Phospholipase activity on *N*-acyl phosphatidylethanolamines is critically dependent on the *N*-acyl chain length

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We have recently shown that an endogenous phospholipase A₂ from bovine erythrocytes does not hydrolyse NAPEs (*N*-acyl L- α -phosphatidylethanolamines), which accumulate remarkably in this system [Florin-Christensen, Suarez, Florin-Christensen, Wainszelbaum, Brown, McElwain and Palmer (2001) Proc. Natl. Acad. Sci. U.S.A. **98**, 7736–7741]. Here we investigate the causes underlying this resistance. *N*-acylation of PE (L- α -phosphatidylethanolamine) results in alteration of charge, head-group volume and conformation, the last two features depending on the *N*-acyl chain length. To evaluate each effect separately, we synthesized NAPEs with selected *N*-acyl chain length. We found that phospholipase A₂ has considerable activity against *N*-acetyl PE, but is poorly active against *N*-butanoyl PE and only marginally active against *N*-hexanoyl PE, whereas the activity is completely lost when *N*-hexadecanoyl PE is presented as a substrate. On

the other hand, *N*-hexanoyl PE does not inhibit phospholipase A₂ activity, suggesting that this substrate fails to enter the hydrophobic channel. Phospholipase C presents a similar, but less sharp pattern. Molecular dynamics simulations of the polar head group of selected NAPEs reveal a substantially increased conformational variability as the *N*-acyl chain grows. This larger conformational space represents an increased impairment limiting the access of these molecules to the active site. Our data indicate that, whereas a change in charge contributes to diminished activity, the most relevant effects come from steric hindrance related to the growth of the *N*-acyl chain.

Key words: *N*-acyl phosphatidylethanolamine, molecular dynamics, phospholipase A₂.

INTRODUCTION

NAPEs (*N*-acyl L- α -phosphatidylethanolamines) occur naturally in diverse biological systems. For example, NAPEs accumulate during cell injury or stress, such as those resulting from ischaemia in animal tissues and dehydration in plant seeds [1,2]. NAPEs may also play signalling roles, as they are the source of *N*-acyl ethanolamides, including anandamide (*N*-arachidonoyl ethanolamide), which binds to CB1 central receptors, acting as a cannabinoid agonist [3]. *N*-Acyl ethanolamides also display cytostatic effects on human breast cancer and rat pheochromocytoma cells [4]. Another interesting aspect worth remarking is that NAPEs are fusogenic, a property shown to be useful for DNA delivery to intact cells [5].

The conformation of NAPEs in a lipid bilayer, as well as their interaction with other lipids, have been the subject of a number of studies in which infrared, Raman [6] and ESR [7] spectroscopies have been used. There is general agreement that for long-chain NAPEs, the *N*-acyl moiety is bent and buried in the hydrophobic phase of the phospholipid bilayer. Moreover, the vertical location of this chain appears to be the same as that adopted by the *O*-acyl chain that esterifies the *sn*-2 position of other phospholipids present in the bilayer [7]. This suggests that important distortions of the phospholipid conformation should take place. By contrast, for short-chain NAPEs, the *N*-acyl chain remains at the level of the glycerol backbone [6], i.e. exposed to the aqueous milieu. In addition, *N*-acylation appears to prevent the formation of intermolecular hydrogen bonds, resulting in increased hydration of the polar head group. NAPEs have been observed in considerable

amounts in the erythrocytes of ruminants [8]. This is a system where we found that endogenous PLA₂ (phospholipase A₂) activity plays an important role in phospholipid composition and its homeostasis [9]. In particular NAPEs are strikingly resistant to PLA₂, a fact that can account for their accumulation in ruminant red blood cells.

