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Probing the role of C-1 ester group in *Naja naja* phospholipase A_2–phospholipid interactions using butanetriol-containing phosphatidylcholine analogues

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To understand the role of the ester moiety of the *sn*-1 acyl chain in phospholipase A_2–glycerophospholipid interactions, we introduced an additional methylene residue between the glycerol C1 and C2 carbon atoms of phosphatidylcholines, and then studied the kinetics of hydrolysis and the binding of such butanetriol-containing phospholipids with *Naja naja* phospholipase A_2. Hydrolysis was monitored by using phospholipids containing a NBD-labelled *sn*-2 acyl chain and binding was ascertained by measuring the protein tryptophan fluorescence. The hydrolysis of butanetriol-containing phospholipids was invariably slower than that of the glycerol-containing phospholipids. In addition, the enzyme binding with the substrate was markedly decreased upon replacing the glycerol residue with the 1,3,4-butanetriol moiety in phosphatidylcholines. These results have been interpreted to suggest that the *sn*-1 ester group in glycerophospholipids could play an important role in phospholipase A_2–phospholipid interactions.

Keywords: phospholipase A_2; phosphatidylcholine; phospholipid conformation; butanetriol analogue.

Cobra venom phospholipase A_2 is a Ca^{2+}-dependent enzyme that catalyses the hydrolysis of the fatty acyl group at the *sn*-2 position of membrane phospholipids. It is composed of a single polypeptide chain of 119 amino acids which contains seven disulphide bonds [1]. The crystal structures of phospholipase A_2 from several sources have been determined [1–3], and the amino acids that form the enzyme catalytic site have been reported to be highly conserved [1]. Also, the structure of the catalytic surface has been shown to be exactly identical regardless of the degree of evolutionary divergence, state of oligomerization, ionic strength, or pH of the crystallization conditions [3,4].

Binding of phospholipase A_2 with phosphatidylcholines involves interactions of the enzyme active site calcium with the phospholipid phosphate oxygen and the *sn*-2 carbonyl oxygen followed by the hydrophobic interactions between the phospholipid fatty acyl chains and the enzyme catalytic site [2–5]. Apart from the *sn*-2 acyl chain [5], the acyl chain at the *sn*-1 position also plays an important role in phospholipase A_2–glycerophospholipid interactions [6,7]. It has been reported that an increase in the hydrophobicity of the *sn*-1 acyl residue increases the affinity between the phospholipid molecule and the enzyme [6]. However, it is not yet known whether the ester moiety (-CO-O-) of the *sn*-1 acyl chain plays any role in phospholipase A_2–glycerophospholipid interactions. To investigate this we introduced one additional methylene residue between the glycerol C1 and C2 carbon atoms and then studied the interactions of the resulting modified phospholipids (Fig. 1) with *Naja naja* phospholipase A_2. Results of these studies indicate that the *sn*-1 ester moiety plays an important role in binding of the enzyme with the substrate.

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Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPBPC, 1,3-dihexadecanoyloxy-(3*R*)-but-4-yl-[2-(trimethylammonium)ethyl] phosphate; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; FAB, fast atom bombardment; C_6-NBD-PC, 1-hexadecanoyl-2-[6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphocholine; C_{12}-NBD-PC, 1-hexadecanoyl-2-[12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyl]-*sn*-glycero-3-phosphocholine; C_6-NBD-bPC, 1-hexadecanoyloxy-3-[6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyloxy]-(3*R*)-but-4-yl-phosphocholine; C_{12}-NBD-bPC, 1-hexadecanoyloxy-3-[12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl)aminododecanoyloxy]-(3*R*)-but-4-yl-phosphocholine.

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MATERIALS AND METHODS

Materials

Phospholipase A_2 from *N. naja* snake venom, *sn*-glycero-3-phosphocholine, 6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoic acid, 12-aminododecanoic acid, Triton X-100, 4-chloro-7-nitrobenz-2-oxa-1,3-diazol, 1,1′-carbonyldiimidazole, EGTA, EDTA and octylglucoside were from Sigma Chemical Co. and were used without further purification. 12-*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) aminododecanoic acid was prepared from 12-aminododecanoic acid using standard procedures.

