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Factors regulating the substrate specificity of cytosolic phospholipase A_2-alpha in vitro

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ABSTRACT

Cytosolic phospholipase A_2 alpha (cPLA$_2\alpha$) plays a key role in signaling in mammalian cells by releasing arachidonic acid (AA) from glycerophospholipids (GPLs) but the factors determining the specificity of cPLA$_2\alpha$ for AA-containing GPLs are not well understood. Accordingly, we investigated those factors by determining the activity of human cPLA$_2\alpha$ towards a multitude of GPL species present in micelles or bilayers. Studies on isomeric PC sets containing a saturated acyl chain of 6 to 24 carbons in the *sn*1 or *sn*2 position in micelles showed an abrupt decrease in hydrolysis when the length of the *sn*1 or *sn*2 chain exceeded 17 carbons suggesting that the acyl binding cavity on the enzyme is of the corresponding length. Notably, the saturated isomer pairs were hydrolyzed identically in micelles as well as in bilayers suggesting promiscuous binding of acyl chains to the active site of cPLA$_2\alpha$. Such promiscuous binding would explain the previous finding that cPLA$_2\alpha$ has both PLA$_1$ and PLA$_2$ activities. Interestingly, increasing the length of either the *sn*1 or *sn*2 acyl chain inhibited the hydrolysis in bilayers far more than that in micelles suggesting that with micelles (loosely packed) substrate accommodation at the active site of cPLA$_2\alpha$ is rate-limiting, while with bilayers (tightly packed) upward movement of the substrate from the bilayer (efflux) is the rate-limiting step. With the AA-containing PCs, the length of the saturated acyl chain also had a much stronger effect on hydrolysis in bilayers *vs.* micelles in agreement with this model. In contrast to saturated PCs, a marked isomer preference was observed for AA-containing PCs both in micelles and bilayers. In conclusion, these data significantly help to understand the mode of action and specificity of cPLA$_2\alpha$.

1. Introduction

Arachidonic acid (AA) is a precursor of prostaglandins and other eicosanoids and thus plays a key role in signaling in mammals, particularly during inflammation [1,2]. AA can be released from various glycerophospholipids (GPLs) by a number of A-type phospholipases (PLAs), including secretory PLAs (sPLA$_2$s), cytosolic Ca^{2+}-dependent PLA$_2$s (cPLA$_2$s) and Ca^{2+}-independent PLA$_2$s (iPLA$_2$s) [3]. However, amongst those enzymes only cPLA$_2\alpha$ (a.k.a. Group IVA PLA$_2$) has a pronounced selectivity towards arachidonate-containing GPLs [4,5]. cPLA$_2\alpha$ is ubiquitously expressed in mammalian tissues and its knockout in mouse greatly reduces the release of AA from GPLs [6,7] thus demonstrating that this protein plays a central role in the production of AA-derived signaling molecules in mouse and probably in the other mammals as well. The crystal structure shows that cPLA$_2\alpha$ consists of two main domains [8]. The N-terminal C2 domain that facilitates the initial association of the enzyme with a membrane in response to increasing intracellular Ca^{2+}, which in turn induces phosphorylation of certain serine residues [9,10]. The second, catalytic domain contains a "lid" that has to move aside for a GPL molecule to enter the active site cavity of cPLA$_2\alpha$ [11].

Due to the pivotal role of cPLA$_2\alpha$ in AA-release, its substrate specificity and regulation has been studied by several groups (e.g., [12–16]). Notably, cPLA$_2\alpha$ is not fully specific for AA-containing GPLs since it also hydrolyses other polyunsaturated GPLs (including those containing a 20:5 residue in the *sn*2 position) as well as other unsaturated species, albeit less efficiently [17]. The enzyme cleaves AA from GPLs containing a *sn*1 ether or ester bond with a similar efficiency, and has also been considered unselective towards the GPL's head group structure [18].

Abbreviations: AA, arachidonic acid; GPL, glycerophospholipid; SUV, small unilamellar vesicles; LPC, lysophosphatidylcholine; cPLA$_2$, Calcium^{2+}-dependent phospholipase A$_2$; PA, phosphatidic acid; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPM, dimyristoyl-phosphatidylmethanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; 16:0/C$_n$-PC, *sn*1–16:0/*sn*2-C$_n$-phosphatidylcholine (n = 6–24); C$_n$/16:0-PC, *sn*1-C$_n$/*sn*2–16:0-phosphatidyl-choline (n = 6–24); 20:4/C$_n$-PC, *sn*1–20:4/*sn*2-C$_n$-phosphatidylcholine (n = 6–24); C$_n$/20:4-PC, *sn*1-C$_n$/*sn*2–20:4-phosphatidylcholine (n = 6–24).

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Yet, cPLA$_2\alpha$ acts as a PLA$_1$, transacylase or lyso-PLA [19–21]. Regarding its lyso-PLA activity, it is intriguing that *sn*1-palmitoyl lyso-PC was hydrolyzed faster than *sn*2-palmitoyl lyso-PC *in vitro* [19]. Such regiospecificity is unexpected, considering that the enzyme is thought to mainly cleave the *sn*2 chain of intact GPLs, implying that binding of the substrate acyl chain to the active site may be promiscuous or that there is more than one lipid binding site on cPLA$_2\alpha$ [4,5].

