

Substrate Efflux Propensity is the Key Determinant of iPLA β -Mediated
Glycerophospholipid Hydrolysis*

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*Running title: *Substrate efflux regulates iPLA β*

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Background: Ca²⁺-independent phospholipase A-beta (iPLA β) has been repeatedly implicated as a homeostatic enzyme.

Results: Activity of iPLA β correlates inversely with the hydrophobicity of the phospholipid substrate.

Conclusion: Efflux of the phospholipid substrate from a membrane mainly determines the activity of iPLA β .

Significance: Results support the role of iPLA β as a homeostatic enzyme which responds to increased phospholipid efflux proposed to occur at particular "critical" compositions.

ABSTRACT

A-type phospholipases (PLAs) are key players in glycerophospholipid (GPL) homeostasis and in mammalian cells, Ca²⁺-independent PLA-beta (iPLA β) in particular has been implicated in this essential process. However, the regulation of this enzyme, which is necessary to avoid futile competition between synthesis and degradation, is not understood. Recently, we provided evidence that the efflux of the substrate molecules from the bilayer is the rate-limiting step in the hydrolysis of GPLs by some secretory (nonhomeostatic) PLAs. To study if this is the case with iPLA β as well, a mass-spectrometric assay was employed to determine the rate of hydrolysis of multiple saturated and unsaturated GPL species in parallel using micelles or vesicle bilayers as the macrosubstrate. With micelles, the hydrolysis decreased with increasing acyl chain length independent of unsaturation and modest discrimination between acyl positional

isomers was observed, presumably due to the differences in the structure of the *sn*1 and *sn*2 acyl binding sites of the protein. In striking contrast, no significant discrimination between positional isomers was observed with bilayers, and the rate of hydrolysis decreased with the acyl chain length logarithmically and far more than with micelles. These data provide compelling evidence that efflux of the substrate molecule from the bilayer, which also decreases monotonously with acyl chain length, is the rate-determining step in iPLA β -mediated hydrolysis of GPLs in membranes. This finding is intriguing as it may help to understand how homeostatic PLAs are regulated and how degradation and biosynthesis are coordinated.

Glycerophospholipid (GPL³) homeostasis is crucial for mammalian cells as indicated by the fact that they maintain the GPL compositions of their membranes within close limits (1) and major compositional deviations are lethal in mice (2). The key processes maintaining GPL homeostasis are biosynthesis, acyl chain remodeling and degradation (3). Regarding degradation, several studies have provided evidence that A-type phospholipases (PLAs) are key players in this process (3-9). Particularly compelling evidence for this comes from studies in which the synthesis of a GPL class was boosted by over-expression of the rate-limiting synthetic enzyme. For instance, over-expression of CTP:phosphocholine cytidylyl-transferase (CT) in HeLa cells increased PC synthesis by 4-5 fold, but the PC content of the cells did not

increase significantly. Instead, a marked increase of glycerophosphocholine, a deacylation product of PC was detected in the cells and also the medium (4, 10). Forcing the synthesis of PE or PS also did not significantly increase the cellular content of these GPLs, but rather increased their degradation mediated by PLAs (11, 12). Conversely, when the synthesis of a GPL class was inhibited, its degradation decreases in proportion, thus maintaining GPL homeostasis. For example, when the synthesis of PE was inhibited by mutating the rate-limiting enzyme ethanolamine phosphotransferase (EPT), the PE content of the cells did not decrease due to reduced degradation (13). In CHO mutants with a partially inactive choline kinase- α , the rate of PC synthesis was reduced 4-fold, but the PC content was normal (14). Collectively, those data demonstrate that in mammalian cells GPL synthesis and degradation are strictly coordinated, and provide strong evidence that the cells contain homeostatic PLA enzyme(s) that selectively degrade the GPL species which are present in excess.

Mammalian cells express numerous PLA proteins (15, 16), but those involved in GPL homeostasis have not been firmly established. However, select members of the Ca²⁺-independent PLA (iPLA) subfamily, particularly iPLA β and iPLA δ , have been implicated in this process (17-20). For instance, it was shown that the activity of iPLA β increased 2-fold in CHO cells when PC synthesis was boosted by over-expressing CT (5), and that treatment of cells with bromoenol lactone (BEL), an inhibitor of iPLA β suppressed PC degradation (4). In addition, we have recently found that knock-down of iPLA β in HeLa cells markedly inhibits the turnover of the major GPL classes as studied by heavy isotope-labeled precursors⁴. Notably, for efficient homeostasis, the catalytic activity of iPLA β should be regulated, since an alteration in its expression is obviously too slow to avoid energy-wasting compositional fluctuations (21). In essence this means that iPLA β must be able to sense the GPL composition of a membrane in order to “know” if a particular GPL is present in excess or not. Although the activity of iPLA β might be regulated indirectly, i.e., by other homeostatic proteins (22), there is no evidence that such a regulation plays a role in GPL homeostasis. Thus, the simplest and the most attractive model is that the membrane GPL composition directly modulates the activity of this enzyme, like it regulates the activity of

biosynthetic enzymes such as CT (23, 24) and PS synthase (25, 26). Consistent with this model, we have recently provided evidence that the activity of some secretory, *nonhomeostatic* PLAs correlates with the upward movement of the GPL substrate from the membrane bilayer (efflux propensity) (27). This finding is intriguing, since efflux propensity has been predicted to change abruptly at particular membrane GPL compositions (21).

To study if substrate efflux propensity regulates the activity of the homeostatic iPLA β as well, we employed a high-throughput mass spectrometric based assay which allowed us to study the hydrolysis of numerous GPL species simultaneously. The key result is that, when using vesicle bilayers as the macrosubstrate, the rate of iPLA β -mediated hydrolysis decreased strongly and monotonously with increasing substrate hydrophobicity, thus strongly suggesting that the activity of iPLA β is by and large determined by the ease of efflux of its substrate from the bilayer. Since efflux of a GPL is predicted to increase strongly when it is present in excess (i.e. over its optimal concentration), these data support strongly the role of iPLA β in GPL homeostasis of mammalian cells.

EXPERIMENTAL PROCEDURES

Lipids and other chemicals - L- α -1-palmitoyl-2-arachidonyl-[1-¹⁴C]-PC (58 μ Ci/ μ mol) was purchased from Perkin-Elmer Life Sciences (Boston, MA), while unlabeled GPLs, lyso-PCs and sphingomyelins (SM) were obtained from Avanti Polar Lipids (Alabaster, AL) and D₃-methyl iodide from Cambridge Isotope Laboratories (Andover, MA). Ethanolamine, phospholipase D (*Streptomyces species*), NP-40 (IGEPAL) and EDTA-free protease inhibitor cocktail were obtained from Sigma (St. Louis, MO). Silica gel 60 thin-layer plates and HPLC-grade solvents were from Merck Millipore (Munich, Germany), Ni-NTA agarose from Qiagen (Germantown, MD), Strep-Tactin superflow resin and Desthiobiotin from IBA-Life Sciences (Goettingen, Germany), BEL and MAFP inhibitors from Cayman Chemicals (Ann Arbor, Michigan), bovine serum albumin standard and Coomassie brilliant blue R-250 solution from Bio-Rad (Sunnyberg, Sweden). Penicillin streptomycin, fetal bovine serum (FBS) and Fungizone were from Gibco Life

Technologies (Bleiswijk, Netherlands). The *sn1-16:0/sn2-C_n-PC* and *sn1-C_n/sn2-16:0-PC* species were synthesized by acylating *sn1-16:0-LPC* with the anhydride of a C_n-fatty acid or *sn1-C_n-LPC* with the anhydride of 16:0-fatty acid, respectively. D₉-labeled PC species were synthesized from their corresponding phosphatidylethanolamines (PE) by methylation with D₃-methyl iodide as described earlier (28), except that benzene was replaced with toluene. The purity of GPLs was confirmed by TLC and mass spectrometry and their concentrations were determined by phosphate assay (29).