General methods

The purity of various reaction intermediates was checked by TLC on silica gel G-60 plates. Homogeneity of phospholipids was established by both TLC and HPLC. TLC plates were developed in chloroform/methanol/water (65 : 25 : 4, v/v/v) and the phospholipid spots were visualized by staining the plate

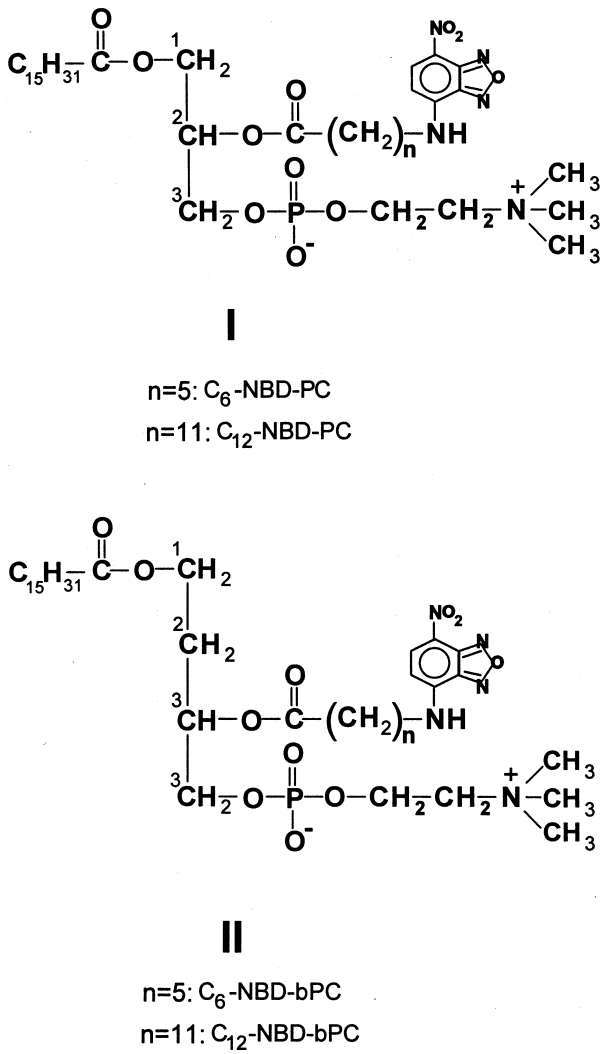

Fig. 1. Molecular structures of phosphatidylcholine analogues in which the glycerol moiety has been replaced by 1,3,4-butanetriol.

with iodine vapour followed by Molybdenum-blue spray [8]. HPLC was performed using a Waters HPLC pump (Model 510) with a Waters R401 differential refractometer or a Waters fluorescence detector (Model 420-AC) interfaced with a Waters System Interface Module. Chromatograms were analysed by using baseline 810 software. Analytical HPLC was performed on an Altex Ultrasphere C18 reverse-phase column (4.6 × 250 mm, particle size 5 μm) using methanol/chloroform/water (87 : 13 : 6, v/v/v) containing 176 mM choline chloride at a flow rate of 1.0 mL·min^{-1} as the elution system; preparative HPLC was carried out on an LKB TSK-ODS-120T semiprep column (7.8 × 300 mm, particle size 10 μm) using methanol/chloroform/water (90 : 10 : 4, v/v/v) at a flow rate of 2 mL·min^{-1} as the eluent.

Phospholipids were purified by silica gel column chromatography, Sephadex LH-20 column (2.5 × 100 cm) chromatography, and preparative HPLC. The Sephadex LH-20 column was eluted with chloroform/methanol (1 : 1, v/v) at a flow rate of ≈ 60 mL·h^{-1}. All of the phospholipids thus purified exhibited single spots on TLC and single peaks on analytical HPLC, and were characterized by NMR and mass spectrometry analysis. ^{1}H-NMR spectra were recorded on a Bruker ACP 300 Fourier transform-NMR spectrometer, and the chemical shifts have been expressed in parts per million down-field from tetramethylsilane. Positive ion fast atom bombardment (FAB) MS was performed on a Jeol JMS-SX 102 FAB mass spectrometer equipped with JMA-DA 6000 data system.