Recently, Mouchlis and coworkers employed computer-aided modeling guided by previous deuterium-exchange mass spectrometry studies to investigate the interactions of PAPC with cPLA$_2\alpha$ [12]. Their data indicated that both the acyl chains of PAPC are fully accommodated inside a hydrophobic pocket containing aromatic residues which may favorably interact with the double bonds of the AA residue. Intriguingly, both the *sn*1 and *sn*2 chain appeared to share a common hydrophobic cavity in the modeled protein. Another simulation study [22] found marked differences in the binding of PAPC vs. DPPC (containing two 16:0 chains) by cPLA$_2\alpha$. While the *sn*2 chain of PAPC was fully embedded inside cPLA$_2\alpha$, both acyl chains of DPPC remained partially outside the protein after the simulation. Although these modeling studies suggested that accommodation of the substrate acyl chains in the active site determines the substrate specificity of cPLA$_2\alpha$, they did not provide information on the role of other factors, particularly that of substrate efflux from the bilayer [23,24], which is probably the rate-limiting step in the hydrolysis of GPLs by several other soluble PLAs [25,26]. Accordingly, we employed a high-throughput mass-spectrometric assay to study to what extent the accommodation of the substrate in the catalytic site of cPLA$_2\alpha$ and substrate efflux contribute to its marked specificity for AA-containing GPLs. Towards this end, the hydrolysis of several systematically constructed sets of GPLs were studied in both micelles and vesicle bilayers. The results suggest that while favorable accommodation of the substrate in the active site greatly contributes to the preferential hydrolysis of AA-containing GPLs by cPLA$_2\alpha$ also the efflux of the substrate from a bilayer plays a significant role. In fact, when the substrate lacked an AA residue, efflux from a bilayer appeared to be the key rate-limiting step of hydrolysis. Promiscuous binding of the acyl chains of a GPL to the active site of cPLA$_2\alpha$ most probably explains why cPLA$_2\alpha$ displays both PLA$_1$ and PLA$_2$ activities.

2. Materials and methods

2.1. Lipids and other chemicals

L-α-1-palmitoyl-2-arachidonoyl-[1-^{14}C]-PC (58 μCi/μmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA), unlabeled GPLs, lyso-PCs and sphingomyelin (SM) were obtained from Avanti Polar Lipids (Alabaster, AL) and D_3-methyl iodide from Cambridge Isotope Laboratories (Andover, MA). Ethanolamine, phospholipase D (Streptomyces species), Triton X-100 and EDTA-free protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Silica gel 60 thin-layer plates and HPLC-grade solvents were obtained from Merck Millipore (Munich, Germany), Strep-Tactin superflow resin and desthiobiotin from IBA-Life Sciences (Göttingen, Germany), bovine serum albumin standard and Coomassie brilliant blue R-250 solution from Bio-Rad (Sundyberg, Sweden). Penicillin, streptomycin and fetal calf serum (FCS) were from Gibco Life Technologies (Bleiswijk, The Netherlands). The primary (sc-454) and secondary (Streptactin HRP-conjugate) antibodies were purchased from Santa Cruz Biotechnology and BioRad respectively. The *sn*1–16:0/*sn*2-C$_n$-PC and *sn*1-C$_n$/*sn*2–16:0-PC species were synthesized by acylating *sn*1–16:0-LPC with the anhydride of a C$_n$-fatty acid or *sn*1-C$_n$-LPC with the anhydride of 16:0-fatty acid, respectively [27]. The *sn*1–20:4/*sn*2-C$_n$-PC and *sn*1-C$_n$/*sn*2–20:4-PC species were synthesized analogously by acylating *sn*1–20:4-LPC with the anhydride of a C$_n$-fatty acid or *sn*1-C$_n$-LPC with the anhydride of 20:4-fatty acid, respectively. The D_9-labeled PC species were synthesized from the corresponding phosphatidylethanolamines by methylation with D_3-methyl iodide [28]. The purity of GPLs was confirmed by TLC and mass spectrometry and their concentrations were determined by using a phosphate assay [29].

2.2. Cloning, expression and purification of human cPLA$_2\alpha$

Full length cPLA$_2\alpha$ cDNA from the clone MGC:126350 IMAGE:40034995 (ImaGenes, GenBank™ BC114340.2) was amplified by PCR using the forward primer 5′-GTCGGTCCGCCA-CCATGTCATTTA TAGATCCTTACCAGCACA-3′ containing a *Rsr*II cleavage site and the reverse primer 5′-CTGGTACCTTAGTGGTGGTGGTG-ATGGTGATGATGAT GATGTTCGAAACGACC-TTCGATTGCTTTGGGTTTACTTAGAAACTCCTTG-3′ containing the *Bst*BI and *Kpn*I cleavage sites. The *Rsr*II- and *Kpn*I-digested PCR product was ligated into pFastBac1-vector (pFB1, Gibco Life Technologies) resulting in pFB1-cPLA$_2\alpha$-His$_{10}$ vector. Replacing the *Bst*BI-*Kpn*I fragment of pFB1-cPLA$_2\alpha$-His$_{10}$ by the annealed oligonucleotides 5′-CGAATGGAGCCACCCGCAGTTCGAG-AAAGGAGGAGGAAG CGGAGGAGGAAGCGGAGGAGGAAGCT-GGAGCCACCCGCAGTT-TGAAAA ATAGGTAC-3′ and 5′-CTA-TTTTTCAA-ACT-GCGGGTGGCTCCAGCTTCCT CCT-CCGCTTCCTCCTCCGCTTCCTCTCCTTTTCGAACTGCG-GGTGGCTCCA TT-3′ produced the pFB1-cPLA$_2\alpha$-StrepIII vector. The inserted sequences and reading frames of the constructs were verified by sequencing. The Bac-to-Bac baculoviral expression system (Gibco) was utilized for bacmid preparation and FuGENE®6 (Promega)-mediated transfection of Sf9 cells was employed to produce the recombinant baculovirus. To express the cPLA$_2\alpha$-StrepIII protein, Sf9 cell suspension (500 mL, ~10^6 cells/mL) was infected with the recombinant baculovirus at the multiplicity of 1. After incubation at 26 °C for 72 h, the cells were washed with ice-cold PBS and resuspended in 50 mL of a lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Triton X-100, 5% glycerol) containing an EDTA-free protease inhibitor cocktail. The cells were then lysed by sonication (6 × 1 min with 30 s intervals, 40% duty cycle) and centrifuged at 50,000 ×*g* for 30 min to pellet the membranes after which the purification was carried out as earlier [26].

