Hexadecylphosphocholine Inhibits Inositol Phosphate Formation and Protein Kinase C Activity

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ABSTRACT

Hexadecylphosphocholine (HePC) inhibits protein kinase C (PKC) from NIH3T3 cells in cell-free extracts with a 50% inhibitory concentration of about 7 μM. Inhibition is competitive with regard to phosphatidylserine with a Ki of 0.59 μM. In order to determine whether HePC affects PKC in intact cells, the bombesin or tetradecanoylphorbolacetate-induced, PKC-mediated activation of the Na+/H+-antiporter was determined. It is demonstrated that HePC causes a drastic inhibition of this enzyme indicating a similar sensitivity of PKC to HePC in intact cells compared to cell-free extracts. In addition to the effects on PKC, treatment of NIH3T3 cells with HePC depresses the bombesin-induced formation of inositol 1,4,5-trisphosphate and the concomitant mobilization of intracellular Ca2+. Dose-response curves for the inhibition of inositol 1,4,5-trisphosphate formation and Ca2+ mobilization reveal 50% inhibitory concentrations of 2 or 5 μM, respectively. Polyphosphorylated phosphoinositides accumulate in HePC-treated cells indicating that the depression of inositol 1,4,5-trisphosphate generation is not caused by an inhibition of phosphoinositide kinases. Addition of bombesin to HePC-treated cells in the presence of LiCl revealed no evidence for an accelerated rate of inositol 1,4,5-trisphosphate turnover by the phospholipid analogue. It is concluded that HePC inhibits phosphoinositide C in intact cells. The data strongly suggest that the growth-inhibitory effect of HePC is at least in part explained by the interference with mitogen signal transduction.

INTRODUCTION

Synthetic phospholipids have been shown to exert antitumor activity (1–3). The compounds most widely used represent 1-O (or S)-alkyl analogues of 2-lysophosphatidylcholine. The detailed mechanism underlying the growth-inhibitory effect of these compounds is not entirely clear. Recent evidence from this and other laboratories indicates that ALPs inhibit Ca2+- and phospholipid-dependent protein kinase (4, 5). The inhibition of tumor cell multiplication occurs at the same drug concentrations as does the depression of this enzyme (4). Considering the essential role of PKC in growth factor signal transduction and cellular proliferation (6) it seemed conceivable to attribute the growth-inhibitory effect of ALP to the blockade of this enzyme. Hexadecylphosphocholine exhibits remarkable antitumor activity in vivo and in vitro (7, 8). This compound differs from the “conventional” ALP by the lack of a glycerol backbone. The mechanism responsible for the growth-inhibitory effect of HePC is unclear. ALP inhibits PKC by competing with the biological activator of this enzyme, i.e., phosphatidylserine (4, 5). The structural differences between ALP and HePC, especially the lack of a diacylglycerol moiety in HePC, suggest a different mechanism of action. It was decided, therefore, to determine whether HePC affects PKC and, if an inhibition can be observed, what the mechanism of inhibition is. Furthermore, HePC as a potential membrane active agent may affect other membrane-bound enzymes besides PKC. In this respect, the phospholipase C or phosphoinositidase C hydrolyzing PIP2 to Ins(1,4,5)P3 and diacylglycerol is of particular interest inasmuch as this enzyme is presumed to be involved in signal transduction of a variety of growth factors (for a recent review see Ref. 9). The studies presented here demonstrate that HePC, beside an inhibition of PKC, depresses the bombesin-induced elevation of Ins(1,4,5)P3.