Cloning, expression and purification of human iPLA β - Full length iPLA β cDNA from the clone DKFZp434A102Q (ImaGenes, GenBank AL080187.1) was amplified by PCR using the forward primer 5'-TTTGGTACCATGCAGT-TCTTTGGACGC-3' and the reverse primer 5'-TTTACCTGGGGGTGAGAGCAGCAGCT-3'. The product was digested with KpnI and AgeI, ligated into the pMT expression vector (Invitrogen Life Technologies) that contains the *Drosophila* metallothionein (MT) -inducible promoter and a C-terminal His₆-tag. Next, the sequence coding for a C-terminal His₁₀-tag was appended to the iPLA β sequence by PCR using pMT-iPLA β -His₆ as template. The forward primer was 5'-GTCGGTCCG-CCACC-ATGCAGTTCTTTGGCCGC-3' containing the RsrII cleavage site while the reverse was 5'-CTGGTACCTTAGTGGTGGTGGTGATGGTGATGATGATG-ATGTTCGAAACGACCTTC-GATGGGTGAGAGCAGCAGCT-3' containing the BstBI and KpnI cleavage sites. The RsrII- and KpnI-digested PCR product was ligated into pFastBac1-vector (pFB1, Gibco Life Technologies) resulting in pFB1-iPLA β -His₁₀. Replacing the BstBI-KpnI fragment of pFB1-iPLA β -His₁₀ by the annealed oligonucleotides 5'-CGAATGGAGCCACCCGCAGTTCGAGA-AAGGAGGAGGAAGCGGAGGAGGAAGCGGAGGAGGAAGCTGGAGCCACCCGCAGTTTGAAAAATAGGTAC-3' and 5'-CTATTTTT-AACTGCGGGTGGCTCCAGCTTCCTCCTC CGCTTCCTCCTCCGCTTCCTCCTTCTC GAACTGCGGGTGGCTCCATT-3' resulted in the pFB1-iPLA β -Strep vector. The inserted sequences and the reading frames of the constructs were verified by sequencing. The Bac-to-Bac baculoviral expression system (Gibco Life Technologies) was then utilized for bacmid preparation and CellFECTIN-mediated

transfection of Sf9 cells to produce the recombinant baculovirus.

To express the iPLA β -StrepIII protein, Sf9 cell suspension (500 ml, $\sim 10^6$ cells/ml) was infected with the recombinant baculovirus at the multiplicity of 1. After incubation for 72 h at 26 °C, 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 mM ATP, 0.5 mM EDTA, 0.5% NP-40, 5% glycerol) containing an EDTA-free protease inhibitor cocktail. The cells were then lysed by sonication (6 \times 1 min with 30 sec intervals, 40% duty cycle) and centrifuged at 50,000 \times g for 1 h to pellet the membranes. The supernatant was collected and poured into a Poly-Prep column (Bio-Rad) containing buffer W (20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 5% glycerol) equilibrated Strep-Tactin superflow resin. The column was then washed with 5 column volumes of buffer W and iPLA β -StrepIII was eluted with 5 \times 1 mL buffer E (= buffer W+2.5 mM Desthiobiotin).

To prepare tag-free iPLA β , the Strep-Tactin column fractions with high PLA activity were pooled and incubated with the Factor Xa protease (Thermo Scientific) at a Factor Xa to protein ratio of 1:100 for 2 hours at 20 °C. After inhibiting any further proteolysis with 1 mM PMSF, the sample was applied to a DG-10 desalting column (Bio-Rad) to exchange the incubation buffer for buffer A (20 mM Tris-HCl, pH 8.0). The sample was then loaded onto a buffer A-equilibrated HiTrap Q HP (GE Healthcare) ion-exchange column (1 mL bed volume) coupled to an AKTA-FPLC system (GE Healthcare Life Sciences, UK). The elution was performed with a gradient of 0 - 1 M NaCl in buffer A at a rate of 1 mL/min with detection at 280 nm. Fractions were pooled based on the elution profile, concentrated on an ultrafiltration device (Amicon Ultra-30k, Millipore) and assayed for PLA activity. The fractions with the highest activity were pooled and stored at -80 °C in the presence of 60% (v/v) glycerol.

Immunoblotting - To assess their iPLA β content, the transfected 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 for 3 min at 800 \times g at 4 °C and 20 μ g of supernatant protein was run on a 10% SDS-polyacrylamide gel. The proteins were then transferred onto a PVDF

membrane (Millipore), treated with 5% defatted dry milk in 50 mM Tris-buffered NaCl (150 mM) containing 0.1% Tween-20. The recombinant iPLA β was detected with an anti-iPLA β (Santa Cruz Biotechnology, 1:100 dilution) or a Streptactin HRP-conjugate antibody (Bio-Rad, 1:10000 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.

Extraction of HeLa microsomal GPLs - HeLa cells were grown on eight 14 cm dishes until ~80% confluent (30). After rinsing with ice-cold PBS, cells were scraped off and centrifuged at 1500 \times g for 3 min. After resuspending in 1 mL of a lysis buffer (25 mM Hepes-NaOH, pH 7.4, 500 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂) followed by incubation on ice for 30 min, the cells were broken with a Dounce homogenizer followed by 10 passes through a 27G needle. The intact cells and nuclei were removed by centrifugation at 1200 \times g for 5 min and the supernatant was centrifuged at 50,000 \times g for 1.5 h at 4 °C to pellet the microsomes. After suspending the microsomes in 1 mL of a buffer constituting 100 mM Hepes-NaOH, pH 7.4, 0.5 M sucrose, any aggregates present were removed by centrifugation at 800 \times g for 10 min. The supernatant containing the microsomes was transferred to a Kimax tube, lipids were extracted (31) and after determination of the GPL content (29) the extract was stored at -20 °C.

Assay of PLA activity - PLA activity of the recombinant iPLA β was determined using a radioactivity assay. The macrosubstrate was prepared by mixing 10,000 cpm of 1-palmitoyl-2-arachidonoyl-1-¹⁴C-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 N₂ stream and then under high vacuum for 1 h followed by addition of 0.5 mL assay buffer (50 mM sodium phosphate, pH 7.4, 10 mM ATP, 0.5% Triton X-100) and vortexing for 2 min at RT. To start the reaction, an aliquot of purified iPLA β was added and the mixture was incubated for 30 min at 37 °C. Aliquots of 50 μ L were removed at intervals and mixed with 2 mL of ice-cold methanol to stop the reaction and the lipids were extracted according to ref. 31, except that 0.1 M HCl was used to maximize lipid recovery. The extract was analyzed on TLC

using hexane/ethyl ether/acetic acid (70:30:1, v/v) as the eluent and radiolabeled PC, LPC and fatty acid were detected with Starion FLA-9000 image scanner (Fujifilm), visualized using Image Reader FLA-9000 software and quantified with the Multigauge analysis software.

Substrate specificity of iPLA β - A mixture of synthetic GPLs or total microsomal lipids, 16:0/18:1-PA and 21:00-SM as an internal standard were mixed in chloroform, dried under a N₂ stream and further under high vacuum for 1 h. Butylated hydroxytoluene (1 mol%) was included as an antioxidant when the mixture contained unsaturated GPLs. To prepare small unilamellar vesicles (SUVs), the dried lipids were dissolved in 20 μ L ethanol and injected into 0.5 mL of the assay buffer (50 mM sodium phosphate, pH 7.4, 10 mM ATP) using a constant-speed Hamilton syringe. To prepare large unilamellar vesicles (LUVs), lipids dried in a glass tube were overlaid with 1 mL of the assay buffer and placed in a water bath at 4 °C for 1 min and then in a water bath at 50 °C for 1 min. This cooling-heating cycle was repeated for four times. The lipid suspension was vortexed for 1 min at RT, then extruded 10 times through two stacked 1.2 μ m polycarbonate membranes and then 10 times through two 0.4 μ m membranes using the Lipex extruder (Vancouver, Canada) pressurized with Argon. The SUV or LUV bilayers were incubated in the presence of purified iPLA β at 37 °C, 50 μ L 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 three times with the theoretical upper phase (31) and taken to dryness under a N₂ stream. The residue was reconstituted in 200 μ L of chloroform/methanol (1:2, v/v) and stored at -20 °C. To study the hydrolysis in micelles, a lipid mixture was dried as above, dispersed in the assay buffer with 1% NP-40 detergent and the assay was conducted as above.