2.3. Immunoblotting

To assess their cPLA$_2\alpha$ content, transfected Sf9 cells were pelleted, washed with ice-cold PBS, resuspended in a lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1.0% deoxycholate, 5 mM EDTA) and left on ice for 30 min. The lysate was centrifuged at 800 ×*g* for 3 min at 4 °C and 20 μg of the supernatant protein was run on a 10% SDS-polyacrylamide gel. The proteins were then transferred onto a PVDF membrane (Millipore) which was treated with 5% defatted dry milk in 50 mM Tris, pH 7.4, 150 mM NaCl containing 0.1% Tween-20. The recombinant cPLA$_2\alpha$ was detected with an *anti*-cPLA$_2\alpha$ (1:2500 dilution) or a Streptactin HRP-conjugate antibody (1:10,000 dilution). After treatment with ECL Plus detection reagent kit (GE Healthcare), the blot was scanned with Starion FLA-9000 image scanner (Fujifilm) and visualized using Image Reader FLA-9000 software.

2.4. GPL mixtures studied

To determine how the structure of the acyl chains and the head group influences GPL hydrolysis by cPLA$_2\alpha$ *in vitro*, we employed a mass-spectrometric assay devised previously [30]. This high-throughput assay allows one to determine the rates of hydrolysis of multiple GPLs present together in a macrosubstrate particle, thus avoiding any bias caused by substrate-dependent variations in the physical properties of the macrosubstrate and protein binding [31,32]. Seven different mixtures of GPL species were studied (Supplementary Table 1). The first mixture ("PC-mix") consisted of 27 PC species including: (*i*) 10 saturated species, (*ii*) 3 monounsaturated species; (*iii*) 6 diunsaturated species; (*iv*) 3 species with four double bonds; (*v*) 3 species with 6 double bonds; (*vi*) one species with eight double bonds and (*vii*) one species with 12 double bonds. The second mixture (16:0/C$_n$-PCs) consisted of 17 PC species containing a 16:0 chain in the *sn*1 position and a saturated chain of 6–

24 carbons in the *sn*2 position. The third mixture (D_9-C_n/16:0-PCs) consisted of acyl chain positional isomers of those in the second mixture. The fourth mixture (20:4/C_n-PCs) consisted of 16 PC species with a 20:4 chain in *sn*1 position and a saturated chain of 6–24 carbons in the *sn*2 position. The fifth mixture (C_n/20:4-PCs) consisted of acyl chain positional isomers of those in the fourth one. The sixth mixture consisted of 18:1/18:1-PC, -PE, -PS, -PG, -PI and -PA. The seventh mixture was identical to the sixth one except for the acyl chain composition (16:0/20:4) of the GPLs. Ten mol% of 21:0-SM (a non-hydrolysable internal standard) and 10 mol% of 16:0/18:1-PA was included in all the mixtures (except 6 and 7) presented to the enzyme either in small unilamellar vesicle (SUV) bilayers or TX-100 detergent micelles.

2.5. Assay of cPLA$_2$α activity

The activity of the human recombinant cPLA$_2$α was determined using the micellar assay with a radiolabeled PC as the substrate. The micellar macrosubstrate was prepared by mixing 10,000 cpm of 1-palmitoyl-2-arachidonyl-1-^{14}C-PC, unlabeled 16:0/18:1-PC and 16:0/18:1-PA (250/25 nmol) in chloroform followed by removal of the solvent under a N_2 stream and subsequently under a high vacuum for 1 h. Then 0.5 mL of the assay buffer (20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 800 μM Triton X-100) was added and the suspension was vortexed for 2 min at RT. An aliquot of purified cPLA$_2$α was added and the mixture was incubated for 30 min at 37 °C. Aliquots of 50 μL were removed at intervals and mixed with 2 mL of ice-cold methanol to stop the reaction and the lipids were extracted according to an established protocol [33] except that 0.1 M HCl was used instead of water to maximize lipid recovery. The extract was analyzed on TLC using hexane/diethyl ether/acetic acid (70:30:1, v/v) as the eluent with PC, LPC and fatty acid bands being detected with a Starion FLA-9000 scanner (Fujifilm) and their radioactivity quantified with the Multigauge software.

2.6. Substrate specificity assay of cPLA$_2$α

A mixture of synthetic GPLs, 16:0/18:1-PA (10 mol%) which greatly stimulates the hydrolysis by enhancing cPLA$_2$α binding to the lipid macrosubstrate [36], and 21:00-SM, a nonhydrolyzable internal standard were mixed in chloroform, dried under a N_2 stream and then under a high vacuum for 1 h. Butylated hydroxytoluene (1 mol%) was included as an antioxidant when the mixture contained unsaturated GPLs. To prepare SUVs, the dried lipids were dissolved in 20 μL of ethanol and the solution was injected into 0.5 mL of the assay buffer (20 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM $CaCl_2$) with a constant-speed Hamilton syringe. This method reproducibly produces small unilamellar vesicles (SUVs) with a fairly narrow size distribution [34,35] and thus the vesicles were not characterized here. SUV were then incubated in the presence of purified cPLA$_2$α at 37 °C, 50 μL aliquots were removed at intervals and mixed with 2 mL of ice-cold methanol to stop the reaction. After addition of 0.8 mL water and 4 mL chloroform, vortexing and removal of the upper phase, the lower phase was washed twice with the theoretical upper phase [33] and taken to dryness under a N_2 stream. The residue was reconstituted in 200 μL of chloroform/methanol (1:2, v/v) and stored at −20 °C. To study the hydrolysis in micelles, the dried lipid mixture was dispersed in the assay buffer containing 1% Triton X-100 by vortexing and the assay was conducted as above.

