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INHIBITION OF PHOSPHOLIPASE Cδ BY HEXADECYLPHOSPHORYLCHOLINE AND LYSOPHOSPHOLIPIDS WITH ANTITUMOR ACTIVITY

TADEUSZ PAWELCZYK* and JOHN M. LOWENSTEIN†
Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254, U.S.A.

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Abstract—The antineoplastic compound hexadecylphosphorylcholine (HPC) was shown to be a highly effective inhibitor of phospholipase Cδ (PLCδ$_1$), with an I_{50} of about 30 nmol/mL (30 μM) in the presence and absence of 200 μM spermine. A number of lysophospholipids, of which HPC can be considered to be a structural analog, also inhibited PLC. Lysosphingomyelin, lysophosphatidylserine, and lysophosphatidylcholine exhibited I_{50} values of 15, 10, and 7 nmol/mL, respectively, in the presence of 200 μM spermine. The I_{50} values were increased to 21–53 nmol/mL in the absence of spermine. *N,N*-Dimethylsphingosine and *N,N,N*-trimethylsphingosine, which inhibit the metastatic potential of human and murine tumor cells, were weak activators of PLCδ$_1$. It is postulated that HPC is more effective as an antineoplastic agent than lysophospholipids because HPC is metabolized slowly, while the lysophospholipids are metabolized rapidly *in vivo*.

Hexadecylphosphorylcholine (HPC‡) is an antineoplastic compound which was synthesized on the basis of the minimum structural requirements for antitumor properties of certain ether phospholipids and their analogs [1, 2]. HPC inhibits the growth of mammary tumors in rats [3–6]. It causes skin tumor regression in human breast cancer patients with skin involvement [7]. Its therapeutic efficacy in several other tumors is currently in phase II studies [8]. HPC also shows antineoplastic activity on HL60, U937, Raji, and K562 leukemia cell lines *in vitro* [6, 9]. For unknown reasons, a number of other types of tumors are not sensitive to HPC [8]. HPC is absorbed readily and its phosphorylcholine moiety is converted into choline and phosphate, indicating that it is hydrolyzed via phosphodi- and monoesterases. A portion of these breakdown products is incorporated into phosphatidylcholine [10]. It has been suggested that HPC exerts it antineoplastic effect by inhibiting protein kinase C (PKC) [11–14], and it was shown that HPC inhibits partially purified PKC from pig brain [15] and NIH 3T3 cells [16]. In addition, HPC inhibits IP_3 formation by NIH 3T3 cells in response to bombesin, a finding which led to the suggestion that HPC may act by inhibiting PLC [16]. We now show that HPC and lysophospholipids are strong inhibitors of pure phospholipase Cδ, subtype δ$_1$ (PLCδ$_1$) *in vitro*.

MATERIALS AND METHODS

Materials. PLCδ$_1$ was prepared from rat liver as described previously [17]. Analyses of tryptic peptides showed this to be the δ$_1$ isoform of PLC (Haber MT and Lowenstein JM, unpublished observations). PLCδ$_1$ from human fibroblasts was expressed in *Escherichia coli* (Ghosh S, Pawelczyk T and Lowenstein JM, unpublished) and purified as described previously [17]. The plasmid containing the cDNA for PLCδ$_1$ was a gift from Dr. J. Knopf, Genetics Institute, Cambridge, MA [18]. HPC was a gift from Dr. P. Hilgard, ASTA-Pharma, Bielefeld, Germany. *N,N*-Dimethylsphingosine and *N,N,N*-trimethylsphingosine were gifts from Dr. S-i. Hakamori, The Biomembrane Institute, Seattle, WA. Phospholipids and lysophospholipids were obtained from Avanti Biochemicals, Birmingham, AL.