Phospholipids

1,3-Dihexadecanoyloxy-*rac*-but-4-yl-[2-(trimethylammonium)-ethyl] phosphate was prepared as described previously [9]. It was treated with *N. naja* snake venom (Sigma) and the resulting 1-hexadecanoyloxy-3-hydroxy-(3*R*)-but-4-yl-[2-(trimethyl-ammonium)ethyl] phosphate was isolated in pure form by following the published procedure [10]. Reaction of this lysolipid with 6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-hexanoic acid or 12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminododecanoic acid in the presence of 1,1′-carbonyldiimida-zole [7] produced 1-hexadecanoyloxy-3-[6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyloxy]-(3*R*)-but-4-yl-[(2-tri-methylammonium) ethyl] phosphate (Fig. 1, C_6-NBD-bPC) or 1-hexadecanoyloxy-3-[12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminododecanoyloxy]-(3*R*)-but-4-yl-[(2-trimethylammonium) ethyl] phosphate (Fig. 1, C_{12}-NBD-bPC). Similarly, 1-hexa-decanoyl- 2-[6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-hexyl]-*sn*-glycero-3-phosphocholine (Fig. 1, C_6-NBD-PC) and 1-hexadecanoyl-2-[12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminododecanoyl]-*sn*-glycero-3-phosphocholine (Fig. 1, C_{12}-NBD-PC) were prepared using 1-hexadecanoyl-*sn*-glycero-3-phosphocholine as the starting material, which in turn was prepared as described earlier [11]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,3-dihexadecanoyloxy-(3*R*)-but-4-yl-[2-(trimethylammonium)ethyl] phosphate (DPBPC) were prepared following our earlier reported methods [9,11]. C_6-NBD-PC: FAB mass, 772 (MH^+); ^{1}H-NMR ($CDCl_3$/CD_3OD) δ, 8.49 (d, J = 9 Hz, 1H), 6.23 (d, J = 9 Hz, 1H), 5.28–5.21 (m, 1H), 4.37–4.29 (b, 2H), 4.17–4.08 (m, 2H), 4.03–3.97 (m, 2H), 3.85–3.75 (b, 2H), 3.30 (s, 9H). C_{12}-NBD-PC: FAB mass, 856 (MH^+); ^{1}H-NMR ($CDCl_3$/CD_3OD) δ, 8.50 (d, J = 9 Hz, 1H), 6.19 (d, J = 9 Hz, 1H), 5.27–5.20 (m, 1H), 4.30–4.20 (b, 2H), 4.18–4.10 (m, 2H), 4.02–3.97 (m, 2H), 3.63–3.56 (b, 2H), 3.22 (s, 9H). C_6-NBD-bPC: FAB mass, 786 (MH^+); ^{1}H-NMR ($CDCl_3$/CD_3OD) δ, 8.44 (d, J = 9 Hz, 1H), 6.14 (d, J = 9 Hz, 1H), 5.08–5.01 (m, 1H), 4.27–4.21 (b,2H), 4.14–4.04 (b, 2H), 4.02–3.99 (m, 2H), 3.58–3.49 (b, 2H), 3.14 (s, 9H), 2.03–1.82 (m, 2H). C_{12}-NBD-bPC: FAB mass, 870 (MH^+); ^{1}H-NMR ($CDCl_3$/CD_3OD) δ, 8.43 (d, J = 9 Hz, 1H), 6.14 (d, J = 9 Hz, 1H), 5.06–5.00 (m, 1H), 4.29–4.21 (b, 2H), 4.16–4.05 (b, 2H), 4.03–4.01 (m, 2H), 3.56–3.46 (b, 2H), 3.14 (s, 9H), 2.04–1.82 (m, 2H).

Phospholipase A_2-catalysed hydrolysis

A measured volume of phospholipid solution in chloroform was dried in a glass tube under a slow jet of N_2 followed by overnight *in vacuo*. To it was added Tris/HCl (25 mM, pH 8.5) containing 4.25 mM Triton X-100, 100 mM KCl and 10 mM Ca^{2+} [6,7]. The suspension was sonicated untill a clear solution was obtained, and incubated for 5 min at 30 °C. The required amount of phospholipase A_2 was added and the incubation at 30 °C continued for various times. The reaction was terminated by adding 15 mM EGTA, freezing at – 20 °C and lyophilization. The residue was dissolved in 200 μL methanol/chloroform (2 : 1, v/v) mixture and centrifuged to sediment any suspended material. A portion (40 μL) of the supernatant was analysed by analytical HPLC using a fluorescence detector. Typical elution profiles of the various reaction mixtures are shown in Fig. 2.

