds inhibited this reaction, and the IC_{50} values were much lower than those required to inhibit histone phosphorylation. The inhibitory potency also decreased as the distance

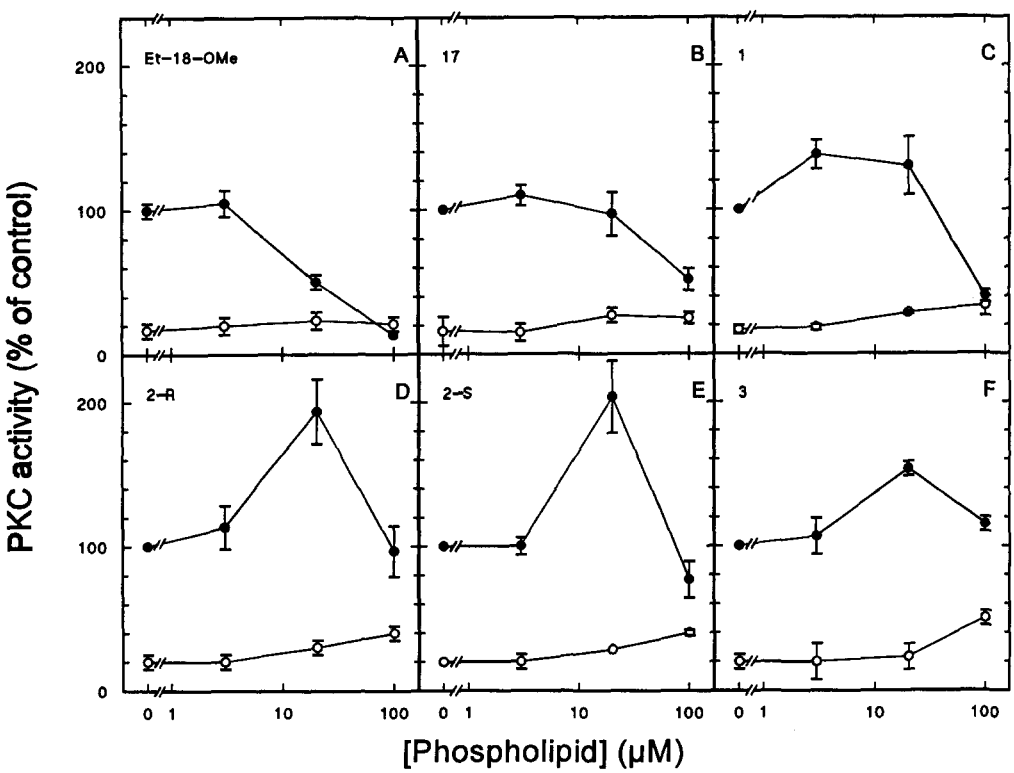


FIG. 1. Effect of different alkyl lysophospholipids on PKC activity in the presence (solid circle) and in the absence (open circle) of activators. The compounds used were (A) ET-18-OMe, (B) 17, (C) 1, (D) 2-R, (E) 2-S and (F) 3. The reaction mixture contained 10 mM $MgCl_2$, 0.2 mg/mL histone H1, 10 μM ATP, 100 μM $CaCl_2$, 5 μg/mL phosphatidylserine and 0.4 μg/mL diacylglycerol when the PKC activators were present. In the assays without activators, 1 mM EGTA was used instead of $CaCl_2$, phosphatidylserine and diacylglycerol. The values represented are the mean of three separate experiments and the error bars show the SEM.

