Electrospray Ionization Mass Spectrometry and Exogenous Heavy Isotope-labeled Lipid Species Provide Detailed Information on Aminophospholipid Acyl Chain Remodeling

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Mammalian cells maintain the phospholipid compositions of their different membranes remarkably constant. Beside de novo synthesis, degradation, and intracellular trafficking, acyl chain remodeling plays an important role in phospholipid homeostasis. However, many key details of this process remain unresolved, largely because of limitations of existing methodologies. Here we describe a novel approach that allows one to study metabolism of individual phospholipid species in unprecedented detail. Forty different phosphatidylethanolamine (PE) or -serine (PS) species with a deuterium-labeled head group were synthesized and introduced to BHK21 or HeLa cells using cyclodextrin-mediated transfer. Their metabolism was then monitored in detail by electrospray ionization mass spectrometry. Atypical PE and PS species were rapidly remodeled at both sn1 and sn2 position, yielding a molecular species profile similar to that of the endogenous PE and PS. In contrast, remodeling of exogenous species identical or similar to major endogenous ones was more limited and much slower. Major differences in remodeling pathways and kinetics were observed between species within a class, as well as between corresponding PE and PS species. These data along with those obtained with pharmacological inhibitors strongly suggest that multiple lipid class-specific A-type phospholipases and acyl transferases are involved in aminophospholipid remodeling. In conclusion, the approach described here provides highly detailed information on remodeling of exogenously added (aminoglycerophospholipids and should thus be very helpful when elucidating the proteins and processes maintaining molecular species homeostasis.

Mammalian cells have been estimated to contain more than 1000 different lipid species (1). Whereas the reasons underlying such diversity are not fully understood, it is well established that the lipid compositions of cell membranes are maintained within close limits. This requires tight coordination of biosynthesis, degradation, and interorganelle trafficking. i.e. the processes that contribute to membrane lipid homeostasis (1). Acyl chain remodeling, i.e. exchange of fatty acid residues (2–5) is yet another process, which is considered to play an important role in phospholipid homeostasis of mammalian (6), yeast (7), and bacterial (8) membranes. Although the precise role of acyl chain remodeling in membrane phospholipid homeostasis remains unresolved, this process is known to be involved in several important phenomena including (i) arachidonic acid-dependent signaling (9), (ii) generation of cardiolipin species required for proper functioning of enzymes in the inner mitochondrial membrane (10, 11), (iii) induced exo- and phagocytosis (12, 13), (iv) synthesis of disaturated phospholipid species necessary for functional alveolar surfactant (14), (v) temperature adaptation of poikilothermic animals (15), and (vi) replacement of oxidized fatty acids of phospholipids (16–18). However, despite its crucial role in several important biological processes, many aspects of phospholipid 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/transacylases are involved? Where does the hydrolysis and reacylation take place? How are remodeling and degradation (turnover) coordinated?

A key problem in resolving these questions has been the lack of suitable methods. Traditionally, phospholipid remodeling has been studied using radiolabeled phospholipid precursors, such as glycerol, choline, or ethanolamine and reversed-phase chromatographic separation of the molecular species (5, 19). More recently, mass spectrometry has been proven to be a convenient tool for such studies (7, 13, 20–22). However, even when labeled precursors are employed, it is virtually impossible to determine the remodeling pathways and kinetics of individual species because numerous molecular species are labeled already during the pulse (7, 20, 23–25). Here, we describe a new approach that provides detailed information on remodeling of aminophospholipids and presumably other glycerophospholipids as well. We synthesized phosphatidylethanolamine (PE) and -serine (PS) molecular species with a deuterium-labeled head group, introduced them one at