Detergent assay for PLCδ. The reaction mixture contained 17 nmol/mL [2-^{3}H]inositol-labeled phosphatidylinositol-4,5-bisphosphate (PIP_2) (1200–1400 dpm/nmol), 2.4 mM sodium deoxycholate, 180 mM NaCl, 100 μM $CaCl_2$, 100 μM EGTA, 50 mM HEPES-NaOH buffer, pH 7.2, and, where indicated, 200 μM spermine [19]. Phospholipids were stored in chloroform–methanol (2:1) under argon. The desired amount of lipid or HPC solution was evaporated to dryness under argon, the reaction mixture was added, and the mixture was sonicated to yield deoxycholate micelles containing the lipid and HPC. The reaction was started by adding PLCδ$_1$ and was run in a final volume of 0.1 mL at 37° for 1 min. In this standard reaction mixture, the concentration of free Ca^{2+} was 2.2 μM as determined by the arsenazo method [20]. Where indicated, free [Ca^{2+}] was varied by changing the proportion of $CaCl_2$ to EGTA in the calcium buffer.

Liposome assay for PLCδ. The reaction mixture contained 25 nmol/mL [2-^{3}H]inositol-labeled PIP_2 (1200–1400 dpm/nmol), 200 nmol/mL of a 9:1 mixture of phosphatidylethanolamine (PE) and phosphatidylcholine (PC), and HPC or a lysophospholipid as indicated in the figure legends.

* On leave from the Medical Academy Gdansk, Poland.
† Corresponding author. Tel. (617) 736-2360; FAX (617) 736-2349.

‡ Abbreviations: HPC, hexadecylphosphorylcholine; SM, sphingomyelin; PS, phosphatidylserine; PC, phosphatidylcholine; PLCδ, phospholipase Cδ; PKC, protein kinase C; PE, phosphatidylethanolamine; PIP_2, phosphatidylinositol-4,5-bisphosphate; and I_{50}, concentration of inhibitor yielding 50% inhibition.

Solutions of the phospholipids and lysophospholipid or HPC were mixed and evaporated to dryness under argon. The residue was sonicated for 10 min in the presence of 180 mM NaCl, 100 μM $CaCl_2$, 100 μM EGTA, and 50 mM HEPES-NaOH buffer, pH 7.2, to yield liposomes. In this standard reaction mixture, the free calcium concentration was 2.2 μM. The reaction was started by adding enzyme and was run in a final volume of 0.1 mL at 37° for 1 min.

In both assays, the reaction was stopped by adding 0.1 mL of 1.2 N HCl, the mixture was vortexed, 0.5 mL chloroform–methanol (2:1) was added, and the mixture was vortexed again. The mixture was allowed to settle, the aqueous layer was separated, and an aliquot was counted. Assay conditions were chosen so that the reaction rate was proportional to time and enzyme concentration. In both assays neither the substrate nor the inhibitor was in true solution. For this reason we prefer to quote their concentrations as nmol/mL. If substrate and inhibitor were in true solution, nmol/mL would become μM. The liposome assay leveled off when about 68% of the 25 nmol/mL of PIP_2 was hydrolyzed. Upon adding 2.4 mM deoxycholate to the reaction mixture, the remainder of the substrate was hydrolyzed to within 98–99% of completion. Thus, in the liposomes used in the assay 17 nmol/mL PIP_2 (68% of 25) faced outward.

RESULTS

Effect of HPC and lysophospholipids on phospholipase Cδ_1 from rat liver. PLCδ_1 was inhibited by HPC and several lysophospholipids. Fifty percent inhibition of PLCδ_1 was observed in the presence of 27 nmol/mL HPC when the enzyme was activated by 200 μM spermine (Fig. 1). In the absence of spermine, 50% inhibition occurred with 34 nmol/mL HPC. This can be compared with the effective dose against rat mammary tumors of 20–150 nmol HPC/g body weight [4]. Spermine is a strong activator of PLCδ_1 [19] and the concentration of 200 μM employed by us is in the physiological range [21]. Lysophospholipids also inhibited PLCδ_1. Fifty percent inhibition by lysophosphatidylcholine (lysoPC), lysophosphatidylserine (lysoPS), and lysosphingomyelin (lysoSM) was observed at 7, 10, and 15 nmol/mL, respectively, in the presence of 200 μM spermine, and at 27, 21, and 53 nmol/mL, respectively, in the absence of spermine (Fig. 1). Under the conditions of the assay, the activity of PLCδ_1 was four times higher in the presence of 200 μM spermine than in its absence.

