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Mass-spectrometric analysis of glycerophospholipid metabolism

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CONTENTS

ORIGINAL PUBLICATIONS

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

I. Hermansson M, Uphoff A, Käkelä R, Somerharju P (2005) Automated quantitative analysis of complex lipidomes by liquid chromatography/mass spectrometry. *Anal Chem 77: 2166-2175.*

II. Hermansson M, Käkelä R, Berghäll M, Lehesjoki AE, Somerharju P, Lahtinen U (2005) Mass spectrometric analysis reveals changes in phospholipid, neutral sphingolipid and sulfatide molecular species in progressive epilepsy with mental retardation, EPMR, brain: a case study. *J Neurochem 95: 609-617.*

III. Kainu V, Hermansson M, Somerharju P (2008) Electrospray ionization mass spectrometry and exogenous heavy isotope-labeled lipid species provide detailed information on aminophospholipid acyl chain remodeling. *J Biol Chem 283: 3676-3687.*

IV. Haimi P, Hermansson M, Batchu KC, Virtanen JA, Somerharju P (2010) Substrate efflux propensity plays a key role in the specificity of secretory A-type phospholipases. *J Biol Chem 285: 751-760*

V. Hermansson M, Hokynar K, Somerharju P (2011) Mechanisms of glycerophospholipid homeostasis in mammalian cells. *Progress in Lipid Research 50: 240-257*.

In addition, some unpublished results are presented.

ABBREVIATIONS

BEL	bromoenollactone
BHK	baby hamster kidney (cells)
CID	collision-induced dissociation
CL	cardiolipin
D	deuterium
DAG	diacylglycerol
Da	dalton
EPMR	progressive epilepsy with mental retardation
ESI	electrospray ionization
GPL	glycerophospholipid
HeLa	human cervical carcinoma (cells)
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
MAFP	methyl arachidonoyl fluorophosphonate
MALDI	matrix-assisted laser desorption ionization
MPIS	multiple precursor ion scanning
MS	mass spectrometry
m/z	mass-to-charge ratio
NL	neutral loss
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phophatidylethanolamine
PEMT	phosphatidylethanolamine N-methyltransferase
PG	phosphatidylglycerol
PI	phosphatidylinositol
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
Q	quadrupole
SM	sphingomyelin
*sn*1	the first carbon of glycerol to which an alkyl chain is esterified/etherified
*sn*2	the second carbon of glycerol to which an acyl chain is esterified
SRM	selected reaction monitoring
TAG	triacylglycerol
TLC	thin-layer chromatography
TOF	time-of-fight
UPLC	ultra-performance liquid chromatography

ABSTRACT

This thesis consists of five parts. In the first part, an automated method for quantitative analysis of phospholipid compositions of cells and tissues by liquid chromatography-mass spectrometry was developed. In the second part, this method was applied to investigate brain lipid compositions of patients with progressive epilepsy with mental retardation (EPMR), caused by mutations in the *CLN8* gene. We were able to show major progressive alterations in brain lipid profiles of EPMR patients which may contribute to disease pathogenesis in those patients. In the third part, a novel approach to investigate the metabolism of single glycerophospholipid molecular species in living cells was developed. This approach was applied to study mechanisms of acyl chain remodeling, *i.e.* the exchange of fatty acyl residues, of aminophospholipids in BHK and HeLa cells. In the fourth part a novel mass-spectrometric approach was developed to investigate the substrate specificity of phospholipases and was utilized to elucidate the specificities of secretory A-type phospholipases in unprecedented detail. We showed that the specificity of those phospholipases depended mainly on the propensity of the substrates to efflux from the membrane and interactions between the substrate and the enzyme catalytic site are secondary. In the fifth part of this thesis, mechanisms of mammalian glycerophospholipid homeostasis were reviewed and novel theoretical considerations presented.

1. INTRODUCTION

Glycerophospholipids (GPLs) form the essential lipid bilayer of all biological membranes and are also intimately involved in signal transduction, regulation of membrane trafficking and many other membrane-related phenomena [1, 2]. GPLs are composed of a glycerol backbone with a polar head group attached to the *sn*3-position *via* a phosphate and hydrocarbon chains linked to the *sn*1 and *sn*2 hydroxyl groups (*cf*. Figure 1). GPLs can be divided into several *classes* (defined by the structure of the polar head group) the major ones being the phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylinositols (PI), phosphatidylserines (PS) and cardiolipins (CL) [3]. Each GPL class consists of numerous *molecular species i.e.* molecules which have the same head group but differ in respect of the hydrocarbon (alkyl) moieties. The alkyl chain at the *sn*2 position is always ester-linked to the glycerol moiety, but the one in the *sn*1 position can be linked *via* an ester, ether or a vinylether bond. Typical length of the chain varies from 14 to 24 carbons and the number of double bonds from 0 to 6. The positions and configurations of the double bonds vary considerably. The alkyl chain in the *sn1* position is typically saturated or monounsaturated, while that in the *sn*2 position is often polyunsaturated.

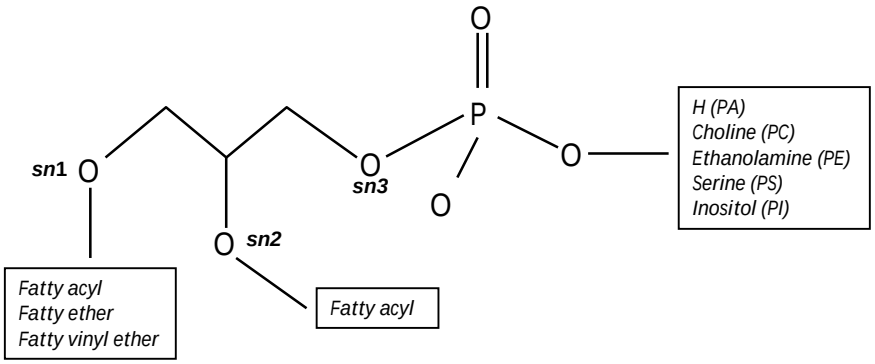


FIGURE 1. *General structure of glycerophospholipids*.

Because of the large number of different alkyl chain combinations, each GPL class consists of numerous structurally different molecular species and thus a eukaryotic cell contains thousands of different GPL molecules [4-7]. The meaning of such diversity is not fully understood, but probably relates to the multiple functions of GPLs [1, 3, 4]. GPLs are the major components of all cellular membranes, but their relative abundances vary significantly from one organelle to another [3]. For example, the inner mitochondrial membrane is enriched in PE, but contains very little PS due to the presence of the PS decarboxylase [8]. On the other hand, PS is abundant in the inner leaflet of the plasma membrane, where it may serve to activate membrane-associated enzymes, such as protein kinase C, as well as assist in membrane fusion [9]. Only CL and bismonoacylglycerophosphate are synthesized at the location they are found, *i.e.* mitochondria and lysosomes, respectively. All other GPLs have to be transported from their

site of synthesis in the ER or the Golgi to other organelles. The mechanisms and regulation of intracellular GPL transport are not well established.

Beside their role as key structural components of membranes, GPLs serve also several other important cellular functions. For example, the GPL precursor lysophosphatidic acid is a potent mitogen [2]. Many GPLs are involved in cellular signaling as sources of arachidonic acid, a precursor of prostaglandins and other leukotrienes [2]. Polyphosphoinositides regulate a plethora of cellular phenomena [10] and PI is part of the GPI-anchors which mediate the association of certain proteins to the outer leaflet of the plasma membrane [11]. Cardiolipin is necessary for proper functioning of the enzymes of inner mitochondrial membrane carrying out oxidative phosphorylation [12].

Higher eukaryotes maintain the concentrations of their membrane GPLs within narrow limits [13], implying that deviations from the optimum composition are deleterious. Consistently, hardly any GPL-related genetic diseases exist in humans, probably because mutations in the key enzymes catalyzing the key biosynthetic or degradative reactions are fatal. This conclusion is supported by the fact that knock-outs of the key enzymes of phospholipid biosynthesis are embryonic-lethal or compromise vital functions in mice [14]. In addition, many GPLs are synthesized via more than one pathway and many key enzymes are encoded by multiple genes [14], which indicates that during evolution mammals have developed alternative mechanisms to ensure adequate synthesis of each GPL under all conditions. Despite the vital importance of GPL homeostasis, information regarding the mechanisms underlying this phenomenon in mammalian cells is scarce. Four processes contribute to the cellular GPL homeostasis: 1) biosynthesis, 2) remodeling, *i.e.* exchange of a fatty acid (FA) residue, 3) degradation and 4) interorganelle transport. Little is known about the coordination of these processes. Such coordination is essential for instance to prevent futile competition between synthesis and degradation. Beside the complexity of the phenomenon itself (see below), understanding the regulation of the key processes has been hampered by the lack of methods allowing detailed and comprehensive analysis of the metabolism of different GPLs and their various intermediates. However, owing to its great resolving power and sensitivity, mass spectrometry (MS) has changed all this by allowing rapid and detailed analysis of complex biological samples. Especially when used together with stable isotope-labeled precursors, MS has opened completely new avenues for studies on GPL homeostasis.

The aim of this thesis was to develop mass-spectrometric methodology for analysis of glycerophospholipids and their metabolism in mammalian cells. These tools were then applied to investigate how mammalian cells maintain the GPL-compositions of their various membranes.

2. REVIEW OF THE LITERATURE

2.1. GLYCEROPHOSPHOLIPID HOMEOSTASIS IN MAMMALIAN CELLS

As discussed above, four different processes contribute to the cellular GPL homeostasis: biosynthesis, remodeling (exchange of fatty acid residues), degradation and interorganelle transport. I will next briefly discuss each of these processes.

2.1.1 Biosynthesis

The synthetic pathways for the major mammalian GPLs have been elucidated [15] and are depicted in Fig. 2. GPL biosynthesis is reviewed in depth in *publication V*, and thus discussed here only briefly. The first GPL in the pathway is phosphatidic acid (PA), which is synthesized from glycerol-3-phosphate *via* sequential acylation of the *sn*1 and *sn*2 hydroxyl groups by glycerol-3-phosphate acyltransferase and acylglycerol-3-phosphate acyltransferase, respectively. Beyond PA the pathway diverts to two main branches: one that leads to formation of the acidic GPLs *i.e.* PI, phosphatidylglycerol (PG) and CL *via* CDP-diacylglycerol; and the other where PA is dephosphorylated by a PA phosphatase (PAP) to diacylglycerol (DAG), which then serves as a precursor of PC, PE and PS. DAG can also be converted to triacylglycerol (TAG) which serves a reservoir or buffer of DAG and fatty acids.

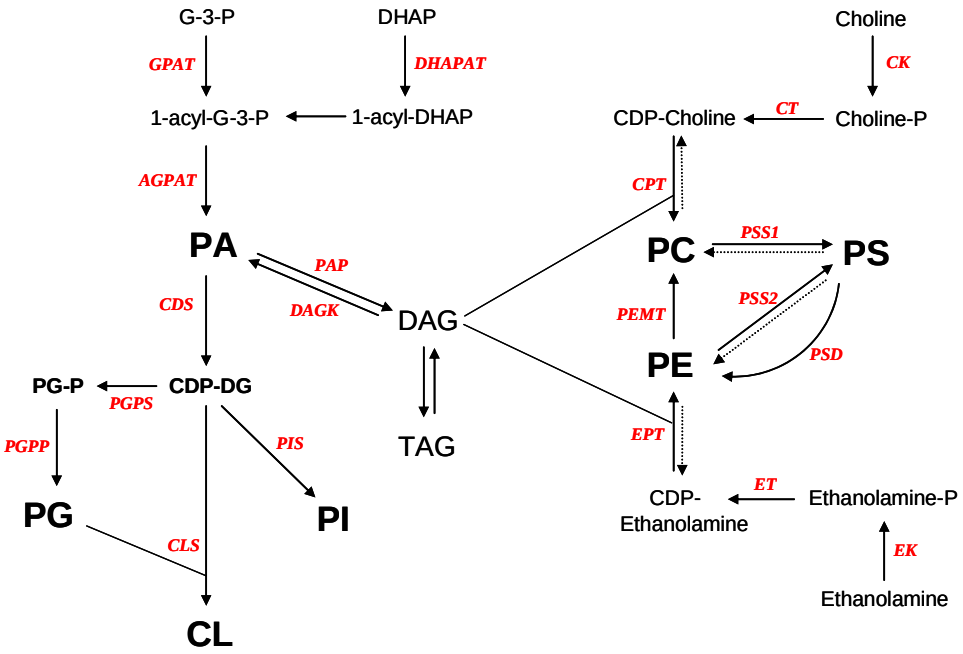


FIGURE 2. *Pathways of glycerophospholipid biosynthesis in mammalian cells.* The key metabolites (in black) and the enzymes catalyzing the respective reactions (in red) are indicated. The abbreviations are specified in *publication V*.

2.1.2. Acyl chain remodeling

After their synthesis *de novo*, most GPL species undergo a process called acyl chain remodeling, which involves phospholipase A-catalyzed removal of a fatty acid from either the *sn*1 or *sn*2 position of the glycerol and acyl transferase or transacylase -mediated reacylation of that position with a different fatty acid (Fig. 3A) [16-19]. GPL remodeling is involved in establishing and maintaining the specific molecular species composition of the various GPL classes. Acyl chain remodeling is required, *e.g.*, for arachidonic acid -dependent signaling [17], activation of enzymes in the inner mitochondrial membrane [20], alveolar surfactant synthesis [21, 22], exo- and phagocytosis [23, 24], repair of oxidized phospholipids [25, 26], and regulation of membrane "fluidity" [27]. Remodeling of CL has received particular attention because defects in this process compromise the functionality of mitochondria [12, 28, 29].