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3 The abbreviations used are: PE, phosphatidylethanolamine; BEL, bromoenol lactone; CD, cyclodextrin; ESI-MS, electrospray ionization mass spectrometry; MAFP, methyl arachidonyl fluorophosphonate; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PLA, phospholipase A; SUV, small unilamellar vesicle; DMEM, Dulbecco’s modified Eagle’s medium; POPC, 1-palmitoyl-2-oleyl-phosphatidylcholine.
a time into BHK21 or HeLa cells using cyclodextrin-enhanced transfer from donor vesicles, and then monitored their conversion to other species using electrospray ionization mass spectrometry (ESI-MS) and head group-specific scanning modes (24, 26). Because initially only a single labeled species is present in the cell, remodeling pathways and kinetics could be resolved in unprecedented detail. As it is likely that the exogenous species are remodeled similarly to endogenous ones, at least qualitatively, this new approach should be very useful for the identification the specific phospholipases and acyl transferases involved in remodeling of (amino)phospholipids.

EXPERIMENTAL PROCEDURES

Lipids and Other Chemicals—Media and reagents for cell culture were obtained from Invitrogen, cholesterol, and other unlabeled lipids from Avanti Polar Lipids (Alabaster, AL), D3-serine and D3-ethanolamine from CIL (Andover, MA), [13C18]-oleic acid, methyl-β-cyclodextrin, hydroxylamine, MAFP, NH4Cl, Triacsin C, and phospholipase D (Streptomyces sp.) from Sigma. S-BEL and R-BEL were from Cayman Europe (Tallinn, Estonia) and the solvents (HPLC-grade) from Merck. The PE and PS species with a deuterium-labeled head group were synthesized from corresponding phosphatidylcholine species and D3-ethanolamine or D3-serine, respectively, using phospholipase D-mediated transphosphatidylation as described previously for unlabeled lipids (27) except that the reaction volume was reduced 5-fold. The products were purified by normal phase HPLC (28), and their purity was confirmed by mass spectrometry. PE and PS labeled both to the fatty acid and head group were prepared by acylating sn1-oleyl- or sn1-palmitoyl-lysoPC by [13C18]oleate (29) followed by conversion of the sn1-oleoyl- and sn1-palmitoyl-[2-13C18]oleoyl-PC to corresponding PE or PS species as described above. The concentrations were determined by phosphate analysis (30).

Cell Culture—Baby hamster kidney (BHK21) cells were grown as previously (31). HeLa-cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Mycoplasma contamination was excluded using an ELISA method.

Introduction of Labeled PS and PE Species to Cells—Cholesterol, 1-palmitoyl-2-oleyl-phosphatidylcholine (POPC), a labeled PE or PS species, and di-22:0-phosphatidylcholine (5:4 1:0.1, mol/mol) were mixed in chloroform, and the solvent was evaporated under a nitrogen stream followed by high vacuum for 1 h. 1 ml of PBS was added, and the sample was probe-sonicated for 3 × 2 min with 30-s intervals. The sample was then centrifuged for 5 min at 3000 × g to pellet any undispersed lipid and probe particles, and the small unilamellar vesicles (SUVs) in supernatant were used as the donors in cell labeling. Cells grown to ~80% confluency on 6-cm dishes were washed twice with DMEM, SUV’s (1 µmol lipid), and methyl-β-cyclodextrin (m-β-CD) (8 mM) in 2 ml of DMEM were added, and the cells were incubated for 1 h at 37 °C. After washing three times with DMEM, the cells were chased in DMEM with or without 10% fetal calf serum up to 24 h, washed with phosphate-buffered saline, scraped into 0.25 M sucrose, and moved to silane-treated screw cap tubes. The lipids were then extracted according to Folch et al. (32), except that the solvent contained 0.1 M HCl. For quantification of the lipid species by mass spectrometry, a mixture of internal standards was added at the one-phase stage of extraction (33). After evaporation of the solvents, the lipids were redissolved in chloroform/methanol (1:2, v/v) and stored at −20 °C.