The molecular determinants of the activity of the different types of phospholipase have been the subject of several structural studies. Crystal structures of these enzymes containing phospholipid analogues have provided information on how the catalytic site is conformed [10–14]. As these enzymes deal with amphiphilic molecules possessing large hydrophobic moieties, they contain hydrophobic channels that interact with the aliphatic chains. Access of the phospholipid molecule to the catalytic site implies its removal from the bilayer or micelle surface into the catalytic site of the enzyme. This transfer depends on adequate interactions with the hydrophobic channel, as well as on the formation of ionic complexes with metal ions present at the catalytic site. If the hydrophobic nature of the phospholipid is altered, as expected from *N*-acylation, substrate removal from the lipid bilayer could be strongly affected. This should be especially important in the case of long-chain *N*-acyl groups. How the phospholipid head group's charge and how steric hindrance at the polar head group influence phospholipase activity are relevant matters for understanding the catalytic mechanisms operating in these enzymes.

In NAPEs, the *N*-acylation bears as its consequence important changes in the physicochemical properties of the phospholipid. Obliteration of the amino group charge converts the zwitterionic

Abbreviations used: NAPE, *N*-acyl L- α -phosphatidylethanolamine; NAPE-2, *N*-acetyl 1,2-dihexadecanoyl L- α -phosphatidylethanolamine; NAPE-4, *N*-butanoyl 1,2-dihexadecanoyl L- α -phosphatidylethanolamine; NAPE-6, *N*-hexanoyl 1,2-dihexadecanoyl L- α -phosphatidylethanolamine; NAPE-16, *N*-hexadecanoyl 1,2-dihexadecanoyl L- α -phosphatidylethanolamine; PE, L- α -phosphatidylethanolamine; PLA₂, phospholipase A₂; PLC, phospholipase C; TFA, trifluoroacetic acid; lyso-*N*-tBOC-PE, lyso-*N*-t-butoxycarbonyl-1-hexadecanoyl PE; MC/SD method, Monte Carlo/stochastic dynamics method.

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parental PE (L- α -phosphatidylethanolamine) into an anionic lipid. Besides, the overall hydrophobicity of the molecule is expected to increase in direct relation to the chain length of the N-acyl group. Concomitantly, this will impose steric hindrance at the level of the head group that might influence catalysis negatively. On the other hand, the increased hydrophobicity could favour the interaction with the hydrophobic channel. These changes can be controlled experimentally by varying the N-acyl group's chain length. Regardless of the choice of this chain, the charge of the head group will be equally affected. In this paper we address these issues for the cases of PLA₂ and PLC (phospholipase C).

EXPERIMENTAL

Materials

Naja naja naja PLA₂, *Bacillus cereus* PLC, 1,2-dihexadecanoyl PE, NAPE-16 (*N*-hexadecanoyl 1,2-dihexadecanoyl L- α -phosphatidylethanolamine), *N,N'*-dimethylaminopyridine, butanoic anhydride, hexanoic anhydride, hexadecanoic anhydride, Hepes, CaCl₂, Triton X-100, Molybdenum Blue, ninhydrin, ZnCl₂ and *N,N'*-dicyclohexylcarbodi-imide were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). TFA (trifluoroacetic acid) was supplied by Riedel de Haën (Seelze-Hannover, Germany). [1-¹⁴C]Hexadecanoic acid (30–60 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Chloroform was refluxed from P₂O₅, distilled before use (boiling point, 59–60 °C) and stored in a brown glass bottle. Triethylamine and acetic anhydride were obtained from E. Merck (Darmstadt, Germany) and were distilled before use.