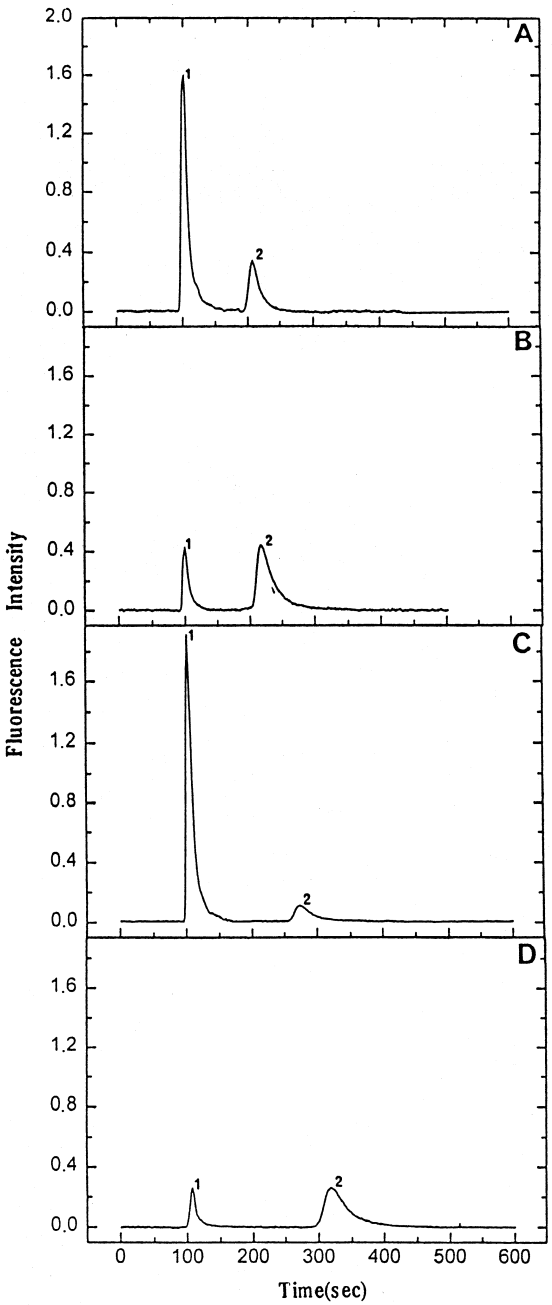

Fig. 2. HPLC profiles of the hydrolysed phospholipids I and II. Peak 1, NBD-containing fatty acid; peak 2, unhydrolysed NBD-containing phospholipid. Panel A, C_6-NBD-PC; panel B, C_6-NBD-bPC; panel C, C_{12}-NBD-PC; panel D, C_{12}-NBD-bPC.

To quantitate the degree of hydrolysis at different times, known amounts of the various fluorescently labelled phospholipids, fluorescently labelled fatty acids and their mixtures were loaded separately on to HPLC, and the total areas under the phospholipid and fatty acid peaks were calculated from the elution profiles. Standard curves were drawn by plotting the peak area on the ordinate and the corresponding quantities on the abscissa; curves were linear up to at least 10 μg of phospholipid and ≈ 1.0 μg of fatty acid. The intensity of the phospholipid peak remained unchanged upon mixing the phospholipid with varying amounts of the fatty acid, but under these conditions, we observed significant broadening of the fatty acid peak without any change in total area covered under this peak. However, in order to avoid any problem, throughout this study we have used the area covered under the unhydrolysed phospholipid peak to calculate the degree of hydrolysis. The peaks were integrated using the ORIGIN™ software that could quantify the peaks to five decimal places.

Kinetic analysis

The kinetic analysis of hydrolysis under conditions where the substrate concentration was varied but the Triton X-100 concentration was kept constant, was performed according to the dual phospholipid model proposed by Hendrickson and Dennis [12] for the action of cobra venom phospholipase A_2 on mixed micelles of phospholipid and the nonionic detergent Triton X-100. According to this model, the water-soluble enzyme binds initially to a phospholipid molecule in the micelle interface. This is followed by binding of the initial enzyme–substrate complex to an additional phospholipid molecule and then catalytic hydrolysis of this bound molecule. This is summarized in the following equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \quad ES + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ESS \xrightarrow{k_3} ES + \text{Product} \qquad (1)$$

The rate equation for this bisubstrate reaction is:

$$\upsilon = V_{max} X_s S_o / K_m K_s + K_m S_o + X_s S_o \qquad (2)$$

where: υ (mol·vol^{-1}·time^{-1}), velocity of steady state (bulk concentration); S_o (mol·vol^{-1}), total phospholipid substrate (bulk concentration); X_s (mole fraction, unitless), total phospholipid substrate (surface concentration); $K_s = k_{-1}/k_1$ (mol·vol^{-1}); $K_m = (k_{-2} + k_3)/k_2$ (mole fraction, unitless), apparent Michaelis constant (suface concentration); $V_{max} = k_3 E_o$ (mol·vol^{-1}·time^{-1}), maximal velocity where E_o (mol·vol^{-1}) is the total enzyme.