2.7. Mass-spectrometric analysis of GPLs

After addition of 25% NH_4OH in water (4% final concentration) the sample was infused to a Micromass Quattro Micro triple-quadrupole mass spectrometer operated as previously [37]. The GPL species were detected using head group-specific precursor or neutral-loss scanning [38]. The spectra were exported to Microsoft Excel and the individual GPL species were quantified using the LIMSA software [39]. Concentrations of the individual lipid species were plotted against time and the relative rate constants of hydrolysis were obtained by fitting a first order exponential decay model to the data. The maximum hydrolysable fraction at t_∞ was constrained to 1.0 with micelles and to 0.67 with SUVs [25]. LC-MS with selective reaction monitoring (SRM) was used when the hydrolysis of PCs in micelles was investigated as this method allows one to divert the (early eluting) detergent to waste thus avoiding the contamination of the mass spectrometer. Waters ACQUITY Ultra Performance LC system equipped with a Waters ACQUITY BEH C_{18} column (1.0 × 100 mm) was used to separate the molecular species using gradient elution. Solvent A was acetonitrile/H_2O (60:40) with 10 mM ammonium formate and 1% NH_4OH, while solvent B was isopropanol/acetonitrile (90:10) containing 10 mM ammonium formate and 1% NH_4OH. The flow rate was 0.13 mL/min and the column temperature 60 °C. Solvent B was set to 40% at injection and increased linearly to 100% in 14 min, remained at this value for 3 min, decreased back to 40% in 1 min and then remained there till the end of the gradient at 20 min. The eluent was infused to the ESI source of Waters Quattro Premier triple-quadrupole mass spectrometer operated in the positive ion mode. The SRM chromatograms were integrated and the relative concentrations of the individual GPL species were calculated using the QuanLynx software (Waters).

2.8. Other procedures

Protein concentration was determined using Lowry [40] or a fluorometric method [41] with BSA as the standard. SDS-PAGE was carried out according to Laemmli et al. [42] and the proteins were stained with Coomassie blue [43].

2.9. Statistical analysis

In all experiments the values are presented as means ± SD of 4 independent experiments and statistical significance was analyzed by student's two-tailed *t*-test and $P < 0.05$ was considered as significant.

3. Results

3.1. Effects of the acyl chain length and unsaturation on PC hydrolysis in micelles and SUV bilayers

We first studied the hydrolysis of 27 PC species ("PC-mix") varying in acyl chain length and/or unsaturation and present simultaneously in detergent micelles (Fig. 1A). Increasing the acyl chain length inhibited the hydrolysis only slightly, except when the acyl chains were very long (>20 carbons). Also the acyl chain unsaturation had only a modest effect except that the PC species with AA in the *sn*2 or both *sn*-positions were hydrolyzed much more rapidly than the more saturated species with an equal total chain length. These data suggest that an AA chain, as expected, strongly increases the affinity of a PC for the substrate binding site of cPLA$_2$α. We next studied hydrolysis of PC-mix in vesicle bilayers (Fig. 1B). Again, the length of the acyl chains had only a modest effect on hydrolysis independent of acyl chain unsaturation. Also otherwise the results were quite similar to those obtained for micelles, i.e., di-20:4-PC was the best substrate followed by the species that contained a single AA chain. For the statistical significance of the differences observed see the legend of Fig. 1.

3.2. Effect of the sn1 or sn2 chain length of PC on hydrolysis in micelles

We first studied the hydrolysis of saturated 16:0/C_n-PCs and D_9-C_n/16:0-PCs present simultaneously in micelles (Fig. 2A). Interestingly, the hydrolysis of both isomeric sets was essentially independent of the acyl chain length until $n = 17$ beyond