MATERIALS AND METHODS

Chemicals. Tissue culture dishes were purchased from Falcon. Serum and culture medium were obtained from Boehringer-Mannheim, Mannheim, Federal Republic of Germany. Phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, all other phospholipids, TPA, fatty acid free BSA, lysine-rich histone H1 (calf thymus type III), 1,2-diolein, L-α-phosphatidyl-L-serine, leupeptin, aprotinin, and bombesin were purchased from Sigma Chemicals, Munich, Federal Republic of Germany. Silica gel-coated glass plates (high performance thin layer chromatography plates, 10 x 20 cm; No. 5641) were from Merck AG, Darmstadt, Federal Republic of Germany. myo-[2-3H]inositol (12.8 Ci/mmol) was from New England Nuclear, Dreieich, Federal Republic of Germany. [-y-32P]ATP (30 Ci/mmol) was supplied by Amersham, Little Chalfont, United Kingdom. Multi-screen H 96-well filtration plates were from Millipore, Austria. DEAE-dextran (DE52) was obtained from Whatman, Clifton, NJ. BCECF and fura-2 were purchased from Molecular Probes, Eugene, OR. Inositol-free DMEM was from Amimed, Basel, Switzerland. Hexadecylphosphocholine was a gift from Dr. P. Hilgard, ASTA-Pharma, Bie elfeld, Federal Republic of Germany. A stock solution of HePC containing 10 mM HePC in 20 mM Tris-HCl, pH 7.4, was prepared and stored at −20°C.

Cell Culture. NIH3T3 cells were grown in DMEM supplemented with 10% FCS in a humidified atmosphere of 95% air with 5% CO2. One day after plating, cells were made quiescent by incubation in inositol-free DMEM containing 0.5% FCS (in the case of inositol phosphate measurement, see below) or DMEM plus 0.5% FCS (for Ca2+ determinations) for 24 h.

Measurement of Cell Proliferation. Cells were plated on 35-mm culture dishes (6-well plates) at 0.5–0.8 x 10⁵ cells/well. Growth was established for 18–24 h and the cell number of representative wells was determined (time 0). The experiments were started by addition of fresh prepared solution of HePC at given concentrations to the cells or equal volumes of Tris-HCl to control cells. After incubation for 60 h, cells were counted with an electronic counter (Coulter Electronics, Luton, United Kingdom). Cellular multiplication (M) was calculated according to the equation...
C represents the number of untreated control cells and T the number of drug-treated cells at time t (h) and time 0 (0 h), respectively. During 60 h, control cells multiplied 5.16 ± 0.4-fold with a population-doubling time of 22.4 ± 1.3 h for cells grown in 10% FCS and 38 ± 3.2 h for cells grown in 5% FCS.

Determination of the Cytosplasmic pH. NIH3T3 cells were grown on coverslips (9 x 18 mm). One day after plating, cells were grown arrested by incubation in low serum (0.5% FCS) for at least 48 h. At this time point average cell number per coverslip was 0.5 to 1 x 10^6 cells. pH_{i} was determined by fluorescence spectroscopy with the use of BCECF. The cells were loaded with BCECF by addition of 2.5 μM BCECF acetoxymethyl ester for 10 min at 37°C. Then the fibroblasts attached to rectangular coverslips were washed twice with a HCO_{3}^- -free HBS. For fluorescence measurements one coverslip supporting the cells was placed into a 1 x 1 x 3-cm³ quartz cuvet containing 2 ml of HBS at 37°C. The coverslip in the cuvet was oriented at a 60 degree angle relative to the excitation beam while 0.6 cm² of the cover glass was illuminated. Fluorescence (excitation 502 nm or 440 nm, emission 530 nm) was detected with a SPEX (Edison, NJ) Fluorolog 2 spectrofluorometer (CM-1) equipped with two excitation monochrometers and a chopper system. The cytoplasmic pH values were calculated from the ratio of the fluorescence intensities I_{503/540}. At the end of each experiment the ratio of the fluorescence intensities I_{503/540} was calibrated to pH_{i} using a nigericin calibration procedure as described (10), with the use of a buffer consisting of 140 mM KCl, 10 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/KOH, and 10 μg/ml nigericin.