General procedures

¹H-NMR spectra were recorded on a Bruker MSL 300 spectrometer. Radioactivity from ¹⁴C was measured with a Wallac 1414 WinSpectral liquid scintillation counter. TLC was routinely performed on 5.0-cm × 1.6-cm plates of silica gel 60 F₂₅₄ (E. Merck). Solvent systems employed were the following: solvent 1, chloroform/methanol/water/acetic acid (130:40:4:1, by vol.); solvent 2, chloroform/methanol (3:1, v/v); solvent 3, chloroform/methanol (14:1, v/v); solvent 4, chloroform/methanol (10:1, v/v). Spots on TLC plates were visualized by fluorescence quenching under illumination with a UV light source (254 nm) and/or staining with the following reagents: Molybdenum Blue (Sigma), iodine vapour or ninhydrin (0.2 % acetone solution). TLC plates containing ¹⁴C-labelled products were covered with Kodak XAR-5 film for autoradiography, and the film was exposed in the dark.

Synthesis and characterization of compounds

Synthesis of *N*-acetyl 1,2-dihexadecanoyl L- α -phosphatidylethanolamine (NAPE-2), *N*-butanoyl 1,2-dihexadecanoyl L- α -phosphatidylethanolamine (NAPE-4) and *N*-hexanoyl 1,2-dihexadecanoyl L- α -phosphatidylethanolamine (NAPE-6) was as follows: 1,2-dihexadecanoyl PE (50 mg, 72 μ mol) and triethylamine (40 μ l, 290 μ mol) were dissolved in anhydrous chloroform (10 ml). To this stirred solution was added dropwise acetic anhydride (20 μ l, 212 μ mol), butanoic anhydride (35 μ l, 215 μ mol) or hexanoic anhydride (50 μ l, 217 μ mol). After 5–6 h at 25 °C, the reaction was essentially complete, as revealed by the disappearance of PE (*R*_F 0.38 by TLC in solvent 2, ninhydrin- and Molybdenum Blue-positive spot) and the appearance of products. At the end, these compounds were purified from the reaction mixture by silica-gel column chromatography. For NAPE-4 and

NAPE-6 excess anhydrides were eluted first with chloroform and products were then eluted with solvent 2 (> 90 % yield). The NAPE-2 reaction mixture was dried first under a high vacuum for 3 h to eliminate acetic acid and excess anhydride and then purified by silica-gel column chromatography: the remaining PE elutes first with solvent 3 and finally NAPE-2 with solvent 4 (> 80 % yield).

NAPE-2: *R*_F 0.32 in solvent 2, ninhydrin-negative, Molybdenum Blue-positive; ¹H-NMR ([²H]chloroform/[²H]methanol, 10:1, v/v, 300 MHz): δ , 0.78 (*m*, 6H), 1.16 (*m*, 48 H), 1.49 (*m*, 4H), 1.88 (*s*, 3H), 2.19 (*t*, 2H), 2.23 (*t*, 2H), 3.30 (broad, 2H), 3.78 (broad, 2H), 4.01–4.30 (complex, 4H), 5, 11 (complex, 1H).

NAPE-4: *R*_F 0.48 in solvent 2, ninhydrin-negative, Molybdenum Blue-positive; ¹H-NMR ([²H]chloroform/[²H]methanol, 10:1, v/v, 300 MHz): δ , 0.77 (*m*, 9H), 1.15 (*m*, 48 H), 1.52 (*m*, 6H), 2.07 (*t*, 2H), 2.18 (*t*, 2H), 2.22 (*t*, 2H), 3.32 (broad, 2H), 3.84 (broad, 2H), 4.00–4.29 (complex, 4H), 5, 11 (complex, 1H).

NAPE-6: *R*_F 0.49 in solvent 2, ninhydrin-negative, Molybdenum Blue-positive; ¹H-NMR ([²H]chloroform/[²H]methanol, 10:1, v/v, 300 MHz): δ , 0.79 (*m*, 9H), 1.16 (*m*, 52 H), 1.50 (*m*, 6H), 2.11 (*t*, 2H), 2.21 (*m*, 4H), 3.32 (broad, 2H), 3.87 (broad, 2H), 4.02–4.30 (complex, 4H), 5, 12 (complex, 1H).