A-type phospholipases (PLAs) are key enzymes of GPL remodeling since they catalyze the committed step of this process. A multitude of PLA proteins exist [30], but the ones involved in GPL remodeling have not been identified. However, Ca^{2+}-independent PLAs (iPLAs) as well as cytosolic phospholipases (cPLAs) could be involved [31-34]. Beside the lack of information on the identity of the PLAs involved, it is unclear how these enzymes recognize the molecular species to be remodeled.

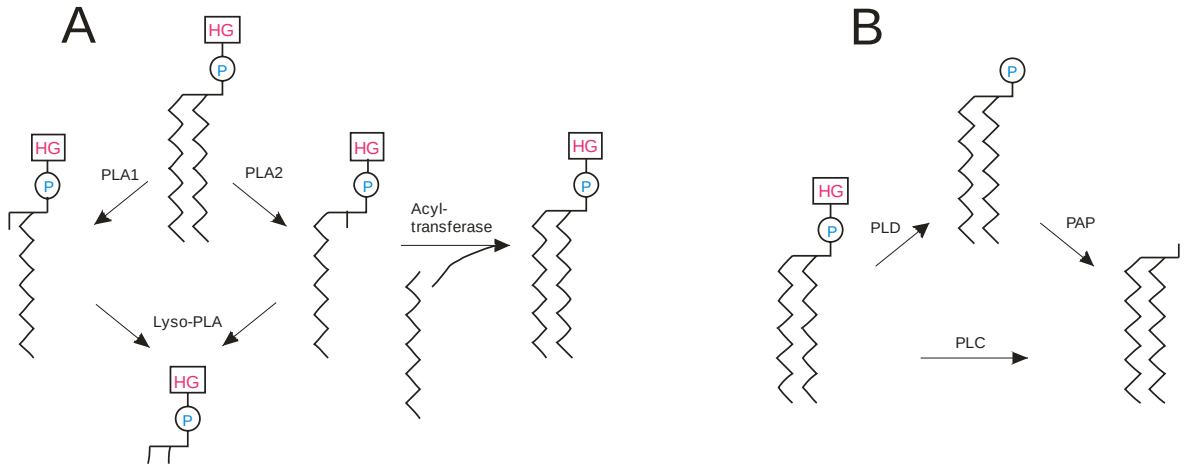


Figure 3. *Phospholipase -catalyzed reactions potentially involved in GPL homeostasis.* A. A-type phospholipases (PLA_1 or PLA_2) cleave either the *sn*1 or *sn*2 ester bond thus producing a lyso-GPL and a free fatty acid. The lysolipid is then either reacylated with a different fatty acid by acyltransferase to complete remodeling, or is degraded by a lysophospholipase to water-soluble lipid "backbone" such as glycerophosphocholine. B. Phospholipase D (PLD) cleaves the bond between the phosphate (P) and the head group (HG) thus producing phosphatidic acid PA, which can be dephosphorylated by phosphatidic acid phosphatase to yield DAG. PLC hydrolyzes the ester bond between the phosphate and the glycerol backbone thus yielding DAG directly.

The lyso-GPLs formed *via* the action of a PLA_1 or PLA_2 can be either reacylated by an acyl transferase to reform an intact phospholipid or they can be hydrolyzed by a lysophospholipase to a water-soluble GPL "backbone" and a fatty acid (Fig. 3A). It is a crucial but yet unresolved question how the fluxes *via* these alternative paths are regulated.

Two families of lysophospholipid acyltransferases that are involved in phospholipid remodeling have been identified (reviewed in [19]). These acyltransferases use acyl-CoA as acyl group donors and show varying specificity towards the acyl-CoA [35] and can also discriminate between lysophospholipids with an ester *vs.* alkyl or alkenyl-linked chain [35, 36]. Beside CoA-dependent acyltransferases, reacylation of lysophospholipids can be accomplished by CoA-independent acyltranferases and transacylases [17, 37, 38]. Little is known of the regulation of acyltransferases and other enzymes involved in acylation of lysoGPLs.

Despite its crucial role in several important biological processes, many aspects of glycerophospholipid remodeling remain unresolved. For example, to what extent does acyl chain remodeling contribute to the steady state phospholipid compositions of cells or their subcellular organelles? What are the molecular characteristics rendering a phospholipid molecule susceptible for remodeling, *i.e.*, how do the phospholipases involved recognize their targets? Are there specific phospholipases for each phospholipid class, or even for different species within a class? Which acyltransferases are involved? Where does the hydrolysis and reacylation take place? How are remodeling and degradation (turnover) coordinated? A key problem in resolving these issues has been the lack of suitable methods.

Early studies on phospholipid remodeling were carried out by incubating cells with radiolabeled GPL precursors *e.g.* fatty acids, glycerol, choline or ethanolamine. The radiolabeled molecular species were then separated by reversed-phase liquid chromatography, followed by chemical or phospholipase-mediated hydrolysis of the acyl residues which were then identified by gas-chromatography [16, 39-41]. Such methods are very laborious and insensitive and provided only limited information of GPL acyl chain remodeling. More recently, MS combined with stable isotope-labeled precursors has proven a convenient tool for such studies [24, 42-45]. However, even with this approach it is impossible to resolve reliably the remodeling pathways and kinetics individual species because numerous molecular species are labeled already during the pulse [42, 43, 46-48]. Thus, novel approaches are required to elucidate the mechanisms of GPL remodeling, including the identification of the specific phospholipases and acyltransferases involved.

2.1.3. Degradation

Degradation plays a major role in GPL homeostasis as indicated by rapid (and selective) turnover of GPLs. For example, the half-life of PC is only 2 - 4 h in actively proliferating cells [49, 50]. GPLs are also hydrolyzed in response to various extracellular stimuli to generate precursors for second messengers [10, 51, 52]. As compared to biosynthesis, surprisingly little is known about degradation of phospholipids. For example, the intracellular site, the enzymes involved

and their relative contributions to GPL turnover remain elusive. It is clear that phospholipids destined to lysosomes are degraded therein, but the contribution of the lysosomal pathway is rather low and thus most of GPL degradation is catalyzed by nonlysosomal phospholipases [53]. Depending on the bond they cleave (Fig. 3), phospholipases are divided in three groups, *i.e.* phospholipases A (PLAs), C (PLCs) and D (PLDs).

PLAs release the fatty acid in the *sn1* or *sn2* position of the glycerol moiety thus producing a lysophospholipid and a free fatty acid. The lysoGPL formed is either reacylated to form new GPL molecule, or is degraded by a lysophospholipase (Fig. 3A). PLAs form a large protein superfamily consisting of 15 different groups [54], and currently 24 different genes encoding for mammalian PLAs have been identified. PLAs play a crucial role a multitude of other cellular phenomena [30]. PLAs are key players in GPL remodeling [13] and probably in GPL class homeostasis as well, since much of GPL turnover seem to be mediated by PLAs [55, 56]. However, the specific PLAs involved here have not been established, albeit some Ca^{2+}-independent PLAs (iPLAs) have been implicated.

PLCs hydrolyze the bond between phosphate and glycerol backbone to produce DAG and a phosphorylated head group (Fig. 3B). PLCs are commonly thought to be involved in signal transduction [57, 58], rather than phospholipid homeostasis. However, some studies support the involvement of PLCs in GPL homeostasis. For example, Minahk and co-workers showed that when [^{3}H]-PC in LDL particles was taken up by rat hepatocytes, 50% of the cell-associated [^{3}H]-PC was hydrolyzed to [^{3}H]-DAG (presumably by a PLC) which was then converted to TAG [59]. Analogous results were obtained for [^{3}H]-PC taken up from HDL particles [60]. PLCs were also implicated in the turnover of PE and PC derived from exogenous [^{3}H]-lysoPC and -PE [61, 62].

PLDs hydrolyze the bond between the phosphate and head group of a GPL thus producing phosphatidic acid (PA) and a free head group (Fig. 3B). Like PLCs, PLDs are considered to be involved mainly in signal transduction [51, 52], and there seems to be no published data linking these proteins to GPL homeostasis. However, many PLDs catalyze *in vitro* transphosphatidylation reactions in which a GPL is converted to another with a different head group. Intriguingly, PS synthases catalyze a reaction analogous to PLD by replacing the group of PC or PE with serine [15] and also catalyze the reverse reaction. Thus they and other PLD-like enzymes could play an important role in GPL homeostasis by catalyzing the interconversions of different GPL classes.

2.1.4. Regulation of GPL metabolism

Remarkably little is known of how GPL synthesis and degradation are coordinated. Such coordination is essential to avoid futile (energy-wasting) competition between these opposite processes. As implied by Fig. 4, the maintenance of GPL homeostasis in mammalian cells is a formidable task because the biosynthesis and degradation of the many different phospholipid classes have to be coordinated precisely.

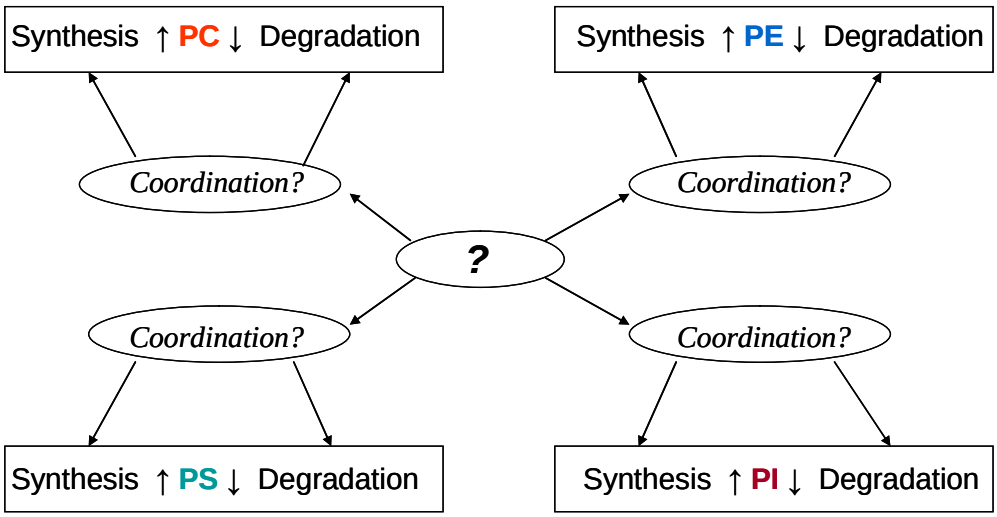


Figure 4. *Hypothetical model for the maintenance of cellular GPL class homeostasis*. The synthesis and degradation each GPL class could be coordinated by specific regulators, which in turn are coordinated by a "grand regulator" (noted by ?). The identities of the regulatory factors remain unknown. Reprinted with permission from *publication V*.

Compelling evidence for strict coordination of GPL synthesis and degradation comes from studies in which the synthesis of a GPL class was boosted by over-expressing the rate limiting enzyme. For instance, overexpression of *CTP:phosphocholine cytidylyltransferase* in HeLa cells increased phosphatidylcholine (PC) synthesis 4-5 fold, but the PC content of the cellular membranes remained essentially constant. However, greatly increased amounts of glycerophosphocholine, a deacylation product of PC (but not of other GPLs) were detected in the cells and the culture medium [62, 63]. Analogously, forcing the synthesis of PE or PS did not significantly increase the cellular content of the lipid, but increased their deacylation [64, 65]. Conversely, when the synthesis of a GPL is downregulated, its degradation decreases in proportion to maintain homeostasis. For example, in CHO mutants with partially inactive *choline kinase α*, the rate of PC synthesis was reduced 4-fold, but the PC content was normal [66]. Analogously, when PE synthesis in cells was strongly inhibited by mutating *ethanolaminephosphotransferase*, the PE did not decrease because its turnover was reduced in proportion [67].

The findings discussed above indicate that GPL synthesis and degradation in mammalian cells are strictly coupled. They provide compelling evidence that mammalian cells contain phospholipases which *selectively* degrade the GPL present in excess, consistent with the model suggesting that specific regulatory circuits exist for each GPL classes (Fig. 3). However, it is not obvious how those phospholipases are regulated so that they degrade only the GPL in excess, but not more. An answer to this intriguing question could be provided by the *superlattice model* to be discussed below.

Superlattice formation - It is possible that composition-dependent changes in the lateral organization of membranes regulates both phospholipid synthesis and degradation, thus coordinating these opposing processes. This idea is based on the predictions of a *superlattice model* (SL-model) of membrane organization (reviewed in [68, 69]). The SL-model is most relevant to GPL homeostasis for two reasons. First, the model predicts the existence of a limited number of "critical" (allowed) compositions, which are energetically more favorable than the intervening compositions. Accordingly, the lipid composition of a membrane has an intrinsic tendency to settle in one of the critical compositions, thus serving as a natural set point. Another important prediction of the SL-model is that when the concentration of a phospholipid exceeds a "critical" value, membrane packing defects appear, since the molecules in excess cannot be accommodated in the existing superlattice. The lipid species in excess would thus be forced to form separate domains and, consequently, domain boundaries with packing defects appear. It is well established that many phospholipases are strongly activated by bilayer packing defects (reviewed in [70]) and thus they could activate homeostatic phospholipases as well, thus leading to degradation of the lipid species present in excess. When the species in excess has been hydrolyzed, the defects disappear and the hydrolysis stops. Accordingly, SL formation could provide a highly accurate regulation and coordination of the putative homeostatic phospholipases acting on different phospholipid classes (Figure 4). Evidence for regulation of a venom PLA_2 by superlattice formation has been obtained [71]. Notably, the predicted abrupt changes in membrane organization could also regulate the activity of key biosynthetic enzymes [68]. The best evidence for that SL formation is involved in phospholipid homeostasis comes from a study showing that the phospholipid compositions of erythrocytes (and platelets) from different species coincide remarkably well with those predicted by the SL-model [72].

