Faculty of Biological and Environmental Sciences
University of Helsinki

Academic Dissertation

Factors Regulating the Substrate Specificity of A-type Phospholipases - A Mass-Spectrometric study

Krishna Chaithanya Batchu

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in Biomedicum 1, Helsinki (Haartmaninkatu 8), lecture hall 2, on 8th January, 2016, at 12 noon.

Helsinki 2016
To my Mom and Dad
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1. ORIGINAL PUBLICATIONS

This thesis is based on the following publications. They are referred to in the text by their roman numerals.

   *Equally contributed


## 2. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>BEL</td>
<td>bromoenollactone</td>
</tr>
<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
</tr>
<tr>
<td>DI</td>
<td>direct infusion</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization</td>
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<tr>
<td>GPL</td>
<td>glycerophospholipid</td>
</tr>
<tr>
<td>HeLa</td>
<td>human cervical carcinoma cells</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MAFP</td>
<td>methyl arachidonoyl fluorophosphonate</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>NL</td>
<td>neutral loss</td>
</tr>
<tr>
<td>PLA</td>
<td>phospholipase</td>
</tr>
<tr>
<td>PIS</td>
<td>phosphatidyl inositol synthase</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidyl choline</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidyl ethanolamine</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidyl serine</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidyl inositol</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidyl amine</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidyl glycerol</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>SMS</td>
<td>sphingomyelin synthase</td>
</tr>
<tr>
<td>SRM</td>
<td>selected reaction monitoring</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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3. ABSTRACT
The aim of this thesis was to study the substrate specificity of A-type phospholipases (PLAs) that belong to different sub-families in order to understand the key factors that regulate their activity. The experimental part of this thesis consists of three studies each one focusing on PLAs that belong to a specific subgroup. In the first study, we developed a mass-spectrometric (MS) assay and implemented it to study in detail the effect of acyl chain length and unsaturation of glycerophospholipids (GPLs) on their hydrolysis by three different secretory PLA2s (sPLA2s) from various sources. The key finding of this study was that efflux of the GPL substrate from the bilayer largely determines the rate of hydrolysis by these PLAs. In micelles, accommodation of the GPL acyl chains in the catalytic active site seems to be more important for substrate specificity. In the next study we used the MS assay developed in study I, to investigate whether substrate efflux propensity regulates the activity of the Ca2+ - independent PLA-Beta (iPLAβ). Our results strongly suggest that the activity of iPLAβ is also determined by the efflux of the GPL substrate from the membrane bilayer. Our last study was on the cytoplasmic PLA-alpha (cPLA2α) that has been implicated in the initiation of the inflammatory lipid-mediator cascade generating eicosanoids and platelet-activating factor. The study was carried out to understand to what extent accommodation in the catalytic site determines specificity for arachidonic acid (AA) and if efflux propensity plays a role in the substrate specificity of cPLA2α. Our results indicate that while accommodation of the substrate in the active site greatly contributes to the preferential hydrolysis of AA-containing GPLs by cPLA2α, efflux of the substrate from a membrane bilayer also plays a significant role. In summary, these studies not only provide information on the factors regulating the substrate specificity of various PLAs but also indicate that lateral arrangement of GPLs could be a key regulator of homeostatic PLAs like iPLAβ.
4. INTRODUCTION

GPLs constitute the major structural lipids in nearly all biological membranes. Typical GPLs consist of a glycerol backbone with varying phosphate containing polar head groups esterified to its \textit{sn}-3 position and an acyl chain moiety of varying lengths esterified to the \textit{sn}-1 and \textit{sn}-2 positions resulting in a complex repertoire of >1000 distinct molecular species. Depending on the structure of the polar head group, GPLs are categorized into various classes, the major ones being phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS) and phosphatidylinositols. Each class in turn consists of a variety of molecular species varying in their fatty acid composition. GPLs are amphipathic that causes them to spontaneously assemble into a bilayer membrane in an aqueous medium. The membranes help in maintaining the integrity of the cell or the organelles by creating a semi-permeable boundary between the outside and inside. In addition, GPLs are also involved in intracellular signaling and membrane trafficking (64, 79, 128 and 312). Although many aspects of GPL metabolism have been established, it is still poorly understood how mammalian cells maintain the relative concentrations of the different GPL classes within close limits. A-type PLAs form a ubiquitous class of enzymes that catalyze the hydrolysis of fatty acids either at the \textit{sn}-1 or \textit{sn}-2 position of the GPL glycerol moiety, thus producing a lyso-GPL and a free fatty acid. Earlier studies have shown evidence that cells contain A-type PLAs that selectively degrade GPLs that are present "in excess" (206, 222). However, the identity of these enzymes is still elusive. Recent data suggest that select members of the Ca\textsuperscript{2+} -independent (iPLA) subfamily, in particular iPLA\textbeta and iPLA\textdelta are involved in membrane GPL homeostasis (19, 50, 96 and 186) but there is little information on what regulates the activity of these putatively homeostatic PLAs. In the present study, we developed a high-throughput mass spectrometry-based assay and used it to study the substrate specificities of various A-type PLAs in an unprecedented detail in order to determine the factors that contribute to the specificity of these enzymes.
5. REVIEW OF THE LITERATURE

5.1 Processes maintaining GPL homeostasis in mammalian cells

GPL homeostasis is crucial for the survival of mammalian cells as indicated by the fact that they maintain GPL class compositions of their membranes within close limits (282, 290). The key cellular processes that contribute to membrane GPL homeostasis in mammals are biosynthesis, acyl chain remodeling and degradation. Each of these will now be briefly discussed.

5.1.1 Biosynthesis

Fig 1. gives an overview of the biosynthetic routes for the production of different classes of GPLs.

Figure 1. Pathways of GPL synthesis in mammalian cells. Key metabolites are represented in purple and the enzymes in the reactions are in orange.
5.1.1.1 Biosynthetic pathways of GPLs

Phosphatidic acid (PA), the common precursor of all GPLs, is the first intermediate that is synthesized \textit{de novo} from glycerol-3-phosphate (G-3-P) through the sequential acylation of the \textit{sn}-1 and \textit{sn}-2 hydroxyl groups by acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyl transferase (LPAAT) respectively (12, 46, 229, 246, 247 and 266). PA can also be synthesized from DAG upon its phosphorylation by the enzyme diacyl glycerol kinase (DAGK) (45, 273). An alternative route, through the acylation of dihydroxyacetone-P and the subsequent reduction of acyl-dihydroxyacetone-P to lyso-PA also contributes to PA synthesis (60, 102, 161). PA serves as a branching point for the synthesis of various classes of GPLs through two pathways. In one branch, PA is converted to CDP-diacylglycerol by CDP-diacylglycerol synthase (CDS) for the formation of PI, PG and CL as the principal products (73). In the other branch, dephosphorylation of PA by phosphatidate phosphatase (PAP) yields DAG that serves as an intermediate precursor for PC and PE \textit{via} the CDP-choline or the CDP-ethanolamine pathway, respectively (38, 39, 56). Alternatively, PE is derived from PS -mediated decarboxylation (191, 287, 292). PS is produced \textit{via} a Ca\textsuperscript{2+}-dependent base-exchange reaction by PSS1 or PSS2 in which serine replaces the existing head group of PC and PE respectively (159, 160, 294).