Effect of the head group of GPL on its spontaneous intervesicle translocation - Spontaneous intermembrane translocation of 18:1/18:1 GPLs with varying head groups was measured as previously described (32). Briefly, negatively charged donor vesicles consisting of an equimolar mixture of 18:1/18:1-PC, -PE, -PS, -PG, -PA, -PI (1335 nmol GPL), D₉-labeled 22:1/22:1-PC donor marker (15 nmol) + tetra-

18:1 cardiolipin (150 nmol) and uncharged acceptor vesicles consisting of 16:0/18:1-PC were both prepared in a buffer constituting 10 mM HEPES-NaOH, pH 7.0, 25 mM KCl, 0.5 mM EDTA by probe sonication on an ice-water bath. The donor and acceptor vesicles (1.5 and 7.5 μ mol of total GPL, respectively) were incubated at 37 °C; aliquots were withdrawn at intervals and applied to a DEAE-Sephacel (Amersham Biosciences) minicolumn to trap the negatively charged donor vesicles. To quantify the 18:1/18:1 GPLs transferred to the acceptor vesicles (in the flow-through) a standard for each GPL class was added before lipid extraction and mass spectrometric analysis.

Mass spectrometric analysis of GPLs - After addition of NH₄OH (4% final concentration) the lipid sample was infused at 6 μ L/min to a Micromass Quattro Micro triple-quadrupole mass spectrometer operated as previously (33). GPL species were detected using head group-specific precursor or neutral-loss scanning (34). The spectra were exported to Microsoft Excel and the individual GPL species were quantified using LIMS software (35). 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 hydrolyzable fraction at t_{∞} was constrained to 1.0 with micelles, to 0.67 with SUVs and 0.5 with LUVs (27).

LC-MS with selective reaction monitoring (SRM) was used when the hydrolysis of microsomal GPLs was investigated. Waters ACQUITY Ultra Performance LC system equipped with a Waters ACQUITY BEH C₁₈ column (1.0 \times 100 mm) was used to separate the molecular species using gradient elution. Solvent A was acetonitrile/H₂O (60:40) with 10 mM ammonium formate and 1% NH₄OH, while solvent B was isopropanol/acetonitrile (90:10) containing 10 mM ammonium formate and 1% NH₄OH. 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 directed to the ESI source of Waters Quattro Premier triple-quadrupole mass spectrometer operated in the positive ion mode. For SRM transitions, proton adducts of the PC, PE, PS and PI species were selected as

the precursors, while the product ion was either the head group (PC, PI) or the diacylglycerol fragment (PE, PS). For quantification of the microsomal lipids 20:1/20:1-PC, 22:1/22:1-PE, 20:1/20:1-PS and 16:0/16:0-PI standards were added during the single-phase stage of extraction. The SRM chromatograms were integrated and the relative concentrations of the individual GPL species were calculated using the QuanLynx software (Waters).

Other procedures - Protein concentration was determined using Lowry (36) or a fluorometric method (37) with BSA as the standard. SDS-PAGE was carried out according to Laemmli *et al.* (38) and the proteins were stained with Coomassie blue (39).

RESULTS

Expression and Purification of Human iPLA β - We initially expressed iPLA β with a C-terminal His₆-tag in *Drosophila* S2 cells and then subjected it to purification on a Ni²⁺-affinity column. Although the preparation was not fully homogenous, iPLA β -His₆ was the main band when visualized on an SDS-PAGE gel and most likely responsible for all the PLA activity present in the preparation. However, to exclude that any of the contaminating protein impurities might bias the specificity data, we constructed iPLA β with a C-terminal His₁₀- or StrepIII-tag (see *Experimental Procedures*). While the His₁₀-tag did not help to purify the enzyme beyond that achieved with the His₆ version, iPLA β -StrepIII was almost pure after affinity chromatography on a Strep-Tactin column. To exclude the possibility that even the minor impurities remaining could bias the hydrolysis data, the StrepIII-tag was cleaved off with the Factor-Xa protease and the tag-free iPLA β was purified on a cation-exchange column. As shown in Fig. 1, an essentially homogenous protein was obtained. The specificity data shown in this manuscript was obtained with this homogenous enzyme, but identical results were obtained with iPLA β -His₆ as well (data not shown). Accordingly, neither the tag nor the degree of purity of iPLA β had a detectable effect on the results.

High-throughput assay of iPLA β substrate specificity - In order to obtain information on how the structure of the acyl chain and the head group structure influences GPL hydrolysis by iPLA β , we employed a high-throughput mass

spectrometric based assay devised previously (30). This assay allows one to determine the rates of hydrolysis of a multitude of GPL species simultaneously. Four different mixtures of synthetic GPL species as well as the GPLs extracted from HeLa cell microsomes were studied here. The first mixture (“PC-mix”) consisted of a total of 25 species i.e., (i) 10 disaturated species with the length of both acyl chains varying from 13 to 22 carbons; (ii) two monounsaturated species (14:0/18:1 and 16:0/18:1); (iii) 6 species with two double bonds, including 16:0/18:2 and 5 dimonounsaturated species with acyl chain lengths varying between 14 to 22 carbons (14:1/14:1, 16:1/16:1, 18:1/18:1, 20:1/20:1 and 22:1/22:1); (iv) two species with four double bonds (16:0/20:4 and 18:0/20:4); (v) three species with 6 double bonds (18:3/18:3, 16:0/22:6 and 18:0/22:6); (vi) one with eight double bonds (20:4/20:4); and (vii) one with 12 double bonds (22:6/22:6). The second mixture (16:0/C_n-PCs) consisted of 17 saturated PC species with a 16:0 *sn1* chain and a *sn2* chain of 6-24 carbons, and the third mixture (D₉-C_n/16:0-PCs) consisted of the positional isomers of the second one. The fourth mixture consisted of six 18:1/18:1 GPLs each with a different polar head group (i.e., 18:1/18:1-PC, -PE, -PS, -PG, -PI and -PA). 21:0-SM (10 mol %) was included as a non-hydrolyzable internal standard in all the mixtures. The lipid mixtures were presented to the enzyme either in the form of SUV or LUV bilayers or in detergent micelles.

Effects of the acyl chain length and unsaturation on PC hydrolysis in micelles - We first studied the hydrolysis of the 25 PC species (“PC-mix”) incorporated in detergent micelles. In the case of fully saturated species the rate of hydrolysis decreased with increasing chain length with some minor deviations from a monotonic behavior (Fig. 2). An analogous trend was observed for the dimonounsaturated species. An inhibitory effect of increasing chain length was observed for the other PC sets as well. Also the number of double bonds had a strong effect on the rate of hydrolysis. This is particularly notable for the species with a total of 36, 38 or 40 acyl chain carbons. For example, 36:6 (18:3/18:3) species was hydrolyzed ~12.3-fold faster than the 36:0 (18:0/18:0) species and the 40:8 (20:4/20:4) species ~14.7-fold faster than the 40:0 (20:0/20:0) species. We propose that the major positive effect of unsaturation in these

cases is, at least in part, due to increased affinity of the substrate for the active site of the enzyme (cf. 30).

*Effect of *sn1* vs. *sn2* chain length of PC on hydrolysis in micelles* - In order to assess the contribution of the individual acyl chains on the rate of hydrolysis of iPLA β , we employed two sets of saturated PC species in which the length of either the *sn1* (C_n/16:0-PCs) or the *sn2* (16:0/C_n-PCs) acyl chain varied from 6 to 24 carbons, while the length of the acyl chain at the other *sn* position was constant at 16 carbons. Since the C_n/16:0-PC species contained a D₉-labeled choline head group while the 16:0/C_n-PC species were unlabeled, both sets could be present together thus eliminating the possibility of any matrix bias. As shown in Fig. 3, variation of the *sn1* versus *sn2* chain length had distinct effects on the rate of hydrolysis. When the length of the *sn1* chain increased, the rate of hydrolysis was first essentially independent of the length until C12 and then decreased in an approximately logarithmic manner. The ratio of the fastest to the slowest rate was ~14.5. In contrast, when the length of the *sn2* chain increased, the rate first increased from C7 till C8 and then declined in a rather monotonous manner. The fastest species was hydrolyzed ~23.7 times more rapidly than the slowest one; a difference that is significantly higher than in the case of C_n/16:0-PC set. Most probably, the observed differences between the two sets of positional isomers are due to the different properties of the *sn1* vs. *sn2* acyl binding sites of iPLA β .

Effects of the acyl chain length and unsaturation on PC hydrolysis in SUV bilayers - We next studied hydrolysis of the “PC-mix” using vesicle bilayers as the macrosubstrate. Independent of acyl chain unsaturation, the rate of hydrolysis decreased monotonously with increasing chain length (Fig. 4). Notably, however, the effect of chain length was far more pronounced than with micelles. For instance, the most rapidly hydrolyzed saturated species (13:0/13:0) was hydrolyzed ~50-fold faster than the slowest one (22:0/22:0). In case of the diunsaturated species, the fastest species (14:1/14:1) was hydrolyzed ~20 fold faster than the slowest one (22:1/22:1). The corresponding differences in micelles were ~7.2 and ~10.6, respectively. Furthermore, the rate increased with increasing number of double bonds for the species with an equal number of total acyl chain

carbons. These data are consistent with the proposition that substrate efflux (hydrophobicity) is the key determinant in the hydrolysis of membrane-bound PC species by iPLA β .