2.2. ANALYSIS OF GLYCEROPHOSPHOLIPID COMPOSITIONS

Since the discovery of phospholipids in the 19th century a multitude of different methods for their analysis has been developed. While most common methods are based on different forms of chromatography, *i.e.*, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC), also spectroscopic methods, especially nuclear magnetic resonance (NMR) spectroscopy, have provided important information about structure of phospholipids and membranes. During the past two decades, mass spectrometry (MS) has emerged as the method of choice for lipid analysis due to its high selectivity, sensitivity as well as simplicity. I will briefly review the main analytical approaches employed to investigate mammalian lipid compositions with a particular emphasis on MS.

2.2.1. Traditional methods

2.2.1.1. Thin-layer chromatography

Thin-layer chromatography (TLC) was one of the first methods used for phospholipid analysis and is still widely used [73]. In TLC the analytes are separated based on their differential partitioning between a liquid mobile phase and a liquid stationary phase on a solid support which usually consists of silica gel particles attached to a glass or plastic plate. The silica can be modified in various ways to improve separation of phospholipid classes [73]. The eluent is a mixture of organic solvents (usually chloroform and methanol) and water with various additives, *i.e.*, salts, acids or bases.

The sample solutions are applied as spots or bands on the TLC-plate which is then placed in chamber containing the solvent eluent. Phospholipid classes will migrate with the eluent and separate from each other due to differential partitioning between the mobile and stationary phases [74]. TLC can be carried out in either one or two dimensions. The latter uses two different solvent mixtures and provides better separation of the classes, but only one sample can be applied to a plate [73, 75].

Iodine vapor is probably most commonly used to visualize phospholipids on TLC plates, but does not work well with saturated lipids. It also covalently modifies the double bonds of unsaturated lipids, which precludes their further analysis by *e.g.*, gas-chromatography or mass-spectrometry [73, 76]. GPLs can be selectively visualized with phosphate stains, the ones based on a molybdate reagent being most common [75, 77]. Fluorescein or rhodamine stains can be used to detect GPLs by UV light. Charring is yet another common method to detect GPLs and other lipids on TLC plates [75, 78].

While TLC is a simple and inexpensive method to separate and quantify of phospholipid classes, it has several shortcomings. The main one is that it does not provide resolution of the individual molecular species. Second, hydrolysis or oxidation of the phospholipids can occur thus biasing the data [79]. TLC also has rather poor sensitivity and low resolution compared to other methods, particularly mass-spectrometry [77]. Finally, TLC cannot be readily automated, although robots for semi-automated lipid analysis exist [73].

2.2.1.2. Gas-liquid chromatography

Gas-liquid chromatography, or more commonly gas chromatography (GC), is another form of partition chromatography in which volatilized compounds are passed in a stream of a carrier gas through a column containing a high boiling point liquid (a stationary phase) on a solid support [80]. The compounds separate based on their differential partitioning between the mobile carrier gas and stationary liquid phase. The most common mode of detection is that based on flame ionization which is highly sensitive and has low background [81]. GC can also be coupled to a MS detector, which provides important additional information on the analytes [82, 83]. Obviously, GC can only be used to analyze compounds that can be vaporized without decomposition. Since GPLs are not volatile, their direct analysis by GC is not possible but they must be first hydrolyzed by PLC to diacylglycerol, which is then derivatized to form a more volatile compound [80].

Typically, GC has been used to determine the fatty acid composition of phospholipid classes after their separation by TLC [84]. While this approach is still in use, it requires large amounts of starting material, is time consuming and laborious. Hence most investigators use alternative techniques, particularly mass-spectrometry. The most useful application of GC today is the analysis of free fatty acids [83].

2.2.1.3 Liquid chromatography

Like TLC, liquid (column) chromatography (LC), separates the analytes based on their differential partitioning between a liquid mobile phase and a liquid stationary phase which resides on the surface of particles packed in a steel or glass column. Modern LC utilizes very small particles, which necessitated the use a relatively high elution pressure, and thus the term "high pressure (or performance) liquid chromatography" (HPLC) was introduced [85]. HPLC was first used to separate phospholipids in 1975 [86] and ever since a vast number of studies have used this method. Recently, so-called ultra performance liquid chromatography (UPLC) was introduced. In UPLC the particles are very small and thus a very high pressure has to be used to obtain a reasonable rate of elution. The major benefits of UPLC are short elution times and high resolution [87].

In LC two different separation modes are used: (*i*) normal phase LC, which separates GPL classes based on the structure of the head group and (*ii*) reverse phase LC (RPLC) which separates the molecular species (mainly) based on the hydrophobicity of the alkyl chains [88]. Full separation of GPL species is not possible with RPLC only, since the molecular species in different lipid classes (*e.g.* PC, PE and PS) tend to coelute. Thus prior separation of the classes by normal-phase LC is necessary, unless mass-spectrometric detection is available (see below).

Traditional LC detection methods are those based on absorbance, refractive index, light-scattering, electrochemical, suppressed conductivity and mass spectrometry [81, 89, 90]. Among these, evaporative light scattering has become popular because its response is nearly independent of the molecular structure [89]. However, MS detection is by far the most sensitive one and also provides structural information not obtainable with any other detection method. Thus, in current GPL analysis LC is mainly used in combination with a MS detector as discussed below (2.2.2.4).

2.2.1.4. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy allows elucidation of GPL structures, as well as their quantitative analysis in complex samples [91, 92]. A particular virtue of NMR is that it allows the analysis of GPL compositions of tissues *in vivo* [92, 93]. Another advantage of NMR is that it allows one to determine the position of the double bond in the alkyl chain and differentiate between *cis/trans* isomers [94]. NMR is also useful for characterizing lipid-lipid and lipid-protein interactions and dynamics in membranes [95, 96]. NMR is not commonly used to analyze complex lipid mixtures, mainly due to its low sensitivity and complication due to overlap of [^{13}C] and [^{1}H]-NMR signals. This problem can be partially circumvented by the use of heteronuclear single quantum coherence NMR which provides a 2-D lipid map [97, 98]. From such maps, differences between samples can be resolved and the differentiating compounds identified and quantified. Thus, heteronuclear single-quantum correlation NMR could be used for *e.g.* lipid biomarker discovery [97]. NMR studies have also been used to demonstrate that choline lipid metabolism is significantly altered in various tumors [99] and thus NMR-based lipidomics may be utilized in diagnosis [100].

2.2.2. MASS-SPECTROMETRIC LIPID ANALYSIS

Advances in mass spectrometry have revolutionized the analysis of lipid compositions of cells and other biomaterials by simplifying the analytical protocol dramatically and by increasing the sensitivity of detection by several orders of magnitude. Instrument development and intense research have brought this method to a level where identification of up to 1000 individual lipid species present in a single sample is possible [101-103]. Consequently, a new field of *lipidomics* emerged. Lipidomics is a branch of metabolomics that aims at the quantitative molecular characterization of the full lipid complement (known as a *lipidome*) of cells, tissues or whole organisms in order to determine the biological functions of those lipids [4, 5, 7, 104-106].

However, as eukaryotes are estimated to contain 10^4 to 10^5 individual molecular lipid species belonging to dozens of lipid classes [6], we are still far from reaching this objective. Two main analytical strategies have been adopted for lipid analysis by MS: (*i*) direct infusion MS (DI-MS) or the so-called "shotgun" approach and (*ii*) LC-MS using on-line LC separation prior to MS analysis [7, 107]. In addition, mass-spectrometric imaging of lipids in tissues has recently become available. These approaches will be discussed in more detail below, after introduction to the basic principles of mass spectrometry.

2.2.2.1 Principles of mass spectrometry

Mass spectrometry is based on separation of gas phase ions in magnetic and electrical fields. An MS instrument typically comprises of four components: *i*) an inlet for sample introduction, *ii*) an ion source to generate ions, *iii*) a mass analyzer and *iv*) a detector. In the ion source, molecules, unless already charged, are converted to positively or negatively charged ions. Then these ions are separated on the basis of their mass-to-charge-ratio (*m/z*) by the mass analyzer before being detected. The primary output of an MS instrument is a mass spectrum where the abundance (or intensity) of the ions are plotted as a function of their *m/z* (FIGURE 5). Additional dimensions to the data can be obtained by, *e.g.*, preseparation of the analytes by LC on-line (FIGURE 6), or by collision-induced dissociation of the analytes (see below).

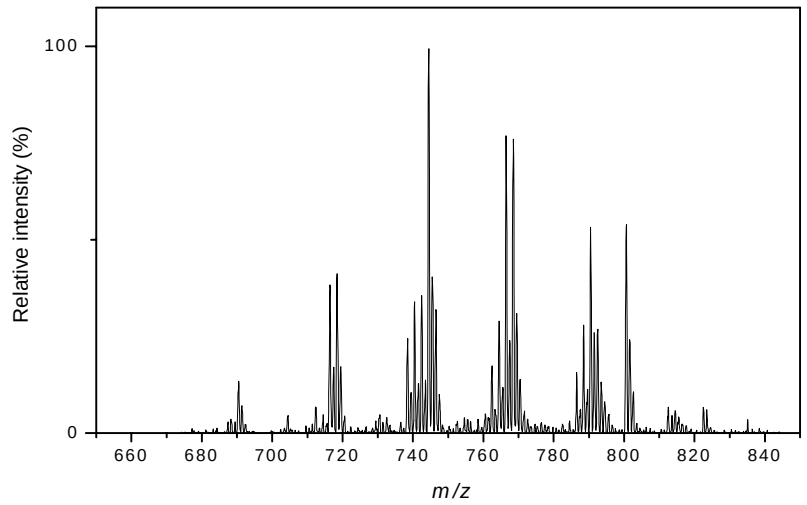


FIGURE 5. *A Mass spectrum.* The primary output of a mass spectrometer is a mass spectrum, where the *m/z* of recorded ions is displayed on the x-axis and their intensity (counts) on the *y*-axis. This spectrum was obtained from a HeLa cell lipid extract by NL141 scanning, which specifically detects diacyl PE species (see below). Unpublished data.

There are numerous types of mass spectrometers with different types of ion sources and/or mass analyzers. Electrospray ionization (ESI), atmospheric pressure chemical ionisation, matrix-assisted laser/desorption ionisation (MALDI) and electron ionisation are the most commonly

used ionisation techniques [108, 109]. The most common mass analyzers are the quadrupole (Q), ion trap, time-of-flight (TOF), Fourier transform ion cyclotron resonance and Orbitrap, each of which has its specific advantages [108, 110]. Typically lipid profiling is conducted on tandem mass spectrometers, which consist of two mass analyzers separated by a collision cell. Structural information on the lipids is obtained by fragmenting them (see below) by collision-induced dissociation (CID). The techniques used to record these fragmentation reactions are called tandem MS (MS/MS) or MS^n. Typical MS/MS instruments include triple quadrupoles and quadrupole-TOFs (QTOF). Ion-trap-type instruments are capable of multistage MS (MS^n), in which multiple consecutive fragmentations can be carried out. Detailed description of all types of ionization methods and MS-analyzers is out of the scope of this thesis, but can be found on recent books and reviews [108, 109]. I will briefly introduce the methods pertinent to the current work, namely electrospray ionization and triple quadrupole mass analyzer.

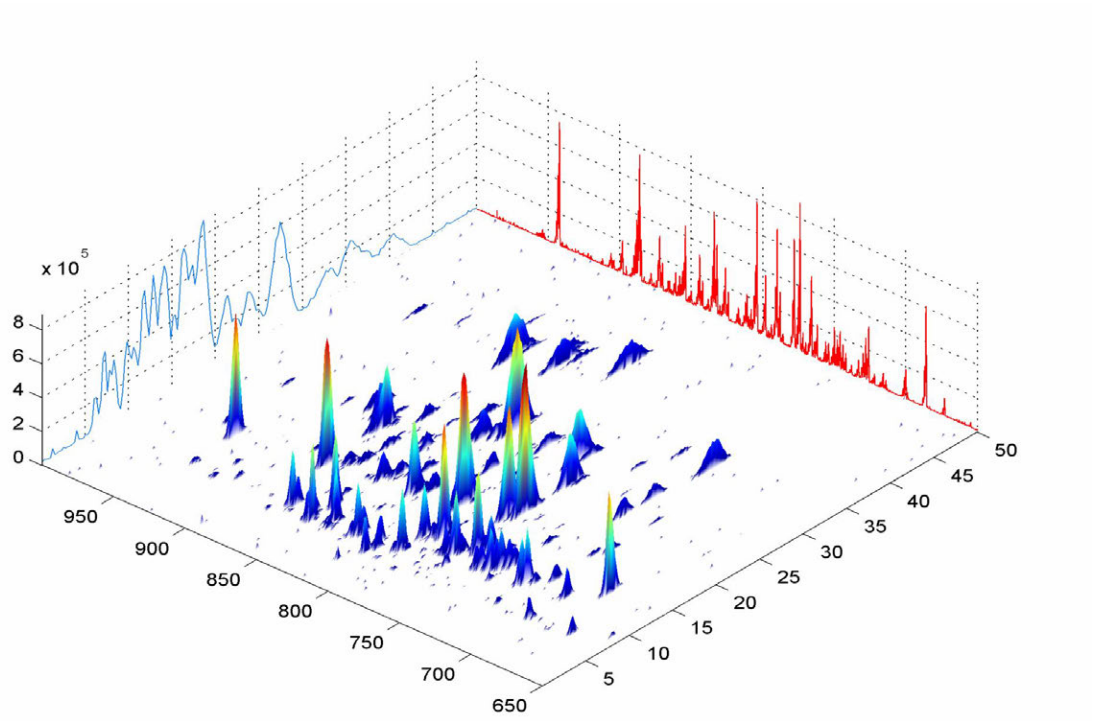


FIGURE 6. *A three-dimensional display of liquid chromatography-mass spectrometric dataset obtained from a mouse brain extract. m/z* is displayed on the x-axis, ion intensity on the y-axis and time on the z-axis. Projection of the time dimension (blue) = total ion chromatogram. Projection of the *m/z* dimension (red) = total ion spectrum. Reprinted with permission from (Hermansson et al. (2005), Anal Chem 77, 2166-75). Copyright (2005) American Chemical society.

