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ALKYLPHOSPHATE ESTERS AS INHIBITORS OF PHOSPHOLIPASE D

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Alkylphosphate esters were shown to be potent inhibitors of phospholipase D. Using phosphatidyl choline/sodium dodecylsulfate (2:1) as substrate, IC_{50} values were determined for alkylphosphocholines of different chain length (C_{10} - C_{18}) and for various octadecylphosphate esters with different polar head groups. The inhibitory potency strongly increased with increasing chain length of the alkyl chain. The substitution of choline for heterocyclic nitrogen compounds or for 2-trimethylarsonio-ethanol also affected the inhibition of phospholipase D. Octadecylphosphocholine proved to be the most efficient inhibitor ($IC_{50} = 6.4\ \mu M$).

Keywords: Phospholipase D, alkylphosphocholine, octadecylphosphate esters, inhibition, substrate

INTRODUCTION

Phospholipase D (PLD, EC 3.1.4.4) occurring in plants, microorganisms and mammalian tissues[1] catalyzes the cleavage of the terminal phosphate diester bond in glycerophospholipids. Its main substrate is phosphatidyl choline (PC) yielding phosphatidic acid and choline as products. In the presence of a primary alcohol, the enzyme is able to transfer the phosphatidic residue to this alcohol, whereby PLD becomes interesting for synthetic purposes.[2,3]

The physiological role of PLD in plants and microorganisms has been unknown, hitherto. Recently, mammalian PLD has attracted much attention, because it was found to play an important role in signal transduction processes.[4] Also the knowledge on the molecular properties of PLD is still very limited; neither

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amino acid sequences nor tertiary structures are known. Recently, the gene sequences of PLD from *Ricinus communis* L.[5] and *Oryza saliva* L.[6] have been published. As is typical for lipolytic enzymes, PLD needs its substrate in aggregated form to become fully active[7,8] and for most of the PLD types calcium ions are essential for activity.[7] Several organic solvents such as diethyl ether or surfactants such as sodium dodecylsulfate (SDS) stimulate the activity of PLD.[7] Phosphatidic acid,[9] phosphatidylinositol,[10] and neutral lipids[11] were found to activate PLD from cabbage and *Streptomyces chromofuscus*, respectively, whereas some metal ions such as Hg^{2+} or Cu^{2+} were reported to inhibit PLD.[12,13] Furthermore, the inhibitory effects of *p*-chloromercuribenzoate[14] and different surfactants[1] were described. Little information, however, is available about the effect of substrate-analogous compounds. Low and Huang reported on the inhibition of glycosylphosphatidylinositol-specific PLD from bovine serum by product analogues.[15]

Alkylphosphocholines such as hexadecylphosphocholine (HPC) represent a group of phospholipid analogues that have gained much importance because of their antineoplastic effects *in vitro* and *in vivo*.[16–18] Recently it was found that structurally related compounds in which choline is substituted for other polar groups could be successfully included into the series of substances with anticancer properties.[19,20] An inhibitory effect of these compounds was shown on different enzymes such as protein kinase C,[21,22] phospholipase C,[23] and CTP:phosphocholine cytidyltransferase.[24]

In the present paper the inhibitory effect of alkylphosphocholines and their analogues on PLD from cabbage is demonstrated. Using alkylphosphocholines with varying alkyl chain length and octadecylphosphate esters with different positively charged alcoholic moieties, the influence of the structure of the hydrophobic part as well as of the head group on the inhibitory properties was studied.

MATERIALS AND METHODS

Materials

Lyophilized PLD (EC 3.1.4.4) from cabbage was purchased from Boehringer, Mannheim. PC (egg yolk), 98.8%, was obtained from Lipoid KG, Ludwigshafen. The alkylphosphate esters D-23402, D-23170, D-19390, D-18506, D-19391, D-21805, D-20133, D-21266 and D-21002 (Tables I and II) were synthesized as described recently.[25–27] SDS (special grade) was a product of Boehringer, Mannheim. All other chemicals were reagent grade and commercially available.

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Protein Determination

The protein content of PLD solutions was determined according to Lowry *et al.*[28]

PLD Activity Towards PC

For the preparation of substrate stock solution, 64.52 μmol (50 mg) of PC dissolved in 500 μl chloroform was evaporated in a centrifugal vacuum evaporator (Jouan). After resuspension of the PC film by 5 μl of double distilled water containing 32.26 μmol SDS, the mixture was heated to 40°C, shaken by a Vortex mixer and sonicated in an ultrasonic bath for 5 min. In the water jacketed reaction cell of the pH-stat titrator VIT 90 (Radiometer) equipped with pH and reference electrodes and a magnetic stirrer, 25–200 μl of substrate stock solution were added to 20 mM sodium acetate buffer, pH 5.7, containing 40 mM $CaCl_2$ and 100 mM NaCl, to give a total volume of 3 ml. After thermal equilibration at 30°C, the reaction was started by the addition of 10–50 μl PLD (5 mg protein/ml) dissolved in 20 mM sodium acetate buffer, pH 5.7, containing 40 mM $CaCl_2$, and followed by pH-stat titration with 10 mM NaOH.