Triton X-100 present in the micelle is assumed to be an inert diluter with negligible affinity for the enzyme. The bulk concentration of Triton X-100 in the micelle, T_o (mol·vol^{-1}), was kept constant and the substrate concentration was varied. The kinetic expression for this case is:

$$\upsilon = V_{max} S_o^2 / K_m K_s T_o + (T_o + K_s) K_m S_o + (K_m + 1) S_o^2 \qquad (3)$$

where $T_o = T_t - T_{mon}$ (mol·vol^{-1}); T_t (mol·vol^{-1}) = the total concentration of Triton X-100 utilized and T_{mon} (mol·vol^{-1}) = the monomer concentration of Triton X-100 present in the mixture, which is assumed to be equal to its critical micellar concentration of 0.24 mM.

The double reciprocal form of this equation is:

$$\frac{1}{\upsilon} = \frac{K_m K_s T_o}{V_{max}} \left(\frac{1}{S_o}\right)^2 + \frac{(T_o + K_s) K_m}{V_{max}} \left(\frac{1}{S_o}\right) + \left(\frac{K_m + 1}{V_{max}}\right) \qquad (4)$$

This equation is in the form of a second order polynomial, $Y = ax^2 + bx + c$. Plots of 1/υ versus 1/S_o curve upward and fit this polynomial. The values of a, b and c were determined by nonlinear regression in the computer program Minsq II (V 1.02), and the values of the kinetic parameters, V_{max}, K_m, binding constant ($K_s = k_{-1}/k_1$) were calculated.

Phospholipase A_2 binding

The affinity of phospholipase A_2 for DPPC and DPBPC was monitored by measuring the protein tryptophan fluorescence (excitation, 296 nm). The excitation and emission slit widths were kept at 10 nm and 2.5 nm, respectively. The emission maxima in the absence of both Ca^{2+} and lipid, in the presence of Ca^{2+} and absence of lipid, and in the presence of both Ca^{2+} and lipid were 354 nm, 352 nm and 347 nm, respectively.

Phospholipids at a concentration of 4 $\mu g \cdot \mu L^{-1}$ were suspended in 25 mM Tris/HCl (pH 8.5) containing 100 mM KCl and 4.25 mM octylglucoside by sonication. Measured aliquots of this suspension were added to a stirred solution of phospholipase A_2 (0.42 μM) in a total volume of 1.5 mL Tris/HCl (25 mM, pH 8.5) containing 100 mM KCl, 4.25 mM octylglucoside and 10 mM $CaCl_2$ in a cuvette at 30 °C. The protein tryptophan fluorescence was measured after each addition (emission, 347 nm). The addition continued until saturation was reached.

RESULTS

Time and concentration-dependent phospholipid hydrolysis by phospholipase A_2

Equimolar amounts of I and II were incubated separately with phospholipase A_2 under identical conditions, and amounts of the unhydrolysed phospholipid at different time intervals was determined by HPLC, as described in Materials and Methods. Figure 3 shows that hydrolysis of C_6-NBD-PC (or C_{12}-NBD-PC) was considerably faster than that of C_6-NBD-bPC (or C_{12}-NBD-bPC). To examine whether this difference between the phospholipid hydrolysis could be maintained even with saturating enzyme concentrations, we first determined the saturating phospholipase A_2 concentrations for C_6-NBD-PC and C_{12}-NBD-PC (44.94 pmol and 37.45 pmol of phospholipase A_2 for 25.91 nmol of C_6-NBD-PC and 23.37 nmol of C_{12}-NBD-PC, respectively), and then used these concentrations to hydrolyse both I and II. Even under these conditions, the hydrolysis of C_6-NBD-PC and C_{12}-NBD-PC were faster than that of C_6-NBD-bPC and C_{12}-NBD-bPC, respectively.

To analyse this problem further, we studied the phospholipid concentration-dependent hydrolysis of C_{12}-NBD-PC and C_{12}-NBD-bPC, and then calculated the various kinetic parameters as

