Enzymatic Hydrolysis of *p*-Nitrophenyl Phosphoryl Derivatives by Phospholipase D

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A series of phosphodiesters of *p*-nitrophenyl phosphoryl derivatives were synthesized and used as a model substrate for phospholipase D (PLD). The phosphodiester substrates were synthesized from *p*-nitrophenyl phosphorodichloridate and corresponding alcohols with different chain lengths and polar groups. To measure the activity of PLD, either spectroscopic method for *p*-nitrophenol or pH-stat titration method was employed. For each substrate, effects of substrate concentration, pH, and Ca^{2+} ion were examined. The kinetic parameters V_{max} for the different substrates were varied depending on the chain lengths or charge of the alcohols. No calcium effect was observed in the hydrolysis of neutral and negatively charged alcohol derivatives, while positively charged choline derivative showed a strong Ca^{2+} ion dependence.

Introduction

Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) catalyzed hydrolytic cleavage (Reaction 1) of the terminal phosphate diester bond of glycerophosphatides containing choline, ethanolamine, or serine, with the formation of phosphatidic acid. Phospholipase D (PLD) also mediates transphosphatidylation (Reaction 2) by which the phosphatidyl group of phosphatidylcholine (PC) is transferred to another alcohols.[1]

$$\text{phosphatidylcholine} + H_2O \rightarrow \text{phosphatidic acid} + \text{choline} \quad (1)$$

$$\text{phosphatidylcholine} + \text{ROH} \rightarrow \text{phosphatidyl-OR} + \text{choline} \quad (2)$$

The catalytic mechanism of PLD had not been well characterized, possibly due to the fact that the enzyme is unstable and had not been purified to homogeneity until very recently.[2] In addition, kinetic studies of phospholipases are often complicated by interfacial interactions.[3] However, following facts are known : Based on exchange reaction between the choline of PC and free [^{14}C] choline, Yang *et al.*[4] suggested that phosphatidyl-enzyme complex exists. And on the basis of stereochemical study of PLD catalyzed reaction involving chirally labeled PC, it was suggested that PLD catalyzes transphosphatidylation with overall retention of configuration at phosphorous.[5] For structure-reactivity relationship, the transphosphatidylation activity of PLD toward various alcohol acceptors was examined.[6] Recently, there was a study about kinetics of PLD with alkyl phosphorylcholines of different chain lengths.[7] It revealed that solubilities of the substrate play an important role in the activity of PLD.

In view of these limited information on the substrate specificity of PLD reaction, we employed another model substate, *p*-nitrophenyl phosphodiesters with different chain lengths or polar head groups. This model system might provide some characteristics of PLD reaction in respect of substrate size as well as charge in the head group.

Experimental

Materials. *p*-Nitrophenyl phosphorodichloridate was purchased from Aldrich. *p*-Nitrophenyl phosphate, bis *p*-nitrophenyl phosphate and TMD-8 column were purchased from Sigma. *p*-Nitrophenol was obtained from Kanto. Thin layer chromatography sheets of silica gel from Merck were used. Crude egg phosphatidylcholine (PC) from Sigma was purified by aluminum oxide column. All other chemicals were reagent grade commercially available.

Enzyme preparation. Phospholipase D was prepared from savoy cabbage homogenate treated by 55 ℃ heat and acetone precipitation procedures. The purification was carried out through Sephadex G-200 column, DEAE cellulose and hydrophobic affinity chromatography.[8] The fractions containing PLD activity were frozen and used as enzyme source.

Synthesis of substrates. The synthetic method for *p*-nitrophenyl phosphorylcholine (p-NPPC) used here was described by Chesebro and Metzger.[9] The choline iodide, *p*-nitrophenyl phosphodichloridate (2 mmoles) and dry quinoline (2 mmoles) were dissolved in 0.5 m*l* of dry acetonitrile, respectively, and mixed together and stirred at 0 ℃ in the dark for 6 hours. Then 1.0 m*l* of pyridine and 0.2 m*l* of H_2O were added and the solution was incubated at room temperature for 30 minutes. After the solvents were evaporated, the residue was dissolved in H_2O and passed through a 40 m*l* TMD-8 column equilibrated with H_2O. The effluent was lyophilized. When the product was analyzed by thin layer chromatography on silica gel using 1-butanol : acetic acid : H_2O (5 : 2 : 4, v/v), only a single component was observed. The *p*-nitrophenyl phosphorylbutyrate (p-NPPB) was also prepared similiary. These compounds were stored dry at room temperature in the dark.