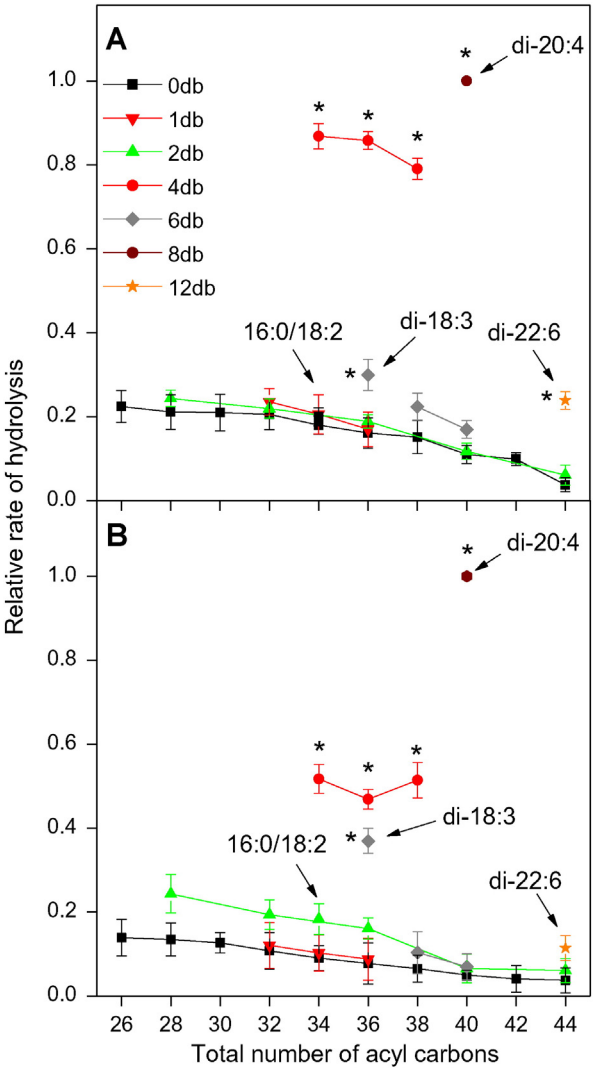

Fig. 1. Hydrolysis of PC species with varying acyl chain length and unsaturation. (A) PC-mix, 16:0/18:1-PA and 21:0-SM (250/25/25 nmol) were dispersed in 0.5 mL of assay buffer containing 1% TX-100 and incubated with 15 μg of cPLA$_2$α for 30 min at 37 °C. Samples of 50 μl were drawn at intervals and the rate of hydrolysis was determined as indicated in *Materials and Methods*. Specific activity calculated based on the hydrolysis of di-20:4-PC was 9.8 nmol/μg protein∗min. (B) Vesicles consisting of PC-mix, 16:0/18:1-PA and 21:0-SM (250/25/25 nmol) were incubated with 10 μg of cPLA$_2$α in 0.5 mL assay buffer for 30 min at 37 °C and the hydrolysis was determined as above. The specific activity calculated based on the hydrolysis of di-20:4 PC was 18 nmol hydrolyzed per μg cPLA2/min. The sets with only a single member are indicated by an arrow. Data are mean ± S.D. of four independent experiments. db = total number of acyl chain double bonds. Statistically significant difference was found between the species marked with an asterisk and the saturated species (0 db) with the same number of total acyl chain carbons.

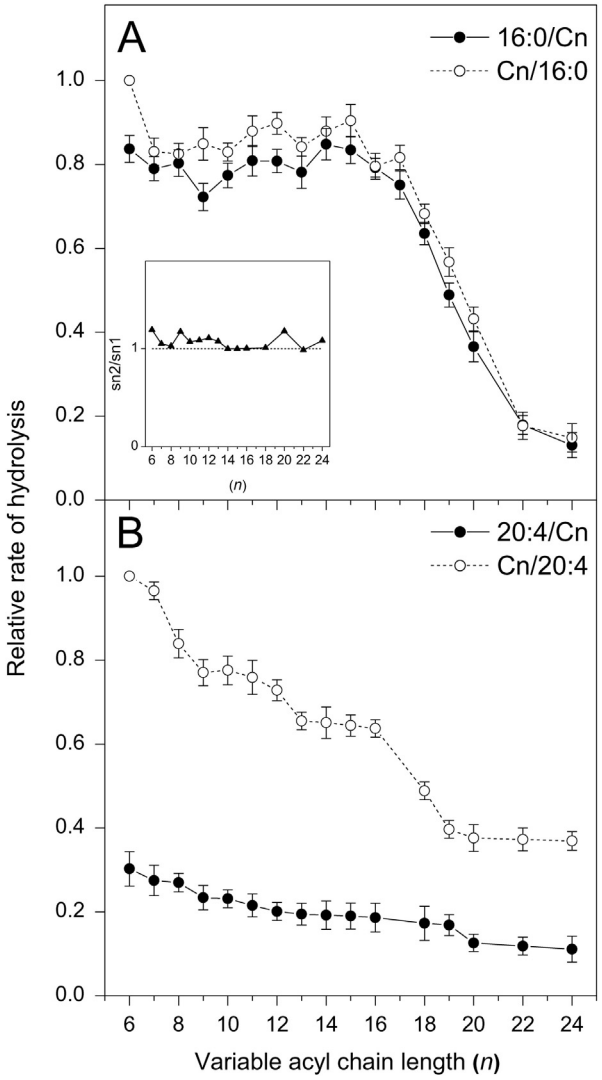

Fig. 2. Effect of the length of the *sn*1 or *sn*2 chain of PC on hydrolysis in Triton X-100 micelles. (A) 16:0/C$_n$-PCs, D$_9$-C$_n$/16:0-PCs, 16:0/20:4-PC, 16:0/18:1-PA and 21:0-SM (112.5/112.5/25/25/25 nmol) were dispersed in 0.5 mL of assay buffer containing 1% TX-100 and then incubated with 24 μg of cPLA$_2$α for 30 min at 37 °C. Specific activity calculated for the most rapidly hydrolysed PC species was 7.2 nmol per μg of cPLA2/min. Statistically significant differences were found only for $n = 6$ and 9. *Inset* shows the isomer preference vs. chain length plot obtained by pair-wise division of the rate of 16:0/C$_n$-PC hydrolysis by that of its D$_9$-C$_n$/16:0-PC isomer. Dotted line at y = 1.0 indicates the absence of isomer preference. Note: no hydrolysis of the saturated PCs was observed in the absence of 16:0/20:4-PC or free AA (data not shown). (B) 20:4/C$_n$-PCs, C$_n$/20:4-PCs, 16:0/18:1-PA and 21:0-SM (125/125/25/25 nmol) were dispersed in 0.5 mL of assay buffer containing 1% TX-100 and then incubated with 15 μg of cPLA$_2$α for 30 min at 37 °C. Specific activity calculated for the most rapidly hydrolysed PC species was 14.5 nmol hydrolyzed per μg cPLA2/min. Data are mean ± S.D. of four independent experiments. Statistically significant differences were found for all the isomer pairs. See the legend of Fig. 1 for the other details.

which a rapid decline was observed. These data are compatible with a model suggesting that (*i*) both the *sn*1 and *sn*2 acyl chains share the same binding cavity in cPLA$_2$α and (*ii*) the length of the cavity is similar to that of a C17 acyl chain. Intriguingly, the saturated PCs were not hydrolyzed significantly (data not shown) unless either 16:0/20:4-PC or free AA was included in the micelles thus suggesting that the AA moiety somehow "primes" the enzyme for the hydrolysis of PCs in micelles. In a notable contrast to the saturated PCs, cPLA$_2$α showed a marked isomer preference for the AA-containing PCs in micelles, i.e., the species containing an AA residue in the *sn*2 position were hydrolyzed ~4-fold faster than their isomers (Fig. 2B). For the statistical significance of the differences observed see the legend of Fig. 2.