¹⁴C-Labelled compounds

1-Hexadecanoyl-2-[1-¹⁴C]hexadecanoyl PE was synthesized by acylation of lyso-N-tBOC-PE (lyso-*N*-t-butoxycarbonyl-1-hexadecanoyl PE) with [1-¹⁴C]hexadecanoic anhydride: 0.1 mCi of [1-¹⁴C]hexadecanoic acid was dissolved in freshly distilled dry chloroform (200 μ l) and cooled in an ice bath. With constant stirring a recently prepared solution of *N,N'*-dicyclohexylcarbodi-imide (0.8 mg, 3.9 μ mol) in a minimum volume of dry chloroform was added dropwise. The reaction flask was gassed with dry nitrogen and capped tightly. After 5 min at 0 °C, the reaction mixture was allowed to warm up to 25 °C. The appearance of a white precipitate of dicyclohexylurea was evident after 10–15 min. After 24 h, the precipitate was removed by filtration through glass wool, and the solution was used immediately in the coupling reaction to lyso-N-tBOC-PE (the synthesis of lyso-N-tBOC-PE was adapted from [15] using PE as a starting material). The coupling method followed here was similar to that described in [16], with minor modifications. Lyso-N-tBOC-PE (2 mg, 3.6 μ mol) was dissolved in dry chloroform, the solvent was then evaporated under a nitrogen stream and traces of water or alcohol were azeotropically removed by washing the sample three times with dry toluene and evaporating this solvent under high vacuum. Lyso-N-tBOC-PE was finally dissolved in dry chloroform. To this solution *N,N'*-dimethylaminopyridine (0.5 mg, 4.1 μ mol) was then added. At this point, the mixture was cooled down in an ice bath before the solution containing the previously prepared anhydride was added dropwise with constant stirring. After 24 h at 25 °C the *N*-t-butoxycarbonyl-1-hexadecanoyl-2-[1-¹⁴C]hexadecanoyl PE was purified from the reaction mixture by silica-gel thin-layer chromatography with solvent 3. Deprotection of the amino group was achieved with TFA. The N-tBOC-PE was dissolved in chloroform (100 μ l) and the solution was cooled to 0 °C in an ice bath. With constant stirring, methanol (5 μ l) and TFA (100 μ l) were added dropwise. The flask was then flushed with nitrogen and capped tightly. After 1.5 h at 0 °C, the solvents were evaporated under a nitrogen stream at 0 °C. Traces of TFA were eliminated by repeated washings with chloroform/methanol (1:1, v/v) and evaporation under high vacuum. Purification of the product was achieved by TLC developed with solvent 2. Acylation of the product ¹⁴C-labelled PE with the appropriate acyl anhydride was scaled down from the procedure

described previously. The ^{14}C -labelled NAPes co-migrate in TLC (solvent 2) with the corresponding unlabelled compounds.