Determination of Cytosolic Ca^{2+} Concentrations. Cells were prepared for cytosolic Ca^{2+} concentration measurements by growing on glass coverslips (9 x 18 mm). Ca^{2+} was determined by fluorescence spectroscopy with the use of fura-2 (11). Cells attached to rectangular coverslips were loaded with fura-2 by incubation in HBS containing 5 μM acetoxymethyl ester for 60 min at 37°C. For fluorescence measurements one coverslip supporting the cells was placed into a 1 x 1 x 3-cm³ quartz cuvet containing 2 ml HBS in the presence of 10 μM EGTA to remove the extracellular free Ca^{2+}. Fluorescence (excitation 350 nm or 385 nm, slit width 1 nm; emission 510 nm, slit width 1 nm) was measured by a SPEX Fluorolog 2 spectrofluorometer (CM-1) equipped with two excitation monochrometers and a chopper system. Values of [Ca^{2+}]_{i} in nM were calculated from the ratio of observed fluorescence intensities (I_{350/385}) of the intracellular fura-2 as

\[ [Ca^{2+}]_{i} = K_{D} \times f_{1} \times \left( \frac{I_{350/385}}{I_{350/385}} - \frac{I_{350/385}}{I_{350/385}} \right) \]

where K_{D} = 225 nm for Ca^{2+}/fura-2 at cytoplasmic ionic conditions. The factors f_{1} = 12.2, I_{385/385} = 26.3 and I_{350/385} = 1 were determined in a separate EGTA-Ca^{2+} calibration procedure as described (11).

Isolation of Inositol Phosphates and Analysis of Phosphatidylinositol. NIH3T3 cells (1.4–1.8 x 10^6) were seeded onto 35-mm culture dishes (6-well plates). After 24 h, cells were washed twice with an incubation buffer (140 mM NaCl, 2.7 mM KCl, 4.6 mM NaH_{2}PO_{4}, 1.7 mM NaHPO_{4}, 1 mM CaCl_{2}, 0.5 mM MgCl_{2}, 5.5 mM glucose, pH 7.4) and incubated for 48 h in 1 ml inositol-free DMEM (1:1, inositol-free DMEM:nonsupplemented essential medium) containing 0.5% FCS in the presence of 10 μM myo-[2-3H]inositol per ml. Cell density at this time was approximately 3–4 x 10^6 cells/dish. The cells were washed again, 1 ml incubation buffer (containing the appropriate BSA and Hepes concentrations) was added, and the cells were incubated for another 2 h. Where indicated, the fibroblasts were stimulated with 10 μM bombesin for 10 s. The incubation was terminated by aspiration of the buffer and addition of 1 ml of ice-cold 15% TCA/dish. After 30 min on ice, the TCA extract was collected. Cell layers were rinsed with 1 ml H_{2}O, and the wash fluid was combined with the initial 1 ml 15% TCA. The TCA present in the extract was removed by four sequential washes with 8 ml diethyl ether, and the water-soluble inositol phosphates were separated by ion exchange chromatography on AG 1 X8, formate form (Bio-Rad, Vienna, Austria) following the procedure of Berriedge and Irvine (12).

Phosphoinositides. The TCA-insoluble fractions were extracted with 2 ml chloroform:methanol:12 n HCl (200:100:1), the dishes were rinsed with 1 ml of the same solution, and the combined extracts were mixed with 1 ml of chloroform and 1 ml of 0.1 n HCl. After centrifugation (600 x g, 10 min), the (lower) hydrophobic phase was transferred to glass vials and dried under a stream of nitrogen at 30°C. The dried pellets were solubilized in 40 μl of chloroform:methanol:water (75:25:1). After a sonication procedure (10 s, 40 W, in a water bath at 25°C), 15 μl were loaded to the high performance thin layer chromatography plate. Phospholipids were analyzed by ascending thin-layer chromatography as described by van Dongen et al. (13). Plates were pretreated with potassium oxalate [1% in methanol:H_{2}O (2:3)]. After 45 min at room temperature, the plates were dried for 2 min at 110°C. Phospholipids were separated using a solvent system composed of chloroform:methanol:ammonia:water, 64:57:4:5:13.5 (v/v). The spots were visualized with a spray containing 3% copper acetate in 8% phosphoric acid until the gel layer became transparent. After being dried for 10 min at 160°C, the plates were scanned with an automatic thin-layer chromatography linear analyzer (Berthold München GmbH, Munich, Federal Republic of Germany) for radioactivity.