Effect of sn1 vs. sn2 chain length of PC on hydrolysis in SUV bilayers - To study how the variation of the length of the *sn1* or *sn2* chains independently affect the rate of hydrolysis in bilayers, D₉-16:0/C_n-PCs and C_n/16:0-PCs were incorporated together in SUVs which were then incubated with iPLA β . As shown in Fig. 5, increase in the length of either the *sn1* or the *sn2* chain reduced the rate of hydrolysis dramatically, i.e. the difference between the fastest and the slowest species was ~125-fold in both isomer sets. In contrast with micelles, no detectable differences in the rate of hydrolysis were observed for the positional isomers. Such monotonous and acyl *sn*-position independent decrease of hydrolysis further supports the notion that efflux of the GPL substrate from the bilayer is the rate-limiting step in hydrolysis of membrane-bound substrates by iPLA β .

Effect of GPL polar head group structure on hydrolysis - We next investigated the effect of the GPL polar head on the rate of hydrolysis by iPLA β in micelles as well as SUV and LUV bilayers. Hydrolysis of 6 different 18:1/18:1 GPLs presented together to the enzyme was compared. With micelles, the best substrate was PE that was hydrolyzed ~3 fold faster than PG and PA. The other GPLs were hydrolyzed at intermediate rates (Fig. 6A). In striking contrast, the GPL species were hydrolyzed similarly in SUV bilayers (Fig. 6B) although PG was hydrolyzed slightly faster and PI slightly slower than the rest. Very similar results were obtained with LUVs (Fig. 6C). These results are consistent with the notion that with bilayers substrate efflux is rate-limiting, while with micelles differences in steric and/or other interactions of the head group with the enzyme catalytic site play a more important role.

Effect of the head group on the intervesicle transfer rate (efflux) - Albeit it seemed likely that the efflux of GPLs from a bilayer is rather independent of the head group structure, we determined this experimentally by measuring the relative rates of spontaneous intervesicle transfer of different head group variants. As shown in Fig. 7, all six GPLs translocated at rather similar rates from the donor to acceptor vesicles. Since the intervesicle

transfer rate can be equated with the rate of efflux (32), these data indicate the head group has only a modest effect on the rate of efflux from a bilayer. This result is again consistent with the idea that the rate of hydrolysis of a bilayer-bound GPL by iPLA β (Fig. 6) mainly depends on its efflux propensity.

Hydrolysis of microsomal GPL species in SUV bilayers - To study the specificity of iPLA β with a more natural macrosubstrate, we extracted lipids from HeLa cell microsomes and reconstituted them in SUV bilayers. Hydrolysis of 14 PC, 14 PE, 11 PS and 9 PI molecular species could be reliably analyzed using LC-MS with SRM detection (see *Experimental Procedures*). As shown in Fig. 8A, increase in total acyl chain length of PC systematically inhibited the hydrolysis independent of the number of double bonds. On the other hand, increasing the number of double bonds increased the rate of hydrolysis when the total acyl chain length was constant. Parallel results were observed for the PE, PS and PI species (Fig. 8B-D), i.e., increasing acyl chain length systematically inhibited hydrolysis, while increasing unsaturation increased it when the chain length was constant. In addition, the head group structure (among PC, PE, PS and PI) had an insignificant effect on the rate of hydrolysis when the total acyl chain length and unsaturation was constant (data not shown). In conclusion, the data for multiple natural GPL species strongly support the proposition that iPLA β -mediated hydrolysis of GPLs closely correlates with their efflux propensities.

DISCUSSION

In case of soluble PLAs like iPLA β , several steps have been proposed necessary for the hydrolysis of a membrane-bound GPL molecule: 1) binding of the enzyme to the membrane, 2) efflux (at least partially) of the substrate molecule from the bilayer, 3) accommodation of the substrate in the catalytic site of the enzyme and 4) cleavage of the acyl-glycerol ester bond (40-42). However, when studying the substrate specificity of a PLA using the present approach, only steps 2 and 3 need to be considered since all GPL species are present simultaneously and are structural homologues, i.e. have an identical chemical structure at the site of cleavage. The requirement for substrate efflux derives from the (unproven) assumption

that soluble PLAs may not penetrate the membrane significantly and thus the catalytic site remains above the membrane surface during their action (40, 43). As indicated by recent hydrogen/deuterium exchange and homology modeling studies (44, 45), this may apply for iPLA β as well. It was proposed that the substrate has to be extracted (i.e., efflux) from the membrane to be hydrolyzed by iPLA β , but this could not be proven due to limitations of the methodology used (46).

Our previous study on 3 *nonhomeostatic* secretory PLAs, i.e., those from bee or snake venom and pig pancreas, strongly suggested that efflux of the substrate is the rate-determining step of hydrolysis of membrane bound GPLs (27). This conclusion was based on that (i) the hydrolysis of homologous PC species in vesicles decreased strongly and monotonously with increasing length of the acyl chains, (ii) identical effect was observed when the length of the *sn1* vs. *sn2* chain was varied, i.e., there was no discrimination between the *sn* positional isomers, and (iii) the rate of hydrolysis correlated closely with the rate of spontaneous intervesicle diffusion which depends on the rate of efflux of the PC species. Importantly, very different results were obtained with detergent micelles i.e., the rate of hydrolysis decreased monotonously with the length of the *sn1* chain, but not with that of the *sn2* chain. For the latter, an optimum length was observed and the effects of the chain length and the number of double bonds varied markedly between the 3 enzymes, most probably reflecting differences in the structure of the catalytic site. Collectively, those data indicated that with micelles, specific interactions of the substrate with the substrate binding site on the enzyme significantly contribute to the rate of hydrolysis, while with bilayers efflux is the main rate-limiting factor. This difference between bilayers and micelles is most probably derived from different packing and intermolecular interactions in these aggregates. In micelles the intermolecular packing is far more loose than in bilayers as shown e.g. by NMR studies (47) and MD simulations (48). Such loose intermolecular packing makes the rate of GPL efflux from micelles several orders of magnitude faster as compared to vesicle bilayers (49) and thus probably not rate-limiting in PLA-mediated hydrolysis. In contrast, tight intermolecular packing of the GPL molecules in bilayers

strongly impede their efflux, thus making it rate-limiting.

Here, we show that iPLA β behaves similarly (but not identically) to the previously studied secretory PLAs which are structurally unrelated and *nonhomeostatic* (15, 16). Most importantly, the rate of hydrolysis of bilayer-bound PC species by iPLA β decreased monotonously with the length of the acyl chains independent of the number of double bonds, and practically no hydrolysis of saturated species took place when the total number of acyl chain carbons exceeded 38 (Fig. 4). On the other hand, increasing the number of acyl chain double bonds increased the rate of hydrolysis markedly. Since substrate efflux rate *decreases* progressively with increasing acyl chain length and *increases* with the number of double bonds (see above, 50), these data strongly support the notion that efflux of the substrate from the bilayer is the rate-determining step in the hydrolysis of GPLs by iPLA β . Consistently, increasing the length of the *sn1* or *sn2* chain of PC in bilayers inhibited the hydrolysis identically (Fig. 5), unlike in micelles where statistically significant differences were observed (Fig. 3). Also, the finding that the head group had a significant effect in micelles, but little if any in vesicles (Fig. 6) is consistent with the efflux being the rate-limiting step with bilayers, since efflux propensity is largely determined by the acyl chain length and unsaturation (51, 52).