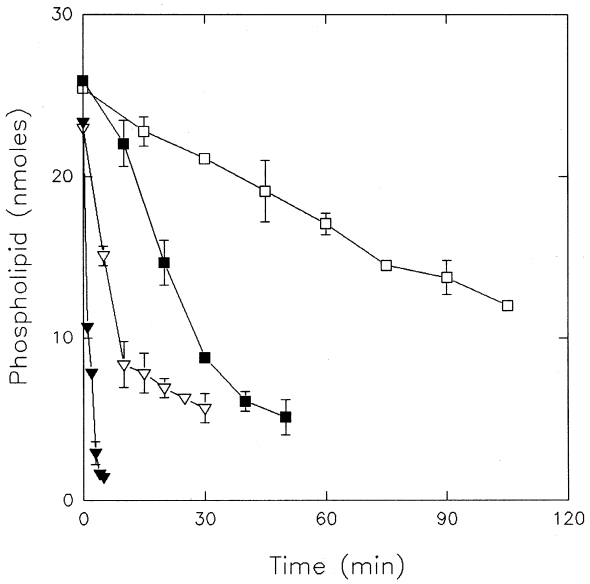

Fig. 3. Time-dependent *N. naja* phospholipase A_2-catalysed hydrolysis of phospholipids I and II. The incubation mixture contained phospholipids dissolved in 70 μL of 25 mM Tris/HCl, 100 mM KCl, 10 mM $CaCl_2$ (pH 8.5) and 4.25 mM Triton X-100. Phospholipase A_2 added was 7.5 pmoles. Incubation was carried out at 30 °C. The reaction was terminated by adding 3.7 μmoles EGTA and immediately freezing at –20 °C. ■, C_6-NBD-PC; □, C_6-NBD-bPC; ▼, C_{12}-NBD-PC; ▽, C_{12}-NBD-bPC. Values shown are the means of at least three observations ± SD.

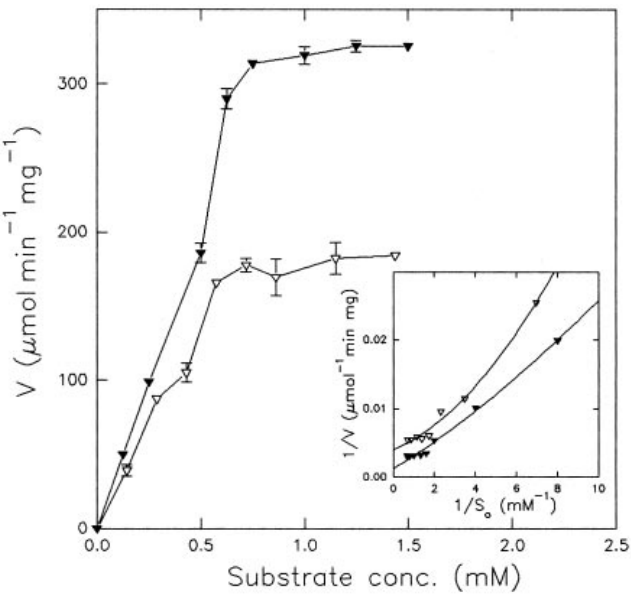

Fig. 4. Rate of hydrolysis versus concentration of C_{12}-NBD-PC and C_{12}-NBD-bPC. The reaction was catalysed by 0.56 pmoles of *N. naja* phospholipase A_2 in 40 μL of 25 mM Tris/HCl, 100 mM KCl, 10 mM $CaCl_2$, pH 8.5, in mixed micelles with 3.35 mM Triton X-100, at 30 °C. ▼, C_{12}-NBD-PC; ▽, C_{12}-NBD-bPC. The values shown are the means of at least three observations ± SD. The inset shows double reciprocal plot. Solid lines, computer fits to polynomial $1/\upsilon = a\,(1/S_o)^2 + b\,(1/S_o) + c$. Constants: ▼, a = 5.934 (± 5.0) × 10^{-5}, b = 1.871 (± 0.44) × 10^{-3}, c = 1.199 (± 0.57) × 10^{-3}, $P = 0.02$; ▽, a = 2.624 (± 0.82) × 10^{-4}, b = 1.258 (± 0.64) × 10^{-3}, c = 4.028 (± 0.83) × 10^{-2}, $P = 0.01$.

given in Materials and methods. Figure 4 shows that the rate of hydrolysis of C_{12}-NBD-PC was faster than that of C_{12}-NBD-bPC. The various kinetic parameters derived for C_{12}-NBD-PC and C_{12}-NBD-bPC as per Eqn (4) are given in the Table 1. The dissociation constant, K_s, of C_{12}-NBD-bPC was almost seven times higher than that of C_{12}-NBD-PC, indicating that the binding of C_{12}-NBD-bPC to phospholipase A_2 is weaker than that of C_{12}-NBD-PC. Furthermore, a higher value of V_{max} (about six times) in case of C_{12}-NBD-PC as compared to C_{12}-NBD-bPC indicates that C_{12}-NBD-PC is a much better substrate for phospholipase A_2 than C_{12}-NBD-bPC.