Preparation of Protein Kinase C. Protein kinase C was partially purified from NIH3T3 cells as described by Walton et al. (14). Cell extracts were prepared from confluent NIH3T3 cells (10-mm cell culture dishes). The medium was discarded, and the cell monolayer was rapidly washed twice with 2 ml of ice-cold wash buffer (20 mM Tris-HCl-150 mM NaCl-5 mM glucose-leupeptin, 20 μg/ml aprotinin, 2 μg/ml, pH 7.4). All subsequent steps were carried out at 4°C. The wash buffer was removed and the cells were collected and resuspended in homogenization buffer [50 mM Tris-HCl-5 mM dithiothreitol-1 mM phenylmethylsulfonyl fluoride-2 mM EGTA-0.1% Triton X-100 (w/v)-20 μg/ml leupeptin-2 μg/ml aprotinin]. The cells were disrupted by a sonication procedure (MSE sonifier at 60 W) and centrifuged at 1,500 x g for 5 min, yielding the crude nuclear pellets and the postnuclear supernatants. The combined supernatants were centrifuged at 100,000 x g for 1 h and the resulting soluble membrane extracts were applied onto DE-52 columns (2.5 x 8 cm; Whatman). Protein was eluted with a gradient from 0.0–0.4 M NaCl in elution buffer (20 mM Tris-HCl-200 mM mercaptoethanol-0.1 mM EGTA-0.1 mM EDTA-2% glycerol, pH 7.4).

Determination of Protein Kinase C Activity. PKC was assayed by measuring the incorporation of 32P from [γ-32P]ATP into histone H1 following the method of Aflab and Hait (15). Reactions [total volume of 125 μl, containing 0.133 μM [γ-32P]ATP (30 Ci/mmol, 40 mM Tris-HCl (pH 7.4), 1 mM CaCl_{2}, 700 μg EGTA, 50 μg histone H1 type III, 0.1 μM L-α-phosphatidyl-l-serine, and 3 μM 1,2-dioctanoyl-rac-glycerol], were set up in a 96-well plate. The assays were started by the addition of [γ-32P]ATP with an eight channel pipettor. After incubation for 15 min at 32°C the reactions were terminated by the addition of 150 μl 20% trichloroacetic acid. The reaction mixtures were transferred to 96-well filter plates and were filtered using a semiautomatic 96-well assay system. The filter disks (type HA) were collected by using a filter punch apparatus (Millipore) and counted in a liquid scintillation counter. PKC activity was calculated as the difference of specific Ca^{2+} and phospholipid-dependent kinase activity and nonspecific phosphotransferase activity (transfer in the presence of EGTA without phospholipids).

RESULTS

Inhibition of Protein Kinase C. Hexadecylphosphocholine inhibits Ca^{2+}, phospholipid-dependent PKC from NIH3T3 cells in cell-free extracts with an IC_{50} of about 7 μM (Fig. 1). The inhibitory effect is competitive with respect to phosphatidylserine with a K_{i} of 0.56 μM (Fig. 2, inset). In order to investigate
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Fig. 1. Effect of HePC on PKC activity. PKC was prepared from NIH3T3 cells as described in "Materials and Methods." The enzyme was incubated for 15 min in the presence of 100 nm phosphatidylserine, 1 mm CaCl₂, and concentrations of HePC (for structure, see inset) as indicated. PKC activity was determined as described in "Materials and Methods." Bars, SEM of three independent experiments.

Fig. 2. Inhibition of PKC by HePC competition with phosphatidylserine (PS). The enzyme was assayed under standard conditions except for various concentrations of phosphatidylserine (as indicated) and HePC (10 μM). Inhibition was competitive with respect to phosphatidylserine with a Kᵢ of 0.59 μM as calculated from double reciprocal plot (inset). Each point represents the mean from 2 independent determinations. A, control; B, 10 μM HePC. DAG, diacylglycerol.