Our previous study on 3 secretory PLAs showed significant differences in the effect of the *sn1* vs. the *sn2* chain length on the rate of hydrolysis with micelles as the macrosubstrate, i.e. the rate of hydrolysis decreased monotonously with the length of the *sn1* chain, while for *sn2* chain an optimum at C8 - C10 was observed for each of the 3 enzymes. Such data suggest that there is a binding site for the *sn2* chain, but not for the *sn1* chain in those enzymes, and that this site interacts only with the first 8 - 10 carbons of the *sn2* chain (27). This conclusion is consistent with the fact that these PLAs hydrolyze the *sn2* ester bond but not the *sn1* as shown in studies with substrate analogues (53, 54). For iPLA β the differences between the *sn1* and *sn2* chain were much smaller, albeit statistically significant (Fig. 3). This result is consistent with the fact that, in contrast to venom PLAs, iPLA β is not specific for the *sn2* or *sn1* ester bond but hydrolyzes both (55, 56). For both

the *sn1* and *sn2* chain the rate started to drop strongly when the chain length exceeded 11 - 13 carbons, which indicates that only the corresponding carboxyterminal carbons in the acyl chains interact with the substrate binding site of iPLA β .

Previously, few studies have investigated the substrate specificity of iPLA β using a limited set of GPL species (41, 57, 58). Although some differences between GPL species were observed, the data are difficult to interpret because (i) mixed GPL/detergent micelles were employed in those studies and (ii) the apparent specificity profile of iPLA β can vary dramatically depending on the detergent/GPL ratio used (59). In addition, the charge of the micelle surface can vary depending on the GPL present, which could bias the data since binding of PLAs to a macrosubstrate is sensitive to the surface charge (60, 61). The present results and previous studies (27, 62-65) clearly show that the properties of the macrosubstrate can have a remarkable effect on the apparent specificity of PLAs.

Could efflux propensity regulate GPL homeostasis? Several studies have indicated that lipids in multicomponent bilayers tend to adopt regular rather than random lateral distributions. This putative behavior, which is formulated in the sc. superlattice/regular distribution model (21, 66), is relevant to GPL homeostasis for two reasons. First, it predicts the existence of a number of "critical" GPL bilayer compositions which are energetically more favorable than the intervening compositions and, therefore, there is an intrinsic tendency for the composition to settle in one of the critical ones. Second, if the composition deviates from a critical one (e.g., due to synthesis of a certain GPL "in excess"), packing defects will appear as the molecules present "in excess" cannot be equally accommodated in the bilayer lattice (i.e., the optimal stoichiometry is perturbed). Those molecules in excess are predicted to have an increased chemical activity and therefore

increased efflux propensity (fugacity), which would make them susceptible for hydrolysis by homeostatic PLAs, as shown here for iPLA β . When molecules present "in excess" have been degraded, hydrolysis is predicted to slow down markedly. This hypothetical model is reminiscent of that proposed to regulate the concentration of cholesterol in the plasma membrane of eukaryotic cells via the formation of putative stoichiometric cholesterol/sphingomyelin complexes (67). However, in the present case "critical compositions" with particular stoichiometries, rather than complexes, are assumed.

Such a "critical composition" -based regulation would be highly accurate and would thus minimize compositional fluctuations (hysteresis) which are energy-wasting due to futile competition between synthesis and degradation (3). Supporting this scenario, it is well established that many PLAs are strongly activated by bilayer packing defects (68-70), and a (venom) PLA seems to respond to predicted critical compositions (71). Importantly, such critical compositions could also regulate GPL biosynthesis (and thus coordinate it with degradation), since the GPL molecules present in excess should have increased chemical activity and could thus potently inhibit the synthetic enzymes. Notably, there is good evidence for such product inhibition in case of e.g. PS synthases 1 and 2 by PS (25, 26) and PI synthase (72).

In conclusion, the present study provides the first experimental evidence that the efflux of substrate from a bilayer is the rate-limiting factor in the hydrolysis of GPL molecules by iPLA β , a putative homeostatic PLA. This finding is intriguing since it suggests, particularly when related to predictions of the superlattice model of lipid lateral distribution, an intriguing model as how iPLA β or other homeostatic PLAs could recognize and hydrolyze the GPLs present in excess thus maintaining GPL homeostasis.

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FOOTNOTES

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³Abbreviations used are: LC-MS, Liquid chromatography-Mass spectrometry; GPL, glycerophospholipid; PLA, phospholipase; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; LPC, lysophosphatidylcholine; PC-mix, an equimolar mixture of 25 PC species as listed in Experimental Procedures; iPLA, Calcium²⁺-independent phospholipase A; SM, sphingomyelin; BEL, bromoenol lactone; MAFP, methoxyarachidonylfluorophosphonate; NP-40, Nonidet P-40; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PVDF, polyvinylidenedifluoride; 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-phosphatidylcholine (*n* = 6-24)

⁴Hermansson M, Hänninen SM, Hokynar K and Somerharju P., unpublished data.

FIGURE LEGENDS

FIGURE 1. SDS-PAGE analysis of tag-free iPLA β after cation-exchange chromatography. The tag was removed from iPLA β -StrepIII by selective proteolysis with Factor Xa and the reaction mixture was submitted to cation-exchange chromatography as described in *Experimental Procedures*. *Panel A* The right column fraction containing all PLA activity was analyzed by SDS-PAGE and the gel was stained with Coomassie blue. *Panel B* The left column shows the PLA containing fraction stained with anti-iPLA β antibody (1:100 dilution). The left column in *Panel A* and the right one in *B* show the marker protein and molecular weights.

FIGURE 2. Hydrolysis of saturated and unsaturated PC species of varying chain length in NP-40 micelles. PC-mix, 16:0/18:1-PA and 21:0-SM (250/25/25 nmol) were dispersed in 0.5 mL of the assay buffer containing 1% NP-40 and incubated with 8.0 μ g of iPLA β for 30 min at 37 °C. Samples of 50 μ l were drawn at intervals and the relative rates of hydrolysis were determined as indicated in *Experimental Procedures*. The relative rate of hydrolysis is plotted against the total number of acyl chain carbons for several series containing a fixed number of acyl chain double bonds. The sets with only a single member are indicated by an arrow. The specific activity for total PC hydrolysis was 18.2 nmol/ μ g protein. Data are mean \pm SD of four independent experiments.

FIGURE 3. Hydrolysis of 16:0/C $_n$ -PC and C $_n$ /16:0-PC species versus the length of the variable acyl chain in NP-40 micelles. D $_9$ -16:0/C $_n$ -PCs, C $_n$ /16:0-PCs, 16:0/18:1-PA and 21:0-SM (125/125/25/25 nmol) were dispersed in 0.5 mL assay buffer containing 1% NP-40 and incubated with 9.5 μ g of iPLA β for 30 min at 37 °C. See legend of Fig. 2 for other details. *Inset* shows an isomer preference plot obtained by pair-wise division of the rate of 16:0/C $_n$ -PC hydrolysis by that of C $_n$ /16:0-PC. Dotted line at $y = 1.0$ indicates the absence of any isomer preference. Pair-wise student's t-test showed that the differences between isomer pairs was significant only when n was 8 (t value = 10.9496, 2 tailed p -value = 0.0016, $n = 4$, 95% CI) or 11 carbons (t value = -3.0906, 2 tailed p -value = 0.0214, $n = 4$, 95% CI). The specific activity for total PC hydrolysis was 20.78 nmol/ μ g protein. Data are mean \pm SD of four independent experiments.

FIGURE 4. Hydrolysis of saturated and unsaturated PC species of varying chain length in SUV bilayers. Vesicles consisting of PC-mix, 16:0/18:1-PA and 21:0-SM (250/25/25 nmol) were incubated with 10.5 μ g of iPLA β in 0.5 mL assay buffer for 30 min at 37 °C. See legend of Fig. 2 for other details. Specific activity for total PC hydrolysis was 11.2 nmol/ μ g protein. Data are mean \pm SD of four independent experiments.

FIGURE 5. Hydrolysis of 16:0/C $_n$ -PC and C $_n$ /16:0-PC species versus the length of the variable acyl chain in SUV bilayers. Vesicles consisting of D $_9$ -16:0/C $_n$ -PCs, C $_n$ /16:0-PCs, 16:0/18:1-PA and 21:0-SM (125/125/25/25 nmol) were incubated with 11.0 μ g of iPLA β in 0.5 mL assay buffer for 30 min at 37 °C. See legend of Fig. 2 for other details. *Inset* shows an isomer preference plot obtained as indicated in the legend of Fig. 3. Pair-wise student's t-test showed that the apparent differences observed were statistically insignificant. The specific activity for total PC hydrolysis was 15.3 nmol/ μ g protein. Data are mean \pm SD of four independent experiments.