3.3. Effect of the sn1 or sn2 chain length of PC on hydrolysis in SUV bilayers

With bilayers, the results were very different from those obtained for micelles. In case of the saturated PCs, the rate of hydrolysis decreased strongly (nearly logarithmically) with the length of either *sn*1 or *sn*2 acyl chain, except that the PC with C6 at the *sn*1 position deviated from this trend (Fig. 3A). In addition, there were generally no significant differences in the hydrolysis of the region- (*sn* position) isomers. These data strongly suggest that substrate efflux propensity mainly determines the rate of hydrolysis of saturated PCs by cPLA$_2$α in bilayers. Unlike in micelles, the hydrolysis proceeded readily in the absence of any

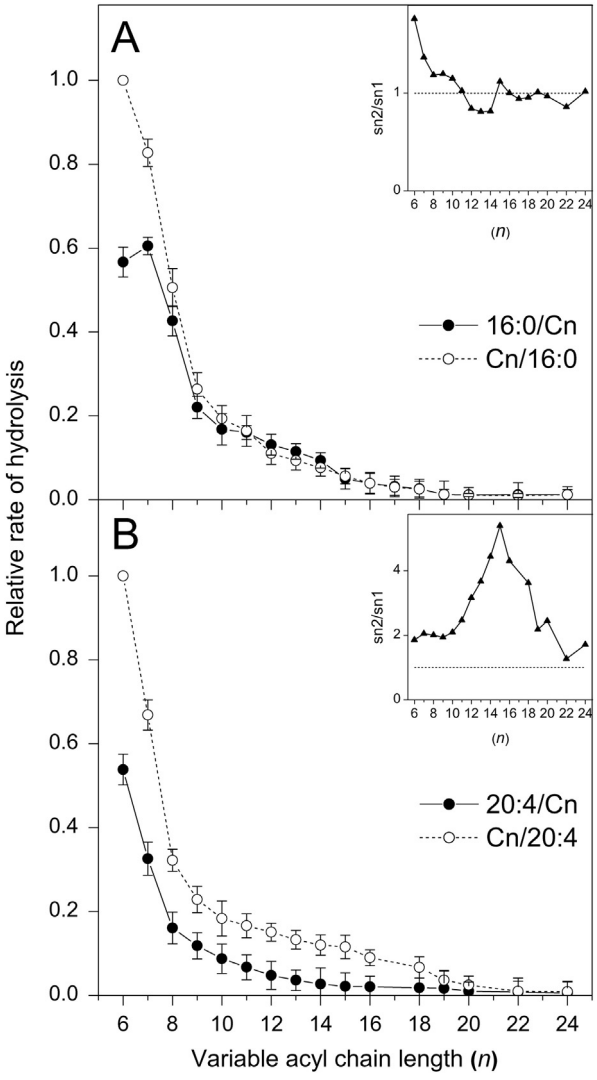

Fig. 3. Effect of the length of *sn*1 or *sn*2 chain of PC on hydrolysis in vesicle bilayers. (A) Vesicles consisting of 16:0/C_n-PCs, D_9-C_n/16:0-PCs, 16:0/18:1-PA and 21:0-SM (125/125/25/25 nmol) were incubated with 18 μg of cPLA$_2\alpha$ in 0.5 mL of the assay buffer for 30 min at 37 °C. Specific activity calculated for the most rapidly hydrolysed PC species was 11 nmol per μg cPLA2/min. Statistically significant differences were found only for $n = 6$ and 7. *Inset* shows an isomer preference plot obtained as indicated in the legend of Fig. 2. (B) 20:4/C_n-PCs, C_n/20:4-PCs, 16:0/18:1-PA and 21:0-SM (125/125/25/25 nmol) incubated with 11 μg of cPLA$_2\alpha$ in 0.5 mL of assay buffer for 30 min at 37 °C. The specific activity calculated for the most rapidly hydrolysed PC species was 21.5 nmol hydrolyzed per μg of cPLA2/min. See legend of Fig. 1 for the other details. Data are mean ± S.D. of four independent experiments. Statistically significant differences were found for $n = 6$–9 and 12 and 13.

added AA-containing PC or free AA. Also in the case of the AA-containing PCs the rate of hydrolysis decreased initially strongly with increasing length of the saturated acyl chain (Fig. 3B), suggesting that substrate efflux contributes to the hydrolysis of the AA-containing PCs as well. However, in striking contrast to the saturated PCs, a marked difference was observed between all the positional isomer pairs, i.e., the PC species with AA in the *sn*2 position were hydrolyzed 2 to 4-fold faster than their isomers. For the statistical significance of the differences observed see the legend of Fig. 3.

3.4. Effect of GPL polar head group structure

Finally, we studied the effect of the polar head group on the rate of hydrolysis of GPLs by cPLA$_2\alpha$ both in micelles and vesicle bilayers. First, a mixture of six 16:0/20:4-GPLs (PC, PE, PS, PI, PG and PA) was incorporated together in micelles or vesicle bilayers. In micelles, PA was hydrolyzed much more rapidly than all the other GPLs (Fig. 4A). Also in bilayers, PA was the best substrate but now, also PG and PC were hydrolyzed significantly faster than the remaining GPLs (Fig. 4B). Very similar results were obtained for the di-18:1 derivatives with both macrosubstrates (Fig. 4C, D), suggesting that the effect of the head group structure is largely independent of the acyl chain composition of the GPL.

3.5. PLA_1 vs. PLA_2 activity of cPLA$_2\alpha$

The hydrolysis data given above is based on the rate of the decrease of each PC peak in the mass spectra and thus does not now allow one to determine the relative contributions of the PLA_1 vs. PLA_2 activities of cPLA$_2\alpha$. In principle, these contributions could be determined for the relative abundances of the lyso-PC species released from C_n/20:4-PCs and 20:4/C_n-PCs. However, this approach is compromised due to the facts that cPLA$_2\alpha$ has a significant lyso-PLA activity [19] and could thus hydrolyze the lyso-PCs produced and, secondly, the rate of lyso-PC hydrolysis is likely to depend on the fatty acid present. Accordingly, it was not feasible to quantify the relative PLA_1 and PLA_2 activities of cPLA$_2\alpha$. Nevertheless, the data for AA-containing PCs suggest that (at least with these PCs) the PLA_2 activity prevails over the PLA_1 activity since only very small amounts of 20:4-lyso-PC was released from C_n/20:4-PCs, while significant amounts of 20:4-lyso-PC was released from 20:4/C_n-PCs (Fig. S2).