whether HePC affects PKC in intact cells, we determined the PKC-mediated activation of the Na⁺/H⁺-antiporter. In accordance with numerous observations in other systems (16, 17), we have shown previously that addition of bombesin or TPA to growth-arrested NIH3T3 fibroblasts leads to a PKC-mediated activation of the Na⁺/H⁺-antiporter resulting in an increase in cytosolic pH, (10). PKC dependency was shown by the sensitivity of the antiporter activity to the PKC inhibitor staurosporine and by the fact that bombesin- or TPA-induced alkalinization is eliminated in PKC-depleted cells (10). Thus, bombesin- or TPA-induced activation of the Na⁺/H⁺-antiporter can be used as an indirect marker for PKC activity in intact cells. The activity of the Na⁺/H⁺-antiporter was determined by measuring the dimethylamiloride-sensitive cytosolic alkalinization. As shown in Fig. 3 HePC depresses the bombesin- or TPA-induced cytosolic alkalinization. A direct inhibition of the Na⁺/H⁺-antiporter by HePC could not be observed (data not shown). The data indicate that concentrations inhibiting PKC in vitro are equally effective in intact cells.

Interference with Inositol 1,4,5-Trisphosphate Formation. In order to investigate whether, in addition to the depression of PKC, HePC interferes with other membrane-associated enzymes which are involved in growth factor signal transduction, we studied the effect of this compound on bombesin-induced inositol 1,4,5-trisphosphate formation. Addition of bombesin to quiescent NIH3T3 cells causes a rapid hydrolysis of PIP₂ to Ins(1,4,5)P₃ and diacylglycerol (18). The kinetics of Ins(1,4,5)P₃ formation after stimulation of growth-arrested cells by bombesin is shown in Fig. 4. Fig. 5 demonstrates that HePC inhibits bombesin-induced Ins(1,4,5)P₃ formation with an IC₅₀ of approximately 2 μM. Hexadecylphosphocholine does not interfere with the phosphorylation of phosphatidylinositol to PIP₂. Fig. 6 demonstrates that both phosphatidylinositol 4-phosphate as well as PIP₂ accumulate in cells incubated in the presence of 10 μM HePC. LiCl was used in order to determine whether the reduction of Ins(1,4,5)P₃ formation by HePC is caused by an accelerated rate of degradation. Li⁺ has been shown to inhibit the inositol phosphatase hydrolyzing inositol 1-phosphate and thereby blocking the recycling of inositol (12). As a result of this blockade, inositol phosphate accumulates in the presence of Li⁺ under conditions which enhance inositol phosphate
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Fig. 4. Ins(1,4,5)P_3 (IP_3) formation after stimulation of growth-arrested cells by bombesin. NIH3T3 cells were grown and prepared for Ins(1,4,5)P_3 analysis as described in "Materials and Methods." Where indicated 1 μM bombesin was added. Each point represents the mean of at least 3 independent experiments ± SE (bars).

Fig. 5. Effect of HePC on bombesin-induced Ins(1,4,5)P_3 formation. NIH3T3 cells were grown and prepared for Ins(1,4,5)P_3 analysis as described in "Materials and Methods." For the last 2 h of the incubation period the cells were kept in 1 ml incubation buffer containing the bovine serum albumin, FCS, and HePC concentrations as indicated. Data are expressed as percentage of the maximal bombesin-induced Ins(1,4,5)P_3 response in corresponding controls in the absence of HePC, i.e., 10–20 s after addition of bombesin (compare Fig. 4). Mean of two independent experiments (0.1% FCS; 0% bovine serum albumin) or the mean ± SE (bars) (n ≥ 3). Inset, effect of HePC on bombesin-induced Ins(1,4,5)P_3 formation in the presence of 0.4 mg/ml BSA.

Fig. 6. Accumulation of phosphatidylinositol 4-phosphate (PIP_2) and phosphatidylinositol 4,5-bisphosphate (PIP_3) in cells treated with HePC. NIH3T3 cells were grown and prepared for phosphatidylinositol analysis as described in "Materials and Methods." Values of phosphatidylinositol 4-phosphate and PIP_2 are calculated as percentage of the control level and represent the mean of at least 3 independent experiments ± SE (bars).