FIGURE 6. Effect of the head group on the hydrolysis of GPL in micelles, SUV and LUV bilayers. Equal amounts of 18:1/18:1-PC, -PE, -PS, -PI, -PG and -PA (250 nmol of total GPL) incorporated to micelles, SUVs or LUVs were incubated with iPLA β (9.0, 12.0 or 28.0 μ g, respectively) in 0.5 mL assay buffer for 30 min at 37 °C. Samples of 50 μ l were drawn at intervals and the relative rate of hydrolysis was determined as indicated in *Experimental Procedures*. The specific activity was 24.99, 18.74 and 8.04 nmol 18:1/18:1-PE hydrolyzed / μ g protein in micelles, SUVs and LUVs, respectively. The values have been normalized to that of the most rapidly hydrolysed GPL in each system. Data are mean \pm SD of four independent experiments.

FIGURE 7. Spontaneous intervesicle transfer of GPLs with different polar head group. Donor vesicles (1335 nmol) consisting of 18:1/18:1-PC, -PE, -PS, -PG, -PA and -PI in equal molar ratio, D $_9$ -

labeled 22:1/22:1-PC (15 nmol) and tetra-18:1 cardiolipin (150 nmol) were mixed with a 5-fold excess of acceptor vesicles consisting of 16:0/18:1-PC and the mixture was incubated at 37 °C. Samples were taken at intervals and the amount of each 18:1/18:1 GPL transferred to the acceptor vesicles was determined as detailed in *Experimental Procedures*. The data are mean \pm S.D. from four independent experiments.

FIGURE 8. Hydrolysis of microsomal GPL species in SUV bilayers. Microsomal lipids (250 nmol GPL) extracted from HeLa cells were reconstituted into SUV bilayers and incubated with 8.0 μ g of iPLA β in 0.5 mL assay buffer for 30 min at 37 °C. The relative rates of hydrolysis were determined as described under *Experimental Procedures*. Data for the most significant PC, PE, PS and PI species are shown in *panels A, B, C* and *D*, respectively. Legend of *panel A* indicates the total number of acyl chain double bonds in the GPL sets. The data are mean \pm S.D. from four independent experiments.