Mass Spectrometry and Data Analysis—After addition of aqueous NH3 (4%), the sample was infused (6 µl/min) into a Micromass Quattro Micro triple-quadrupole mass spectrometer operated as previously (33). The D₄-labeled and unlabeled PE species were selectively detected using constant neutral-loss (NL) scanning of 145 and 141, respectively, in the positive ion mode (34). The D₃-labeled and unlabeled PS species were detected using NL of 90 and 87, respectively, in the negative ion mode. The peaks were identified and quantified using the recently developed LIMSA software (35). Quantification was carried out using three internal standards for both PE and PS (36). For identification of the acyl chain sn-positions of the endogenous species, as well as the remodeling products derived from the exogenous species, PE and PS classes were isolated by normal phase HPLC (28), and the individual species were then subjected to collisionally activated dissociation (CAD) and product ion analysis. Although some previous and ESI-MS studies have indicated that it is possible to identify the sn-positions of the acyl chain of phospholipids by collisional fragmentation and product ion scanning (37–39), controversial data exist (39, 40). Therefore, we carried out detailed studies with several synthetic standards to establish the acyl chain and sn-position-dependent fragmentation patterns of PE and PS. We then isolated the PE and PS classes from BHK21 cells using normal phase HPLC (28) and determined the sn position of the individual species based on the data obtained with standards as detailed in Supplemental Note S1.

Other Determinations—Cholesterol (41) and protein (42) were determined as described previously.
22:0/22:0-PC, which is too hydrophobic to be efficiently transferred by cyclodextrins, was detected in cells (not shown).

M-β-CD is known to extract cholesterol from cells (46), which could compromise some cellular functions. However, we found that if 50 mol% of cholesterol was included in donor vesicles, virtually no change in cellular cholesterol content was observed during the incubation (not shown), as expected from earlier studies (47). A modest elevation of POPC, the major component of the donor vesicles, was observed in the cells after labeling, but its level was normalized during the 24-h chase. No other significant changes in the endogenous lipid composition were observed. Neither did the labeling procedure compromise cell viability as judged from Trypan Blue exclusion and lactate dehydrogenase assays (not shown) as well as by the metabolic data discussed below. Importantly, the less hydrophobic species, e.g. 14:1/14:1-PE and -PS, could also be introduced to cells without m-β-CD albeit in significantly lesser amounts. Their remodeling was essentially identical to that observed in the presence of m-β-CD (not shown), thus indicating that the metabolism of exogenous PE or PS is not significantly influenced by the presence of m-β-CD or by the amount of lipid introduced into cells.

Remodeling of Exogenous Phosphatidylethanolamine Species—To study how the structure and the sn position of the acyl chains affects the metabolism of PE, we synthesized a variety of D₄-labeled PE species, introduced them into BHK21 cells and

FIGURE 1. Remodeling of different exogenous PE species in BHK21 cells. A D₄-labeled PE species was introduced into cells and then chased for up to 24 h as outlined under “Experimental Procedures.” 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, and their m/z positions, as well as that of the main final remodeling product (18:1/18:1-PE) are marked with dashed vertical lines. 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. A, 14:0/14:0-PE; B, 14:1/14:1-PE; C, 18:3/18:3-PE; D, 18:0/22:6-PE; E, 18:0/18:1-PE; F, 18:1/18:1-PE.
then determined their remodeling pathways and kinetics using ESI-MS as detailed under "Experimental Procedures." Experiments carried out with 20 different exogenous PE species showed that remodeling of atypical (i.e. endogenously absent or minor) PE species was rapid and extensive, whereas those similar or identical to major endogenous ones were remodeled to a lesser degree (Figs. 1–3).

Fig. 1 shows spectra for cells labeled with 14:0/14:0-PE and chased for 0, 3, 7, or 24 h. The metabolism of this atypical PE species was extremely rapid since a variety of labeled species, many of them more abundant than the precursor itself, were detected already after the 1-h pulse. During the chase, the precursor and most of the early remodeling products disappeared completely, and the profile of labeled species became similar to that of the endogenous ones by 24 h. Based on the relative abundance of the precursor and its remodeling products versus chase time (Fig. 2 A) as well as information on the sn-position of the acyl chains (see Supplemental Note S1), the remodeling pathways of 14:0/14:0-PE could be deduced (Supplemental Fig. S1 A).