4. Discussion

Hydrolysis of a membrane-bound GPL molecule by cPLA$_2\alpha$, or another soluble PLA, requires the following steps: 1) binding of the enzyme superficially to the membrane surface, 2) efflux (i.e., upward movement) of the GPL molecule from the macrosubstrate, 3) accommodation of the substrate in the binding site of the enzyme and 4) cleavage of an acyl-glycerol ester bond and 5) release of the products [20]. Although there is a plethora of data showing that cPLA$_2\alpha$ plays a key role in the liberation of AA from GPLs, there is only limited information on how those factors contribute to the preferential hydrolysis of AA-containing GPLs by this enzyme. Here, several systematically constructed sets of GPLs were employed to determine the importance of the steps 2 and 3. To this end, we studied the hydrolysis both in micelles and vesicle bilayers because the former are loosely packed or disordered [44–46], which dramatically enhances lipid efflux [47–49] as compared to bilayers in which the intermolecular interactions are much stronger and thus the chemical activity of the lipids much lower [50]. Based on these results, it can be predicted that substrate accommodation in the active site of the enzyme (step 3) is much more important with micelles, while efflux (step 2) is more important in bilayers. Our previous studies with several other PLAs are in full agreement with these predictions [25,26]. Regarding the methodology, since the lipid species under study were present together in the same macrosubstrate, the relative rates of hydrolysis could be determined without the need to vary the substrate concentration, which would not be the case had the species been presented to the enzyme in separate vesicles [18,31].

With PC-mix, a complex mixture of PC molecules varying in acyl chain length and unsaturation, a marked preference for the species containing an AA chain was observed both with micelles and bilayers (Fig. 1). This result is in accordance with the known preference of cPLA$_2\alpha$ for GPLs contain an AA residue. The very rapid hydrolysis of di-20:4-PC suggests that the active site of cPLA$_2\alpha$ can readily accommodate two AA chains simultaneously. In fact, the enzyme seems to accommodate even two docosahexanoyl (22:6) chains, albeit apparently not as well as two AA chains (Fig. 1A). The hydrolysis of the PC species containing 22:6 chain(s) is not consistent with a previous study suggesting that such lipids are not hydrolyzed by cPLA$_2\alpha$ [17].

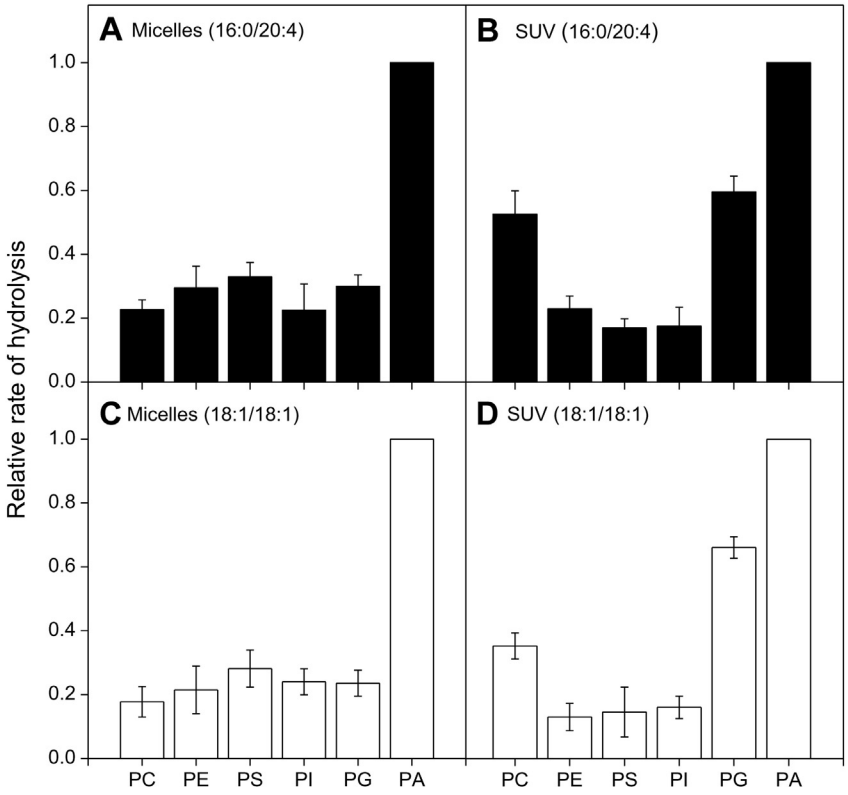

Fig. 4. Effect of the head group on GPL hydrolysis in micelles or SUV bilayers. Hydrolysis of 16:0/20:4-PE, -PS, -PI, -PG and -PA in micelles (A) or bilayers (B). Hydrolysis of 18:1/18:1-PE, -PS, -PI, -PG and -PA in micelles (C) or bilayers (D). Equimolar amounts of each GPL (250 nmol of total GPL) together with 21:0-SM (25 nmol) was incubated with 21 (A), 12 (B), 30 (C) or 18 μg (D) of cPLA$_2$α and the hydrolysis was monitored as specified in the legend of Fig. 1. The calculated specific activity was 8.0 nmol (A), 15.5 nmol (B), 4.7 nmol (C) or 8.5 nmol (D) of PA hydrolyzed per μg cPLA2/min, respectively. The differences between PA and the other GPLs are statistically significant (all panels) as it that between PC and PG vs. PE, PS and PI (panels B and D only).