Electrospray ionization (ESI) is the most commonly used ionization technique in mass spectrometric analysis of lipids [111]. In ESI, ions are generated at atmospheric pressure by introducing analytes in solvent through a small capillary set to high potential (voltage) relative to the MS analyzer entrance [109, 112]. A strong electric field drives formation of a fine spray of charged droplets [113, 114]. Evaporation of the solvent progressively increases the charge density at the surface of those droplets leading to their fission to smaller droplets and, eventually, gas phase ions which are pulled to the MS-analyzer by the electric field [113, 114].

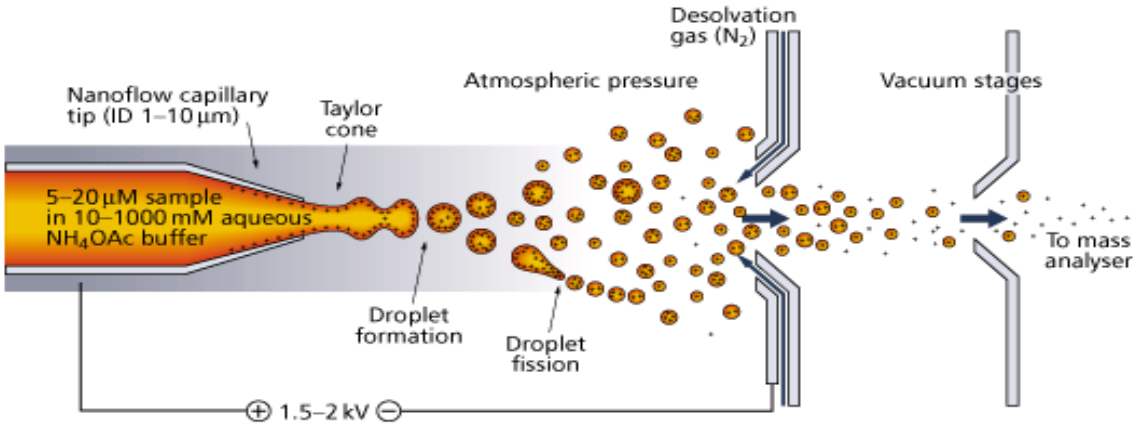


FIGURE 7. *The mechanism of electrospray ionization.*

Partitioning of the molecules between the surface and the interior of the droplets depends on their polarity and other properties [115, 116]. As the ionization process occurs at the surface of the droplet [117], such a behavior results in unequal ionization of molecules in complex mixtures. Furthermore, easily ionizable impurities, such as salts, detergents or other surface-active molecules can cause significant suppression in ionization of the analytes because such molecules tend displace the analytes from the surface of the droplets thus reducing the efficiency of their ionization [118]. This “ion suppression” complicates quantification, since the ion intensity does not depend solely on the concentration of the analyte, but is also affected by the concentrations of other molecules in the sample [115]. Beside impurities, also analytes can suppress the ionization of each other [117] and thus it is useful to operate at as low sample concentrations as possible.

In the positive ionization mode, ESI produces protonated molecular cations ($[M + H]^+$) or adducted cations (*e.g.* $[M + Na]^+$ and $[M + NH_4]^+$), while deprotonated molecular anions ($[M – H]^–$) and anion adducts (*e.g.* $[M + Cl]^–$ and $[M + CH3COO]^–$) are observed in the negative mode.

In the case of phospholipids, the zwitterionic PC, SM and PE are best analyzed as $[M+H]^+$ ions and the acidic phospholipids, PS, PI, PA, PG and CL as $[M-H]^-$ ions.

Triple quadrupole mass spectrometer has three quadrupoles (Q) in sequence (Figure 8). The first and third quadrupoles (Q1&Q3) act as mass filters that separate ions, while the second quadrupole (Q2) functions as a collision cell where ions can be fragmented upon collision with neutral gas molecules, *e.g.* argon. A quadrupole is composed of four precisely matched parallel metal rods (Figure 8). Direct current and radio frequency potentials are applied to these rods to produce an oscillating electric field. By varying the direct current and radio frequency potentials, ions of a specific *m/z* value is allowed pass through the quadrupole. Quadrupoles can be operated in three modes: 1) all ions with *m/z* within a certain range (*e.g.* 500-1000) can be transmitted simultaneously, 2) ions of a narrow *m/z* range (*e.g.* 1 Da) can pass the analyzer or 3) ions are sequentially passed through the quadrupole in small intervals (*e.g.* 0.2 Da), *i.e.* the analyzer "scans".

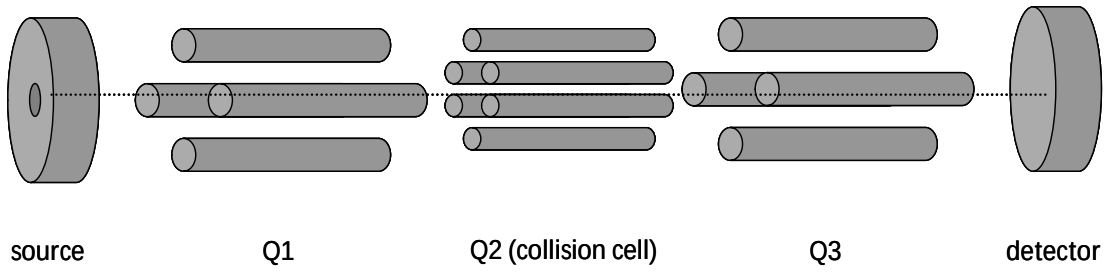


FIGURE 8. *A diagram of a triple quadrupole mass spectrometer.*

A triple quadrupole mass spectrometer is particularly well suited for lipidomics because it allows for several modes of MS/MS, including product ion, precursor ion and neutral loss scanning as well as selected reaction monitoring (Figure 9). In product ion scanning, an ion with a given *m/z* is selected by MS1 (Q1), fragmented in the collision cell by collision-induced dissociation (CID) and daughter (product) ions formed are analyzed by scanning MS2 (Q3). In precursor ion scanning, MS2 is set to transmit a defined fragment ion, *e.g.* a phosphocholine ion with a *m/z* of 184 while MS1 is scanned. In this way only the molecules that give the select fragment ion are detected, *i.e.* PC and SM in this case. In a neutral loss scan MS1 and MS2 are scanned in tandem with a constant offset, *e.g.* 141 Da, to detect the loss of the phosphoethanolamine group from PE. To obtain maximum sensitivity, selected reaction monitoring (SRM) can be performed. In SRM the MS1 transmits only the precursor ion of interest and MS2 transmits only a specific fragment ion. As neither quadrupole is scanned, time is not wasted on the acquisition of irrelevant ions and thus a high sensitivity of detection can be achieved. Multiple SRMs can be programmed in the instrument software, thus allowing for multiple reaction monitoring.

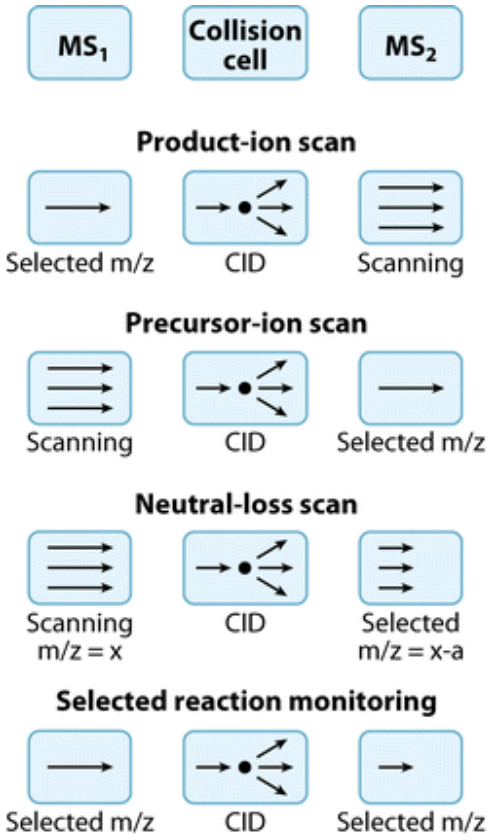
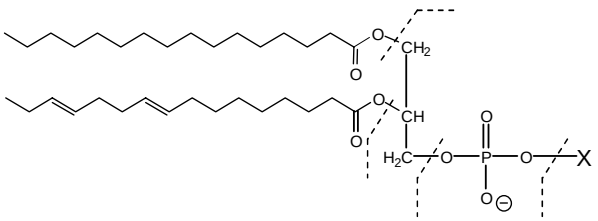


FIGURE 9. *The MS/MS modes available in a triple quadrupole mass analyzer.*

2.2.2.2. Fragmentation of GPLs

GPLs consist of a polar head group, the glycerol backbone and two alkyl chains and CID of GPLs creates many informative fragments (Figure 10). Those deriving from the head group are particularly useful since they allow for specific detection of the various GPL classes based on specific precursor ion or neutral loss scans. As noted above, CID of $[M+H]^+$ ions of PC generates a characteristic phosphocholine head group fragment ion with a *m/z* 184 and thus, PC molecules can be detected by scanning for the precursors of *m/z* 184 on a tandem MS instrument [119, 120]. However, since also SM gives this characteristic fragment, SM are also detected by such scans [119]. $[M+H]^+$ ions of PE and PS lose the head group (phosphoethanolamine and -serine, respectively) as a neutral fragment thus allowing their specific detection by scanning for neutral loss of 141 or 185 Da, respectively [119, 120]. Fragmentation of molecular anions of PI in the negative mode produces a negatively charged phosphoinositol fragment with *m/z* 241 [119, 120], thus allowing selective detection of PI by scanning for the precursors of *m/z* 241. Also PS can be detected in the negative mode by scanning for the neutral loss of 87 Da, *i.e.* serine. [119]. A specific scanning mode exists also for phosphatidylglycerol [121], but unfortunately not for *e.g.* PA or CL [121]. These lipids can be

detected by scanning for the precursor of glycerophosphate (*m/z* 153) in the negative mode, but also other negatively charged lipids are detected using this mode.



X	Phospholipid
Choline	Phosphatidylcholine (PC)
Ethanolamine	Phosphatidylethanolamine (PE)
Serine	Phosphatidylserine (PS)
Inositol	Phosphatidylinositol (PI)

Figure 10: *Fragmentation of glycerophospholipids by CID provides structural information and allows specific detection of most phospholipid classes.* The dotted lines indicate the most labile bonds from which fragmentation most frequently occurs.

Although the MS/MS scans described above allow for selective and sensitive detection of GPL species belonging to the various GPL classes, they do not provide direct information on the alkyl substituents or their *sn* positions, but only on the total number of carbon atoms and double bonds present in the molecule. Thus, such scans fail to distinguish between isomeric (*e.g.* species with same acyl substituents but in reverse *sn* positions, like 16:1/18:1 and 18:1/16:1) and isobaric species (*e.g.* species with same total carbon double bond number but different acyl moieties, like 18:1/18:1 and 18:0/18:2). Such information can be obtained by analyzing the products formed upon CID-induced cleavage of the ester bonds linking alkyl chains to the glycerol moiety (Figure 10). CID of molecular anions or anion adducts of GPLs yields three main types of product ions: *i*) fatty acid carboxylate anions, *ii*) lysoPL formed upon neutral loss of a fatty acid residue as a ketene and *iii*) a lysoPL-like lipid due to neutral loss of a free fatty acid [121]. The relative intensities of these fragments depend on the *sn* position of the fatty acid moiety in the glycerol backbone and can thus be used to assess their *sn* positions [121]. Identification of the positions of the double bonds in the GPL acyl chains by ESI-MS is generally not straight-forward. However, MS^n of GPL lithium adducts [122] and chemical derivatization by osmium tetroxide [123] or ozone [124, 125] have been successfully used to identify the double bond positions in phospholipids.

2.2.2.3. Direct infusion mass spectrometry

Direct infusion MS of a crude lipid extracts is often called “shotgun lipidomics”. Three principal shotgun approaches are commonly used. One of these is based on so-called intrasource separation of lipid classes [126], which takes advantage of the varying propensity of the different lipid classes to form ions in an electric field. By judicious choice of solvents, additives

and source polarity, all major lipid classes can be directly quantified without chromatographic separation. Tandem MS, *e.g.* product ion/neutral loss scanning, allows identification of the alkyl residues of the quantified species and estimation the ratio of isobaric molecular species [126].

Another commonly used approach relies on quantification of the GPL classes and species by head group-specific scanning modes *i.e.* precursor ion and neutral loss scanning discussed above [119, 120]. This methodology is highly sensitive for identifying and quantifying the major phospholipids, but does not allow selective detection of some lipid classes, *e.g.* PA, CL and neutral lipids, for which there is no specific detection mode based on fragmentation. Also, the method does not provide direct information on the fatty acyl constituents which need to be identified by separate product ion neutral loss scans.