Figure 1

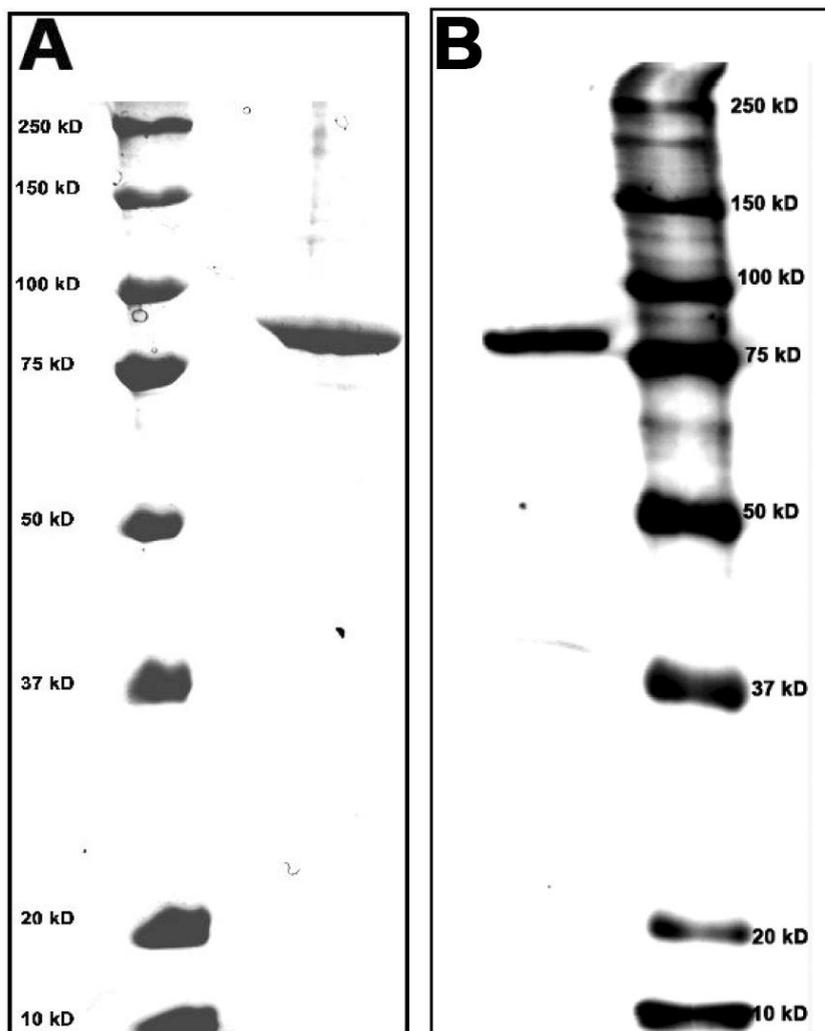


Figure 2

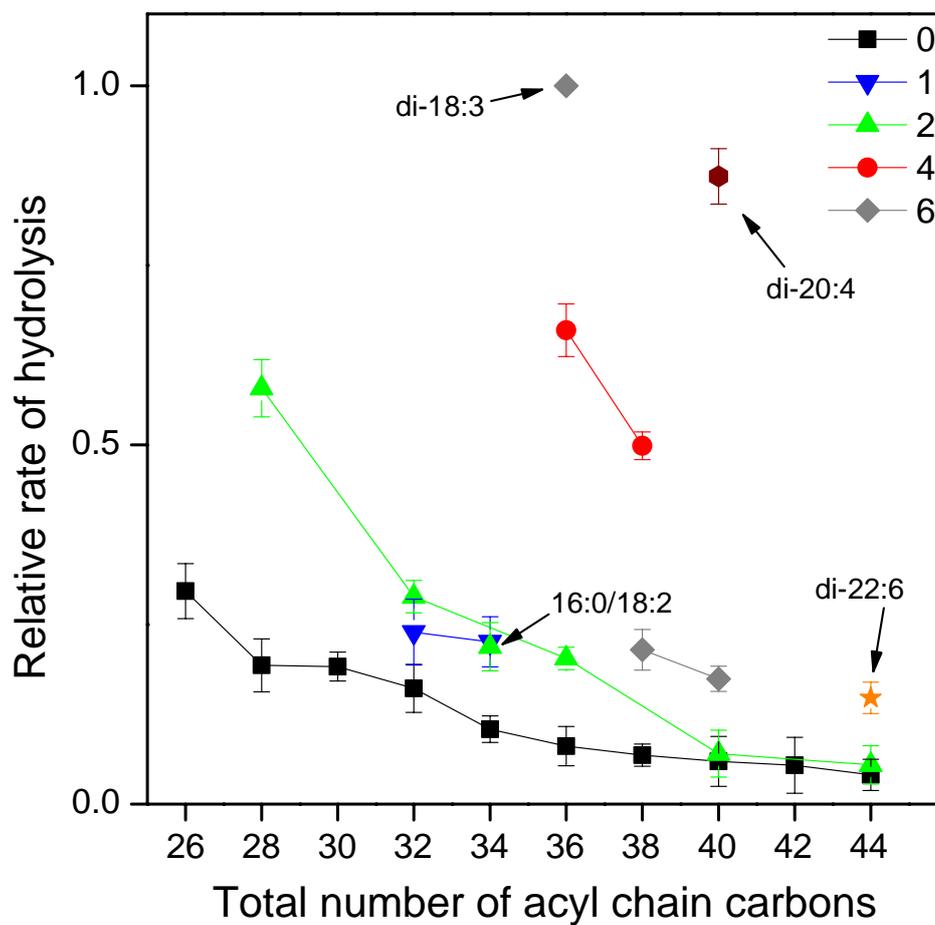


Figure 3

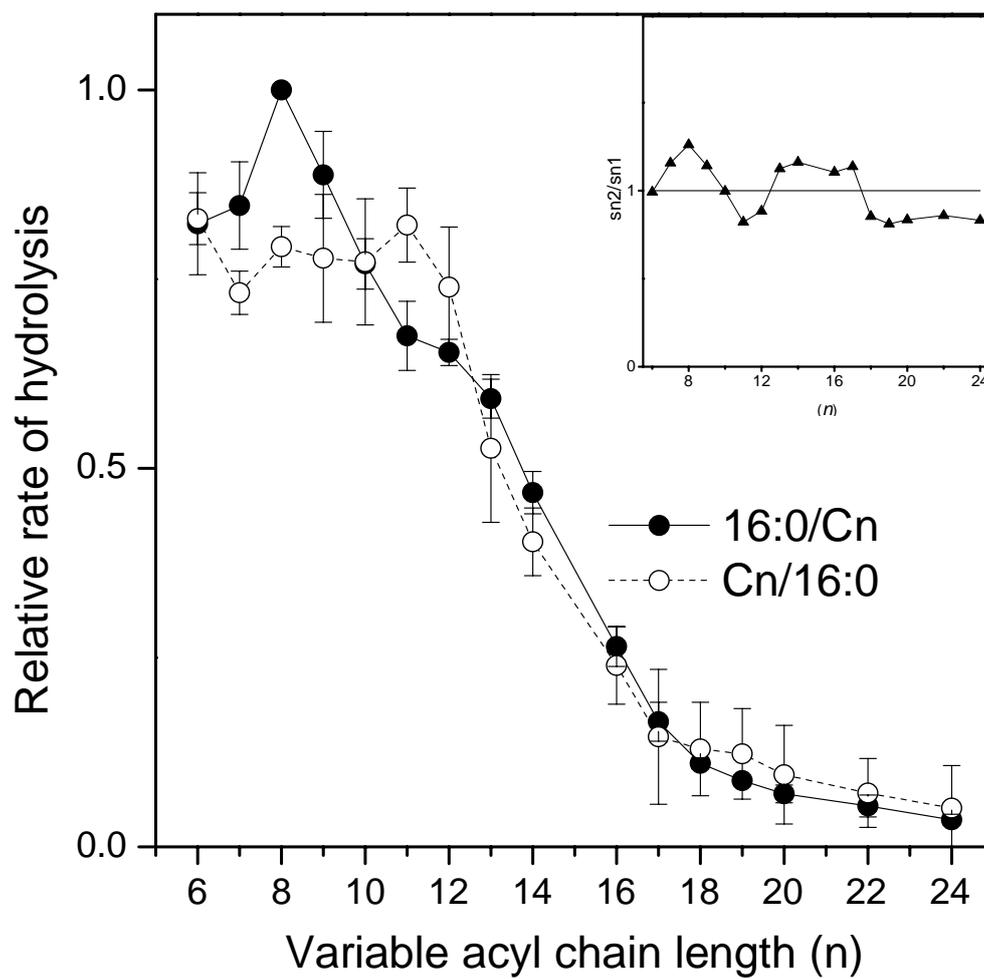


Figure 4

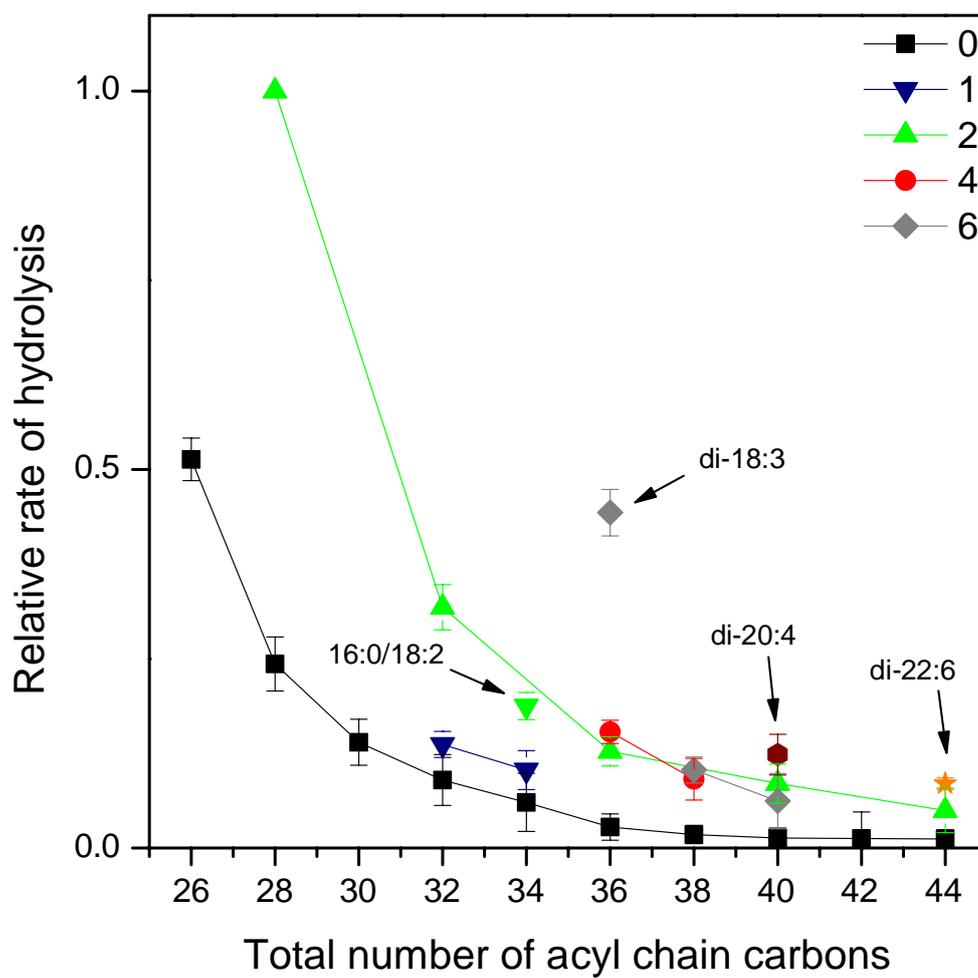


Figure 5

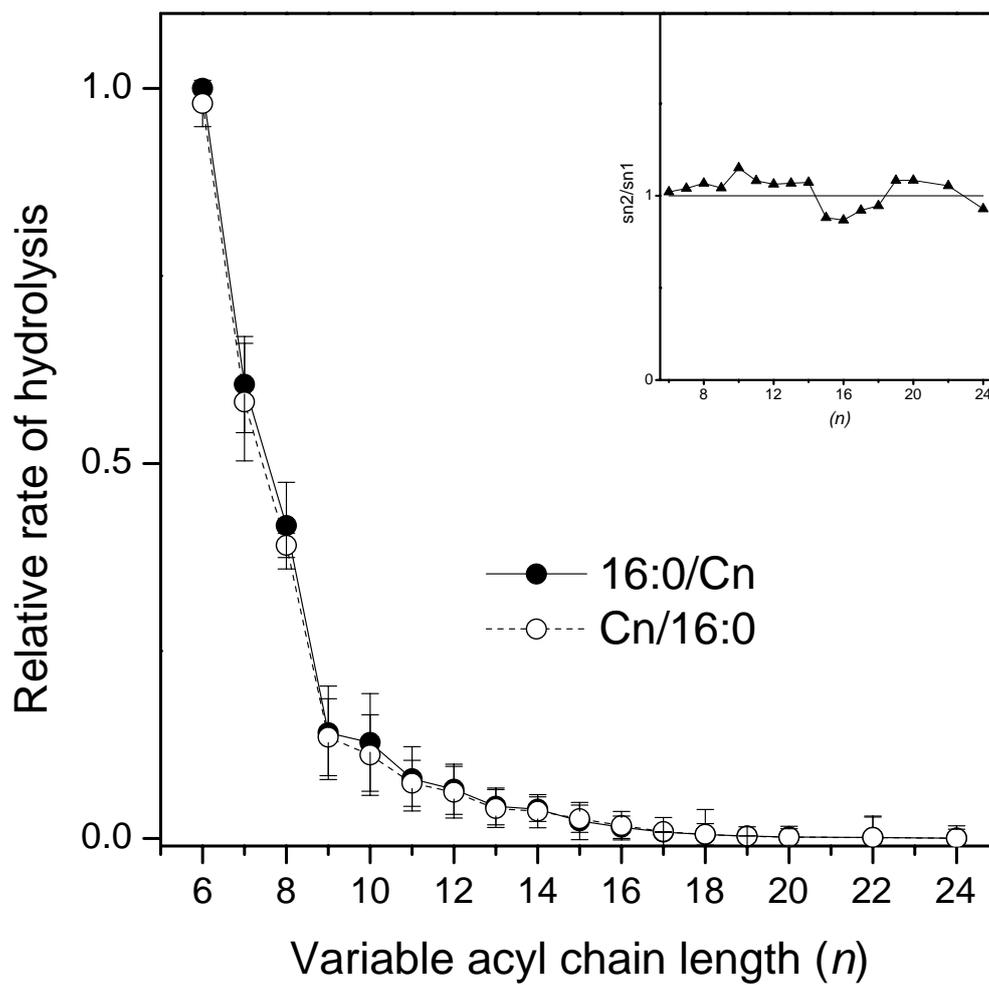


Figure 6

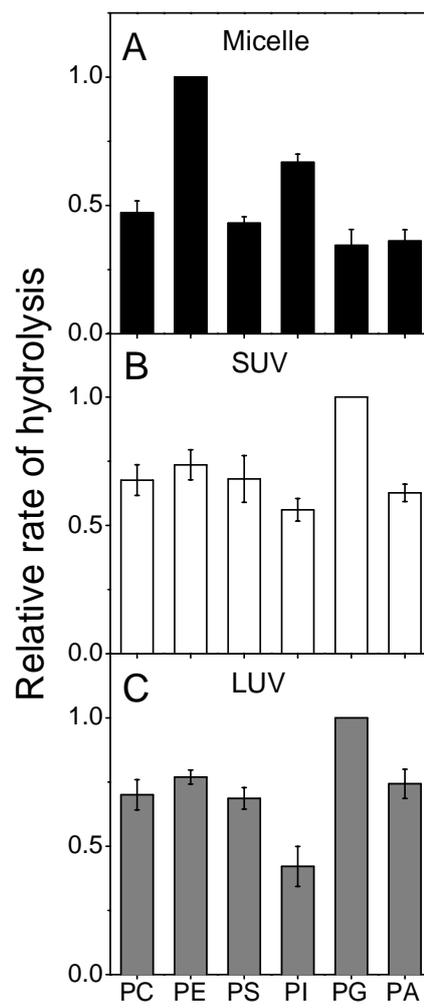