The hydrolysis of 16:0/C$_n$-PCs and C$_n$/16:0-PCs followed a very similar pattern in micelles, i.e., the rate was nearly constant until the length of the *sn*1 or *sn*2 chain reached 17 carbons after which a fairly steep decline was observed (Fig. 2A). These data strongly suggest that the length of the hydrophobic cavity on cPLA$_2$α accommodating the *sn*1 and *sn*2 chains corresponds to that of a straight C17 chain. While this may seem unexpected considering the accommodation of a 20:4 (or 22:6) acyl chain, it should be noted that the *effective* length of a 20:4 (and 22:6) chain in the *sn*2 position of a diacylglyceride (and thus a GPL molecule) is less than that of a 18 carbon saturated chain in the *sn*1 position due to the particular conformation of those polyunsaturated chains [51].

Interestingly, the close similarity of the hydrolysis vs. chain length plots obtained for 16:0/C$_n$-PCs and C$_n$/16:0-PCs implies that binding of the acyl chains of saturated PCs to the active site of cPLA$_2$α is promiscuous, i.e., the *sn*1 and *sn*2 chains do not have specific binding sites in the enzyme. This conclusion is consistent with previous modeling studies suggesting that the two acyl chains of PC share a common hydrophobic cavity rather than two separate cavities in cPLA$_2$α [12]. Supporting this interpretation, in a previous study the activity of three secretory PLAs (sPLA$_2$s), which do not have any significant PLA$_1$ activity [52], was very differently influenced by the length of the *sn*1 or *sn*2 acyl chain [25]. In a notable contrast to the saturated PCs, cPLA$_2$α showed here a marked isomer preference for the AA-containing PCs in micelles, i.e., the species containing an AA residue in the *sn*2 position were hydrolyzed ~4-fold faster than their isomers (Fig. 2B). The faster hydrolysis of the PC isomers with an AA in the *sn*2 position is probably due to the fact that such molecules have a higher affinity for the enzyme because (at least in part) of the more favorable interactions of the double bonds of AA with the aromatic residues in the binding pocket of the enzyme [12]. The slower hydrolysis of the isomers containing AA in the *sn*1 position is probably due to that the (presumed) affinity of the AA's double bonds for those same aromatic amino acid residues in the binding cavity forces the isomeric PC molecules to adopt a distorted or twisted conformation which is energetically unfavorable and thus impedes their hydrolysis. Such promiscuous binding of the *sn*1 and *sn*2 acyl chains of PC probably explains why cPLA$_2$α has both PLA$_2$ and PLA$_1$ activities.

In bilayers a very similar, nearly logarithmic drop of hydrolysis vs. acyl chain length was observed for both 16:0/C$_n$-PCs and C$_n$/16:0-PCs (Fig. 3A). This result closely resembles the data obtained for four other PLAs previously [25,26] and suggests that substrate efflux is rate-limiting in the hydrolysis of these saturated species in bilayers, in contrast to micelles. Notably, an initial, precipitous drop in the hydrolysis vs. increasing chain length plots was observed for the AA-containing PCs as well (Fig. 3B), thus suggesting that substrate efflux also contributes significantly to the activity of cPLA$_2$α towards AA-containing PCs. However, the significantly faster hydrolysis of the isomers with an AA residue in the *sn*2 position suggests that also the fit of the substrate to the binding site on the enzyme contributes to its activity.

Intriguingly, hydrolysis of 16:0/C$_n$-PCs and C$_n$/16:0-PCs in micelles (but not in bilayers) required that some 16:0/20:4-PC was initially present. However, the hydrolysis of these saturated species continued even when all 16:0/20:4-PC had been hydrolyzed or when free AA was included in the micelles instead of AA-containing PC. These findings imply that AA acts as an activator of cPLA$_2$α in micelles. Whether AA (*i*) increases binding of cPLA$_2$α to the micelles by modulating their physical properties or (*ii*) if AA is an allosteric activator opening the lid covering the active site [11] or if (*iii*) AA has to bind to the enzyme's active site to initiate each catalytic cycle is not clear. Previously, incorporation of an AA-containing PC or PI to DMPM vesicles was found to enhance DMPM hydrolysis by cPLA$_2$α and it was suggested the AA-containing lipid acts as an allosteric effector of the enzyme or favorably modulates the physical properties of the bilayer [13]. It has also been

suggested that the bilayer surface to which cPLA$_2\alpha$ binds, acts as an allosteric activator of the protein [12].

Regarding the head group specificity of cPLA$_2\alpha$, we found that PA was a much better substrate than the other GPLs in micelles (Fig. 4), i.e., when the accommodation at the active site of the enzyme is more critical than the efflux. This result suggests that the region of cPLA$_2\alpha$ where the substrate's head group binds is spatially restrictive or, alternatively, the binding of PA is promoted by its ability (not shared by the other GPLs studied) to possess two negative charges in its head group. In vesicle bilayers PC and PG were hydrolyzed almost as well as PA. Since PC and PG efflux faster from bilayers than PA [53], these data support the notion that with bilayers, substrate efflux contributes significantly to hydrolysis.

In conclusion, the present study provides novel information on the factors determining the substrate specificity of cPLA$_2\alpha$. First, our working model suggests that while the rate-limiting step in the hydrolysis of saturated PCs is their efflux from a bilayer, the rate of hydrolysis of the AA-containing PCs is determined both by the substrate efflux and its high affinity for the active site of cPLA$_2\alpha$. However, further experiments are needed to test this interpretation. Second, promiscuous binding of the *sn*1 and *sn*2 acyl chains of the substrate to the active site probably explains why cPLA$_2\alpha$ has both PLA$_1$ and PLA$_2$ activities. Third, with micelles (but not bilayers) AA was necessary for the hydrolysis of saturated PCs, possibly because it displaces the lid domain that otherwise blocks the access of the substrate to the active site.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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