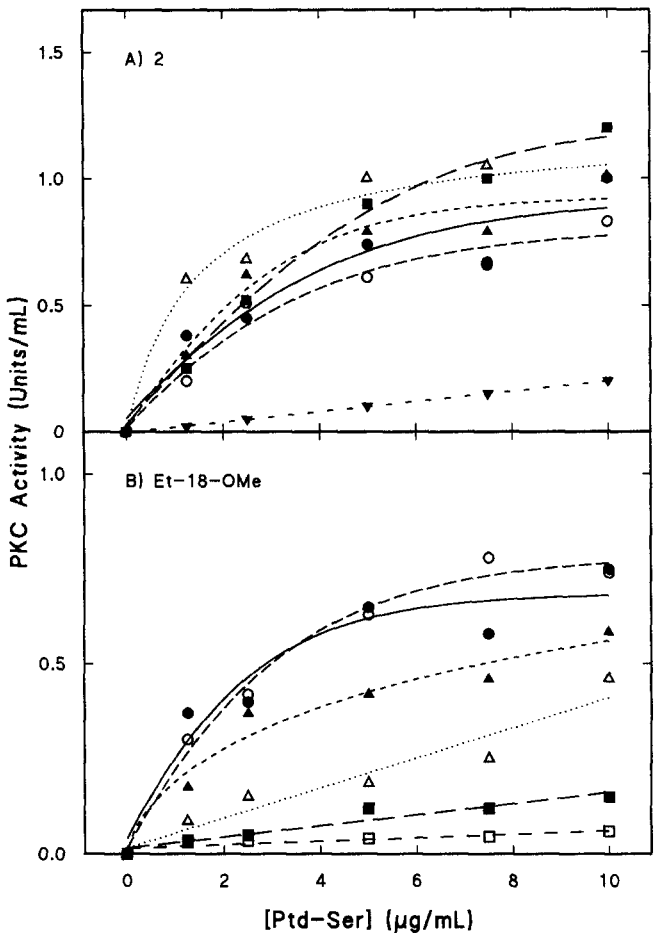


FIG. 2. Influence of ALPs on the phosphatidylserine dependence of PKC. Total PKC activity was assayed by using histone H1 as substrate in the presence of 0.4 μg/mL diacylglycerol, 100 μM $CaCl_2$ and different concentrations of phosphatidylserine. Assays were carried out either in the absence (solid circle) or in the presence of 5 μM (open circle),10 μM (solid triangle), 20 μM (open triangle), 40 μM (solid square), 80 μM (open square) or 100 μM (solid upside-down triangle) of ALPs. The ALPs assayed were compound 2 (A) as the racemic mixture and ET-18-OMe (B).

between the carbonyl group and the *sn*-1 position of glycerol increased, but, interestingly, no stimulatory effect was observed with any of these compounds. A previous study has shown that they possessed important antiproliferative activity on three different human tumor cell lines, including HL-60 cells and membranolytic activity [1]. When the values obtained in that study were plotted against the position of the carbonyl group in the C-1 chain (inset in Fig. 3) and compared with the IC_{50} values for the inhibition of autophosphorylation of PKC, a very similar profile was obtained. Furthermore, the IC_{50} values for growth inhibition of HL-60 cells and for inhibition of the autophosphorylation of PKC were within the same range of concentrations, both being lower than the MNEC. This result supports the idea that changes in the autophosphorylation activity of PKC could be linked to the cytotoxic effect of these analogues.

Some quaternary ammonium derivatives of alkyl glycerols with cytotoxic effect on HL-60 cells are also potent inhibitors of histone phosphorylation by PKC [9, 10]. Replacement of the phosphocholine group of ET-18-OMe by a pentamethylene trimethylammonium group (compound **19**) maintained the cytotoxicity of the analogue, and the simultaneous introduction of a ketone group in C-7 of the alkyl chain (compound **6**) slightly increased this biological effect [1]. As expected from the data with similar compounds, the pentamethyleneammonium analogue **19** inhib-