Based on the results shown in Figs. 1 and 2, as well as those obtained for 14 other PE species (not shown), exogenous PE species could be grouped in four main categories according to the kinetics and/or the order of sn1 versus sn2 remodeling (Fig. 3, A–D). 1) PE species containing a saturated sn2 chain were remodeled very rapidly (Fig. 3 A) and initially always at the sn2 position by substituting the saturated chain (mainly) for 18:1. The unsaturated/saturated species (18:1/14:0, 18:1/16:0, and 18:1/18:0) were not remodeled further (not shown), but the sn1 chain of the saturated/saturated species (14:0/14:0, 16:0/16:0, 14:0/18:0, and 18:0/14:0) was subsequently exchanged for 18:1 (see Figs. 1 A and 2 A). 2) Unsaturated PE species differed greatly from each other in terms of remodeling pathways and kinetics (Fig. 3 B). The polyunsaturated species (18:2/18:2, 18:3/18:3, and 20:4/20:4) were remodeled rapidly at the sn1 position and subsequently at sn2 one (see Figs. 1 C and 2 C). 14:1/14:1-PE was also rapidly remodeled, but at both sn positions in parallel (Figs. 1 B and 2 B). Remodeling of 16:1/16:1-PE was analogous to that of 14:1/14:1-PE, albeit slower (Fig. 3 B), while 18:1/18:1-PE was hardly remodeled (Figs. 1 F and 2 F), probably because it is the major endogenous PE species in BHK21 cells. 3) Saturated/polyunsaturated PE species (14:0/20:4, 16:0/20:4, 18:0/20:4, and 18:0/22:6) were remodeled quite rapidly (Fig. 3 C) and at both sn-positions in parallel by replacing either of the acyl chains with oleate (see Figs. 1 D and 2 D). The initial remodeling products were then converted to 18:1/18:1 via exchange of the remaining original sn1 or sn2 acyl chain. 4) Saturated/monounsaturated PE species (14:0/18:1, 16:0/18:1, and 18:0/18:1) were remodeled quite slowly (Fig. 3 D) and exclusively at sn1 position producing mainly 18:1/18:1 (see Figs. 1 E and 2 E).

Notably, the turnover of the total pool of labeled PE was quite similar for all exogenous species studied (not shown), indicat-
ing that selective precursor degradation, i.e. conversion to water soluble products, does not significantly contribute to the observed differences in the remodeling kinetics. Remodeling of several PE species was also studied in HeLa cells with analogous results (Supplemental Fig. S2).

Remodeling of Exogenous Phosphatidylserine Species—We next studied remodeling of exogenous PS species in BHK21 cells to see if the lipid head group affects remodeling pathways and kinetics. Analogously to the corresponding PE species, 14:0/14:0-PS was first converted to 14:0/18:1, which was then further remodeled to 18:0/18:1 and 18:1/18:1 (Fig. 4A), the two major endogenous PS species in these cells. Consequently, the profile of labeled species became similar to that of the endogenous ones (Fig. 4) by 24 h of chase. Notably, remodeling of 14:0/14:0-PS was significantly slower than that of corresponding PE species (see Fig. 3, A and E).