5.1.1.2 Regulation of Biosynthesis of PC

In mammals, PC is synthesized \textit{via} two pathways, the CDP-choline pathway in all the tissues or the PEMT (phosphatidylethanolamine N-methyltransferase) pathway in the liver (94, 284, 285). The CDP-choline pathway consists of 3 steps: First, choline is phosphorylated in an ATP-dependent manner by choline kinase (CK-\textalpha{} or CK-\textbeta{}) (8, 307). Under certain conditions, this step might be rate-limiting in PC synthesis (133, 134, 206). In the second step, the enzyme CTP: phosphocholine cytidylyltransferase (CT) facilitates the conversion of phosphocholine to CDP-choline (264, 313). Two mammalian isoforms of CT (CT-\textalpha{}, CT-\textbeta{}) exist, encoded by \textit{Pcyt1a} or \textit{Pcyt1b} respectively (267, 145). CT-\textalpha{} is a soluble protein that is present both in the cytoplasm as well as in the nucleus; it contains a nuclear localization sequence (NLS) (55, 125, 149 and 178). The CT-\textbeta{} variant lacks the NLS, so it has been found only in the cytoplasm (148, 178, 298). Global deletion of \textit{Pcyt1a} in mice resulted in embryonic lethality (297), on the other hand tissue specific \textit{Pcyt1a} inactivation especially in lung (270), liver (137), macrophages (314) or B-lymphocytes (80) did not seem to have a lethal effect. CT-\textbeta{} knockout mice grow normally, but display gonadal deformation resulting in sterility (136, 145). Another study showed that \textit{Pcyt1b} disruption caused impairment in axon branching (286). Collectively these results suggest that CT-\textalpha{} has a more dominant role in PC synthesis than CT-\textbeta{}. In the final step, the phosphocholine moiety is added to a diacylglycerol moiety in a reaction catalyzed by CDP-choline: 1, 2-diaclylglycerol cholinephosphotransferase (CPT) (189). In humans, two genes coding for enzymes with CPT activity have been identified i.e. \textit{CPT1} and \textit{CEPT1}. The former encodes for a CDP choline specific enzyme (CPT) while the latter encodes for CEPT that has specificity for both CDP-choline as well as CDP-ethanolamine (113, 114).
The PEMT pathway is primarily found in the liver (285). PEMT is an intrinsic membrane protein that catalyzes three sequential reactions through which PE is converted to PC (284). The PEMT pathway and the CDP-choline pathway might be reciprocally regulated (57, 295). The PEMT pathway contributes to ~30% of the PC synthesized in the liver, when there is a limited supply of choline (67, 263, 284). It has also been suggested that this pathway is critical in lipoprotein synthesis as it provides PC for VLDL formation (208-210, 284). PEMT^-/- mice survive and grow normally, however if they are fed a choline-deficient diet, the liver fails and mice die within three days (172).

Previous studies have proposed that regulation of CT activity, the rate-limiting enzyme in PC biosynthesis, is modulated by a physical property of the lipid membrane leaflets termed curvature elastic stress or membrane torque tension which depends on the membrane lipid composition (59, 253). Indeed the activity of CT-α increased with increasing elastic stress in agreement with this model (14). However, there is no evidence that curvature elastic stress can regulate other biosynthetic enzymes or homeostatic PLAs.

### 5.1.1.3 Regulation of Biosynthesis of PE

The CDP-ethanolamine pathway is analogous to the CDP-choline pathway and consists of three enzymatic steps. In the first step, ethanolamine kinase (EK1/EK2) catalyzes ATP-dependent phosphorylation of ethanolamine, thus producing phosphoethanolamine (8, 181, 269). In the next step, phosphoethanolamine reacts with cytidine triphosphate (CTP) to form CDP-phosphoethanolamine (CDP-EA) through the action of the cytosolic protein CTP:phosphoethanolamine cytidyl transferase (ET) (16, 32). Very little is known about the regulatory mechanism of the CDP-ethanolamine pathway. Disruption of the gene encoding ET, (pcyt2) resulted in embryonic lethality in mice (89). Mice heterozygous for pcyt2 accumulated fat both in the liver and the adipose tissue due to increased lipogenesis and reduced utilization of fat as a source of energy (89). Further, liver-specific ET knockout in mice resulted in normal growth, however defects in hepatic lipid metabolism was observed (168). This reaction is considered to be the rate-limiting step in the Kennedy pathway (32, 263). In the final step, CDP-EA reacts with DAG catalyzed by the enzyme CDP-ethanolamine diacylglycerol ethanolamine phosphotransferase (EPT1) to yield the end product PE (113, 114).

The decarboxylation pathway is considered to be the major route for synthesis of PE species with a polyunsaturated acyl chain at the sn-2 position (35, 288, 291). PS is first transported from its site of synthesis to the inner mitochondrial membrane (IMM), where phosphatidylserine decarboxylase (PSD) catalyzes the decarboxylation reaction to form PE. This transportation of PS from one organelle to another is considered to be the rate-limiting step in the PSD pathway (111, 293). However the precise mechanisms involved in this process still remain to be elucidated. Deletion of pisd, the gene encoding for PSD in mice, results in their death between 8-10 days of embryonic development (255). In PSD-deficient cells, mitochondria appear to be fragmented,
apparently shaped and diffuse, indicating reduced levels of mitochondrial PE thus being the cause for an impairment in mitochondrial development (255). All these data suggest that the PSD pathway is crucial for normal development.

### 5.1.1.4 Regulation of Biosynthesis of PS

In mammalian cells, PS is synthesized from either PC or PE through a base-exchange reaction catalyzed by phosphatidylserine synthases, PSS1 or PSS2 (288, 289). Both these proteins are localized in ER domains in close association with the mitochondrial membrane (MAM) (258). Over-expression studies have shown that PSS2, but not PSS1, is subject to product (PS) inhibition (9). Incubation of CHO cells with exogenous PS vesicles inhibited both the synthetizing enzymes (159). *In vitro* assays showed that purified PSS1 but not PSS2 was resistant to product inhibition for reasons unknown (271). Disruption of genes encoding PSS1 and PSS2 in mice were shown to cause death *in-utero*, in contrast mice lacking either one of them did not have any obvious defects (9, 29).

### 5.1.1.5 Regulation of Biosynthesis of PI and PG

Two steps are involved in the synthesis of PI. In the first step, CDP-diacylglycerol synthase (CDS1 or CDS2) catalyzes the generation of CDP-diacylglycerol (CDP-DAG) from PA and CTP (110, 243). In the next step, condensation of myo-inositol with CDP-DAG is catalyzed by PI synthase (PIS), an ER associated protein (7). Studies by Imai and Gershengorn have shown that PI synthase activities might be subject to product inhibition. PI synthesis was strongly inhibited up to 84 to 91% when the ER and PM from rat pituitary GH3 cells were incubated with exogenously added PI. Similar inhibition was observed when the PI content in intact cells was increased (131). When CDS1 and PIS were over-expressed in COS-7 cells, a 25- and 8- fold increase in their activities was observed, however there was no significant enhancement in PI synthesis and at the same time the cellular content of both PI and CDP-diacylglycerol did not increase as well (180).

In PG synthesis, PGP synthase (PGPS) first catalyzes the committed step by combining G-3-P and CDP-DAG thus resulting in the formation of phosphatidylglycerol phosphate (PGP). In the second step, PGP is dephosphorylated by PGP phosphatase (PGPS) to form PG (148, 290). Reduced PGPS activity in CHO cells resulted in a 90% decrease in PG content and an impairment of mitochondrial morphology and function (212, 213). PGPS could be a rate-limiting enzyme in PG synthesis based on the observation that upon its over-expression in CHO cells, there was a 2.5-fold increase in cellular PG content (147).

### 5.1.2 Acyl chain remodeling in GPLs

The newly synthesized GPLs (PC and PE) through the *de novo* pathways typically possess a saturated fatty acid at the *sn*-1 position and a mono- or a di-enoic fatty acid at the *sn*-2 position (281). In the following step, one or both acyl residues can be exchanged in for others in a reaction (remodeling) catalyzed by a combination of multiple
enzymatic activities through PLAs, lysophospholipid acyl transferases (LPLATs) and transacylases (120, 165, 236). Acyl chain remodeling takes place through two routes. Route 1 involves PLAs and acyl transferases and route 2 involves transacylases.

5.1.2.1 Remodeling through route 1

In this route, fatty acid incorporation into GPLs is dependent on the availability of lyso-GPL acceptors maintained by the continuing action of a multitude of PLAs, of which not many have been identified. However iPLAβ has been thought to be involved in GPL remodeling (17, 20, 21, 43 and 301). In addition, the Group VIB Ca^{2+}-independent PLA₂-gamma (iPLA₂γ) has been implicated in this process (196). Yet another PLA possibly involved in GPL remodeling is cPLA₂γ (11, 157, 256).