PLD Activity Towards HPC

HPC (D-18506) was dissolved in 20 mM sodium acetate buffer, pH 5.7, containing 40 mM $CaCl_2$ and used as substrate stock solution. The enzyme assay was performed by analogy to that described above using PC as substrate.

Inhibition Studies

PLD activity towards PC was determined in the presence of various concentrations of inhibitor, which was added to the substrate solution before starting the reaction with enzyme. If not indicated otherwise, the PC concentration in these assays was 0.860 mM. IC_{50} values were estimated from the duplicate activity values of at least five different inhibitor concentrations using the double-logarithmic plot of $v/v_o(1 - v/v_o)$ against the inhibitor concentration, where v and v_o are the initial rates in the presence and in the absence of inhibitor. In this plot the intersection of the curve with the abscissa corresponds to Log IC_{50}. The standard deviation of the activity values was within 10%.

RESULTS AND DISCUSSION

Since the catalytic behaviour of lipolytic enzymes such as PLD strongly depends on the physical state of substrate aggregates, their kinetic analysis is much more

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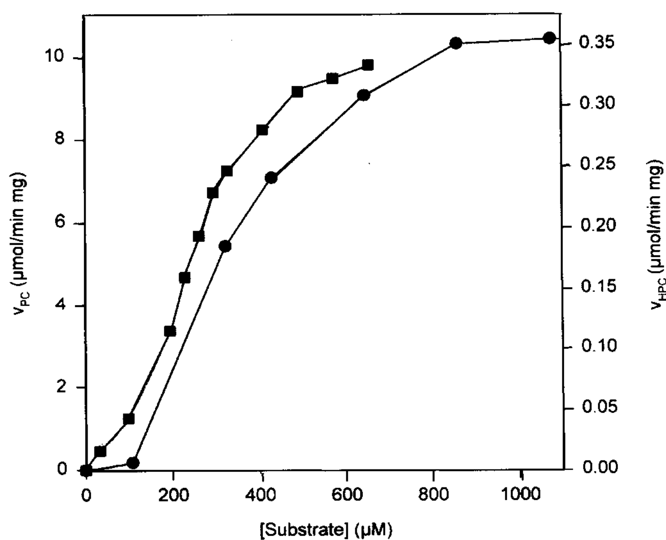

FIGURE 1 v/S diagram for PLD. v_{PC} and v_{HPC} are the initial rates of the hydrolysis of PC/SDS (•) and HPC (■).

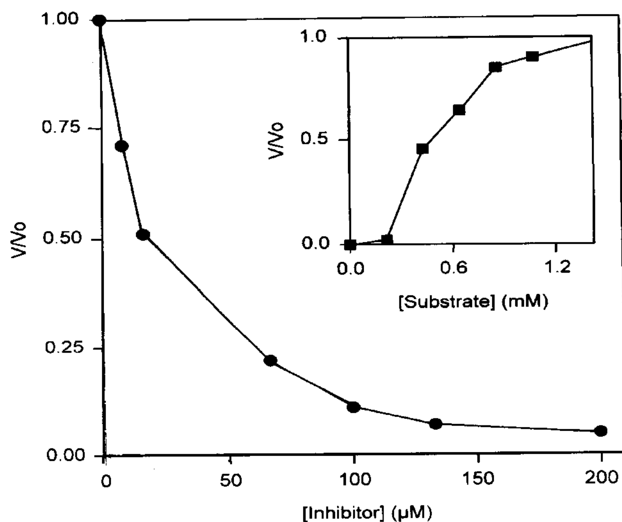

FIGURE 2 Inhibition of PLD by HPC as a function of inhibitor and substrate concentration. v/v_o are the initial rates related to those in the absence of HPC. The concentration of the substrate PC/SDS in the main diagram was 0.86/0.43 mM. The concentration of the inhibitor HPC in the insert was 9.25 μM.

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difficult than for other hydrolytic enzymes. Even inhibitory effects cannot be evaluated as usual but may be caused by factors different from direct interactions between inhibitor and enzyme. Thus, the inhibitor can affect the activity indirectly by influencing the process of interfacial binding. The choice of assay conditions is, therefore, especially important for inhibition experiments. In accordance with other authors,[7] PC/SDS in the molar ratio of 2:1 was found to yield optimum initial rates of hydrolysis and was used as substrate in the present inhibition studies. Figure 1 shows the $v/[S]$ characteristics for this substrate in the absence of inhibitors.