The synthesis of *p*-nitrophenyl phosphorylalcohols (p-NPPAs) was carried out with the same procedure of p-NPPC except the separation steps. The alcohols employed were butanol (A4), heptanol (A7), and decanol (A10). After the incubation with H_2O at room temperature, the reaction mixture was extracted with 1 volume of chloroform. This step was repeated three times. The solvent of remaining layer was

evaporated. The residue was developed by thin layer chromatography on silica gel using acetonitrile : acetic acid (10 : 1, v/v). The spots were detected by UV-254 nm lamp and scrapped off from the plate. Their R_f values were between 0.6 and 0.8. The product was extracted from silica gel using chloroform : methanol (1 : 1, v/v). The concentration of prepared material was determined by phosphorous microdetermination method.[10]

Assay of phospholipase D. The assay method is based on the spectrophotometric measurement of *p*-nitrophenol, one of the end products of *p*-nitrophenyl phosphoryl derivatives. The substrate was dissolved in 0.3 m*l* 50 mM MES buffer (pH 6.5) containing 12 mM $CaCl_2$. The appropriate amount of enzyme solution containing 3-8 μg of protein was added and the reaction mixture was thoroughly mixed. Then it was incubated at 47 ℃ for 15 minutes. The reaction was stopped by cooling to 0 ℃ in an ice water bath. To produce the color of *p*-nitrophenol, 0.25 m*l* of reaction mixture was diluted with 1 m*l* of 70 mM Tris-HCl buffer (pH 8.0). Then the absorbance increased due to the enzymatic hydrolysis was measured at 405 nm.

In some experiment the change in H^+ ion concentration induced by the enzymatic cleavage of the ester bond of substrate was determined by pH-stat titration using a autotitroprocessor (Metrohrm, 670 Titroprocessor). For the assay of the phospholipase D activity toward p-NPPC, the reaction mixture contained 10 mM p-NPPC in 3 m*l* 0.1 mM MES with 12 mM $CaCl_2$. The mixture was preincubated, then 50 μ*l* of enzyme solution was added. The pH of the reaction mixture was adjusted to 6.5 and maintained at this value by adding 0.014 N NaOH solution at 47 ℃.

Results and Discussion

Determination of hydrolysis site in p-NPPC. *p*-Nitrophenyl phosphorylcholine has two possible hydrolysis sites, *p*-nitrophenyl site and choline site. The hydrolysis site was determined by comparing the results from the two different detection methods. When 30 μmol p-NPPC were hydrolyzed by the enzyme, 921 nmol *p*-nitrophenol was detected spectrophotometically. In the same reaction, 943 nmol acid was generated as detected by the pH-stat titration. The two values agreed within 2.5%. Therefore, it was safely assumed that PLD hydrolyzed p-NPPC preferentialy at the *p*-nitrophenyl site. This hydrolytic site is not the same site as the natural phospholipid such as PC. The cleavage site of PC was known to occur in P-O bond at the choline site.[11]

Kinetic parameters. The kinetic constants for p-NPPAs with different chain lengths were determined by varying the concentration of the substrate, in a range of 0-14 mM (Fig. 1). The apparent K_m and V_{max} values were obtained from Lineweaver-Burk plot. For p-NPPA4, a typical hyperbolic saturation curve was obtained. But inhibition appeared at high concentrations in the hydrolysis of p-NPPA7 and p-NPPA10. In the case of p-NPPA7 and p-NPPA10, the concentration range which showed the inhibition was not included in Lineweaver-Burk plot. This inhibition of hydrolysis at high p-NPPA7 concentration disappeared when SDS was added. The K_m values under the presence of SDS did not change significantly (data not shown). The K_m values were 3.45, 0.687, 2.625 mM for p-NPPA4, p-NPPA7 and p-NPPA10, respectively (Table 1). The K_m value of PC was 0.85 mM. The V_{max} was highest for p-NPPA4 and decreased as the chain length of alkyl group was increased. This result coincided with the result of Ohno *et al.*[7], showing that the higher