To further confirm that the affinity of diacyl glycerophospholipids for phospholipase A_2 is higher than that of butanetriol-containing phospholipids the change in protein tryptophan fluorescence at different phospholipid concentrations was measured. The tryptophan fluorescence was measured after adding varying concentrations of mixed micelles of DPPC or DPBPC with octylglucoside to phospholipase A_2, as described in Materials and methods. Figure 5 shows that about 2.6 times more DPBPC is required to reach the half maximal binding as compared with DPPC.

Table 1. Kinetic parameters. V_{max} (maximal velocity), K_m (apparent Michaelis constant), K_s (dissociation constant for the enzyme-mixed micelle complex) for phospholipase A_2-catalysed hydrolysis of C_{12}-NBD-PC and C_{12}-NBD-bPC at 30 °C in 25 mM Tris/HCl containing 100 mM KCl, 10 mM $CaCl_2$, pH 8.5, in mixed micelles with 3.35 mM Triton X-100.

Substrate	V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	K_m (mole fraction)	K_s (μM)
C_{12}-NBD-PC	1657.21 (± 98.63)	0.987 (± 0.186)	32.04 (± 2.96)
C12-NBD-bPC	273.92 (± 21.07)	0.103 (± 0.010)	223.6 (± 8.69)

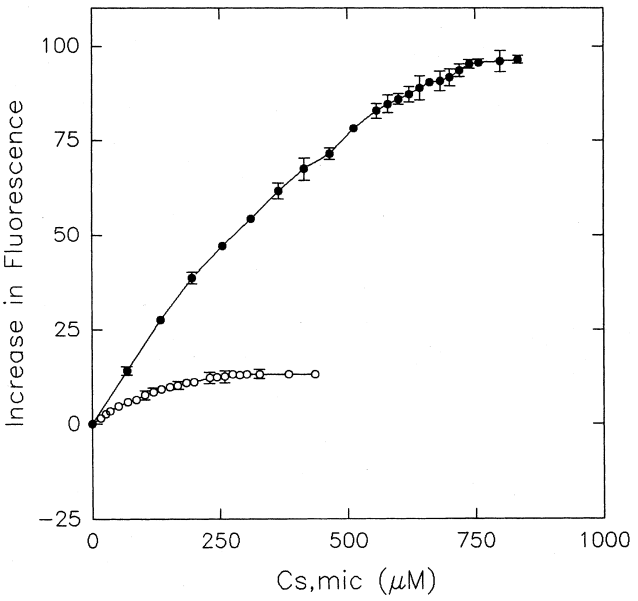

Fig. 5. Fluorometric titration of the *N. naja* phospholipase A_2 with micellar DPPC (○) and DPBPC (●) at 30 °C. The enzyme was dissolved in 25 mM Tris/HCl, 100 mM KCl, 10 mM $CaCl_2$ (pH 8.5). The fluorescence change at 347 nm was plotted as a function of the molar concentration of the substrate in the micellar form (Cs, mic). The protein concentration was 4.2×10^{-7} M. Values shown are the means of three observations ± SD.

Effect of butanetriol-containing phosphatidylcholine analogues on phospholipase A_2-catalysed phosphatidylcholine hydrolysis

In order to ascertain whether butanetriol-containing phosphatidylcholine analogues can competitively inhibit the phospholipase A_2-catalysed hydrolysis of phosphatidylcholines, we analysed the effects of DPPC and DPBPC on C_{12}-NBD-PC hydrolysis. This hydrolysis was not affected much by mixing increasing quantities of DPPC with C_{12}-NBD-PC, but it was decreased appreciably when the amount of DPBPC was increased to 2–3 times than that of C_{12}-NBD-PC (Fig. 6).