The inhibition of PLCδ_1 was also observed in the liposome assay, which contained no detergent (Fig. 2). HPC, lysoSM, lysoPS, and lysoPC all showed an I_{50} of 32–35 nmol/mL, concentrations which were similar to those observed in the detergent assay. All experiments shown were repeated three or four times. The *average* standard deviation for all points was ± 3.2%. This places error bars on or just outside the points in the figures; for this reason error bars have been omitted.

Inhibition of PLCδ_1 by 30 μM HPC was constant between 0.1 and 4 μM free Ca^{2+} (not shown), which

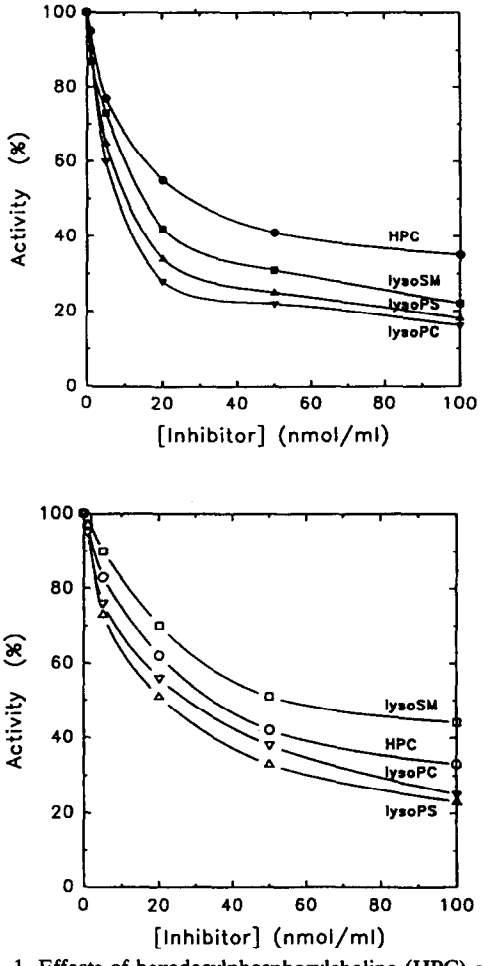

Fig. 1. Effects of hexadecylphosphorylcholine (HPC) and lysophospholipids on the activity of PLCδ_1 using the detergent assay. The reaction mixture was as described in Materials and Methods. Rat liver enzyme was used. *Solid symbols* in the upper panel show activity in the presence of 200 μM spermine; *open symbols* in the lower panel show activity in the absence of spermine. In the absence of inhibitors, the activity of PLCδ_1 was 3.31 nmol/mL/min in the presence of 200 μM spermine and 0.82 nmol/mL/min in its absence. The average standard deviation for all points was ± 3.2% (N = 3 or 4).

spans the physiological range of Ca^{2+} concentrations [22]. PLCδ_1 exhibited Michaelis–Menten kinetics; the inhibition by HPC was of the mixed type (Fig. 3).