The third shotgun approach is based on multiple precursor ion and neutral loss scanning (MPIS). MIPS is a powerful tool for analysis of GPL compositions, since it allows for simultaneous identification of the head group as well as the acyl substituents and their *sn* positions [127-129]. MPIS is nowadays often used together with automated chip-based nanospray ESI-source (Advion NanoMate) and a high resolution QTOF or Orbitrap tandem mass spectrometer [101, 130, 131]. The sample is infused at a very slow flow rate (few nl/min), which provides a reduced background noise and extended acquisition time, which allows for many products ion scans from a small sample. Nanospray ionization is also less prone to suppression effects, presumably because the smaller size of the droplets allows a larger fraction of the ions to occupy the surface and thus enter the analyzer [117, 129]. The product ion spectra can be searched for structure specific fragment ions or neutral losses that correspond to characteristic fragments of a lipid molecule, *e.g.* fatty acid or head group. The high resolution of TOF or Orbitrap mass analyzers allows the fragment ion or neutral loss to be accurately specified, which reduces misidentification of some lipids that could occur with instruments operating at a lower resolution [127]. The MPIS experiments produce very complex data sets which require specific software for the analysis [6, 128].

The shotgun approaches are relatively simple and have the advantage over LC-MS that the electrospray conditions remain constant, *i.e.* the solvent composition, matrix and sample concentration which can affect the ionization of analytes do not change during the run. Thus quantification of the lipids is more straightforward with this approach. However, this technique suffers from ion suppression and the inability to distinguish many isomeric or isobaric species. Consequently, if one is after the best sensitivity and selectivity, the LC-MS approach is often a better choice.

2.2.2.4. Liquid chromatography-mass spectrometry

Another commonly used approach for lipid analysis employs liquid chromatography with mass spectrometric detection (LC–MS) [132, 133] in which a HPLC (or UPLC) instrument equipped with a normal or reverse-phase column is coupled to an ESI-MS-instrument. A particular benefit of LC is that solvent entering the MS instrument at any given time contains only a few species,

which minimizes ion suppression and reduces interferences by isomers, isobars, and isotopes [7, 133]. This improves the sensitivity of detection as compared to DI-MS, thus allowing more facile detection of minor species. Another important advantage of LC-MS is that elution order of the analytes from the LC column provides information which can help in identification of lipids [132].

Normal phase LC provides separation of the GPLs based mainly on their head group [134-136]. Since all molecular species in a class eluted within a narrow time window together with the internal standards, accuracy of quantification is improved as compared to RP-LC where there is a wide spread of elution times in a class (see below). Usually, normal phase LC-MS is carried out in negative mode, which allows facile detection of PA, PE, PS, PI, CL as [M-H]- ions. PC and SM can be detected as formate or chloride adducts [134-136].

Reversed-phase LC separates GPL molecules based on their fatty acyl chains rather than the head groups. Thus RP-LC can separate more molecular species than normal phase [135]. However, there is often a major overlap between species in the different classes (and standards), which can significantly bias the data due to ion suppression effects. In the case that gradient elution is used solvent composition-dependent changes in ionization can also cause bias unless corrected for.

In LC-MS, GPLs are typically first quantified using MS scanning. Identification is then carried out in a second chromatographic run using MS/MS [137]. Alternatively, data-dependent acquisition on modern high-resolution instruments capable of MS^n [138] or MS^E [139, 140] allow for simultaneous quantification and identification of the GPL molecules in a single run.

A drawback of LC-MS analysis of GPLs has been that a rather long time (often more than an hour) is needed for the LC separation. However, the recent introduction of UPLC equipment has reduced the time of separation to 10 min or less, thus making the acquisition time comparable to that required the DI-MS based shotgun analysis.

2.2.2.5. Lipid imaging by MS

With modified MS instruments it is possible to image the distribution of numerous lipid species in cells or tissues. Several different ionization methods have been explored in lipid imaging, including secondary ion MS (SIMS), MALDI and desorption ESI [141-143]. SIMS has the best resolution (<50 nm) and has been utilized to investigate the transport of [^{13}C]-labeled oleate in an adipocyte [144]. Unfortunately, this method effectively fragments GPLs and thus imaging intact GPLs is currently not feasible [142, 144, 145]. However, cholesterol can be imaged with SIMS [145]. MALDI-based MS imaging does not allows imaging of subcellular compartments yet, but is readily applicable for imaging of GPLs and other lipids in tissue slices [143]. The distribution of lipids in brain and kidney as well as in an entire mouse has been studied by MALDI-imaging MS [143]. However, as with MALDI-MS in general, these data are biased due to

unequal ionization of the different lipid classes due to ion suppression by the matrix compound. Also Na^+ and K^+ adducts of the GPLs often interfere with data interpretation [143].

2.2.2.6 Quantification of GPLs by ESI-MS

Quantitative MS is best carried out using a stable isotope-labeled standard for each analyte [79]. This, however, is not feasible in global lipidomics since such standards are not available for each of the hundreds of different GPL species present in a typical mammalian cell. Consequently, accurate quantification of GPLs by ESI-MS is not straight-forward and several issues need to be considered. Typically, the intensity of ions deriving from any GPL species is not directly proportional to the concentration of that species. Several factors, such as the chain length and unsaturation of the acyl substituents and particularly the head group affect the instrument response [120]. In tandem MS, also the collision energy has a marked effect on the instrument response, [129, 146] and need to be accounted for. For accurate quantification of lipids by MS, it is essential to use internal standards for which the instrument response is similar to that of the analytes. For best accuracy, several standards for each lipid class should be included [120, 133]. According to some reports, phospholipid acyl chain length or unsaturation has no effect on the ionisation efficiency at low total lipid concentrations and, therefore, inclusion of a single standard for each lipid class would be adequate, provided that the sample concentration is low [126, 146, 147]. This, however, limits the number of species that can be reliably quantified.

Most of the elements have more than one stable isotope which differ in mass due to the presence of different number of neutrons. In particular, the abundance of the [^{13}C] is fairly high (1% of carbon isotopes) and thus the MS spectra of all organic compounds display multiple, major isotope peaks [148]. Therefore, it is necessary to correct the so-called [^{13}C] isotope effect, which varies with the number of carbon atoms present in the molecule [148]. Furthermore, many species within a lipid class are separated by only two mass units (one double bond), which leads to significant overlap of their isotopic patterns. For accurate quantification this overlap needs to be corrected for [148].

2.3. ANALYSIS OF GLYCEROPHOSPHOLIPID METABOLISM

Measuring of steady-state concentration of GPL in cells or tissues generally offers little information regarding their metabolism or its regulation (homeostasis). What are required are means to monitor time-dependent changes in the concentrations of GPLs and their metabolites. To achieve this, the investigator must use some kind of label and possess means to detect the label. Commonly used labels include radioisotopes, stable heavy isotopes and fluorescent tags. The benefits and drawbacks of each of these are briefly discussed next.

2.3.1. RADIOLABELING

Studies of Chaikoff and co-workers in the late 1930's [149-151] established radiolabeling as a powerful tool to investigate GPL metabolism. Since radioisotopes differ from normal atoms only in the number of neutrons present in their nuclei, compounds labeled with such isotopes are considered chemically identical to endogenous ones and thus radiolabeled GPLs are thought to faithfully report on the metabolism of the unlabeled molecules. However, the so-called isotope effect can cause deviating behavior in some cases. Radiolabeled compounds allowed Eugene Kennedy and co-workers to elucidate the biosynthetic pathways of GPLs [152-159] and has since been a key tool to investigate GPL metabolism, probably because their use is relatively straight-forward and does not require expensive equipment.

The most commonly used radioisotopes [^{3}H], [^{14}C] and [^{32}P] are used to label GPL precursors, which are then incubated with cells and become incorporated to GPLs and their metabolites. Typically head group precursors, such as choline, ethanolamine, or serine labeled with one of the isotopes is used to study GPL synthesis and turnover. Metabolism of the DAG-moiety of GPLs can be studied by radiolabeled glycerol, acetate or fatty acids.

The labeled lipids (and/or their metabolites) are separated usually by TLC (or HPLC) and detected by autoradiography [160], liquid scintillation counting [161] or a phosphorimager [162]. The latter two methods provide quantitative information on the radioactivity of the separated compounds.

The main benefit of radiolabeling is the sensitivity of detection [161], which allows one to use short labeling times. However, it should be noted that the limiting factor in metabolic studies is not the detection of radioactivity, but rather the determination of the chemical amount of the unlabeled species. This is because determination of the *specific activity* (*i.e.* units of radioactivity per mole of compound) is often required for meaningful interpretation of metabolic data. Although it is possible to label the cellular GPLs to an equal degree using long-term (>48 h) incubation with [^{32}P] [163], thus circumventing the need to determine their contents chemically, this approach has not gained popularity due to the short half-life of [^{32}P] and particularly the safety issues of the high energy β-radiation emitted by this radioisotope.

Radiolabeling is particularly problematic when studying the metabolism of GPL molecular species, since one has to separate the species by reverse-phase HPLC, collect fractions and determine their radioactivity and phosphate content, which is laborious and insensitive. An on-line radioactivity detector simplifies the process somewhat [164] [39], but dos no eliminate the sensitivity problem. An additional complication is that the radiolabeled precursors are often metabolized to various compounds which can then incorporate to GPLs via other routes. For example, while radiolabeled serine mainly incorporates to the head group of PS, also other parts of the PS molecule become eventually labeled due to metabolism of serine to acetate, formate and glycine, which are incorporated to the glycerol and fatty acid moieties of GPLs via multiple pathways [165, 166]. Tedious control experiments (including phospholipase mediated hydrolysis) are necessary to account for such "false" labeling. There are innumerable studies

using radiolabeled compounds to study GPL metabolism, but their review is outside the scope of this thesis.

2.3.2. FLUORESCENT LIPIDS

Fluorescent tags can be used to monitor GPL metabolism, but have found limited use for two main reasons. First, the fluorescent tag is bulky and can thus severely bias the data. There is no simple way to account for such deviating behavior. The other problem is that the fluorescent tag, such as nitrobenzoxadiazole (NBD), dipyrromethene difluoride (BODIPY) typically attached to an acyl chain, increases the polarity of the parent molecule to such a degree that they are rapidly degraded by (unknown) housekeeping phospholipases [167-171]. Pyrene is hydrophobic and thus GPLs with a pyrene-labeled acyl chain are not rapidly degraded [172, 173] and are metabolized similarly to their endogenous counterparts. For instance, a pyrene-PA was converted into diacylglycerol, triacylglycerol and PC and a pyrene-PS was decarboxylated into PE [173]. Also, pyrene-labeled fatty acids were readily incorporated to GPLs of BHK21 cells [174]. Nevertheless, the pyrene moiety can certainly lead to deviating metabolic behavior as indicated by the fact that phospholipases can be very sensitive to the structure of the GPL acyl chains (see 5.3 and 5.4). GPLs with an acyl chain with several conjugated double bonds in the acyl chain ("polyene-lipids") have been suggested to behave very similarly to natural ones [175], but this is uncertain as these derivatives have been employed only in few studies.

2.3.3. STABLE ISOTOPE-LABELED PRECURSORS

The most recent advancement in the analysis of GPL metabolism has been the utilization of stable heavy isotope labels, mainly [^{2}H] (deuterium, D), [^{13}C], [^{15}N] or [^{18}O]. Glycerol, fatty acids and head group precursors labeled with a stable isotope are commercially available at affordable prices, thus making stable isotope -labeling of phospholipids feasible. With tandem-MS both labeled and unlabeled GPLs can be determined in parallel by adjusting a single parameter, *e.g.* the product ion mass [176, 177] or the neutral loss (Figure 11). Hence, pre-existing and newly synthesized GPLs can be readily distinguished and quantified [178]. Furthermore, MS allows one to establish in which part of the molecule the label is located. Thus labeling of a "wrong" part of the molecule due to metabolism of the precursor *via* alternative pathways does not bias data interpretation, unlike when radiolabeled precursors are used. Most notably, however, stable isotope labeling allows facile tracing the metabolism of individual GPL-species, which is hard to achieve with the radiolabeling approach. Yet another advantage of stable isotope-labeling is that it is possible to perform lipid metabolic studies in human subjects as no obvious health issues are involved.

The first studies on GPL metabolism with stable isotope -labeling typically employed GC-MS analysis. For example, incorporation of [^{18}O] from $H_2{}^{18}O$ to the acyl carbonyl of GPLs was used to investigate acyl chain remodeling of GPLs in rat hepatocytes [179] and mouse macrophages

[180]. Analysis of [^{13}C]-acetate incorporation to fatty acids by GC-MS and thermospray-LC-MS was utilized to show that *de novo* hepatic lipogenesis is a quantitatively minor pathway in man [181]. PC and DAG turnover in HL60 cells was investigated by incubating cells with [^{13}C]-palmitate, followed by GC-MS analysis [182].