Based on experiments with 19 different labeled PS species we could conclude that, similarly to PE, atypical (i.e. endogenously absent) PS species were rapidly remodeled to species corresponding to the major endogenous ones i.e. mainly 18:0/18:1 or 18:1/18:1, whereas the PS species similar or identical to the endogenous ones were remodeled much less and more slowly (Figs. 3 and 4). Notably, however, the remodeling kinetics and pathways of PS species often differed markedly from those found for corresponding PE species (see below). 1) PS species containing a saturated sn2 chain were initially remodeled at the sn2 position, but in contrast to the corresponding PEs, only at a moderate rate (Fig. 3E). The saturated/saturated PS species (14:0/14:0, 16:0/16:0, 14:0/18:0) were converted mainly to 14:0/18:1, 16:0/18:1, and 14:0/18:1, respectively (see Fig. 4A), which were remodeled further via exchange of the sn1 chain for 18:0/18:1. Unlike these species, 18:0/14:0-PS was directly converted to 18:0/18:1 and not remodeled further (not shown). Remodeling of the monounsaturated/saturated PS species (18:1/14:0, 18:1/16:0, and 18:1/18:0) was slower than that of the disaturated ones (Fig. 4F). Unlike the corresponding PE, which was hardly remodeled, exogenous 18:1/18:1-PS was converted to 18:0/18:1 (Fig. 4F). 3) Saturated/polyunsaturated PS species. Compared with the corresponding PEs, remodeling of 16:0/20:4-, 18:0/20:4-, and 18:0/22:6-PS species was clearly slower (Fig. 3F). 16:0/20:4-PS was remodeled at both sn positions in parallel (not shown) like the corresponding PE species. On the other hand, 18:0/20:4- and 18:0/22:6-PS were remodeled exclusively...
at sn2 yielding 18:0/18:1 (Fig. 4D), which differs significantly from the corresponding PE species (see Fig. 1D). 4) Saturated/monounsaturated PS species. As the corresponding PE, 16:0/18:1-PS was remodeled quite slowly (Fig. 3H) and exclusively at sn1 position to yield mainly 18:1/18:1. However, significant amounts of 18:0/18:1 was also formed, which was not the case with PE. Also diverging from the corresponding PE, remodeling of exogenous 18:0/18:1-PS was almost non-existent (Fig. 4E), probably because it is the main endogenous PS species in BHK21 cells.

Because the comparison of the remodeling kinetics of corresponding PS and PE species is complicated by that different PS species are decarboxylated at different rates (31, 34), we also carried out control experiments in the presence of 1 mM hydroxylamine, which blocks the PS decarboxylase without compromising cell viability (48). The results from these studies (not shown) were consistent with the conclusions drawn above regarding PS remodeling.

In summary, because both the remodeling pathways and kinetics of most PS species differed significantly from those of corresponding PEs, the remodeling PLAs and acylating enzymes acting on these two aminophospholipids in BHK21 cells are likely to be different.

Effect of PLA Inhibitors on Remodeling of PE—The data presented above demonstrate that both PLA₁ and PLA₂ activities are involved in remodeling of exogenous PE. Whereas little is known of the PLA₁s potentially involved in remodeling of phospholipids, several types of PLA₂s have been proposed to partic-
ipate in this process. Particularly the Group VIA Ca\(^{2+}\)-independent phospholipase iPLA\(_2\)/H\(_{9252}\) has been frequently implicated (49), albeit contradictory data have been obtained with some cell types (50). Also certain Group IV cytosolic phospholipases (cPLAs) may participate in phospholipid remodeling (51, 52).

To complement the pathway data indicating the involvement of multiple PLAs in aminophospholipid remodeling, we studied the effects of methyl arachidonyl fluorophosphonate (MAFP) and R- and S-enantiomers of bromoenol lactone (BEL) on remodeling of select PE species in BHK21 cells. MAFP effectively inhibits all cPLA\(_2\)s as well as iPLA\(_2\)/H\(_{9252}\) (53), while S-BEL and R-BEL should inhibit iPLA\(_2\)/H\(_{9253}\) and iPLA\(_2\)/H\(_{9252}\), respectively, but not cPLA\(_2\)s (54).

Fig. 5A displays a spectrum for cells labeled with 14:1/14:1-PE and then chased for 3 h in the absence of inhibitors. As can be seen, the precursor has virtually disappeared and significant

![ESI-MS/MS spectra of labeled PE and PS molecular species from cells treated with different inhibitors.](image-url)
PLA2s are involved in remodeling of exogenous PE species: one 16:0/20:4-PE (Fig. 6)

Effectively none of the inhibitors had any effect on remodeling of 14:0/18:0-PE and 16:0/20:4-PE.

In the case of 14:0/18:0-PE, neither R- nor S-BEL had any significant effect, whereas MAFP inhibited modestly (Fig. 5). Rather than showing multiple spectra for each, the fraction of the precursor of the total labeled species was calculated. All data are mean ± S.D. of three independent experiments. When not visible, the error bars are smaller than the symbol. See text and the legend of Fig. 5 for further details.