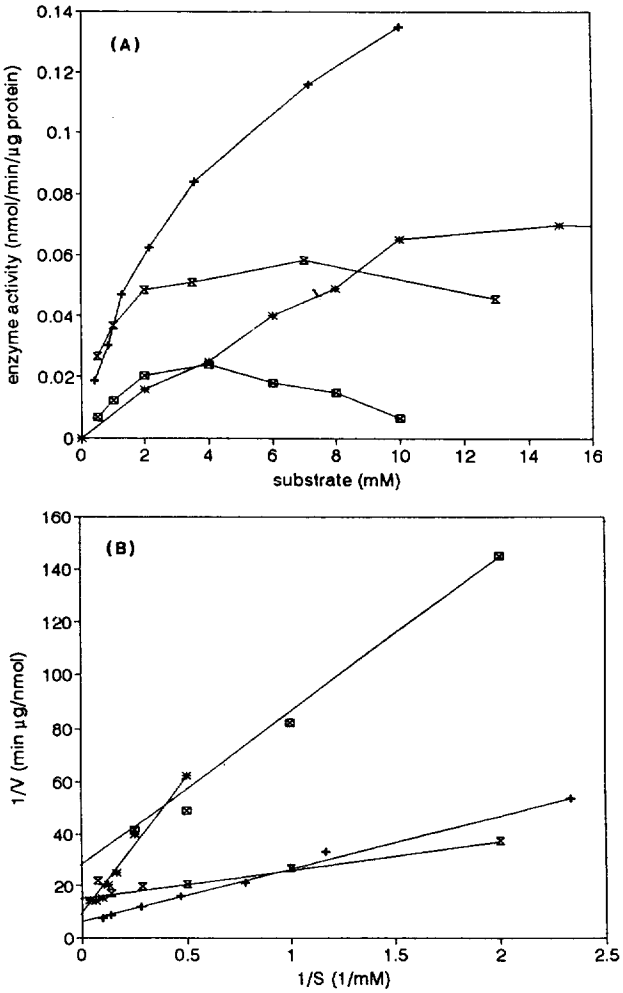

Figure 1. (A) Effect of substrates p-NPPAs and p-NPPB concentrations on the activity of PLD. The quantity of released *p*-nitrophenol was measured.
-✳-, p-NPPB; -+-, p-NPPA4; -⊠-, p-NPPA7; -⊠-, p-NPPA10
(B) Lineweaver-Burk plots of substrate dependence of (A).

Table 1. Kinetic parameters for PLD-catalyzed hydrolysis of p-nitrophenyl phosphoryl derivatives

Substrate	K_m (mM)	V_{max} (nmol/min/μg protein)
p-NPPA4	3.45	0.165
p-NPPA7	0.687	0.063
p-NPPA10	2.625	0.044
p-NPPC	165	0.233
p-NPPB	13.51	0.019
bis p-NPP	0.85	0.044

Abbreviation of substrate : p-NPPA4, *p*-nitrophenyl phosphorylbutanol; p-NPPA7, *p*-nitrophenyl phosphorylheptanol; p-NPPA10, *p*-nitrophenyl phosphoryldecanol; p-NPPC, *p*-nitrophenyl phosphoryl butyrate; bis p-NPP, bis *p*-nitrophenyl phosphate.

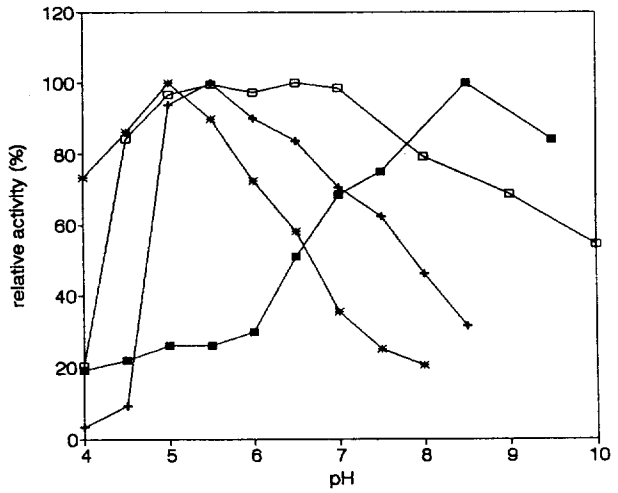

Figure 2. Effect of pH on the activity of PLD toward various substrates. The buffers used were 50 mM acetate buffer between pH 4 and pH 5.5, 50 mM MES buffer between pH 5.5 and pH 7.0 and 50 mM Tris-HCl buffer between pH 7.0 and pH 10. -■-, p-NPPC; -+-, p-NPPA4; -✳-, p-NPPB; -⊟-, bis p-NPP