The key pathways involved in the incorporation of fatty acids into the glycerol backbone of GPLs are the acyl-CoA:glycerol-3-phosphate (GP) acyl transferase and acyl-CoA:1-acyl-GP acyl transferase systems for \textit{de novo} synthesis (124, 164, 244) both of which show strict fatty acid specificity (3, 10, 118 and 144). A number of acyl transferases (LPLATs) incorporate PUFAs into the \textit{sn}-2 position of lyso-GPLs which act as acceptors. For instance, lyso-PC acyltransferase (LPCAT1) incorporates saturated fatty acids such as 16:0 into PC to form disaturated PC, a component of pulmonary surfactant (49, 204). LPCAT2 incorporates 20:4 into PC to form platelet-activating factor and its precursor (245). LPCAT3 exhibits a broad substrate specificity i.e. PUFAs like 18:2 and 20:4 are incorporated into PC, PE and PS (121, 188, 311 and 316). LPCAT4 preferentially incorporates 18:1(n-9) into PC and PE (121). Lyso-PI acyltransferase1 (LPIAT1) is unique in that it incorporates 20:4 into only PI (167). The finding that LPCAT3 is a major acyltransferase that incorporates unsaturated fatty acids such as 18:1(n-9), 18:2 and 20:4 into PC, suggests that it has a major role in determining the level of GPL unsaturation. The rapid turnover of \textit{sn}-2 acyl moiety of GPLs was described by Lands as a remodeling pathway (164) and is attributed to the concerted activation of PLAs and LPLATs (81, 202). Recently, another LPLAT family distinct from the other LPLAT families has been identified (121). These enzymes do not possess the LPAAT motif. The key enzymes that exist under this category are: mLPCAT3, mLPCAT4 and mLPEAT1. MLPCAT3 acylates LPC, LPE and LPS to PC, PE and PS respectively with polyunsaturated acyl CoA's (18:2 and 20:4). mLPCAT4 has LPCAT and LPEAT activities preferring oleoyl-CoA (18:1) and then finally mLPEAT1 shows LPEAT and LPSAT activities preferring oleoyl-CoA (18:1) (122).

5.1.2.1 Remodeling through route 2

Along route 2, acyl chain exchange (remodeling) is carried out by either the CoA-dependent or the CoA-independent transacylation reactions wherein a GPL serves as an acyl chain donor and a lyso-GPL as an acceptor (62, 309). In a CoA-dependent transacylation process, mainly 20:4, 18:2 or 18:0 containing acyl chain of a GPL is transferred to an LPC acceptor in the presence of CoA (309). Studies with the 18:0 specific CoA-T transacylase
showed that it has a restricted acyl donor specificity by using PA or PI but not PE or PS (123). On the other hand, CoA-independent transacylation (CoA-IT) catalyzes transfer of long chained fatty acids (C20, C22) that are polyunsaturated from a donor GPL to an ether bond containing lyso-GPL in the absence of any cofactors (such as CoA) leading to the formation of alkylacyl-PC, alkylacyl-PE or alkenylacyl-PE (33, 53, 54).

5.1.2.2 Phospholipases (PLAs)
PLAs are classified based on their site of attack on the GPL that could either be hydrolysis of the 1-acyl ester (PLA1) or the 2-acyl ester (PLA2) (235). PLAs that can hydrolyze both the acyl chains are called PLA-B (61, 288). Other classes of PLA exist, that could cleave the glycerophosphate bond (PLC) or the base group (PLD) (76, 261). Both these enzymes are called phosphodiesterases. Finally, there are enzymes called lyso-PLAs that can catalyze the removal of the acyl chain from monoacyl(lyso)-GPLs (18, 279, 280).

PLCs induce breakdown of GPL by hydrolyzing the bond between phosphate and the glycerol backbone to form the phosphorylated head group and sn-1,2-diacylglycerol (DAG) that further goes on to activate protein kinase C (PKC) (30, 207). In all, mammalian PLCs that are involved in inositol lipid signaling pathways constitute 13 members that are classified into 6 isotypes (88, 261) based on their structures. Other studies have revealed that unknown players carry out PLC-mediated hydrolysis of PC and PE which in a way is tempting to implicate them in GPL homeostasis (31, 72, 77, 78, 242, 306 and 315). Sphingomyelin synthases (SMS1 and 2) represent another class of enzymes that enable the transfer of a phosphocholine moiety from PC onto ceramide to produce sphingomyelin and diacylglycerol (DAG). SMS1 localizes at the Golgi while SMS2 localizes both at the Golgi and the plasma membrane (260).

Mammalian PLDs are ubiquitous in nature (141). They preferentially hydrolyze PC or PE to yield PA and free choline or ethanolamine respectively (22, 234, 265). Mammalian cells harbor two isoforms of PLD: PLD1 and PLD2 both of which contain two HKD motifs that are critical for enzymatic catalysis (217). The PLD pathway has been implicated in the regulation of insulin signaling, extracellular Ca\textsuperscript{2+} entry into the cells and PI4P 5-kinase activation of mTor amongst others (86, 141, 203). Although in principle, members of the PLD family could be involved in GPL homeostasis, there is hardly any evidence for this.

Regarding degradation, several studies have provided evidence that PLAs are key players in this process (15, 25, 91, 115, 196, 283 and 302). PLAs are ubiquitous in nature and have diverse roles to play from membrane homeostasis, lipid mediator production to signal transduction (41). They vary in the site of action on the GPLs, physiological function, mode of action and finally regulation (41, 48, 163).
5.1.3 Coordination of synthesis and degradation
Although it is clear that GPL homeostasis is maintained through the precise coordination of GPL biosynthesis and degradation, it is quite unclear how this is accomplished. Such coordination is essential in order to avoid futile competition between these opposing processes. Strong support for strict coordination, comes from studies demonstrating that enhancing the synthesis of a GPL class increased its degradation in proportion. In one study, over-expression of cytidylyl-transferase (CT) in HeLa cells led to an increase in the synthesis of PC by 4-5 fold, but no significant increase in the PC content was observed, rather the content of GPC, the deacylation product of PC greatly increased (15, 296). A similar outcome was observed in case of PS and PE (179, 257). Conversely, inhibition of synthesis of a GPL, inhibited its degradation as well obviously to maintain GPL homeostasis. For instance, 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 its reduced degradation (222). These data demonstrate that in mammalian cells, GPL synthesis and degradation are coordinated, and provide strong evidence that the cells contain homeostatic PLAs that selectively degrade the GPL species when produced in excess. Mammalian cells express numerous A-type PLAs (41, 198) and those involved in GPL homeostasis have not been firmly established. Some studies have indicated that members of the Ca\(^{2+}\)-independent PLA (iPLA) subfamily, particularly iPLA\(\beta\) and iPLA\(\delta\) could be involved in this process. (19, 50, 96 and 186).

5.1.3 Secretory PLAs (sPLA\(_2\)s)
sPLA\(_2\)s represent a group of low molecular weight enzymes that require Ca\(^{2+}\) at mM concentrations for their activity. To date, 11 mammalian isoforms of sPLA\(_2\) have been identified (68, 163). In addition, other members exist that have been isolated from different venoms (184, 225).

5.1.3.1 Structure and Properties
PLA\(_2\)s belonging to the groups I, II, V and X are considered as conventional sPLA\(_2\)s and are closely related (235, 278). They share a highly conserved catalytic domain (His-Asp dyad), a Ca\(^{2+}\)-binding loop (XCGXGG) and 6-8 conserved disulfide bridges that contribute to a high degree of stability to the enzymes. On the other hand, PLAs that belong to the groups III and XII are structurally different from those conventional sPLAs as deduced from their protein sequences (58, 106, 238). The molecular weight of sPLA\(_2\)s range from ~13 to 19 kD except for the Group III isoform that has a mass of 55 kD. An aspect that is most common for all sPLA\(_2\)s is that they share a ~15 Å deep active site slot that could fit a single GPL molecule positioned in such a way that the enzyme-susceptible ester is next to the catalytic residues (90, 92, 138 and 139).
Most of the extracellular sPLAs require high Ca\textsuperscript{2+} concentrations (mM) to operate effectively on membrane-bound GPLs, a phenomenon known as "interfacial activation" (92). It has been suggested that "interfacial activation" could largely be a result of the specific conformation assumed by the substrate in an aggregated form (240). Earlier studies have also shown that GPL substrate must be in an aggregated form (i.e; micellar or bilayer) for optimal enzyme activity (190, 220). The transfer of a GPL molecule from an ordered interface to the active site requires less energy than with monomeric substrates as their conformation is random. A recent study by Qin et al demonstrated through modeling studies that in the presence of zwitter ionic membranes, sPLA\textsubscript{2s} favor the hopping mechanism (223). Crystal structures of a few of these enzymes (members of Groups IB, IIA and X) have been solved both with and without a bound transition state analogue or an inhibitor (71, 215, 238-240 and 248). It is assumed that the catalytic mechanism for sPLA\textsubscript{2}-catalyzed ester degradation is initiated by the His-Asp dyad in a Ca\textsuperscript{2+} -dependent manner through the abstraction of a proton from an incoming water molecule followed by a nucleophilic attack on the carbonyl-carbon at the sn-2 position of the GPL thereby leading to the rate-limiting formation of a putative tetrahedral intermediate. Next, protonation of the sn-2 oxygen mediated through His48 in concert with the productive collapse of the tetrahedral intermediate leads to the formation of a free fatty acid and a lyso-GPL (184).
It has been established that mammalian sPLA₂s carry out important biological functions such as dietary GPL digestion [IB] (231, 277), host defense against parasitic, bacterial and viral infections [IIA, V, X, XII] (163) and eicosanoid mediated inflammation [X] (112). Several reports have speculated a role for various mammalian sPLA₂s in a wide range of diseases that include pancreatic acinar carcinoma [IB] (47), arthritis, atherosclerosis, sepsis, cancer [IIA and V] (36, 198, 233 and 272), chronic obstructive pulmonary disease [IID] (130) and asthma [IID] (6). There is no evidence from any of the earlier studies that sPLA₂s are involved in GPL homeostasis.