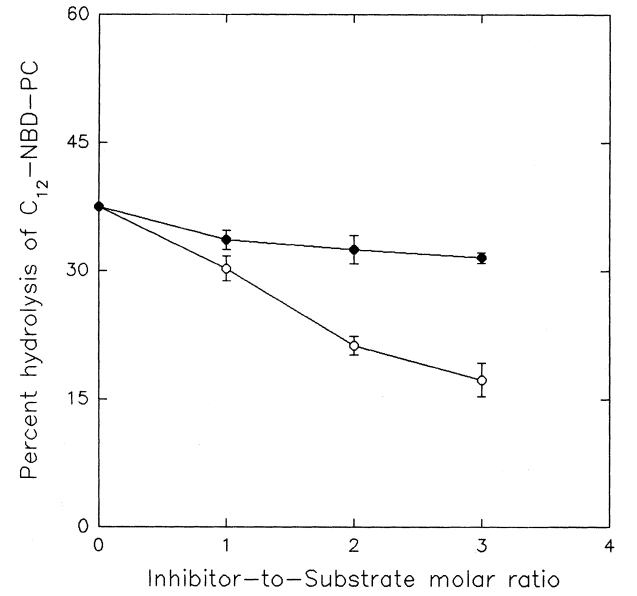

Fig. 6. Effect of DPPC and DPBPC on *N. naja* phospholipase A_2-catalysed hydrolysis of C_{12}-NBD-PC. The incubation mixture contained phospholipids dissolved in 40 μL of 25 mM Tris/HCl, 100 mM KCl, 10 mM $CaCl_2$ (pH 8.5) and 4.25 mM Triton X-100. Phospholipase A_2 added was 0.56 pmoles. The amount of C_{12}-NBD-PC used was 30 nmoles. The incubation was carried out for 5 min at 30 °C. The reaction was terminated by adding 3.7 μmoles EGTA and immediately freezing at −20 °C. ●, DPPC; ○, DPBPC. Values shown are the means of three observations ± SD.

DISCUSSION

This study demonstrates that the phospholipase A_2-catalysed hydrolysis of phosphatidylcholines is rendered slow by an insertion of one methylene residue between the carbons that carry the two acyl chains in these phospholipids. This difference in phospholipid hydrolysis cannot be attributed to the differences in aggregation states of the fluorescent phospholipids [13,14] because the hydrolysis has been carried out in mixed micelles containing a large excess of Triton X-100 [5], under identical conditions, and monitored only after extracting the reaction mixture with organic solvents and quantifying the amounts of unhydrolysed phospholipid by HPLC. In using this assay we observed complete linearity at least up to 10 μg of I or II even in the presence of fluorescent derivatives of C_6 or C_{12} fatty acids. The slower hydrolysis of C_6-NBD-PC (or C_6-NBD-bPC) as compared to C_{12}-NBD-PC (or C_{12}-NBD-bPC), may be attributed to poor binding of the substrate to phospholipase A_2 [6,7].

It appears that the observed differences between the hydrolysis rates of I and II are not due to differences between the physical states of these two types of phospholipid but could arise from differences between the structures of these lipids in the mixed micelles. This is supported further by our present finding that the binding of the phospholipid–octylglucoside mixed micelles with phospholipase A_2 is decreased significantly by replacing DPPC with DPBPC in these micelles. In addition, it is supported by our present observation that the hydrolysis of C_{12}-NBD-PC by phospholipase A_2 is inhibited competetively by mixing with it a two- to threefold excess of DPBPC.

High resolution ^1H and ^{13}C-NMR studies on the racemic form of DPBPC have shown that the conformational preference around the butanetriol C2–C3 bond in this phospholipid is almost identical to that observed for the glycerol C1–C2 bond in DPPC [9]. Based on this observation, it has been suggested that structurally, the C2 methylene residue in the butanetriol backbone of the phospholipid analogue represents the proximal beginning of the primary acyl chain [9]. As the preferred conformation of phosphatidylcholines in micelles or bilayers is such that the *sn*-2 ester group aligns at the micelle (or bilayer) interface whereas the *sn*-1 ester group remains almost perpendicular to the *sn*-2 ester region [15–17], introduction of an additional methylene residue between the glycerol C1 and C2 carbon atoms in DPPC should lead to an altered phospholipid structure in the interface region due to pushing of the *sn*-1 ester group further into the hydrophobic interior of the micelles, which in turn could affect the binding as well as the catalytic efficiency of phospholipase A_2. That this is the case is confirmed well by our present observations.

The butanetriol-containing phospholipid analogues differ from natural phosphatidylcholines in that the distance between the two oxygen ester groups is increased by introducing an additional methylene residue between the glycerol C1 and C2 carbon atoms. This structural change in phosphatidylcholines could affect not only the hydration pattern of the micellar interface but also the binding of the *sn*-1 acyl chain with the enzyme catalytic surface [2,5]. Because hydrophobic interactions, due to their non-specific nature, between the *sn*-1 acyl chain and the enzyme [1,2,5] should not be greatly affected by replacing DPPC with

DPBPC as the substrate, it is likely that the ester moiety of the *sn*-1 acyl chain interacts specifically, directly or indirectly, with the enzyme active site [5]. From these results, we conclude that the C1 ester moiety in DPPC could play a crucial role in its productive interactions with *N. naja* phospholipase A_2, which is quite consistent with the earlier studies in which we reported slower hydrolysis of 1-*O*-hexadecyl-2-fatty acyl-*sn*-glycero-3-phosphocholines, as compared with egg phosphatidylcholines with *N. naja* phospholipase A_2 [18,19].

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