Enzymic assays

A thin film of each phospholipid assayed as a substrate was prepared as follows. A chloroform stock solution of each NAPE (approx. 60 000 c.p.m.) and Triton X-100 was evaporated under a nitrogen stream in the bottom of a 5.6-mm $\times$ 50-mm tube. Final phospholipid and detergent concentration were as indicated in Figures 1 and 2. Traces of organic solvents were thoroughly eliminated after leaving the samples for 30 min under high vacuum. In the end, each phospholipid was solubilized in the appropriate buffers (25 mM Hepes/1 mM CaCl_2 , pH 8.0, for the PLA_2 assay, and with the addition of 25 μM ZnCl_2 for the PLC assay). The samples were then heated to 100 $^\circ\text{C}$ for 40 s and finally sonicated at 30 $^\circ\text{C}$ for 5 min in a bath sonicator (Branson 2200; Branson Ultrasonics). After repeating these last two steps, complete solubilization of the phospholipid substrates was verified by the quantitative recovery in the clear suspension of the radioactivity originally present in the sample. Enzymic reactions (50 μl final volume) were started by the addition of PLA_2 (68 ng) or PLC (22 ng) by appropriate dilution of enzyme stock solutions (PLA_2 , 0.33 mg/ml in 50 mM borate buffer, pH 8.5; PLC, 1.9 mg/ml in 30 mM Tris/borate buffer, pH 7.1) in the corresponding assay buffers, added with BSA (1 mg/ml final concentration). Both phospholipase assays were run at 30 $^\circ\text{C}$. The protein concentration of stock phospholipase solutions was determined by the method described by Bradford [17]. Initial rates of hydrolysis were determined by removing 5- μl aliquots from each reaction mixture and by adding 1 M HCl (10 μl) to stop the reaction. The latest time point was selected so that no more than 15 % of the phospholipid substrate was hydrolysed. Under these conditions, a linear dependence of product yield was observed as regards time of reaction and enzyme concentration. After the reaction was stopped, the samples were dried under high vacuum, dissolved in solvent 3 (15 μl), loaded at 1 cm from the bottom of a 5-cm $\times$ 1.6-cm TLC plate, and developed with a two-solvent system: firstly with solvent 1 and secondly with solvent 3. Under these conditions, NAPes and PE migrate with R_f values of approx. 0.7–0.82, whereas dihexadecanoyl glycerol and hexadecanoic acid do so showing R_f values of 0.98 and 0.94, respectively. Direct quantification of the radioactivity present on the TLC plates was achieved by scraping the silica off the glass and counting the samples by liquid scintillation. Alternatively, radioactivity was quantified by the use of image plates (LE storage phosphor screen, Storm 820; Amersham Biosciences).

Molecular modelling

Molecular mechanics calculations were carried out with MacroModel 5.5 and BatchMin 5.5 [18] running on an SGI O2 workstation (R10000, 320 MB RAM, 54 GB hard disk) under the Irix 6.5 operating system. We used the MM2* force field (the version of Allinger's MM2 force field as implemented in MacroModel). The electrostatic equation used by MM2* uses partial charges and Coulomb's law, instead of the standard dipole–dipole electrostatics. All calculations were carried out neglecting the ionization of the functional groups. The electrostatic interaction cut-off was set to 50 $\text{\AA}$. In order to simulate the conformational ensemble of the *N*-acyl polar head group we applied a mixed MC/SD method (Monte Carlo/stochastic dynamics method) [19]. In the course of the simulation, every SD time step is followed by an MC step. In all cases, the total productive simulation time was 9 ns after an initial equilibration period of 50 ps. A time step

Figure 1 The presence of the *N*-acyl group in NAPes impairs phospholipase activity in a fashion dependent on its chain length

The activities of *N. naja naja* PLA_2 (A) and *B. cereus* PLC (B) towards PE and NAPes were measured. The bars show enzymic activity with respect to PE (100 %). Under the assay conditions, the specific activities of PLA_2 and PLC towards this substrate were 22 and 37 $\mu\text{mol/min}$ per mg, respectively. In these assays substrate concentration was 1 mM and Triton X-100 was 30 mM; other experimental conditions were as described in the Experimental section. In addition, the effect of a variable concentration of NAPE-6 on PLA_2 hydrolysis of PE was measured (A, inset). For this last experiment 0.2 mM ^{14}C -labelled dihexadecanoyl PE and unlabelled NAPE-6 were incorporated in a mixed micellar system with 30 mM Triton X-100.

of 1.5 fs was used for the SD part. All atoms belonging to the polar head, with the exception of the phosphorus and the glycerol phosphate oxygen, were allowed to move. 600 000 conformers were sampled for each compound, resulting from an MC/SD simulation run at 300 K under vacuum.