Considering the simplicity of studying stable isotope labeling of GPLs by ESI-MS/MS, this method has been adopted in surprisingly few studies. The first ESI-MS-based study on GPL metabolism utilized metabolic labeling with deuteriated choline and ethanolamine combined with specific MS/MS scans to demonstrate that the two pathways for PC synthesis (*cf.* Figure 1) produce different molecular species in rat hepatocytes [176]. A similar approach was adopted to study the substrate specificities of the enzymes of the respective PC-synthesis pathways in yeast [43, 183, 184]. The substrate specificity of human PC synthesis was investigated by MS/MS detection of labeled PC formed in CHO cells (over)expressing human CEPT [185]. PI and PC turnover and acyl chain remodeling in a variety of mammalian cell lines and tissues was investigated by pulse-chase labeling with deuteriated choline and inositol followed by ESI-MS/MS detection of labeled and unlabeled PI and PC species [48, 177, 186]. Binder et al. investigated the effect of cholesterol or low density lipoprotein-loading on PC, PE and PS synthesis in human fibroblasts using deuteriated head group precursors [42]. They showed PC synthesis was enhanced and synthesis of PE down-regulated in cells loaded with cholesterol, free fatty acid or lipoproteins. Loading also modified the molecular species profiles of the cells. Transport of PS from the endoplasmic reticulum to mitochondria in BHK cells was investigated using labeling with deuteriated serine and detection of labeled PS and PE species by ESI-MS/MS [187]. Kainu et al. used exogenous deuterium-labeled PS species to study transport of PS from the plasma membrane to mitochondria [188]. As noted above, stable isotopes can be used to probe lipid metabolism also in humans because of their (presumed) safety. For example, D_9-choline was used to investigate lung surfactant PC synthesis and turnover in human volunteers [189].

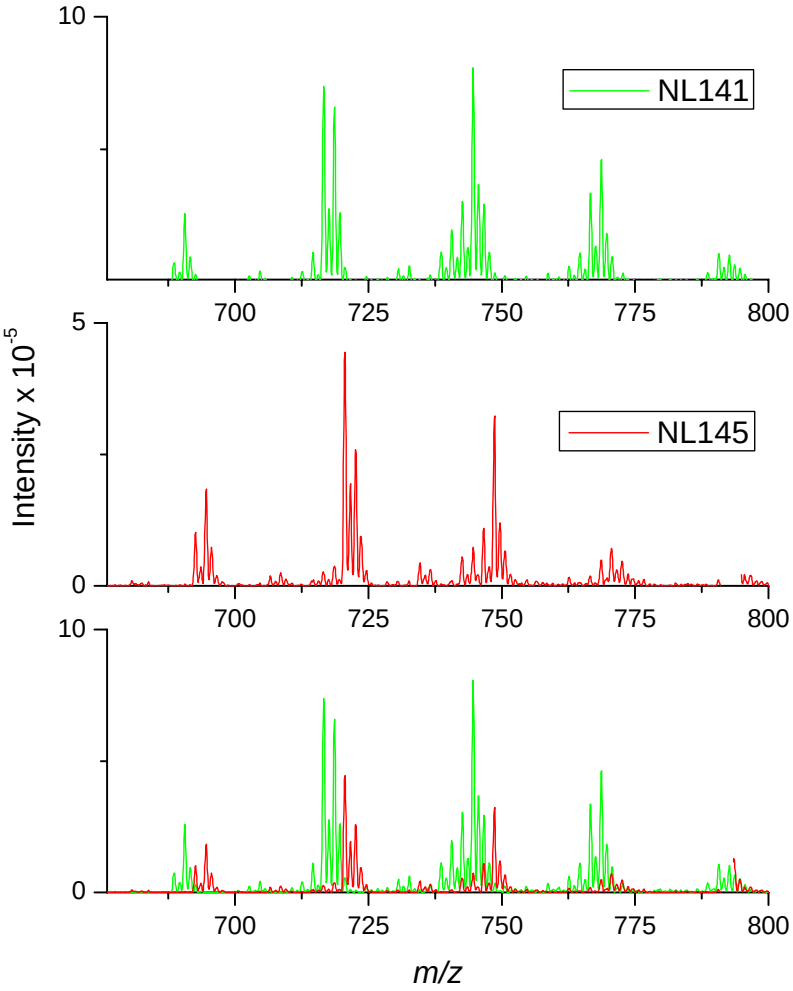


FIGURE 11: *ESI-MS/MS allows for simple detection of unlabeled and D_4-labeled PE species.* HeLa cells were incubated with 100 µg/ml D_4-ethanolamine for 8 hours, the lipids were then extracted and the extract was infused to an ESI-MS instrument. Unlabeled and D4-labeled PE-species were detected by using head group-specific neutral loss scanning. (A) NL141-spectrum corresponding to unlabeled PE. (B) NL145-spectra corresponding to D_4-labeled PE. (C) Overlay of the NL141 and NL145 spectra. Note that the D_4-labeled PE species are 4 mass units heavier than the corresponding unlabeled ones. Unpublished data.

3. AIMS OF THE PRESENT STUDY

The main goals of this study were to develop tools to investigate glycerophospholipid metabolism in mammalian cells. More specific aims were as follows:

- To develop an automated method for the analysis of phospholipid compositions of complex biological samples
- To test whether imbalance in brain lipid metabolism could contribute to the pathophysiology of progressive epilepsy with mental retardation
- To develop means to probe the glycerophospholipid acyl chain remodeling process in living cells
- To investigate the factors contributing to the substrate specificity of A-type phospholipases, which are presumably key players in maintenance of cellular glycerophospholipid homeostasis
- To draw together the current knowledge of the processes contributing to the maintenance of mammalian glycerophospholipid homeostasis to identify “blind spots” of current glycerophospholipid research

4. EXPERIMENTAL PROCEDURES

The experimental procedures used in the original articles and unpublished data are summarized in the table below. Either the reference or the number of the original publication in which the method has been described is indicated.

Method	Reference
Cell culture of CHO, BHK and HeLa cells	*I,III*
Labeling of cells with D_4-ethanolamine	*III*
Labeling of cells with exogenous deuterium-labeled phospholipids	*III*
Parameters used in mass spectrometry	*I, III, IV*
Liquid chromatography-mass spectrometry	*I,II*
Synthesis of MS standards	*I,III,IV*
Synthesis of deuterium-labeled PE and PS	*III*
Synthesis of deuterium-labeled PCs	*IV*
Synthesis of Cn/16:0-PC ands 16:0/Cn-PCs	*IV*
Lipid purification by HLPC	[88]
PLA specificity assay	*IV*
Lipid extraction	[190]
Protein determination	[191]
Phosphorus determination	[192]
Cholesterol determination	[193]

5. RESULTS AND DISCUSSION

The results of this thesis are presented in the four original research articles (I-IV) and one review (V).

The first paper (*I*) describes an automated method for analysis of complex lipidomes based on liquid chromatography-mass spectrometry. Application of the automated method is presented in the following paper (*II*), which reports alterations in cerebral lipid composition of patients with progressive epilepsy with mental retardation, EPMR.

In the third paper (*III*), a novel approach to study GPL metabolism in cultured cells was developed and applied to investigate acyl chain remodeling of PE and PS

The fourth paper (*IV*) provides novel information on the mechanisms underlying the substrate specificity of secretory A-type phospholipases

In the fifth publication (*V*) the mechanisms of GPL-homeostasis in mammalian cells are reviewed and discussed and additional theoretical considerations presented.

5.1. AUTOMATED QUANTITATIVE ANALYSIS OF COMPLEX LIPIDOMES (*I*)

At the time this work was initiated, it was becoming apparent that ESI-MS was becoming the method of choice in lipid analysis or "lipidomics". However, the data analysis methods were not up to par with the vast amounts of data produced by MS, but was rate-limiting due to the extensive manual work required. Consequently, we decided to develop an automated method that allows unattended identification and quantification of lipid molecular species of all the major lipid classes from a two-dimensional chromatographic/mass spectrometric data set (Figure 12). Such an automatic method was considered a necessity for any large-scale routine lipidomics application.

We chose to carry out the on-line chromatographic separations isocratically on a diol-modified silica column using hexane/isopropanol/water-based mobile phase with formic acid and triethylamine as additives. This system allowed the detection of all major glycerophospholipid classes and many sphingolipid classes, either as $[M - H]^-$ or $[M + HCOO]^-$ ions (Fig. 12A,B). In addition, it provided a nearly symmetric peaks (Fig. 12C) and a good retention time stability (±2%), which are very useful for a computerized data analysis.

The computerized data analysis software is a key novel feature of this study and consists of three main tasks: *(i)* finding and fitting of the relevant chromatographic signals, *(ii)* assignment of the signals to specific lipid species, and *(iii)* quantification of those species. These issues are now discussed in more detail.

The first task is to extract all relevant signals from the data. This is achieved by sequentially fitting a signal model (a mass-dependent generic isotope pattern) to the highest peak, subtracting the fit from the data, and repeating the process until all significant signals are detected. This is probably the first study to fit LC–MS data in its native two-dimensional format. Such fitting makes the deconvolution and integration of complex signal patterns more accurate than if only spectral data is used [136, 194]. This is because even when signals of different lipid species overlap in the mass dimension, they are usually (at least partially) separated in time (Fig. 12C). The quality of fitting obtained was very good (Fig 12E,F).

The second task the program carries out is the assignment of the signals (peaks) to lipid species. The assignment relies on the identification of at least two lipid species (reference compounds) in a reference chromatogram by the operator. The algorithm first finds the reference compounds based on (i) their given relative retention times within the respective class and (ii) the relative retention time order of lipid classes. In the next step, all signals that have the correct *m*/*z* and lie in the predicted retention time window are assigned to lipid species found in the database.

In the MS chromatogram shown in Fig. 12A, the automatic method detected 136 lipid species belonging to 11 different lipid classes (color coded in Fig. 12B). Among the 136 automatically assigned signals only 3 were found incorrect, while 2 signals were assigned to 2 alternative species one of which was the correct one. Tests of reproducibility showed that all of the species representing >3 pmol were assigned correctly at least 20 times out of 22 (*I, Fig S3*). Even many less abundant species (>1 pmol) were reliably found (≥19/22). Statistical analysis indicated that signals exceeding 10000 counts, *i.e.* ~2–4 pmol, were correctly assigned in >95% of the replicates (*I, Fig. S5*).

The third and final task of the software is to quantify the found (assigned) lipid species. For accurate quantification of GPLs by MS, it is essential to use internal standards for which the instrument response is as similar as possible to that of the analytes [120]. Previous studies had indicated that the response factor is very sensitive to variations in head group structure as well as acyl chain length and unsaturation, particularly at higher total lipid concentrations [120]. Thus, for best accuracy, several standards for each lipid class should be included. To avoid the complication of obtaining several standards for each lipid class, some investigators have chosen to operate at low total lipid concentration. While relatively accurate results could be obtained this way, we prefer to include several internal standards per lipid class since (*i*) a greater number of minor lipid species can be analyzed when using higher total lipid concentrations and (*ii*) and there is no need to strictly control the total lipid concentration. Yet another important benefit of several standards for this method is that they assist in the assignment of the analytes. The software first adjusts the signal according to the compound-specific isotope pattern by dividing the area of the first isotope by its theoretical relative abundance. Then an instrument response versus *m*/*z* -function is derived based on the intensities obtained for the internal standards [119, 120] and finally, this function is used to calculate the amounts of the analytes.

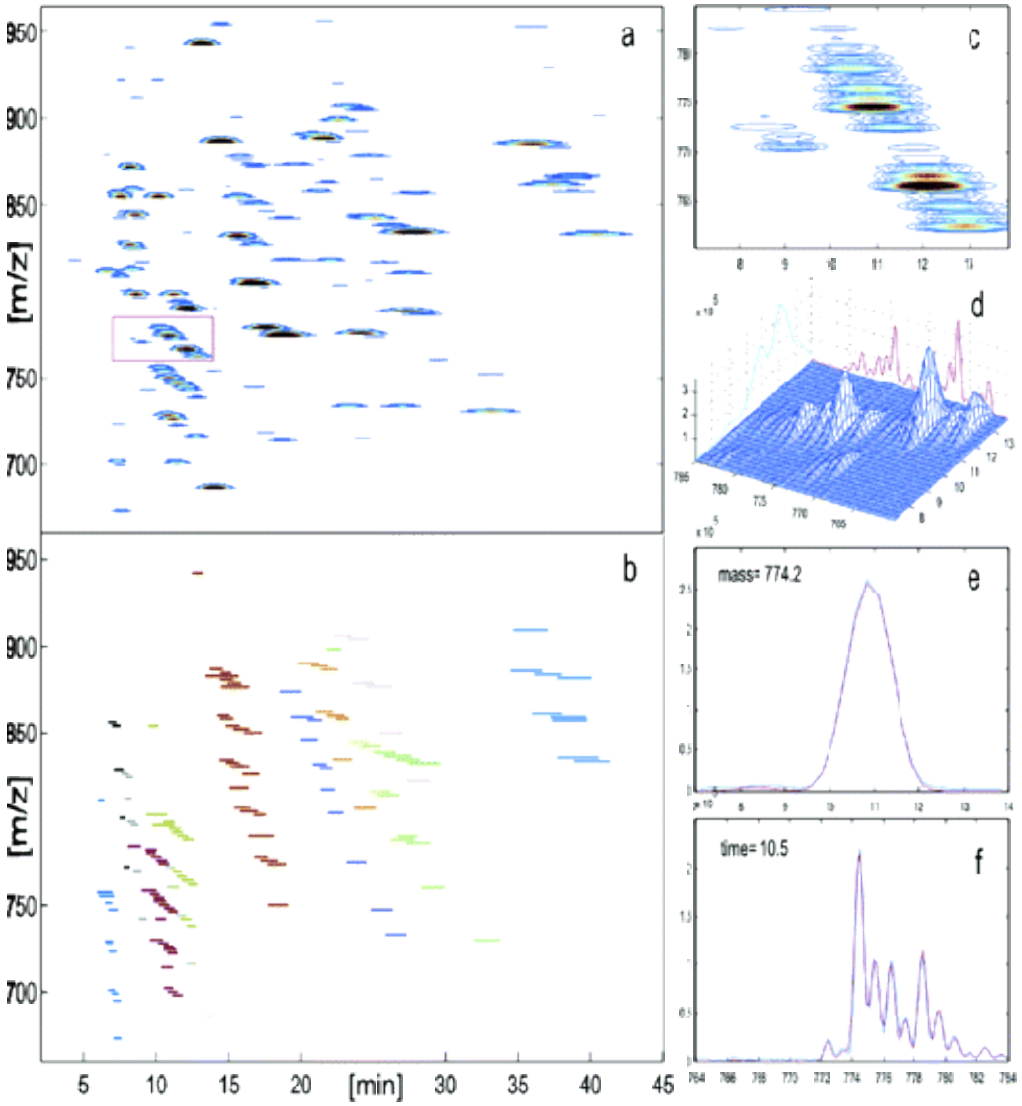


FIGURE 12.*Two-dimensional display of LC–MS data obtained for mouse brain lipid extract.* (a) Negative ion mode 2-D LC–MS data. (b) Assignment of the signals shown in panel a. The lipid classes from left to right are as follows: blue at ~7 min, PA; black, Galactosylceramide; gray, α-hydroxygalactosylceramide; violet, PE plasmalogens, green at ~12 min, PE; brown, PC; blue at ~25 min, SM; dark yellow, sulfatides; gray at ~28 min, α-hydroxysulfatides; green at ~27 min, PS and blue at ~38 min, PI. (c) Enlarged view of the region indicated by the rectangle in panel a. (d) A 3D view of the data in panel c showing the projections in the time (left wall) and mass (right wall) directions. (e) Recorded (blue line) and fitted (red line) ion chromatogram for *m/z* 774.2 (PE alkenyl-40:6) at 7–14 min. (f) Recorded (blue line) and fitted (red line) mass spectra at 10.5 min corresponding to the area shown in panel a. Reprinted with permission from (Hermansson et al. (2005), Anal Chem 77, 2166-75). Copyright (2005) American Chemical society.