Collectively, these data suggest that remodeling of exogenous PS involves at least three different of PLA2s: one which is strongly inhibited with MAFP and by R- and S-BEL; another which is strongly inhibited by S-BEL, but not with R-BEL and MAFP; and a third one which is not inhibited by any of the inhibitors. In conclusion, the inhibitor data strongly support the pathway data indicating that several different PLAs, capable of distinguishing both the lipid head group as well as the acyl chains, participate in remodeling of aminophospholipids in BHK21 cells.

Involvement of Acyl-CoA-dependent and -independent Acyltransferases in Remodeling of Exogenous PE and PS Species—To investigate if multiple types of acylating enzymes are involved in remodeling of exogenous PE and PS species, we studied the effect of Triacsin C, a potent inhibitor of long chain acyl-CoA synthase (55). As shown in Fig. 6A, Triacsin C had no notable effect on the formation of secondary remodeling products (Fig. 5F), as observed for the corresponding PE species. However, in marked contrast to corresponding PE, both R-BEL and S-BEL strongly inhibited the remodeling of 14:1/14:1-PS with virtually identical efficiency (Figs. 5, H and I, and 6D). Unlike 14:1/14:1-PS, MAFP had no effect on remodeling of 14:0/18:0-PS, whereas it was nearly eliminated by S-BEL. R-BEL had only a minor effect (Fig. 6E). None of the PLA2 inhibitors affected remodeling of 16:0/20:4-PS (Fig. 6F) as was observed for the corresponding PE. Collectively, these data suggest that remodeling of exogenous PS involves at least three different of PLA2s: one which is strongly inhibited with MAFP and by R- and S-BEL; another which is strongly inhibited by S-BEL, but not with R-BEL and MAFP, and a third one which is not inhibited by any of the inhibitors. In conclusion, the inhibitor data strongly support the pathway data indicating that several different PLAs, capable of distinguishing both the lipid head group as well as the acyl chains, participate in remodeling of aminophospholipids in BHK21 cells.
Aminophospholipid Acyl Chain Remodeling

In contrast to PE, remodeling of all PS species studied was markedly inhibited by Triacsin C (Fig. 6, D–F). 14:1/14:1 PS was remodeled to 18:0/14:1, 18:1/14:1, and 16:0/14:1 indicating that remodeling of the sn2, but not the sn1 position of PS was inhibited (Fig. 5). Parallel results were obtained for several other PS species studied (not shown). Accordingly, acyl-CoA-dependent acyltransferases or transacylases are implicated in sn2 remodeling of PS, whereas sn1 remodeling appears to be acyl-CoA-independent.

In summary, because Triacsin C modulated sn1 remodeling of PE but not of PS and, conversely, sn2 remodeling of PS but not of PE, it appears that the enzymes reacylating the sn1 and sn2 positions of PE are probably different from each other, as well from those reacylating the corresponding positions of PS.

Accumulation of Remodeling Products Corresponding to the Major Endogenous PE and PS Species Is Due to Their Slow Hydrolysis Rather Than Acyl Chain Recycling—In principle, the accumulation of 18:1/18:1-PE and 18:0/18:1-PS as the main end products of remodeling of exogenous PE and PS species, respectively, could be due to 1) their slow hydrolysis by remodeling PLAs or 2) rapid hydrolysis followed by rapid reacylation with identical fatty acids. To choose between these options, we synthesized 16:0/13C18-18:1-D4PE, i.e. a species in which both the head group and the sn2 acyl chain is labeled, and then studied its metabolism in BHK21 cells. As shown in Fig. 7A, this doubly labeled PE was metabolized slowly to either 18:1/13C18:1-D4PE or 16:0/18:1-D4PE via exchange of the sn1 or sn2 chain, respectively. At later chase times, some 18:1/18:1-D4PE appeared indicating that both original fatty acids had been replaced (Fig. 7A). The 18:1/13C18-1, 16:0/18:1 and 18:1/18:1 species increased at comparable rates, indicating that both acyl chains turn over with similar kinetics. Notably, this turnover was much slower than that of atypical PE species (see Figs. 1–3), because the precursor still represented >40% of the total D4-labeled PE after 24h chase (Fig. 7A). Whereas we cannot exclude the possibility that the unlabeled sn1-palmitate would be recy-