RESULTS AND DISCUSSION

Phospholipase activity is highly dependent on NAPE *N*-acyl chain length

N-acylation of the head group of PE brings about multiple alterations to the parental PE as regards the interaction with phospholipases. They include a change of charge, increased steric hindrance because of the presence of the *N*-acyl chain itself and loss of hydrogen bonding at the polar head group. *N*-acylation can also distort the conformation adopted by the phospholipid in the lipid bilayer. Thus, in NAPes possessing *N*-acyl groups of eight carbons or fewer, these moieties remain at the level of the glycerol backbone. At variance with this, for longer *N*-acyl chains it has been shown that they are placed parallel to the *O*-acyl chains, i.e. buried inside the hydrophobic phase of the lipid bilayer [7].

Here we examine which factors are the most relevant with regard to phospholipase activity. To this end, we prepared NAPes in which the *N*-acyl group is 2, 4, 6 or 16 carbon atoms long. The results presented in Figure 1 show that *N*-acetylation reduces the activity of PLA_2 to about one-eighth of that shown towards

Figure 2 Interfacial catalysis of PLA₂ and PLC toward NAPE-2 and NAPE-4

The activities of *N. naja naja* PLA₂ towards NAPE-2 (**A**) and NAPE-4 (**B**), and of *B. cereus* PLC towards NAPE-2 (**C**) and NAPE-4 (**D**) were measured at three substrate molar fractions (○, $X = 0.091$; □, $X = 0.048$; △, $X = 0.032$). Saturation values of hyperbolic fittings to these data were plotted against the molar fraction assayed in each case (insets to panels **A–D**). A separate set of experiments was run where the concentration of NAPE was kept constant at 0.5 mM and the activities of PLA₂ (**E**) and PLC (**F**) were measured at a variable concentration of Triton X-100 (NAPE-2, □; NAPE-4, ■). Other experimental conditions were as described in the Experimental section.

the parental PE. The effect on PLC is less marked, with about one-fifth of the activity remaining. The fact that considerable PLA₂ activity remains towards this substrate indicates that the change in charge, from a zwitterionic to an anionic phospholipid, causes a major impact on the enzymic activity, but not its complete abrogation. On the other hand, PLA₂ activity drops dramatically in the case of NAPE-4. This lipid shows the same charge as NAPE-2 and probably similar hydration of the polar head group. Further addition of two carbon atoms in NAPE-6 reduces the activity almost completely. Not surprisingly, NAPE-16 is fully resistant to PLA₂ hydrolysis. The results with PLC indicate that this enzyme is less discriminating with regard to substrate structure. This agrees with previous work [20] and probably reflects the shallower penetration of the substrate into this enzyme.

We tested whether NAPE-6, aside from behaving as a very poor substrate, could act as a competitive inhibitor. No effect was observed on PLA₂ hydrolysis of PE (inset to Figure 1A), suggesting that NAPE-6 is simply unable to enter the hydrophobic

pocket leading to the catalytic site of the phospholipase. On the contrary, activation is evident, an effect probably due to the change of the interfacial charge as the concentration of the negatively charged NAPE-6 is increased.

A study of activity of both phospholipases was performed with NAPE-2 and NAPE-4 (Figure 2). Interfacial catalysis can be divided into two basic processes; firstly the adsorption of enzyme to the lipid–water interface and secondly the substrate binding to the enzyme. The first step is usually described as a function of the bulk concentration of substrate, while the second one depends on the interfacial concentration of substrate, which can be approximately described by its molar fraction with respect to detergent [21]. Therefore, two sets of experiments were carried out. The first one used a constant ratio of NAPE to detergent while increasing the bulk concentration of NAPE (Figures 2A–2D). Here we employed three molar fractions of substrate (X_{NAPE} , 0.091, 0.048 and 0.032). From these curves the affinity of each enzyme for the interface can be drawn from the fit of the data to

Figure 3 N-acyl chain growth increases the volume of the dome explored by the terminal methyl group