Decanoyl and palmitoyl carnitine at concentrations of up to 100 nmol/mL did not inhibit PLCδ_1 under conditions where HPC inhibited strongly (Fig. 4). Carnitine, like phosphorylcholine, is a zwitterion. Carnitine esters, like HPC, have detergent-like properties. The observation that decanoyl and palmitoyl carnitine did not affect PLCδ_1 activity indicates that the inhibition of the enzyme by HPC is not due to a non-specific detergent effect. *N,N*-Dimethylsphingosine and *N,N,N*-trimethylsphingosine, but not sphingosine itself, inhibit the

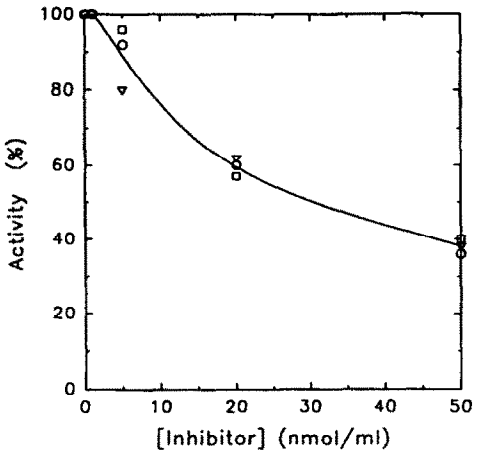

Fig. 2. Effects of HPC, lysoSM and lysoPC on the activity of PLCδ_1 using the liposome assay. The reaction mixture was as described in Materials and Methods. Rat liver enzyme was used. It contained 200 nmol/mL of a 4:1 mixture of PE and PC, 25 nmol/mL [^{3}H]PIP_2, and HPC or lysophospholipid as indicated. The symbols are as for Fig. 1, *lower panel*. One hundred percent activity was 1.20 nmol/mL/min. Standard deviations were as for Fig. 1.

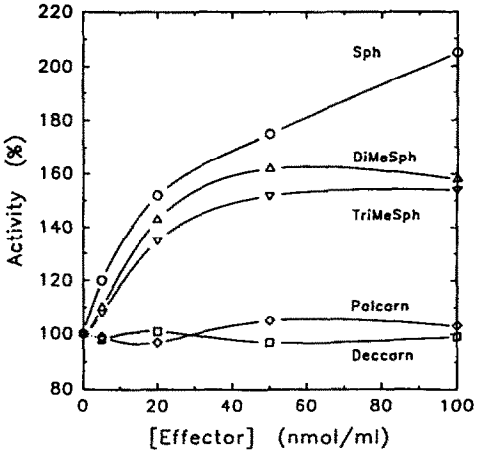

Fig. 4. Effects of carnitine esters, sphingosine and *N*-methylsphingosines on PLCδ_1. The detergent assay was used as described in Materials and Methods except that spermine was omitted and the human fibroblast enzyme was used. One hundred percent activity was 1.4 nmol/mL/min. Abbreviations: Sph, sphingosine; DiMeSph, *N,N*-dimethylsphingosine; TriMeSph, *N,N,N*-trimethylsphingosine; Palcarn, palmitoyl-L-carnitine; and Deccarn, decanoyl-L-carnitine.

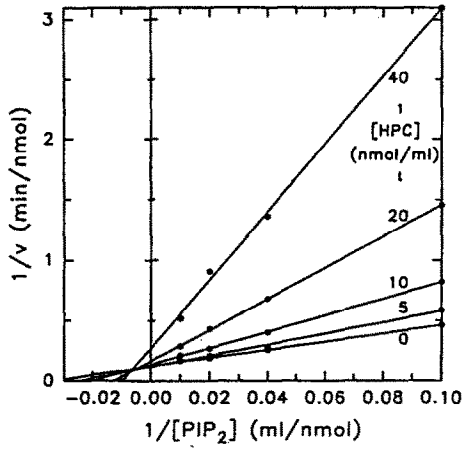

Fig. 3. Effect of HPC on PLCδ_1 using the detergent assay. Conditions were as described in the legend to Fig. 1, *upper panel*, except that the human fibroblast enzyme was used and that the HPC concentration was varied as shown.

metastatic potential of human and murine tumor cells [23, 24]. The *N,N*-dimethyl compound is a strong inhibitor of PKC and a weak activator of v-src and c-src kinase [25] as well as an activator of epidermal growth factor receptor autophosphorylation [26]. *N,N*-Dimethylsphingosine is an endogenous metabolite of A431 carcinoma cells [26]. It was therefore of interest to test whether either or both of these substances affected PLCδ_1. Figure 4 shows that both compounds were weak *activators* of the enzyme, in keeping with our previous finding that sphingosine is a weak activator [27]. These results show that a tertiary or quaternary ammonium group alone is not sufficient to produce inhibition of PLCδ_1 and that an additional functional group, such as a phosphodiester group, seems to be required.