Table 1. Effect of LiCl on the depression by HePC of bombesin-induced inositol phosphate formation

<table>
<thead>
<tr>
<th>HePC (μM)</th>
<th>-Bombesin</th>
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<td>lithium</td>
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<tr>
<td>0</td>
<td>7,161 ± 1,064</td>
<td>9,785 ± 2,197</td>
<td>42,333 ± 7,518</td>
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<tr>
<td>5</td>
<td>8,534 ± 2,030</td>
<td>8,309 ± 2,446</td>
<td>17,166 ± 1,700</td>
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<tr>
<td>10</td>
<td>10,546 ± 2,197</td>
<td>10,373 ± 2,101</td>
<td>13,000 ± 2,343</td>
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The data presented here demonstrate that HePC inhibits PKC activity in cell-free extracts as well as in intact cells. The mechanism of inhibition of PKC by HePC is similar to the mode of action of ALP, namely a competition with the biological activator phosphatidylserine. This is remarkable considering the lack of a diacylglycerol moiety in HePC. These results are in agreement with data reported by Zheng et al. (19) which were published during the preparation of the manuscript. There

**DISCUSSION**

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is no competition of HePC with diacylglycerol (data not shown). The apparent $K_i$ for HePC of 0.59 $\mu$M is also very similar to the $K_i$ obtained with other synthetic ALP (4). In view of the strong competition with phosphatidylserine it seemed questionable whether the active, membrane-bound PKC would be affected by HePC in intact cells. The influence of HePC on PKC in intact cells was determined by the bombesin- or TPA-induced PKC-mediated activation of Na$^+$/H$^+$-antiporter. The inhibition by HePC of this enzyme clearly indicates that drug concentrations depressing the enzyme activity in cell-free extracts are also effective in intact cells. PKC is, however, not the only target of HePC. As shown here, HePC depresses the bombesin-induced inositol phosphate formation and consequently attenuates the release of intracellular Ca$^{2+}$. We presume that this effect is caused by an inhibition of phosphoinositidase C. This assumption is bound on the observation that (a) the formation of the phosphoinositidase C substrate, PIP$_3$, is not inhibited and (b) the rate of Ins(1,4,5)P$_3$ degradation was not found to be enhanced. Hexadecylphosphocholine has been shown to be accepted as a substrate by some phospholipase C-type enzymes (8). It has not yet been studied in any detail if and to what extent HePC competes with biological substrates for different phospholipase C enzymes. An interference with the phosphatidylinositol 4,5-bisphosphate-specific phospholipase C, in particular, has not yet been investigated. However, the rate of hydrolysis of HePC by a bacterial phospholipase C was found to be 10$^4$ times slower compared to the rate of lecithin degradation. It is conceivable, therefore, that HePC acts as a competitive inhibitor for some phospholipase C-type enzymes. Alternatively, HePC may indirectly inhibit phospholipase C activity by disturbing the phospholipid environment of the enzyme. Studies on purified phospholipase C or phosphoinositidase C are required in order to elucidate the mechanism of action of the inhibitory effect of HePC. The inhibition of phosphoinositidase C by HePC may contribute to the blockade of PKC in intact cells by reducing the formation of diacylglycerol, the biological activator of this enzyme. It should be emphasized that HePC-treated cells exhibit normal levels of cytosolic Ca$^{2+}$ even in the presence of 5 $\mu$M HePC in serum-free media. This observation strongly argues against extensive membrane disintegration or leakage as a consequence of HePC treatment. In view of the data presented here we suggest that the antitumor activity of HePC is at least in part caused by an inhibition of mitogenic signal transduction and second messenger generation. Independent and simultaneous studies by Seewald et al. (20) revealed that ether lipids exert similar effects on mitogen-induced Ca$^{2+}$ mobilization and inositol phosphate generation in intact cells by reducing the formation of diacylglycerol, the biological activator of this enzyme. It should be emphasized that HePC-treated cells exhibit normal levels of cytosolic Ca$^{2+}$ even in the presence of 5 $\mu$M HePC in serum-free media. This observation strongly argues against extensive membrane disintegration or leakage as a consequence of HePC treatment. In view of the data presented here we suggest that the antitumor activity of HePC is at least in part caused by an inhibition of mitogenic signal transduction and second messenger generation. Independent and simultaneous studies by Seewald et al. (20) revealed that ether lipids exert similar effects on mitogen-induced Ca$^{2+}$ mobilization and inositol phosphate generation as HePC. Thus despite structural differences the biological effects of HePC and ether lipids appear to be rather similar.

REFERENCES


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