The results obtained with mixtures of synthetic standards as well as with biological samples indicated that the automated method was both accurate and reproducible (*I, Fig. 2*). With the

MS instrument used, the detection limit was 2-4 pmol and the linear range covered more than 3 orders of magnitude (*I, Fig. 3*).

The automated method does not allow one to quantify isobaric species with different fatty acid residues or positional isomers. However, the data (*I, Fig. 4*) indicate that this would be possible, since partial in-source fragmentation of the GPLs produced abundant fatty acyl carboxylate anions and lyso-GPL fragments "coeluting" with the parent lipid, thus allowing identification of the fatty acid residues and their *sn* positions.

In conclusion, using the computerized method described in *publication I*, more than 130 polar lipid species could be automatically quantified from biological samples with good accuracy and reproducibility. The method was found to be generally applicable as samples of very different compositions could be analyzed reliably. Up to 35 samples could be analyzed in a day. Thus, this method would be useful particularly in studies requiring analyses of large sample sets. The main drawback of the method in its present form is that the software is command-line driven and thus requires some expertise in computing.

5.2. CHARACTERIZATION OF BRAIN LIPIDOMES OF EPMR-PATIENTS (*II*)

Progressive epilepsy with mental retardation (EPMR) is an inherited neurodegenerative disorder caused by mutations in the *CLN8* gene [195]. *CLN8* encodes a transmembrane protein that localizes to the endoplasmic reticulum. The function of CLN8 is unknown, but it is linked to a large eukaryotic protein family of TLC domain homologues with postulated functions in lipid synthesis, transport or sensing [196].

To investigate whether CLN8-deficiency caused alterations in the lipid compositions of the brain of EPMR-patients we applied the quantitative lipidomics approach described in *publication I* to analyze GPL, neutral sphingolipid and sulfatide compositions in cerebral samples of two EPMR patients with a progressive and advanced state of the disease as well as age-matched controls. The progressive state brain had reduced levels of ceramide, galactosyl- and lactosylceramide, sulfatide (*II, Fig. 1*) and cholesterol (*II*, see text), as well as a decreased levels of long fatty acyl chain -containing species (*II, Figs. 3-6*). In GPLs, an increase in species containing polyunsaturated acyl chains was detected, especially in PS and PE (*II, Fig. 2*). By contrast, saturated and monounsaturated species were overrepresented in PS, PE and PI in the advanced state brain (*II, Figs. 2 & S1*). These changes in brain sphingo- and phospholipid profiles could compromise membrane structure, vesicular trafficking or neurotransmission or could increase lipid peroxidation, and may thus contribute to the progression of EPMR. Consistently, a recent publication indicates that mice lacking *Cln8* have reduced levels of galactolipids and significant myelin defects in the brain [197].

5.3. AMINOPHOSPHOLIPID ACYL CHAIN REMODELING (*III*)

To investigate the mechanisms of GPL acyl chain remodeling we devised a novel approach which involves *1*) synthesis of GPL with a deuterium-labeled head group, *2*) introduction of a labeled GPL from donor vesicles to cells using of methyl-β-cyclodextrin as a carrier and *3*) ESI-MS/MS analysis of its metabolism. The key advantage of using intact exogenous GPLs rather than labeled precursors is that, initially, only a *single* labeled species is present in the cell. Thus the remodeling pathways and kinetics as well as turnover of the individual lipid species could be determined in a far more detailed manner than had been possible previously. Using this novel approach we conducted an extensive study involving >30 different exogenous PE and PS species to study GPL acyl chain remodeling in BHK and HeLa cells.

One of the key findings of this study was that all PE and PS species were remodeled in BHK and HeLa cells, but the extent of remodeling, as well as the turnover rates, varied markedly between species. Intriguingly, unnatural PE and PS-species (those *not* present endogenously), were remodeled rapidly at both *sn*1 and *sn*2 position, eventually to molecular species similar to the endogenous ones (*III, Figs 1,3,4*). For example, unnatural 14:1/14:1-PE and PS species were rapidly remodeled to natural species such as 18:1/18:1-PE and 18:0/18:1-PS, respectively (*III*, *Fig. 1&4*). In contrast, remodeling of exogenous species identical or similar to major endogenous ones was limited and much slower (*III*, *Fig. 1&4*).

Because PLAs catalyze the first, committed step of the remodeling process, these data mean that those PLAs recognize minor structural differences in the GPL substrate. Both PLA_1 and PLA_2 activities must be involved, since most exogenous PE and PS species were remodeled both at *sn*1 and *sn*2 positions, in agreement with earlier studies [40, 179]. A novel finding is that both the kinetics and the sequence of the *sn*1 vs. *sn*2 remodeling are very sensitive to the phospholipid acyl chain and head group structure. The remarkable selectivity of the PLAs is indicated by that very different remodeling pathways and kinetics were observed for PE and PS positional isomers, *i.e.* species containing identical fatty acids (*e.g.*, myristate and oleate) but in reversed *sn* positions. While 14:0/18:1-PE was slowly remodeled at the *sn*1 position to yield 18:1/18:1-PE (Fig. 13), its positional isomer 18:1/14:0-PE was very rapidly remodeled at the *sn*2 position, yielding a multitude of different species (Fig. 13). These data show that acyl chain remodeling and selective turnover play an important role in GPL homeostasis in mammalian cells. Interestingly, PE species containing saturated acyl chains at *sn*2 position were exceptionally good substrates for the remodeling PLAs, as such species were very rapidly remodeled (Fig. 13 *& III, Fig 3*). While the reason for this is currently unclear, this is consistent with the fact that mammalian PE does not contain saturated acyl chains at *sn*2 position [198, 199].

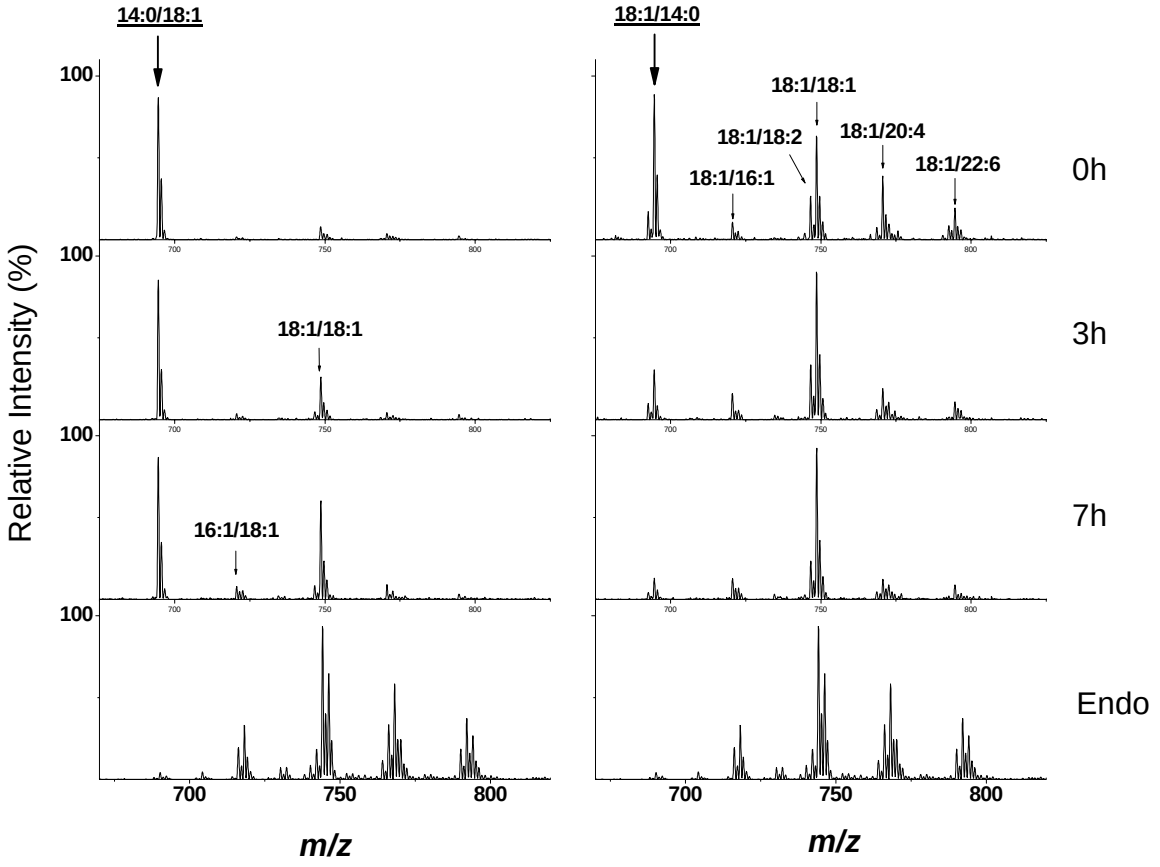


FIGURE 13. *Remodeling of exogenous 14:0/18:1-D_4PE 18:1/14:0-D_4PE species in BHK21 cells*. A D_4-labeled PE species was introduced into cells and then chased for up to 24 h as outlined in *Paper III*. After lipid extraction, ESI-MS/MS spectra were obtained using neural loss of 145 (*labeled species*) or 141 (*unlabeled species, endo*). The duration of chase is indicated on the *right*. The precursors are *underlined* in the *uppermost panels*. The spectra have been normalized to the highest peak, and the x-axis scales have been adjusted to facilitate comparison of the labeled and unlabeled species. Unpublished data.

Earlier studies have implicated several PLA_2 enzymes in the remodeling process [32, 34, 200, 201]. Our studies with pharmacological inhibitors indicated that a $cPLA_2$, $iPLA_2\beta$ and $iPLA_2\gamma$ are involved in the remodeling PE and PS (*III*, *Fig. 5&6*). However, also other, unidentified, PLAs seem to participate in this process. Consistently with our data, existence of head group-specific PLAs in mammalian cells have been obtained previously [24, 202]. However, results obtained with pharmacological inhibitors should be considered only tentative since they are not fully specific for a particular PLA [203-206]. RNA interference [207] is more specific and will be used in our future studies.

To investigate the enzymes involved in reacylation of lysoPS and -PE we studied the effect of Triacsin C, an inhibitor of acyl-CoA synthases [208]. Triacsin C inhibited remodeling of all PS, but

none of the PE species tested (*III*, *Fig 5&6*), thus suggesting that acyl-CoA-dependent acyltransferases are involved in remodeling of PS. Consistently, previous studies have shown that reacylation of the *sn*2 position of lyso-PS occurs by acyl-CoA-dependent acyltransferases [209, 210]. Notably, Triacsin C did not inhibit the remodeling of any PE species and thus reacylation of lysoPE is probably catalyzed by transacylases as also indicated by previous studies [17, 211].

The enzymes implicated in PL remodeling have been localized to several intracellular locations including the ER, the Golgi, mitochondria, nucleus and peroxisomes [30]. Thus it is an intriguing possibility that the organelles harbor a unique set of remodeling enzymes, which could contribute to their specific lipid compositions [3]. Such organelle -specific remodeling has been found for endonuclear PI and PC [177].

Previously several other methods, *e.g.* phospholipid transfer protein-mediated transport [212, 213] and vesicle fusion via virus receptors have been used to introduce lipids to cells [214], but these methods have various complications and have been rarely used. LysoGPLs are readily taken up by cells and reacylated therein [215]. They allow one to introduce phospholipids with a particular (labeled) head group to cells. However, the reacylation process is not fully selective regarding the acyl chain added to the lysolipid and therefore this approach does not allow introduction of a specific molecular species to cells. This is feasible by using methyl-β-cyclodextrin to carry intact exogenous GPLs from vesicles to cells as done in the present study. This method is very efficient, simple and gentle to cells [188] and will thus be helpful in future studies on cellular GPL metabolism.