cled, i.e. hydrolyzed and reacylated with an identical fatty acid, this seems improbable because only a very minor fraction of exogenous 18:1/13C18:1-D4PE was converted to 16:0/18:1 (see Fig. 7B); i.e. there is no strong tendency to add a 16:0 chain to the sn1 position of PE.

We also studied the metabolism of doubly labeled 18:1/13C18:1-D4PE with very similar results. After 24 h, only ~30% of the sn2-chain had turned over, as indicated by conversion of the precursor to 18:1/18:1-D4PE (Fig. 7B). Analogous experiments carried out with 16:0/13C18:1-D4PS and 18:1/13C18:1-D4PS gave parallel results (data not shown).

In conclusion, these results strongly support the notion that remodeling PLAs and PLAS display a low activity toward major endogenous PE and PS species, such as 16:0/18:1 and 18:1/18:1, meaning that the kinetics remodeling are determined by the substrate specificity of those PLAs. On the other hand, the specificities of acyl transferases/ transacylases are crucial as they, together with the availability of different fatty acids, strongly influence the spectrum of remodeling products eventually formed.

Remodeling of Endogenously Synthesized PE Species—When BHK21 cells were briefly labeled with D4-ethanolamine, which incorporates to PE via the CDP-ethanolamine pathway, the profile of newly synthesized D4-PE species was initially quite different from that of pre-existing (unlabeled) PE (Supplemental Fig. S3). However, when the cells were chased in the presence of unlabeled ethanolamine, the labeled PE species were rapidly remodeled to yield a molecular species profile similar to that of unlabeled PE. After a 24-h chase, labeled PE consisted mainly of 18:1/18:1 and few other species. These data resemble those obtained for many exogenous species, thus indicating that the latter are remodeling similarly to the endogenous ones. However, it is not possible to compare the remodeling of exogenous and endogenous species in detail because, as mentioned under the Introduction, the remodeling pathways or kinetics of individual molecular species synthesized endogenously cannot be resolved due to simultaneous labeling of multiple species.

Recycling of Labeled Head Group Does Not Bias the Remodeling Data—The fact that very different profiles were obtained for different exogenous PE and PS species makes it unlikely that recycling of labeled head group would significantly contribute to the profiles of labeled species and would thus bias interpretation of the data. Would the labeled species derive mainly from head group recycling rather than acyl chain remodeling, identical profiles should be obtained for all exogenous species (due to use of the same endogenous DG precursor pool), which clearly was not the case. Nevertheless, to fully exclude this, we studied metabolism of 14:1/14:1-D4PE in the presence of unlabeled ethanolamine (1.6 mM), which should effectively dilute
out the labeled ethanolamine eventually released from the exogenous species and thus prevent its reincorporation to PE. As shown in Supplemental Fig. S4, the profile of labeled PE species was essentially identical to the control lacking ethanolamine, thus confirming that recycling of labeled ethanolamine does not bias the interpretation of the remodeling data. Such studies were not carried out with PS, because serine is an intrinsically important component of the cell culture medium used.

**DISCUSSION**

The present study demonstrates that far more detailed data on aminophospholipid remodeling pathways and kinetics can be obtained by ESI-MS/MS using exogenous phospholipidues compared with the use of labeled ethanolamine or serine as the precursors. This is because of the fact that only a single, rather than multiple, labeled species is initially present. Another key finding of this study is that among the 40 different PE and PS species studied in BHK21 cells the atypical (endogenously absent) ones were rapidly remodeled to endogenously abundant ones, while the exogenous species similar or identical to the endogenous were remodeled only to a limited degree. Analogous results were obtained for all exogenous PE and PS species tested with HeLa cells, whose endogenous PE and PS profiles differ significantly from those of BHK21 cells.