To measure the volume accessible to the polar head, the position of the terminal methyl group with respect to the phosphorus atom was determined by using two parameters (α and d), as defined in (A). The statistical distribution of these parameters over an ensemble of 600 000 structures sampled during an MC/SD simulation is pictured in (B) and (C). The most populated values (mode) of the distribution of d are: NAPE-2, 7.3 Å; NAPE-4, 8.2 Å; and NAPE-6, 10.5 Å. Correlation plots between α and d were derived from a subset of 1000 structures randomly selected from the MC/SD output: (D) NAPE-2, (E) NAPE-4 and (F) NAPE-6.

a hyperbolic model. No significant difference between NAPE-2 and NAPE-4 was observed regarding the interfacial affinities of both enzymes (K_s NAPE-2-PLA₂, 0.2–0.4 mM; K_s NAPE-4-PLA₂, 0.3–0.5 mM; K_s NAPE-2-PLC, 0.5–0.7 mM; K_s NAPE-4-PLC, 0.3–0.4 mM). The saturation parameter inferred from these curves indicates the maximum velocity that the enzyme could attain at a given substrate molar fraction, i.e. when all the enzyme becomes bound to the interface. When this parameter is plotted against the molar fraction assayed (see Figures 2A–2D, insets) an approximately linear behaviour is observed. This is a consequence of the increase in the substrate surface concentration. On the other hand, no hint of curvature can be envisaged, indicating that the K_m for these substrates is beyond the molar fractions assayed. Indeed, to preserve the nature of the micellar system, the substrate/detergent ratio was never higher than 1:6.

We ran a second kind of experiment where the bulk concentration of NAPE was kept constant while the concentration of Triton X-100 was varied (Figures 2E and 2F). In these experiments the molar fraction of substrate as well as the bulk micellar concen-

tration changed, and therefore the results reflect both the binding of the enzyme to the interface and the affinity of the enzyme for the substrate. Here again, in all cases linear behaviour was observed with no sign of saturation. However, both enzymes showed a remarkable difference in activity between NAPE-2 and NAPE-4, as becomes evident from the ratio of the slopes of these curves. NAPE-2 was 6.9-fold and 1.6-fold better than NAPE-4 as a substrate of PLA₂ and PLC, respectively.

In summary, the affinity of both enzymes to the lipid–water interface is similar for both NAPes herein studied and the K_m is beyond the upper limit of molar fraction attainable without compromising the integrity of the micellar system. On the other hand, the specific activities of both phospholipases towards NAPE-2 are higher than those measured towards NAPE-4, PLA₂ being more sensitive to substrate head-group modification than PLC.

The fact that activity is barely detectable against NAPE-6 implies that steric hindrance at the polar head, but not insertion into the bilayer, is enough to prevent activity. What is the nature of the steric hindrance caused by N-acyl chains of different

lengths? This concept entails a description of the conformational space accessible to the aliphatic chain. Of all possible conformations, only a few are suitable for entering the catalytic site. It could be expected that, as the head group elongates, the number of unfavourable conformations increase in such a measure that, at the extreme, the probability of substrate–enzyme complex formation becomes negligible. In other words, the entropy of activation for the entry step of the substrate into the enzyme would depend dramatically on N-acyl chain length. In connection with this, for PLA₂, the substrate amino group is located almost at the protein surface opposite to the membrane-binding surface. At this position, any group attached to the nitrogen atom, regardless of its bulk, would protrude towards the aqueous milieu, without necessarily destabilizing the enzyme–substrate complex. Therefore, the effect herein observed originates before the substrate–enzyme complex could form, i.e. at the substrate-entry step.