5.4. PHOSPHOLIPASE SUBSTRATE SPECIFICITY (*IV*)

As indicated above, A-type phospholipases (PLAs) are key players in GPL homeostasis. Therefore, it is important to establish the factors determining their specificity. In the past, different approaches have been adopted to investigate the substrate specificity of these enzymes. Initially, vesicles consisting of a single phospholipid species were used the macrosubstrate. This, however, is not a good approach, since the rate of hydrolysis can reflects more the affinity of the PLA for the vesicles (which varies depending on phospholipid used), rather than the substrate specificity proper [216]. More reliable information can obtained by comparing hydrolysis of two lipids labeled with different radioisotopes and present simultaneously in the macrosubstrate [216]. However, this approach is not practical when a large number of species are to be studied. Also liposomes made of a mixture natural lipids have been used as the macrosubstrate [217, 218], but only limited information on the effects on acyl chain length, unsaturation and *sn* position on PLA activity can be obtained.

Mass spectrometry provides a powerful means to analyze PLA specificity by allowing one to study the hydrolysis of a multitude of species in parallel, as shown in a recent study where the hydrolysis of the phospholipids present in lipoprotein particles by secretory PLA was

investigated [219, 220]. Here, we used an analogous approach but used micelles and small and large unilamellar vesicles as the macrosubstrate.

The macrosubstrate contained mixture of PC species and 5 mol% of phosphatidylglycerol to provide negative charge increasing the affinity of the enzymes for the macrosubstrate. In addition, a sphingomyelin species was included as a nonhydrolyzable internal standard. Three different mixtures of PC species were studied. One consisted of 27 PC species with varying acyl chain length (13 - 22 carbons) and the number of double bonds (0 - 12). The other mixture consisted of 18 saturated PC species with a 16:0 *sn1* chain and a *sn*2 chain of variable number of carbons (16:0/Cn-PCs; n = 6-24), while the third one (Cn/16:0-PCs) consisted of the positional isomers of those in the second mixture. The Cn/16:0-PC species contained a D_9-labeled choline head group, while the 16:0/Cn-PC species were unlabeled. Both sets were present together in same vesicles or micelles, thus allowing us to study the hydrolysis of the positional isomers under identical conditions. After addition of PLA, aliquots were removed at intervals from the reaction mixture and concentrations of the individual PC species relative to the non-hydrolyzable SM-standard were determined by MS. (*IV, Fig. 1A*) shows exemplary data before and after incubation with *bee* PLA_2. Major differences in the rate of hydrolysis between PC species are obvious (*IV, Fig. 1B*).

Figure (*IV, Fig. 2*) shows the relative rates of hydrolysis of PC with varying acyl chain length and unsaturation by the three PLAs in octylglucoside micelles. Each enzyme responded differently to the number of acyl chain double bond and their position, presumably due differences in their substrate binding sites (*IV*, *Fig. 2*). Hydrolysis by bee PLA_2 increased markedly with increasing acyl chain unsaturation and the arachidonic acid -containing species were especially good substrates for this enzyme. The cobra PLA_2 preferred species with a 22:6 or 18:3 chain, while the porcine PLA_2 favored short diunsaturated species as well as the 16:0/18:2 and di-18:3 species. These data suggest that the acyl-binding sites of the three PLA_2s differ significantly, which is to be expected based on the 3-D structures of these proteins [221, 222]. Experiments with the positional isomers indicated that the length of the *sn2* chain was more critical than that of the *sn1* chain (Figure 14). Peaking of the activity when the length of the *sn2* chain is 9-10 carbons indicates that this equals the dimension of the sn2 chain binding cavity these enzymes, in agreement with previous crystallographic data [222, 223].

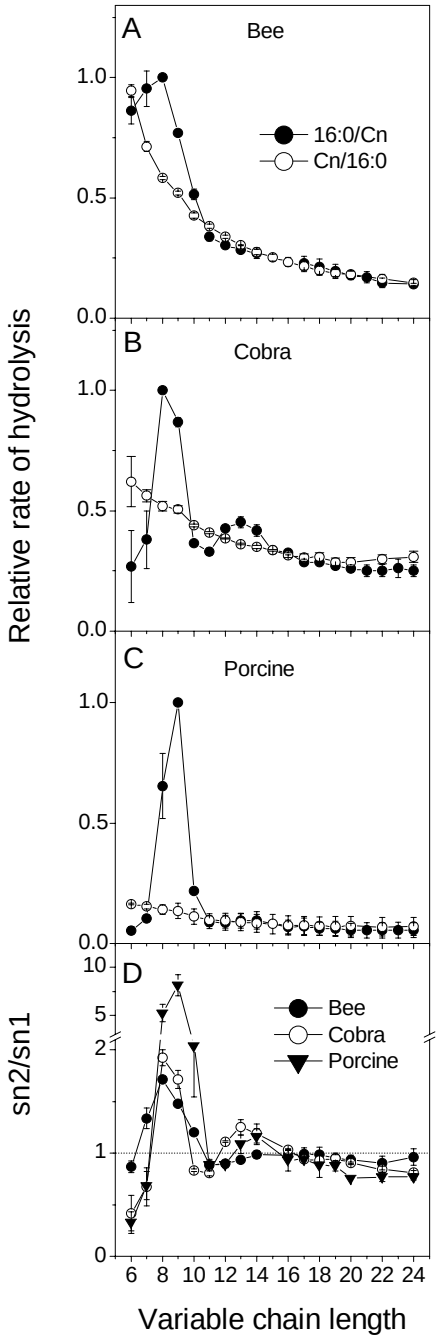


FIGURE 14. *PLA2-mediated hydrolysis of 16:0/C_n-PC and C_n/16:0-PC species in octylglucoside micelles vs. the length of the variable acyl chain.* D_9-16:0/C_n-PCs, C_n/16:0-PCs, POPG, and 17:0-SM were dispersed in 30 mm octylglucoside and incubated with bee PLA_2 (*A*), cobra PLA_2 (*B*), or porcine PLA_2 (*C*) at 37 °C. Relative rates of hydrolysis were determined as described in *paper IV* and are plotted against the number of acyl chain carbons in the variable chain for both isomeric sets. (*D*) The isomer preference was obtained from data in panels *A–C* by pairwise division of the rate of 16:0/C_n-PC hydrolysis by that of C_n/16:0-PC. The *dotted line* at y = 1.0 indicates the absence of isomer preference. The data shown are mean ± S.D. of three independent experiments. Reprinted with permission from original *publication IV*.

Intriguingly, the specificity profiles obtained with vesicles for the 3 PLA_2s differed markedly from those obtained with micelles (*IV*, *Fig. 3*). In particular, the difference between the slowest *vs*. fastest rate was far more pronounced (up to 100-fold) than in micelles (10-fold). The rate of hydrolysis of both saturated and diunsaturated PC species decreased strongly and monotonously with increasing acyl chain length. In addition, the hydrolysis increased with increasing degree of unsaturation (*IV, Fig. 3*).

Strikingly, no discrimination between positional isomers was observed with vesicles as the macrosubstrate, and hydrolysis decreased far more with increasing chain length than with micelles (*IV, Fig. 5*). This very strong dependency on the chain length as well as the lack of discrimination between positional isomers strongly suggests that the efflux of the phospholipid substrate is a key factor in PLA specificity acting on membranes. Supporting this, acyl chain structure influenced hydrolysis and spontaneous intervesicle transfer (which correlates with lipid efflux propensity) in an analogous manner (Figure 15). Studies with cross-linkable phospholipids support the notion that efflux propensity is a key factor regulating the hydrolysis of membrane bound substrates by secretory PLA_2s [224, 225].

We conclude that substrate efflux propensity plays a more important role in the specificity of secretory PLAs than commonly thought, and speculate that it could regulate the hydrolysis on GPLs by homeostatic phospholipases as well.

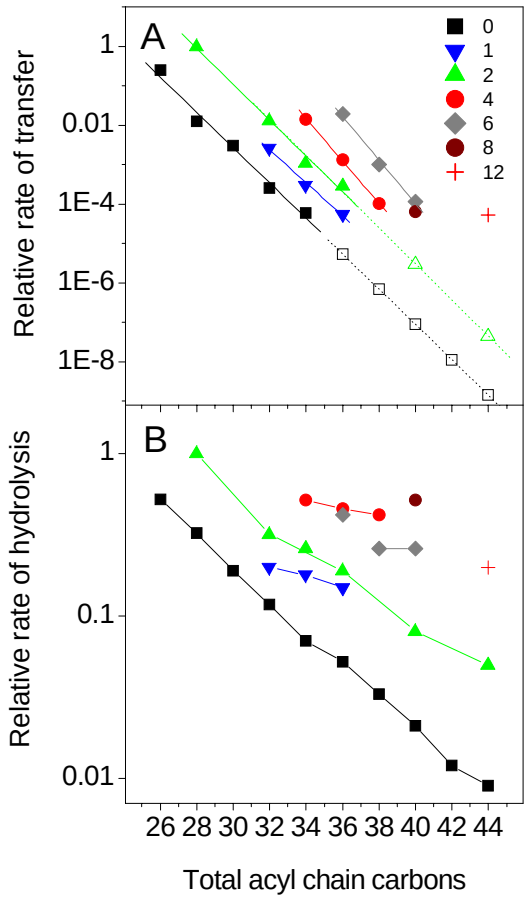


FIGURE 15. *Spontaneous intervesicle transfer of PC species correlates with their rate of hydrolysis.* (*A*) Relative transfer rates for the PC species were determined as described in *publication IV* and are plotted on a logarithmic scale against the total acyl chain carbons for species with different number of double bonds in the acyl chains. The *lines* are linear fits to data. The *dashed lines* are extrapolations. (*B*) Semilogarithmic plot of the rate of hydrolysis by bee PLA_2 of the species in PC-mix27 *versus* the total number of acyl chain carbons. Note that *the* y-scales are very different in *A* and *B*. Reprinted with permission from original *publication IV*.

6. CONCLUSIONS AND PERSPECTIVES

Methods for analysis of cellular glycerophospholipid compositions and metabolism were developed and the processes contributing to maintenance of cellular glycerophospholipid homeostasis reviewed. I will briefly sum the key conclusions and indicate future directions that may be taken based on these studies.

1) The developed automated liquid chromatography-mass spectrometric method was found effective and reliable for analysis of complex lipidomes from cells and tissues. Further development of the software to suit modern high-resolution mass spectrometers as well as inclusion of a user-friendly graphical interface would improve the usability and adaptability of the method.

2) Mass-spectrometric lipid profiling of cerebral samples from patients with progressive epilepsy with mental retardation (EPMR) revealed major progressive changes in neural lipids. Thus, impairment of brain lipid metabolism could contribute to the progression of the disease. Further studies on the function of the CLN8-protein are needed to establish whether impaired lipid metabolism is a primary or a secondary event in pathophysiology of EPMR.

3) A novel approach to investigate GPL acyl chain remodeling in living cells was developed. We showed that the remodeling pathways and kinetics of GPL remodeling are highly dependent on the acyl chain composition as well as the polar head group of the GPL molecule. The remodeling process involves multiple distinct phospholipases targeting both *sn*1 and *sn*2 positions and various acyltransferases and transacylases. The data obtained lays a basis for future studies to elucidate the specific enzymes involved in GPL remodeling, as will be detailed below.

4) A mass-spectrometric high-throughput assay to probe phospholipase substrate specificity was developed. As a proof-of-the-principle we elucidated the specificities of three secretory A-type phospholipases and showed that their specificity depends mainly on the propensity of the phospholipid substrate to efflux from the membrane while interactions between the substrate and the catalytic site are secondary. We speculate that this could be the case with homeostatic (unidentified) intracellular phospholipases as well. Once identified and available as relatively pure preparates the assay described here can be utilized to determine the specificities of the homeostatic phospholipases to gain insight to their mode of action.

5) Processes contributing to maintenance of glycerophospholipid homeostasis in mammalian cells, *i.e.* biosynthesis, degradation, acyl chain remodeling and intracellular transport, were reviewed. It was established that while the enzymes responsible for GPL biosynthesis are well elucidated, their regulation is still poorly understood, probably because many of them are transmembrane proteins, the purification and reconstitution of which is exceedingly difficult. Surprisingly little is known about GPL degradation and the enzymes involved

remain largely unknown despite the fact that over 50 different phospholipases have been characterized in mammals. Also, while it is established that synthesis and degradation are coordinately regulated, probably to prevent futile competition between these opposing processes, it is not understood how such regulation is accomplished. Finally, while intercellular GPL transport is essential for maintenance of the GPL compositions of organellar membranes, the mechanisms of this process are far from established. In conclusion, lipid scientists have plenty of work ahead.

6) Introduction of exogenous heavy isotope-labeled phospholipids to cells combined with mass spectrometry employing class-specific scans is a powerful tool to study phospholipid metabolism. As indicated by chapter *5.3*, explicit and highly detailed data on metabolism (*e.g.* remodeling and degradation) of a single GPL molecular species can be obtained by using this approach. Our subsequent studies indicated that the method is also very useful when studying intracellular transport of GPLs [188]. Furthermore, we showed that introduction of exogenous GPLs to cells using cyclodextrin as carrier enables extensive manipulation of the GPL composition of mammalian cells without compromising cell integrity or viability [188]. Such manipulation has not been possible previously. Thus, it can be envisioned that the approach, when combined e.g. with RNAi-based knock-down of various enzymes putatively involved in synthesis, degradation, remodeling and transport of GPLs, allows one to answer many of the open questions listed above.

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