PLCδ_1 from human fibroblasts was expressed in *E. coli* and then purified as described previously [17]. The properties of the fibroblast enzyme were very similar or identical to PLCδ_1 purified from rat liver with respect to activation by spermine and calcium ions and inhibition by sphingomyelin [27] and HPC.

DISCUSSION

The action of HPC *in vivo* appears to be similar to that of certain lysophospholipids which inhibit the growth of tumor cells, including lysoSM, and sulfogalactosylsphingosine [28]. Some lysosphingolipids are inhibitors of protein kinase C, including, in decreasing order of effectiveness, lysoGM$_2$, galactosylsphingosine, and sulfogalactosylsphingosine [11, 29, 30]. Sphingosine and sphinganine also inhibit PKC in cell extracts and intact cells [30–32] and they inhibit multistage carcinogenesis in C3H 10T½ cells treated with radiation and phorbol myristoyl acetate [33]. LysoPS causes mast cell activation in mice [34, 35]. Lysophosphatidic acid applied to human fibroblasts in culture evokes the formation of inositol-1,4,5-triphosphate and a rise in free Ca^{2+}. This effect is probably mediated via cell surface receptors which trigger GTP-dependent PIP_2 breakdown [36]. In other words, the observed activation of PLC by lysophosphatidic acid is an indirect effect. HPC is probably more effective as an antineoplastic agent

than lysophospholipids, because it is broken down more slowly [10, 12].

HPC may also affect other phospholipases. Micelles containing HPC bind phospholipase A_2 from snake venoms [37, 38] and pancreas [39], indicating that HPC is recognized by this class of phospholipases, presumably because of its resemblance to lysoPC.

Phospholipases normally act at lipid–water interfaces. A phospholipase inhibitor may act by direct inhibition of the enzyme, or by binding to the substrate at the lipid–water interface, or by disturbing the manner in which the substrate faces into the aqueous phase. To obviate artifacts, we used two assays which present substrate and inhibitor to the enzyme from micelles and from small liposomes. Deoxycholate forms micelles containing about 14 molecules per micelle. The concentration of deoxycholate used in the detergent micelle assay was 2.4 mM, which is equivalent to 171 μM deoxycholate micelles. This means that on an average 1 micelle in 10 contained a molecule of substrate or inhibitor when these were present at 17 nmol/mL ($\equiv 17\ \mu$M), while the others contained none. Moreover, at these concentrations, on an average 1 micelle in 100 contained both substrate and inhibitor. Under such conditions the interfacial concentration of deoxycholate is hardly affected and the surface concentrations of substrate and inhibitor are constant, namely one molecule per micelle [40].

In the case of the liposome assay, the concentration of phospholipid was 200 nmol/mL. Assuming that each liposome contains about 4000 molecules of phospholipid [41], this is equivalent to a liposome concentration of approximately 50 nM. When substrate was present at a bulk concentration of 25 nmol/mL ($\equiv 25\ \mu$M), each liposome contained about 500 molecules of PIP_2, of which $500 \times 17/25 = 340$ faced outward. Our observation that the detergent micelle and liposome assays yielded very similar results (Figs. 1 and 2) indicates that the interfacial concentrations of substrate and inhibitors are proportional to their bulk concentration. This makes it unlikely that the results are artifacts of the detergent-like effects of HPC or lysophospholipids. Decanoyl and palmitoyl carnitine did not inhibit or activate PLCδ_1 at concentrations up to 100 nmol/mL (Fig. 4), confirming that our results are not due to non-specific detergent effects.

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