Molecular modelling of NAPes reveals an increased dynamic volume of the polar head group

To simulate the conformational space explored by the polar head of NAPes we used a molecular modelling approach. This space was calculated using a mixed MC/SD simulation. To illustrate the excursions of the N-acyl chain we evaluated the relative position of the methyl terminal group with respect to the phosphorus atom. This position was determined in polar co-ordinates (d and α) as defined in Figure 3(A). The statistical distributions of these parameters over the conformational ensemble of the polar heads of Nape-2, Nape-4 and Nape-6 are depicted in Figures 3(B) and 3(C). A clear bimodal distribution for α is found in all cases. This arises from conformational restrictions associated with the allowed regions of the dihedral angle O-P-O-C. The distribution of d for Nape-2 also shows a clear bimodal form. By contrast, this behaviour fades away for Nape-4 and Nape-6, as chain growth geometrically multiplies the conformational space available (Figure 3C). A random sample of 1000 structures of each compound was used to demonstrate that no correlation exists between α and d (Figures 3D–3F). These plots show a marked increase in the spread of values, reflecting the growth of the conformational space. With this information, the actual position of the methyl group relative to the phosphorus atom can be calculated. When this procedure is applied to the contour of the correlation graphics, a picture of the volume explored by the polar head can be envisaged (Figure 4). This alternative representation clearly shows the effect of the growth in length of the N-acyl chain on the volume of the conformational space.

Hypothetically, accessing the catalytic site is only possible for those conformations in which the N-acyl chain adopts a relatively linear arrangement, thus enabling it to avoid the steric constraints imposed by the hydrophobic channel. Our previous work [20] showed that a phenyl group generates considerable impediment to the entry of the chain, thus providing a measure for the effective width of the channel. Extended conformations are obviously those in which the terminal methyl group lies farthest away from the phosphorus atom and, therefore, they are those located at or near the surface of the head-group dome. Growth of the conformational space implies a progressive reduction of the surface/volume ratio. Since the volume represents the total number of possible structures, whereas the surface measures the number of extended conformations, it becomes evident how the growth of the chain restricts the number of favourable conformations, thus impairing enzymic activity. Moreover, among all extended conformations, only those adequately oriented will complete a successful entry into the channel. This orientation factor will be

Figure 4 Expansion of the head-group conformational space as a consequence of the growth of the N-acyl chain

A contour was defined including all points in each correlation plot of α and d (Figures 2D–2F). Then these contours were transformed to Cartesian co-ordinates to render the solids of revolution around the central phosphorus atom shown here. Each volume represents the dynamic space available to the N-acyl chain: (A) Nape-2, (B) Nape-4 and (C) Nape-6. To avoid artifactual distortions due to van-der-Waals interactions with the glycerol moiety, we choose to consider in the simulations exclusively the head-group atoms up to the glycerol phosphate oxygen. On the other hand, if the glycerol moiety were taken into account, some restrictions to the mobility of the chain due to steric hindrance would induce distortions in shape at the base of the volume. The bars indicate an estimate of the atomic distance at the narrowest point of the hydrophobic channel, which amounts to about 7 Å, i.e. the distance of closest approach between atoms belonging to Leu-6 and Trp-18 in the crystallographic structure of *N. naja atra* PLA₂. The central point indicates the position of the phosphorus atom.

all the more restrictive for longer substrates, since they can miss the access easier than the shorter ones, i.e. with smaller variations of its orientation.

In conclusion, this work demonstrates that beyond six carbon atoms, N-acyl PEs are no longer substrates for *N. naja naja* PLA₂, in agreement with our previous observation that *N*-hexadecanoyl PE is not attacked by bovine erythrocyte PLA₂ [9]. The reasons underlying the loss of activity can be traced essentially to the activation entropy for substrate entry derived from the decrease in favourable-to-possible conformations as chain length grows. This factor reduces the chances of access to the catalytic site. Ligand flexibility in other systems has also been demonstrated to play an important role in binding to proteins [22–24]. Our work also shows that partitioning of the N-acyl chain into the hydrophobic phase is not required for the loss of activity. In addition, N-acyl PEs can attain resistance to enzymic hydrolysis, while gaining anionic properties that make them potentially useful for such purposes as the preparation of non-degradable liposomes with controlled charge density.

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