5.1.4 Ca²⁺-independent PLAs (iPLAs)
iPLAs are also known as the Group VI PLAs or patatin-like PLAs and consist of seven members, that include iPLAβ [VIA-1 and -2] (PLA₂GVIA, PLA₂G6, PNPLA9), iPLA₂γ (PLA₂GVIB, PNPLA8), iPLA₂δ (PLA₂GVIC, NTE, PNPLA6), iPLA₂ε (PLA₂GVID, Adiponutrin, PNPLA3), iPLA₂ζ (PLA₂GVIE, Desnutrin, ATGL, PNPLA2), iPLA₂η (PLA₂GVIF, GS2). In addition, three more isoforms have been identified (iPLA₂φ, iPLA₂ι, and iPLA₂κ) but then not much is known about them so they haven’t been assigned to this group yet (150, 157, 195, 197, 301 and 303). Members of this family carry out substrate hydrolysis through a catalytic dyad (Ser-Asp) and do not require Ca²⁺ for their activity (4, 19, 194). No crystal structure for any of these isoforms is available at the moment. Interestingly, the isoforms: iPLAβ and iPLA₂γ possess a long N-terminal domain which is speculated to facilitate protein-protein interactions and membrane spanning. In addition, both these variants are true PLAs, while the other members mainly hydrolyze triglycerides or act as transacylases (226).

5.1.4.1 Structure and Properties of iPLAβ
The Group VIA PLA gene yields at least 5 splice variants (VIA-1, VIA-2, VIA-3, VIA-Ankyrin1 and VIA-Ankyrin2) of which the 85 kD (VIA-1) and 88 kD (VIA-2) forms are catalytically active and manifest PLA₁/PLA₂, lyso-PLAs and transacylase activities (175, 182, 303 and 305). The full-length variant of iPLAβ protein is of 752 amino acids containing 7 ankyrin repeats, a 10-40 amino acid residue spacer linkage and a catalytic domain that harbors the GTSTG stretch (267). Earlier studies suggest that the ankyrin repeats maybe crucial for protein-protein interactions and it is suspected that the splice variant, GVIA-Ankyrin1 acts as a negative regulator of iPLAβ by blocking the formation of active tetramers of iPLAβ (166). iPLAβ also contains other domains such as an ATP binding consensus motif (GGGVKG) that allows its regulation by ATP, an N-terminal caspase-3 cleavage site (DVTD), a putative bipartite nuclear localization sequence (KREFGEHTKMTDVKKPK) and a calmodulin-binding stretch (AWSEMVGIQYFR) that helps it to form a signaling complex with CaMKIIβ (1, 41, 84, 109, 140, 175, 185, 274 and 303). The hydrolysis of GPL substrate proceeds through an acyl-enzyme intermediate (as in sPLA₂s) except that here serine acts as a nucleophile after aspartic acid has abstracted a proton from it (175, 194). It has been observed that iPLAβ seems to mediate "interfacial catalysis" by acting in the scooting mode (194). Deuterium exchange experiments in combination with molecular dynamic (MD) simulations revealed that five different regions in iPLAβ facilitate its binding to the membrane, one of which (708-730 residues) is located in the
The first proposed role for iPLAβ was that of in GPL remodeling (20). Other studies employing iPLAβ-deficient mice suggest that the enzyme is involved in a number of major cellular events such as apoptosis (13), cell proliferation (262), insulin secretion (23), bone formation (227) and sperm development (24). In addition, iPLAβ has also been implicated in a large number of diseases that include various neurological disorders such as infantile neuroaxonal dystrophy (INAD), multiple sclerosis, alzheimers and also hypertensive heart failure (5, 28).

5.1.5 Cytosolic PLAs (cPLAs)
The cPLA family constitute six paralogs (PLA2GIVA, B, C, D, E and F) which are also referred to as cPLA2 α, β, γ, δ, ε, ζ respectively and they all share the lipase consensus sequence GXSGS (11, 152, 211, 218, 252 and 275). The C2 domain that facilitates membrane binding is also conserved in all the members except in cPLA2γ which is unique in that it exhibits Ca2+-independent PLA2 activity (11).
5.1.5.1 Structure and Properties of cPLA₂α

The cPLA₂α enzyme is ubiquitously expressed and contains 749 amino acids and has a molecular weight of 85.2 kD (52, 158). cPLA₂α was first identified in human platelets in 1986 (154), and was later cloned and sequenced in 1991 (51, 155). It is the only member of the cPLA family that exhibits selectivity for AA (51, 93). In addition, it also possesses PLA₁, lyso-PLA as well as transacylase activities (230). The X-ray crystal structure of cPLA₂α has been resolved (69). The crystal structure shows that cPLA₂α consists of two major domains i.e, (i) an N-terminal Ca²⁺-dependent C2 domain (CaLB) that was found to be essential for the initial association of the enzyme with a membrane (95, 154, 249) in response to increasing intracellular Ca²⁺, that in turn induces phosphorylation of certain serine residues, (ii) a catalytic domain that is composed of a hydrolase core and a novel cap region within which a lid exists, that has to move aside for a GPL molecule to enter the active site cavity of cPLA₂α (42, 194).

cPLA₂α utilizes a similar catalytic mechanism as in iPLAβ as they share the same catalytic active site residues (Ser/Asp) (17, 170). In addition to Ca²⁺, activation of cPLA₂α has also been shown to be regulated through phosphorylation on its multiple serine residues (505, 515, 727) by MAPKs and Ca²⁺/calmodulin-dependent protein kinase II (119, 169, 174 and 224). Other studies have shown lipid messengers: ceramide-1-phosphate and phosphatidylinositol (4,5) bisphosphate to play a role in enzyme activation upon binding to it (254).

Figure 4. Ribbon diagram of cPLA₂α. The C2 domain is shown in green and the Ca²⁺ atoms in red. The “cap” region is in purple while the flexible linker between the C2 and catalytic domains is colored red. (Adapted with permission from: Dessen, A, et al. (1999), Cell, 349-360)
Several studies have established that cPLA$_2$α plays a central role in triggering AA release and eicosanoid production (2, 37, 41, 152 and 276). Using cPLA$_2$α knockout mice to generate disease models, it has been shown that the enzyme is involved in a number of physiological and pathological diseases such as arthritis (1999), inflammatory bone resorption (193), pulmonary fibrosis (200) acute respiratory distress syndrome (200, 201), autoimmune encephalomyelitis (187) and diabetes (214).

5.1.6 Factors determining substrate specificity of soluble PLAs

An accepted model of the catalytic cycle of soluble PLAs takes places in 4 steps (69, 239, 241 and 268):

1.) Binding of the enzyme to the membrane surface through its interfacial binding region,
2.) Upward movement (efflux) of the GPL molecule from the macrosubstrate,
3.) Accommodation of the substrate in the catalytic active site,
4.) Cleavage of the acyl-glycerol ester bond, with subsequent release of products.