In the first series of inhibition studies, the inhibitory potencies of alkylphosphocholines containing alkyl groups of different chain length (C_{10} to C_{18}) were determined as a function of the inhibitor concentration. As demonstrated for HPC (Figure 2), the activity of PLD is drastically reduced with increasing concentration of the alkylphosphocholines. Table I demonstrates that the inhibitory effects strongly increased with the chain length of the alkyl groups. The IC_{50} or the α_{50} values, which give the molar inhihitor concentration or the molar ratio of inhibitor [I] to substrate [S], respectively, where activity was reduced by 50%, decreased by a factor 20 when the alkyl chain is increased from 10 to 18 C atoms.

In the second series of inhibitors studied, the most effective octadecylphosphoryl moiety from the first series was combined with various polar alcohols. These head groups are positively charged, similar to choline in the alkylphosphocholines, but contain heterocyclic groups or arsenic. The results in Table II show that the polar head group also influences the inhibitory potency but less than the hydrophobic chain. The inhibitor with the choline group shows the highest inhibitory effect. The IC_{50} or α_{50} values differ by a maximum factor of 7.

In all cases the inhibition was reversible as could be concluded from dilution experiments (results not shown). Since the kinetics of PLD do not obey the Michaelis-Menten type (Figure 1), the usual approaches to decide between competitive and other types of inhibition could not be applied. An indication that the inhibitor is a competitor of the substrate was provided by measuring the dependence of the inhibition effects on substrate concentration. As demonstrated in the insert of Figure 2 for HPC, the inhibitory effect decreases with increasing PC concentrations.

In the absence of PC, PLD was also shown to possess a small hydrolytic activity towards alkylphosphocholines as demonstrated for HPC in Figure 1. The maximum specific activity towards this substrate amounted to about 3% of the maximum specific activity towards PC/SDS, whereas the concentrations of half-maximum activity were roughly in the same range as with PC/SDS. The ability to hydrolyze alkylphosphocholines (C_{14} to C_{22}) was also described for the enzyme, from savoy cabbage.[29] Our inhibition studies, however, were not interfered with by the substrate function of the alkylphosphate esters since they were performed at concentrations where hydrolysis was not observable.

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TABLE I Inhibition of phospholipase D by alkylphosphocholines with different chain lengths

Inhibitor	IC_{50} (μM)	α_{50} [I]:[S]
D-23402 $CH_3-(CH_2)_9-O-P(=O)(O^-)-O-CH_2-CH_2-N^+(CH_3)_3$	141.67	$16.46 \cdot 10^{-2}$
D-23170 $CH_3-(CH_2)_{11}-O-P(=O)(O^-)-O-CH_2-CH_2-N^+(CH_3)_3$	101.74	$11.83 \cdot 10^{-2}$
D-19390 $CH_3-(CH_2)_{13}-O-P(=O)(O^-)-O-CH_2-CH_2-N^+(CH_3)_3$	40.66	$4.73 \cdot 10^{-2}$
D-18506 $CH_3-(CH_2)_{15}-O-P(=O)(O^-)-O-CH_2-CH_2-N^+(CH_3)_3$	13.00	$1.51 \cdot 10^{-2}$
D-19391 $CH_3-(CH_2)_{17}-O-P(=O)(O^-)-O-CH_2-CH_2-N^+(CH_3)_3$	6.43	$0.75 \cdot 10^{-2}$

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TABLE II Inhibition of phospholipase D by octadecylphosphate esters with various head groups

Inhibitor	IC_{50} (μM)	α_{50} [I]:[S]
D-19391 $CH_3-(CH_2)_{17}-O-P(=O)(O^-)-O-CH_2-CH_2-N^+(CH_3)_3$	6.43	$0.75 \cdot 10^{-2}$
D-21805 $CH_3-(CH_2)_{17}-O-P(=O)(O^-)-O-CH_2-CH_2-As^+(CH_3)_3$	25.90	$3.01 \cdot 10^{-2}$
D-21002 $CH_3-(CH_2)_{17}-O-P(=O)(O^-)-O-$(azepan-4-yl ring, $N^+(CH_3)_2$)	27.69	$3.22 \cdot 10^{-2}$
D-21266 $CH_3-(CH_2)_{17}-O-P(=O)(O^-)-O-$(piperidin-4-yl ring, $N^+(CH_3)_2$)	34.05	$3.96 \cdot 10^{-2}$
D-20133 $CH_3-(CH_2)_{17}-O-P(=O)(O^-)-O-CH_2-CH_2-N^+(CH_3)$(piperidinium ring)	44.62	$5.19 \cdot 10^{-2}$

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Because of the limited knowledge on the kinetics and mechanism of PLD, the present data do not allow unambigious conclusions on the inhibition mechanism. Three arguments, however, suggest that the inhibition is specific and might be based on molecular interactions between the inhibitor molecules and the catalytic site: (1) the inhibitors are efficient at low concentrations in comparison with the PC concentration, (2) the inhibitors are able to function as substrates and, (3) small differences in the molecular structure of the inhibitors induce great effects in their inhibitory properties.

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