Figure 7

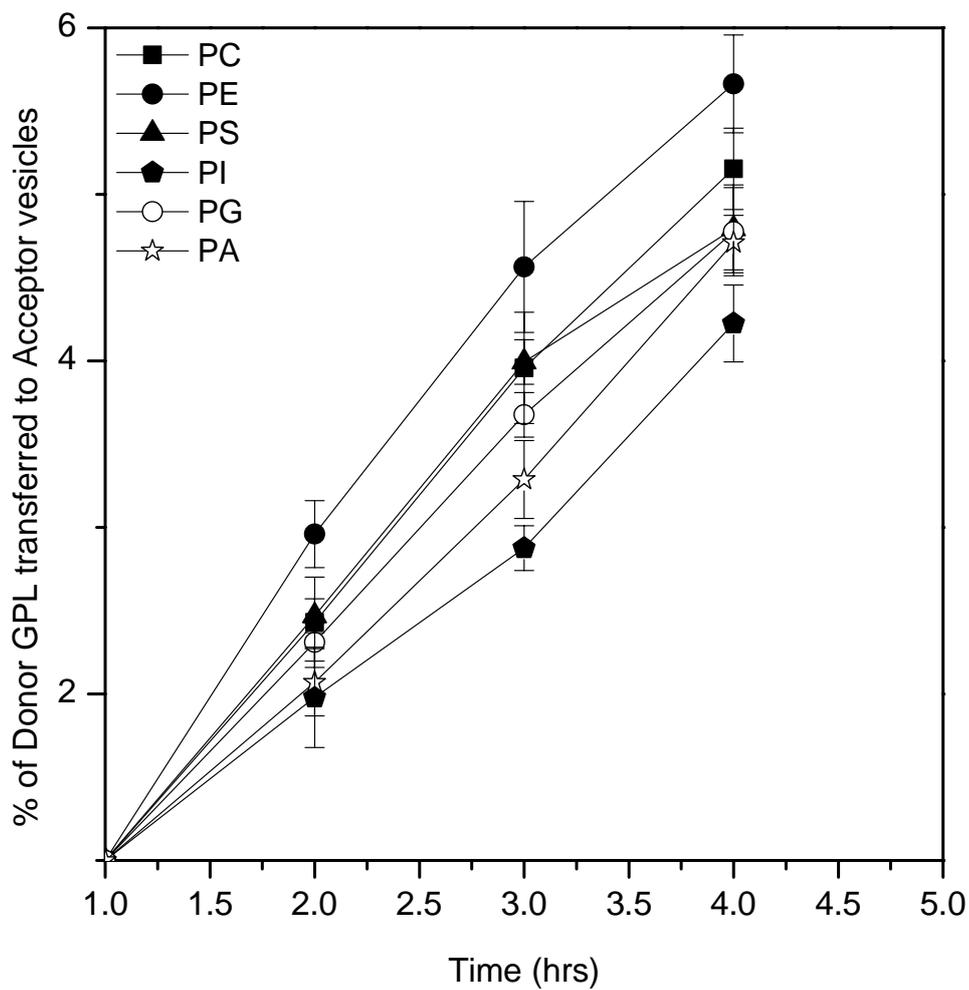
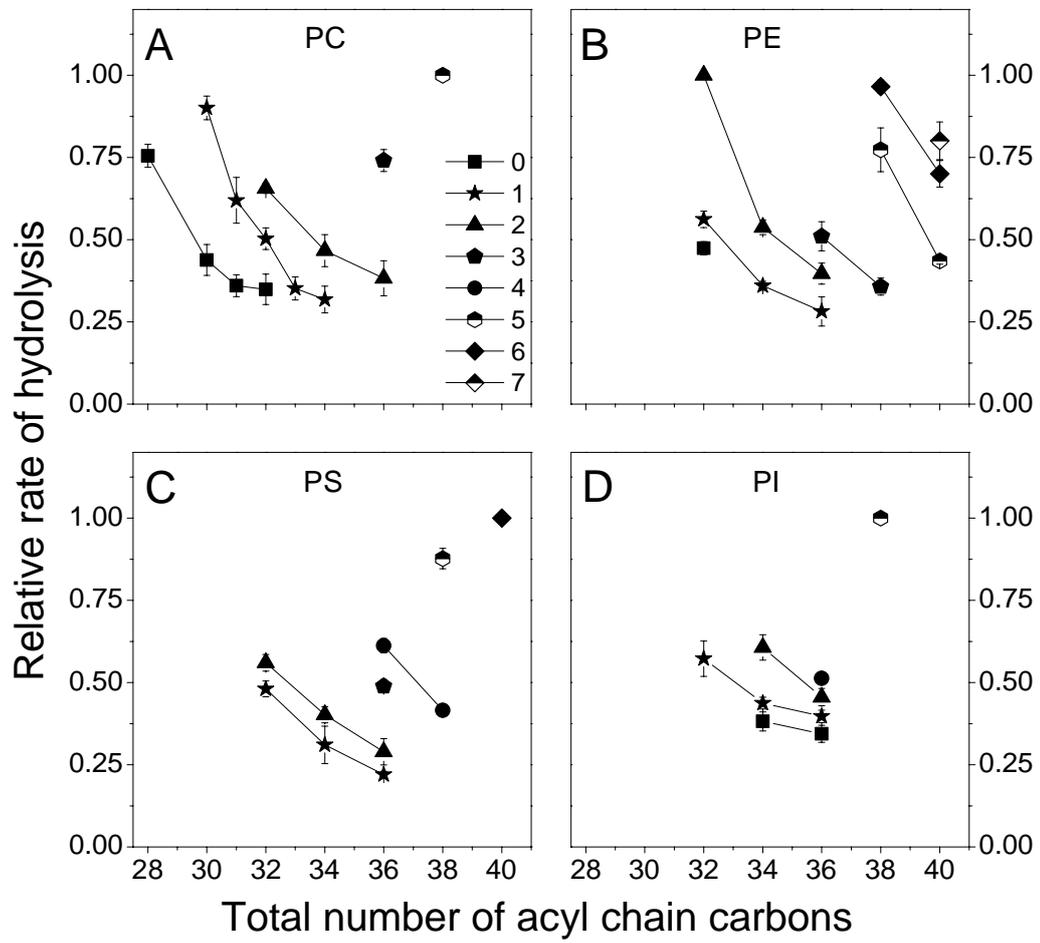


Figure 8

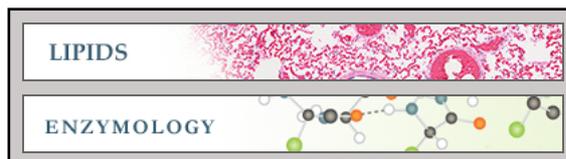


Lipids:

Substrate efflux propensity is the key determinant of iPLA- β -mediated glycerophospholipid hydrolysis

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