![Figure 5. Representation of the accepted model of the catalytic cycle of soluble PLAs](image)

Among these, only steps 2 and 3 determine the substrate specificity of the enzyme. (See below)

5.1.6.1 Substrate accommodation at the catalytic site

Previous studies have demonstrated that interaction of the GPL acyl chains with the active site cavity is critical especially in micelles (65, 171). Significant differences were observed between associations of sn-1 vs. sn-2 acyl chains with various PLAs (219). White et al. found that when a truncated version of a nonhydrolyzable PC in a micellar macrosubstrate was presented to cobra sPLA$_2$, a tighter interaction with the sn-2 acyl chain vs. the sn-1 one was observed (300). Further, Yu and Dennis showed that cobra sPLA$_2$ favors GPL analogues constituting truncated acyl chains with a steady increase when the length of the sn-2 chain reached 9 carbons after which there was an abrupt drop (310). Similar results were obtained by Roberts and colleagues who showed that when the chain length was varied in the sn-2 chain, the variant with 8 carbons was the best substrate for cobra sPLA$_2$ (232). All these results suggest that accommodation at the active site determines the rate of hydrolysis in micelles.
5.1.6.2 Substrate efflux from the lipid bilayer
Importance of an upward movement of the substrate molecule (efflux) from a membrane bilayer to the active site has been studied using cross-linkable and polymerized GPLs (250, 308). That substrate efflux, is necessary for a GPL substrate to be hydrolyzed by a PLA is based on the assumption that soluble PLAs may not be able to penetrate the membrane significantly and therefore the catalytic site remains well above the interface (90, 175). In addition, recent studies employing hydrogen/deuterium exchange and MD simulations have proposed that the substrate molecule has to be extracted (i.e. efflux) from the membrane in order for it to reach the active site for its hydrolysis (40, 126, 127). These findings strongly suggest that efflux propensity could be rate-limiting in the hydrolysis of GPL substrate by PLAs in vesicle bilayers.

5.1.7.1 Superlattice model
As mentioned earlier, GPL synthesis and degradation are processes that must be closely controlled and strictly coordinated in order to maintain membrane lipid homeostasis. However the mechanisms underlying these processes remain largely obscure. Previous studies (63) have indicated that there is a strong possibility that the biophysical properties of the bilayer could regulate these processes. One of the models that has been proposed with such a possibility is the superlattice model which proposes that amphipathic lipids have a tendency to adopt regular, superlattice distributions rather than random lateral distributions in multicomponent bilayers (251). This model is relevant in the context of regulation of membrane GPL homeostasis for two reasons. First the model predicts the existence of a limited number of "critical" or allowed GPL bilayer compositions that are energetically more favorable (membrane free energy at a local minimum) than the intervening ones and therefore there is an intrinsic tendency for the composition to settle in one of the critical compositions. A simple mechanism as this, would minimize wasteful compositional fluctuations. Second, whenever the concentration of a GPL exceeds a critical value in a bilayer, packing defects appear as the molecules "in excess" could not be accommodated in an arrangement predicted by the superlattice model. Thus molecules "in excess" would consequently form segregated domains where they are more loosely packed. Those loosely packed molecules are predicted to have an increased chemical activity and therefore increased fugacity (escape tendency) which would thus make them susceptible to hydrolysis by homeostatic PLAs. Once the GPLs "in excess" are hydrolyzed, the loosely packed domains at the lattice boundaries disappear, thereby greatly reducing the availability of GPLs to the homeostatic PLAs. Thus the superlattice formation would provide a highly accurate mechanism for the regulation of membrane lipid compositions. Supporting this model of regulation has been reported in which the hydrolysis of PC in PC/Cholesterol bilayers by venom sPLA2 was more efficient at intervening compositions than in the allowed ones (129).
5.2 Mass-spectrometric analysis of lipids

Lipid compositions of mammalian cells are enormously complex and thus nearly impossible to resolve with analytical techniques such as thin layer chromatography (TLC), however recent advances in mass spectrometry (MS) lipidomics has greatly extended the analytical capabilities both in terms of sensitivity and selectivity thus facilitating accurate identification and quantification of a multitude of lipid species simultaneously (103, 104, 105). Present lipidomic analyses rely on soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) (176). Analysis of lipids by ESI is based on the ability of lipids to acquire a positive or negative charge when in solution. ESI has been preferred over other ionization techniques such as MALDI (87, 97) and atmospheric pressure ionization for lipid analysis (44, 132). Separation of ions is obtained by various analyzers, such as the ion trap or a multiple quadrupole. ESI-MS is carried out either by direct infusion of the sample into the mass-spectrometer or by combining the MS with a liquid chromatography equipment (LC).

5.2.1 Direct infusion and class specific scanning

Direct infusion (DI) method is also known as shotgun lipidomics method (34, 153). Shotgun lipidomics was coined for the first time by Han & Gross in 1994 and can evade a number of difficulties that could arise from chromatographic problems, such as non-specific absorption (104). In addition, the shotgun approach in combination with class specific fragmentation strategies can detect most GPLs with high sensitivity and specificity as they are readily ionized by either protonation to form (M+H)$^+$ or deprotonation to form (M-H). Precursor-ion scanning (PIS) is achieved by first introducing the ions formed in the ion source into the first quadrupole analyzer (Q1), which is scanned over a wide mass range corresponding to various GPLs. The molecules passing through Q1 are subjected to collision-induced dissociation (CID) in the 2nd quadrupole (Q2) or in the collision cell. Finally, Q3 transmits the mass of the selected fragment. Profiling of GPLs by neutral loss scanning (NLS) is used to detect PS and PE. In a neutral loss scan, Q1 and Q3 analyzers scan the same mass range, except that in Q3 the mass range is lowered by the mass of a neutral fragment e.g. 87 Da to detect the loss of the phosphoserine group from PS. It should be noted that the mass accuracy of the mass spectrometer (MS) during NL scanning mainly depends on the mass difference between Q1 and Q3 analyzers and also the mass accuracy of these two analyzers. This method has its own disadvantages primarily that isobaric species cannot be quantified unless subjected to fragmentation. Further, quantification of molecular species that have low level concentrations could be a difficult task to perform (107).
5.2.2 Liquid chromatography-mass spectrometry (LC-MS)
In order to obtain maximum sensitivity of detection for lipid species that are low in abundance, selected reaction monitoring (SRM) in a triple quadrupole instrument is often carried out (98). Normal phase LC column is employed to separate individual lipid classes and a reverse-phase column is used to resolve molecular species in a lipid class based on the different hydrophobicities of different acyl chain residues of GPLs. LC-MS is often more sensitive than DI-MS because it allows separation of signal by suppressing impurities and competing analytes. But then there are also a number of difficulties that could limit usage of LC-MS while carrying out large scale analysis of lipids primarily due to the mobile phase compositions that can introduce errors in ionization efficiency (99, 192, 151). Major drawbacks include an increase in analysis time, cost of analyzing and in addition, loss of lipids on the column which is not an unusual event (66).

5.2.3 Data Analysis
MS analysis of lipids generates vast amounts of data, processing of which requires proper software tools. Accordingly, a number of software packages have been developed that carry out multiple data-processing tasks such as spectral filtering, peak detection, normalization and quantification (75, 85, 108, 116 and 135). In our laboratory, a software termed LIMSA was developed for DI-MS data analysis. It is an add-in program that runs on EXCEL, it serves as an interface to process data from individual full MS and tandem MS spectra (100). For LC-MS data as well, programs have been developed that take into consideration both the elution times and the determined mass of individual molecular species (74, 117, 146, 221 and 237).
6. AIMS OF THE PRESENT STUDY
The main objective of the present study was to elucidate the factors regulating the substrate specificities of A-type phospholipases (PLAs) by using high-throughput mass spectrometry as a key tool. Specific aims for individual publications were as follows:

1. To develop a mass-spectrometric method to determine the key factors that regulate the substrate specificity of nonhomeostatic PLAs. (A proof of principle study)

2. To study if substrate efflux propensity regulates the activity of iPLAβ, a putative player in GPL homeostasis.

3. To study the key factors that regulate the substrate specificity of cPLA2α, which has a crucial role in liberating arachidonic acid, a precursor of eicosanoids from GPLs.
# 7. EXPERIMENTAL PROCEDURES

## 7.1 List of the experimental methods used in this thesis work (Table 1)

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7.2 PLA activity assay
A mixture of synthetic GPLs, 16:0/18:1-PA and 21:0-SM (internal standard) were mixed in chloroform, dried under a N\textsubscript{2} stream and further under high vacuum for 1 h. To prepare small unilamellar vesicles (SUVs), the dried lipids were dissolved in 20 \( \mu \text{L} \) ethanol and injected into 0.5 mL of the assay buffer (20 mM Tris-HCl, pH 7.5, 5\% glycerol) using a constant-speed Hamilton syringe. The SUV bilayers were then incubated in the presence of the purified PLA at 37 °C, 50 \( \mu \text{L} \) 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 twice with the theoretical upper phase and taken to dryness under a N\textsubscript{2} stream. The residue was reconstituted in 200 \( \mu \text{L} \) of chloroform/methanol (1:2, v/v) and stored at -20 °C. To prepare large unilamellar vesicles (LUVs), lipids were dried in a glass tube, 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 4 times. The lipid suspension was vortexed for 1 min at room temperature then extruded 10 times through two stacked 1.2 \( \mu \text{m} \) polycarbonate membranes and then 10 times through two 0.4 \( \mu \text{m} \) membranes using an extruder pressurized with Argon. To study the hydrolysis in micelles, a dried lipid mixture was first dispersed in the assay buffer containing 1\% Triton X-100 and the assay was conducted as above.