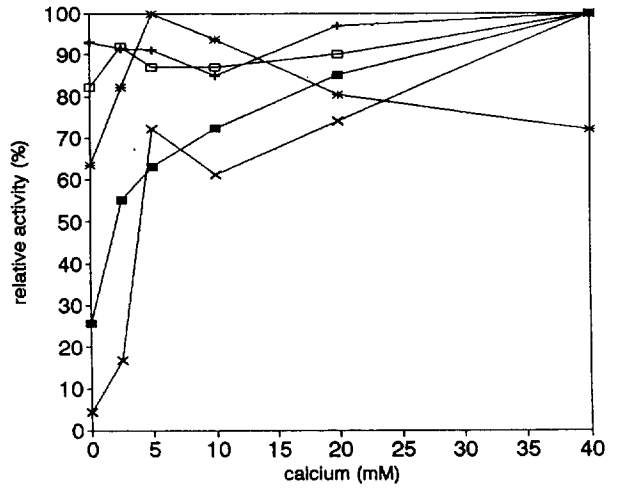

Figure 3. Effect of calcium ion concentration on the activity of PLD toward various substrates.
-✳-, PC; -■-, p-NPPC -+-, p-NPPA4; -✳-, p-NPPB: -⊟-, bis p-NPP

rate of hydrolysis was observed in shorter chain alkyl phosphorylcholines. When the observed values of V_{max} were compared to that of PC, those values are only about 0.5-1%. The large difference in these hydrolysis rates probably reflect that the *p*-nitrophenyl phosphodiesters are rather poor substrate than natural phospholipids which contain at least two long chain acyl groups in the molecules. The K_m and V_{max} values for p-NPPC, p-NPPB and bis-p-NPP were also obtained and summarized together in Table 1. The K_m value of p-NPPC was very large but V_{max} value was higher than those of p-NPPAs. The negatively charged p-NPPB showed relatively large K_m values and at the same time the smallest V_{max} value, particulary when compared with p-NPP4. This indicates that the negatively charged derivatives are not good substrate for the PLD.

Other properties of PLD. It has been known that the optimum pH for the hydrolysis of PC is between 5.5 and 6.5 And there were some studies showing that the apparent pH optimum changes with different substrates and Ca^{2+} concentration.[3,12] Fig. 2 showed that pH optimum for p-NPPC moved to pH 8.5. The hydrolysis rate of bis p-NPP was maintained high over a broad acidic pH range. The p-NPPA4, which has no charged group, showed similar pattern as PC in it's pH optimum. The negatively charged p-NPPB showed relatively sharp pH optimum of 5.0. Therefore, it is likely that the substrates with different charges might affect their affinities to the binding site and affected the pH-reactivity profile accordingly.

It has been assumed that Ca^{2+} ion is essential to the activity of PLD. However, when we examined Ca^{2+} effect on different substrate, it revealed some differences (Fig. 3). As expected as in PC, the hydrolysis of p-NPPC showed positive effect by Ca^{2+} ion. The hydrolysis rate increased rapidly at Ca^{2+} concentration up to 10 mM and maintained at that level. But it had no effects on the hydrolysis of p-NPPAs and bis p-NPP, which have no charged group in their alcoholic side. In the case of p-NPPB, it's hydrolytic activity seems to be dependent more on Ca^{2+} than the one of p-NPPAs with an optimum activity near 5 mM Ca^{2+}. From these data, although the substrates employed here are not good substrate as expected, it could suggest that the presence of Ca^{2+} is not essential for the catalytic activity of PLD. Another word a possible role of calcium ion in the hydrolytic reaction seems to be involved in some interactions between substrate and enzyme rather than a direct binding of Ca^{2+} to the enzyme, since effects of Ca^{2+} on PLD reaction depend largely on the substrates examined. Collectively it is supposed that the variations of pH optimum and Ca^{2+} activation in the PLD reaction could be a reflection of physical state of substrate in the presence of some effector molecules.

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