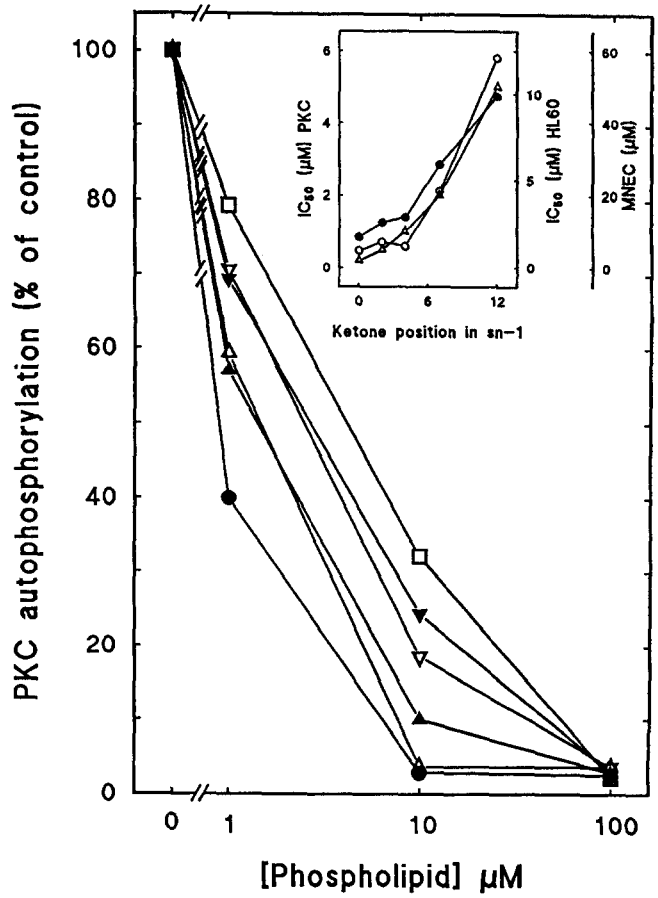


FIG. 3. Effect of different ALPs on PKC autophosphorylation. The ALPs used were ET-18-OMe (solid circle), compound 17 (open triangle), 1 (solid triangle), 2-R (open upside-down triangle), 2-S (solid upside-down triangle) and 3 (open square). The autophosphorylation conditions were 10 mM $MgCl_2$, 10 μM ATP, 100 μM $CaCl_2$, 5 μg/mL phosphatidylserine and 0.4 μg/mL diacylglycerol when the PKC activators were present. In the assays without activators, 1 mM EGTA was used instead of $CaCl_2$, phosphatidylserine and diacylglycerol. The autophosphorylated samples were analyzed by SDS-PAGE and the autophosphorylation quantified by densitometry of the autoradiograph. The values represented are the mean of 3–5 separate experiments and the error bars show the SEM. The inset shows the correlation between the position of the ketone group in the *sn*-1 chain of the ALP analogues, the IC_{50} for PKC autophosphorylation (solid circle) and the values for the IC_{50} for HL-60 cells (open circle) and the MNEC (open triangle) reported by Fos *et al.* [1] for the same compounds.

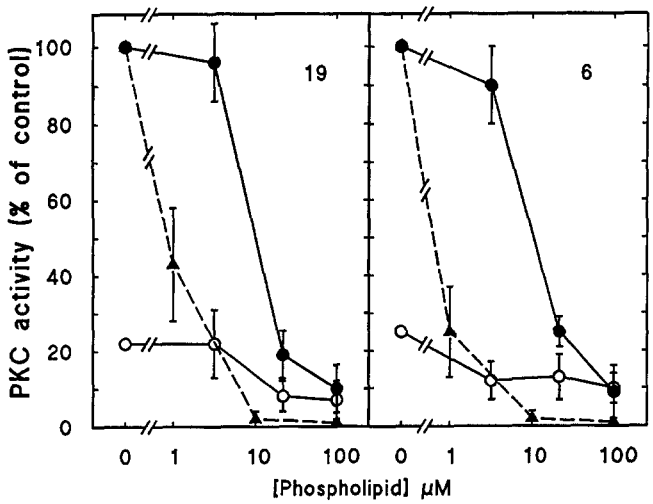


FIG. 4. Effect of compounds 19 and 6 on PKC activity, using histone H1 as substrate, with (solid circle) and without activators (open circle), and on PKC autophosphorylation (solid triangle). The histone kinase activity in the presence and in the absence of phospholipids was determined as in Fig. 1, and the PKC autophosphorylation was assayed as in Fig. 3. The values represented are the mean of three separate experiments and the error bars show the SEM.

ited histone phosphorylation by PKC (Fig. 4) as efficiently as ET-18-OMe. Unexpectedly, its analogue with a ketone group in C-7 (compound **6**) was also a strong inhibitor of histone phosphorylation (Fig. 4). This effect is in contrast with what was observed with the phosphocholine analogues, where the presence of the carbonyl group rendered the compound an activator of PKC (Figs. 1A,D,E, 2). Compounds **6** and **19** were also potent inhibitors of PKC autophosphorylation, with IC_{50} values in the range of 0.7–0.9 μM. This result agrees with their marked cytotoxic effects on HL-60 cells and their low MNEC values [1].

In conclusion, our data suggest that the effects of these analogues on PKC autophosphorylation reflect better than the inhibition of histone phosphorylation the biological potency of the ALP analogues as cytotoxic agents.

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