7.3 Mass-spectrometry and Data Analysis
After addition of 25\% NH\textsubscript{4}OH in water (4\% final concentration) the sample was infused to a Micromass Quattro Micro triple-quadrupole mass spectrometer (116). The GPL species were detected using head group-specific precursor or neutral-loss scanning (111). The spectra were exported to Microsoft Excel and the individual GPL species were quantified using the LIMSA software (100). Concentrations of the individual lipid species were plotted against time and the relative rate constants of hydrolysis were obtained by fitting a first order decay model to the data. The maximum hydrolysable fraction at \( t_\infty \) was constrained to 1.0 with micelles and to 0.67 with SUVs (101). LC-MS with selective reaction monitoring (SRM) was carried out using a Waters ACQUITY Ultra Performance LC system equipped with a Waters ACQUITY BEH C\textsubscript{18} column (1.0 \( \times \) 100 mm) to separate the molecular species using gradient elution. The eluent was directed 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 (27).
8. RESULTS AND DISCUSSION

8.1 The factors that regulate the substrate specificities of sPLA₂s determined by a novel mass-spectrometric assay.

Traditionally, evaluation of the specificity of PLA₂s was done by measuring the rate of hydrolysis of a few radiolabeled substrates individually. Such an approach is tedious if multiple species are to be studied. In addition, variations in enzyme activity could arise from physical properties of the substrate determined by the lipid under study, such as membrane surface charge and fluidity. To improve this, specificity could be studied by comparing the hydrolysis of two GPLs that have different isotope labels (92), but even this approach is tedious when studying multiple GPL species (70). In this case a mass-spectrometric approach is much more convenient as it allows one to study a multitude of GPLs simultaneously. Here a high-throughput mass-spectrometric assay was developed to determine the effect of acyl chain length and unsaturation of GPLs on the rate of hydrolysis by three different sPLA₂s in micelles and vesicle bilayers.

Three different mixtures of synthetic GPL species were used. The first mixture (“PC-mix”) consisted of a total of 27 species i.e., (i) 10 disaturated species with the length of both acyl chains varying from 13 to 22 carbons; (ii) three 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) three 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 (C₉/16:0-PCs) consisted of 18 saturated PC species with a 16:0 sn-1 chain and a sn-2 chain of 6-24 carbons, and the third mixture (D₀-16:0/C₇-PCs) consisted of the positional isomers of the second one. Each mixture also contained, 21:0-SM, a non-hydrolyzable internal standard and 5 mol% of POPA (for it gives a negative charge to the macrosubstrate that helps increase the affinity of the enzyme). The lipid mixtures were presented to the enzyme either in the form of vesicle bilayers or detergent micelles.

8.1.1 Effect of the acyl chain length and unsaturation (“PC-mix”)

8.1.1.1 Micelles

Each of these enzymes showed different specificities with micelles, small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs). SUVs measure between 20 and 100 nm in size and LUVs between 100 and 400 nm. With bee sPLA₂, a decrease in hydrolysis rate took place with increasing acyl chain length both with saturated and diunsaturated PC sets. Similarly, hydrolysis by cobra sPLA₂ modestly decreased with increasing acyl chain length. In the case of porcine sPLA₂, this occurred mainly with the diunsaturated species. On the other hand, increase in the number of double bonds in the acyl chain led to an increase in the rate of hydrolysis by the bee sPLA₂ (Fig 5, left panel). A notable observation was that the PC species containing AA were hydrolyzed more rapidly than the
other PC sets. With cobra sPLA₂, substrate unsaturation did not have any effect on the hydrolysis rate except for those that contained 18:3 and 22:6 fatty acids. Again with porcine sPLA₂, substrate unsaturation had a major effect on the short di-unsaturated species and also on species containing 16:0/18:2 and 18:3/18:3 fatty acids.

8.1.1.2 Vesicle bilayers
The specificity profiles obtained with these three enzymes in SUV bilayers were very different from that in micelles. First with bee sPLA₂, there was a clear difference in the rate of hydrolysis that was far more pronounced than it was in micelles (Fig 5, right panel). For instance, the difference between the most slowly hydrolyzed and the most rapidly hydrolyzed species was ~100 fold vs. 10-fold in micelles. Substrate unsaturation seemed to have an effect on the rate of hydrolysis but then again AA containing species were hydrolyzed much faster as was observed in micelles. Analogous results were obtained with LUVs. As with cobra sPLA₂, the length of the acyl chain had a much stronger effect on the rate of hydrolysis with the saturated and diunsaturated PC sets than that in micelles. A similar trend was observed with LUVs. Also with porcine sPLA₂, the inhibitory effect of the increasing acyl chain length was much stronger in SUVs than in micelles. Collectively, these data indicate that the acyl binding sites for each of these PLAs differ from one another quite significantly, which is in agreement with crystal structure data (71, 215, 238, 239 and 248).
FIGURE 6. Hydrolysis of saturated and unsaturated PC species by bee, cobra or porcine sPLA$_{2}$s in octylglucoside micelles (Left panel) and SUVs (Right panel). Relative rates of hydrolysis are plotted against the total number of acyl chain carbons for several sets of PC species containing a fixed number of acyl chain double bonds.
8.1.2 Effect of \( sn-1 \) vs. \( sn-2 \) chain length

To get more information on the contribution of the individual acyl chains on the rate of hydrolysis, two different sets of saturated PC species, one containing a D\( _9 \) labeled head group and the other one unlabeled were studied. The length of either the \( sn-1 \) (C\(_n\)/16:0-PCs) or the \( sn-2 \) (D\( _\gamma \)-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 16:0/C\(_n\)-PC species contained a D\( _9 \)-labeled choline head group while the C\(_n\)/16:0-PC species were unlabeled, both sets could be present together thus eliminating the possibility of any matrix bias. Experiments with these positional isomers presented in micelles showed significant differences in the hydrolysis rates, i.e. the rate of hydrolysis decreased monotonously with the length of the \( sn-1 \) chain, while for \( sn-2 \) chain an optimum at C8 - C10 was observed for each of the 3 enzymes (Fig 6, left panel). These data suggest that those enzymes contain a binding site for the \( sn-2 \) chain, but not for the \( sn-1 \) chain thus allowing the \( sn-2 \) chain to interact more tightly. This conclusion is consistent with both crystallographic and NMR data from studies obtained from cobra sPLA\(_2\) complexed with substrate analogues (219, 300). In contrast to micelles, an identical effect was observed when the length of the \( sn-1 \) vs. \( sn-2 \) chain was varied in vesicle bilayers (Fig 6, right panel). There was no significant discrimination between the positional isomers, suggesting that translocation of the GPL substrate from the bilayer to the active site mainly determines the rate of hydrolysis of membrane-bound GPLs. This conclusion is in agreement with previous data on hydrolysis of cross-linkable or polymerizable lipids by venom sPLA\(_2\) (250, 308).

The key finding of this study is that efflux propensity seems to be a much more important factor in the hydrolysis of membrane-bound GPL molecules by sPLA\(_2\)s than previously thought. Accommodation of the GPL acyl chains at the substrate binding site of the enzyme also seems to contribute to the specificity of the sPLA\(_2\)s studied here, as indicated by the data obtained with micelles.
FIGURE 7. Hydrolysis of D_{9-16:0}/C_n-PCs and C_n/16:0-PCs in octylglucoside micelles (Left panel) and SUV bilayers (Right panel) by bee, cobra or porcine sPLA_{2}s. The relative rate of hydrolysis is plotted against the number of acyl chain carbons in the variable chain for both isomeric sets.
8.2 Substrate specificity of iPLAβ, a putatively involved player in GPL homeostasis

The high-throughput mass-spectrometric assay developed in the first study was implemented here to determine if efflux of the substrate molecule from the bilayer is the rate-limiting step in GPL hydrolysis by iPLAβ like in the case of sPLA2s (see above). A mixture of six 18:1/18:1 GPLs each with a different head group was also included in this study.

8.2.1 Effect of acyl chain length and unsaturation (“PC-mix”)

8.2.1.1 Micelles

We first studied the relative rates of hydrolysis of PC species with varying acyl chain length and unsaturation. With micelles as the macrosubstrate, the rate of hydrolysis decreased with increasing chain length which was observed across all the different PC series and an increase in acyl chain unsaturation increased the rate of hydrolysis markedly (Fig 7, left panel). In particular, the effect of an increase in unsaturation can be noticed in species with a total of 36, 38 or 40 acyl chain carbons that are hydrolyzed at a faster rate than their saturated counterparts. The cause for this could be in part due to specific interactions of such species with the substrate binding site on the enzyme.

8.2.1.2 Vesicle bilayers

As observed with micelles, an increasing acyl chain length led to a decrease in the rate of hydrolysis with vesicles. The effect of chain length was however more pronounced with vesicle bilayers (Fig 7, right panel). With the saturated series, the difference between the slowest and the fastest species was ~50-fold in bilayers while it was only ~7-fold in micelles. Increasing number of double bonds increased the rate in hydrolysis when the chain length was constant. These data strongly support the idea that acyl chain hydrophobicity is a key factor in iPLAβ-mediated GPL hydrolysis.

The differences between micelles and vesicle bilayers probably derive from different packing i.e. intermolecular interactions in these two macrosubstrates. MD simulations (183) and NMR studies (299) have demonstrated that the intermolecular packing in micelles is looser than in bilayers. In addition the efflux of the substrate molecule from micelles is several fold faster as compared to vesicle bilayers (205). These data support the idea that substrate efflux is the key determinant in the hydrolysis of membrane-bound PC species by iPLAβ.
8.2.2. Effect of sn-1 vs. sn-2 chain length

8.2.2.1 Micelles

To study how the variation of the length of the sn-1 or sn-2 chains independently affect the rate of hydrolysis, both the isomer sets, D_{9y}-16:0/C_{n}-PCs and C_{n}/16:0-PCs were incubated with iPLAβ. With micelles as the macrosubstrate, an increase in the length of the sn-1 chain, did not have any effect on the rate of hydrolysis at least until C12 and then a decrease in a logarithmic manner was observed (Fig 8, left panel). On the other hand increasing the sn-2 chain led to an increase in the hydrolysis rate first from C7-C8 and then declined in a rather monotonous manner. The ratio between the slowest and the fastest hydrolyzed species was ~14.5 and ~23.7 for the D_{9y}-16:0/C_{n}-PC and C_{n}/16:0-PC series respectively. It could be possible that the differences observed between the two sets of positional isomers might be due to the different properties of the sn-1 vs. sn-2 acyl binding sites of iPLAβ. Therefore, unlike the sPLA_{2}s, iPLAβ hydolizes both the acyl chains as has been suggested in previous works (1, 305).
8.2.2.2. Vesicle bilayers

With vesicles bilayers, while increasing the length of acyl chain in either \textit{sn}-1 or the \textit{sn}-2 position caused a dramatic decrease in the rate of hydrolysis (Fig. 8, right panel), no significant differences in hydrolysis were observed for either positional isomer. Such strong and monotonous decrease of hydrolysis further supports the notion that efflux of the GPL substrate from the bilayer is the rate-limiting step in the hydrolysis of membrane-bound substrates by iPLAβ. The fact that there were no significant differences observed between the positional isomers agrees with recent hydrogen/deuterium and homology modeling studies suggesting that efflux of the substrate from the membrane is necessary for its hydrolysis by iPLAβ (126, 127).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig9}
\caption{Hydrolysis of D\textsubscript{e}-16:0/Cn-PCs and Cn/16:0-PCs in TX-100 micelles (Left panel) and SUV bilayers (Right panel) by iPLAβ. Relative rates of hydrolysis are plotted against the number of acyl chain carbons in the variable chain for both isomeric sets.}
\end{figure}

8.2.3 Effect of GPL polar head group structure

In order to determine the effect of the polar head on the rate of hydrolysis, a mixture constituting six 18:1/18:1 GPLs each with a different polar head group was presented to iPLAβ in SUV, LUV or micelle matrices. With micelles, clear differences in hydrolysis were observed between the different GPLs (Fig 9). These differences probably reflect the structural properties of the active site of iPLAβ. In contrast, all the GPL species were hydrolyzed at similar rates in bilayers. To assess the accuracy of the effect of the head group, spontaneous intermembrane translocation assay was carried out as it is the best way to measure those effects. In this assay, the rate of transfer was determined by monitoring the movement of a lipid from two vesicle populations, from charged donor to neutral acceptor vesicles (143).
FIGURE 10. Hydrolysis of 18:1/18:1 PC, PE, PS, PI, PG and PA by iPLAβ in micelles, SUV and LUV bilayers. The data is normalized to that of the most rapidly hydrolyzed GPL in each system.
8.3 Substrate specificity of cPLA₂α, an enzyme releasing AA from GPLs.

A number of studies have demonstrated that cPLA₂α has selectivity toward arachidonoyl containing GPLs (41, 156). Here we studied to which degree this specificity is determined by accommodation vs. efflux. For this we included in this study also 20:4 containing molecular species GPL in two isomeric PC sets (Cn/20:4-PCs; n=6-24) and (20:4/Cn-PC; n=6-24).

8.3.1 Effect of the acyl chain length and unsaturation (“PC-mix”)

8.3.1.1 Micelles

In micelles, the unsaturation of the acyl chains of PC had very little effect on the rate of hydrolysis except when one or two 20:4 containing acyl chains were present (Fig 10A). The slightly faster hydrolysis of PCs containing two vs. one arachidonoyl chain may be due to faster efflux of the former species. This may also be the reason for the faster hydrolysis of the di-18:3 and di-22:6 species vs. the more saturated species with the same number of acyl chain carbons. Increasing the acyl chain length had no effect on the rate of hydrolysis except when the acyl chain was very long (Cn=44). Collectively, these data indicate that accommodation at the active site is more important than efflux for hydrolysis by cPLA₂α in micelles which is as expected. Results also indicate that the active site can accommodate two AA chains simultaneously.

8.3.1.2 Vesicle bilayers

In vesicle bilayers the rate of hydrolysis for species with 20:4 at the sn-2 position was slower (6-fold difference between micelles and vesicles) indicating that efflux is a more important determinant in vesicles (Fig 10B). Di-20:4 was hydrolyzed relatively much faster (than GPLs containing AA only at the sn-2 position) in vesicles vs. micelles suggesting that efflux is a relatively more important factor in the former case. Data from this experiment are in contrast to our earlier data from sPLA₂β and iPLAβ with which a stronger inhibitory effect with increasing hydrophobicity was observed (27, 101). This could probably be due to the reason that cPLA₂α penetrates more significantly into the bilayer in which case the substrate doesn’t need to efflux much in order to reach the active site which might not be the case with the other PLAs.
FIGURE 11. Hydrolysis of saturated and unsaturated PC species by cPLA₂α in TX-100 micelles (Top panel) and SUVs (Bottom panel). Relative rates of hydrolysis are plotted against the total number of acyl chain carbons for several sets of PC species containing a fixed number of acyl chain double bonds.
8.3.2 Effect of sn-1 vs. sn-2 chain length

Micelles
When no AA was present in a PC, no significant isomer preference was observed in micelles. Increasing the length of the saturated acyl chain in either position had hardly any effect on the rate of hydrolysis by cPLA$_2\alpha$ until C17, beyond which a rapid decrease in hydrolysis rate occurred thus suggesting that both the sn-1 and sn-2 acyl chains share a similar binding cavity equalling a length of a C17 acyl chain. This is similar to the result observed for iPLA$_\beta$ (27) except that there the hydrolysis decreased rapidly already beyond C12 (Fig 11A, left panel), thus indicating a shorter acyl binding site in that PLA. The results from the 20:4 containing PC isomer sets were remarkably different (Fig 11B, left panel). The species with 20:4 in the sn-2 position of PC were hydrolyzed approx. 4-fold faster than their positional isomers with 20:4 in the sn-1 position independent of the length of the acyl chain indicating regiospecificity while with the saturated positional isomers there seems to be “sloppy” binding i.e.; both the chains can occupy the same binding domain. These findings are consistent with previous data indicating that cPLA$_2\alpha$ prefers sn-1-saturated/sn-2-20:4 species in a regiospecific manner while binding of disaturated PCs is promiscuous (95, 173, 194 and 228). Hydrolysis dropped significantly when the sn-1 chain was longer that C16-C17, which may correspond to the length of the acyl binding site on the enzyme. All these data suggest that accommodation at the active site is the key determinant of hydrolysis of GPL substrate by cPLA$_2\alpha$ in micelles.

Vesicle bilayers
With the saturated PC sets, hydrolysis decreased strongly and monotonously in an identical fashion with the length of either acyl chain length except for one PC with a short C6 at the sn-1 position that deviated from this trend (Fig. 11A, right panel). These data are similar to those observed for the other PLAs studied and suggest that efflux of the GPL substrate is the key factor determining the rate of hydrolysis in vesicle bilayers. The rate of hydrolysis in the PC isomer set containing 20:4 also diminished with increasing chain length, underlining the fact that efflux is an important factor in the hydrolysis of those species as well (Fig 11B, right panel). However, the species with 20:4 in the sn-2 position were hydrolyzed much faster than their positional isomers (approx. 2 to 4 fold difference) indicating that, in addition to efflux there is more regiospecific orientation of the acyl chain probably allowing the AA double bonds have favorable interactions with certain aromatic residues in the binding pocket of cPLA$_2\alpha$ (194).
FIGURE 12. Hydrolysis of D_{9}-16:0/Cn-PCs and Cn/16:0-PCs (left, upper panel) and 20:4/C_{n}-PCs + C_{n}/20:4-PCs (left, lower panel) in TX-100 micelles by cPLA_{2}. Hydrolysis of D_{9}-16:0/Cn-PCs and Cn/16:0-PCs (right, upper panel) and 20:4/C_{n}-PCs + C_{n}/20:4-PCs (right, lower panel) in SUV bilayers by cPLA_{2}. Relative rates of hydrolysis are plotted against the number of acyl chain carbons in the variable chain for both isomeric sets.

8.3.3 Effect of GPL polar head group structure

Micelles
The rate of hydrolysis of six different 16:0/20:4 GPLs (PC, PE, PS, PI, PG and PI) by cPLA_{2} was compared in micelles. The fastest hydrolyzed GPL was PA while the remaining GPLs were hydrolyzed at much lower and almost comparable rates (Fig. 12A). Very similar data was obtained for the 18:1/8:1 derivatives. The reason could be that PA has a smaller head group than the ones in the other GPLs which helps in binding the GPL substrate to the active site. An earlier study indicated that cPLA_{2} has significant preference (approx. 4-7 fold) for PG over PE and PC (PA and PI were not present) in Triton X-100 micelles (173, 304) which is consistent with the present data.
Vesicles

Also in vesicle bilayers, PA was the most rapidly hydrolyzed GPL but then PG and PC were hydrolyzed significantly faster than the remaining three GPLs that had similar rates of hydrolysis (Fig 12B). These smaller differences between some GPLs in vesicles vs. micelles suggest that efflux propensity may be a more important determinant of hydrolysis of GPLs by cPLA$_2$α in vesicle bilayers. Efflux rate depends on the head group as indicated by our studies on spontaneous transfer rate (Study II). All these data indicate that the contribution of the head group is largely independent of the acyl chain composition.

**FIGURE 13.** Hydrolysis of 16:0/20:4 or 18:1/18:1 molecular species from different GPL classes by cPLA$_2$α in micelles (left/upper and lower panels) and SUVs (right/upper and lower panels). Data normalized to that of the most rapidly hydrolyzed GPL in each system.
8.3.4. Biological and clinical relevance

The present study provides novel information on factors determining the substrate specificity of PLAs that belong to different sub-families. Prior to these studies, the relative contributions of the PLA active site structure and substrate efflux propensity on GPL hydrolysis were poorly understood. Such information is necessary to fully understand the mode of action of various PLAs as well as to establish why PLAs preferentially hydrolyze certain GPL species. The secretory PLAs (sPLA2s) studied here are most probably involved in penetration of toxins to an animal body. While the biological implications of the substrate specificities of the sPLA2 studied here are not obvious, the rapid hydrolysis of 20:4 or 22:6-containing PC species by bee and snake sPLAs, respectively, might contribute to the toxicity of the venom by increasing the production of bioactive metabolites derived of the 20:4 or 20:6 fatty acids.

iPLAβ are involved in a number of cellular processes particularly in GPL homeostasis which depends on the coordination of GPL synthesis and degradation by a yet unknown mechanism. The present data indicate that efflux of the substrate from a membrane is critical for GPL hydrolysis by iPLAβ, which is intriguing considering that efflux propensity, has been predicted to depend critically on the ratio of different lipids. Thus the present data support the involvement of iPLAβ in GPL degradation (homeostasis) in mammalian cells. iPLAβ is also involved in several metabolic disorders in humans. The present study, by improving considerably on our knowledge on the mode of action and the active site structure could help design novel inhibitors to treat disorders involving an increased activity of iPLAβ.

Regarding cPLAα, our study shows that this enzyme hydrolyzes either the sn-1 or sn-2 ester bond of a GPL depending on the fatty acyl residues present in the substrate, and that this is most probably due to promiscuous (“sloppy”) binding of the substrate’s acyl chain to the active site of cPLA2α. This, along with other data on the structure of the active site of cPLAα should be very helpful in designing new, more efficient inhibitors for this enzyme which plays a key role in the release of AA, the precursor of prostaglandins and other second messengers in mammalian cells.

In summary, the present data should be helpful for basic research attempting to resolve the biological functions of mammalian PLAs. In addition, they should also assist in studies attempting to develop novel drugs targeting the PLAs involved in several common metabolic diseases or disorders.
9. CONCLUSIONS

Substrate specificities of different PLAs were studied in this thesis. The major conclusions drawn were:

1. In the first study, a mass-spectrometric high-throughput assay was developed which allows us to monitor the hydrolysis of a multitude of GPL molecular species simultaneously. Specificities of three different sPLA$_2$s were elucidated by this method. The key finding in this study was that efflux of the GPL substrate from the bilayer largely determines the rate of hydrolysis. In micelles, accommodation of the GPL acyl chains in the catalytic active site seems to contribute to substrate specificity.

2. Our second study gives the first experimental evidence that efflux of the substrate from the bilayer is the rate-limiting factor in the hydrolysis of GPL molecules by iPLA$_\beta$. We observed that the activity of the enzyme correlates inversely with the hydrophobicity of the substrate.

3. In the final study on cPLA$_2$α, the key finding is that accommodation of the substrate molecule in the active site of the enzyme is crucial for the preferential hydrolysis of AA-containing GPLs along with efflux of the substrate from the bilayer whereas in the case of GPL substrate lacking AA, efflux from the bilayer seemed to be determining the rate of hydrolysis.
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11. REFERENCES


3. Akesson, B., Elovson, J., and Arvidson, G. (1970) Initial incorporation into rat liver glycerolipids of intraportally injected (9,10-3H2)palmitic acid. Biochimica et Biophysica Acta 218, 44-56


Research 50 Suppl, S225-230


role for stored curvature strain energy. *Biochemistry* 40, 10522-10531


acyltransferase with broad substrate specificity. *Genes to Cells* **13**, 879-888


227. Ramanadham, S., Yarasheski, K. E., Silva, M. J., Wohltmann, M., Novack, D. V., Christiansen, B., Tu, X.,


complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2. The Journal of Biological Chemistry 277, 48535-48549