The accumulation of the 18:1/18:1-PE and 18:0/18:1-PS species as the main remodeling end products in BHK21 cells appears to be mainly due to: 1) their slow hydrolysis by remodeling PLAs and 2) preferential acylation of the lysoPE with oleate and lysoPS with oleate or stearate (Figs. 1, 4, and 7). Thus PLAs, by catalyzing the committed step, set the pace of remodeling of exogenous (and most probably endogenous) PE and PS species. However, the specificity of the acylating enzymes and the composition of the FA donor pools play a crucial role in determining the steady state profile of molecular species in the cells.

Most exogenous PE and PS species were remodeled both at sn1 and sn2 positions in agreement with earlier studies (5, 56) and therefore both PLA1 and PLA2 activities must be involved. A novel finding is that both the kinetics and the sequence of the sn1 versus sn2 remodeling are very sensitive to the phospholipid acyl chain and head group structure. The PLA1s acting on PE seemingly prefer saturated and unsaturated chains, except for 18:1, which is not effectively cleaved (Figs. 1–3). In contrast, the PLA1s acting on PS seem to have a low affinity for saturated chains, particularly 18:0, as indicated by slow remodeling of the sn1-saturated PS species (Figs. 3 and 4). On the other hand, the PLA2s acting on PE seem to avidly cleave saturated chains, but poorly 18:1, while the PLA2s acting on PS seem to cleave preferentially short monounsaturated and polyunsaturated chains (Figs. 3 and 4).

The fact that structurally very similar aminophospholipid species, including positional isomers, were often remodeled with very different kinetics (see Fig. 3) strongly suggests that the susceptibility for remodeling is largely determined by the substrate specificities of the PLAs, rather than by e.g. differences in intracellular trafficking or partitioning between membrane domains.

While little is known of the PLA1s potentially involved in remodeling of phospholipids, several types of PLA2s, particularly members of the iPLA2 and cPLA2 families, have been implicated in this process (49, 51, 52), albeit most studies have concentrated on PC. The involvement of both iPLAs and cPLAs in aminophospholipid remodeling is supported by the present data obtained with the PLA1 inhibitors MAFP, R-BEL, and S-BEL. However, these data imply that also other activities are involved. The observed differences in the effects of these inhibitors on remodeling of six different PE and PS species strongly support the involvement of multiple selective PLA1s, capable of distinguishing both the phospholipid acyl chains and head group, in aminophospholipid remodeling. However, more specific tools, such as RNA interference or other protein knockdown methods are needed to establish the identity of the specific proteins involved.

Triacsin C, an inhibitor of acyl-CoA synthase, did not inhibit remodeling of any PE species studied and thus reacylation of both sn positions of PE probably occurs by transacylation. Triacsin C also did not inhibit reacylation of the sn1 position of PS, thus again implicating CoA-independent transacylases. However, it markedly inhibited sn2 reacylation of PS, which is thus probably mediated by acyl-CoA-dependent acyltransferases/transacylases. CoA-dependent and -independent acylation of lyso-PS has been observed in vitro (57, 58). Because the sn1 position of PS was preferentially reacylated with 18:0 as found previously in vitro (59), whereas that of PE was mainly reacylated with 18:1 or 16:1, it is likely that different transacylases mediate the sn1 acylation of PS versus PE. This is consistent with previous in vitro data (6, 60). In summary, at least three different enzyme activities appear to catalyze reacylation of aminophospholipids in BHK21 cells.

Beside the specificity of PLAs and acylating enzymes the pools of free/esterified fatty acids obviously also play an important role in determining the molecular species eventually formed, as shown by the remarkable changes in the phospholipid species profiles obtained by feeding different fatty acids to cells (61). Phospholipases and acyl transferases/transacylases putatively involved in phospholipid remodeling have been localized to the endoplasmic reticulum, the Golgi, mitochondria, nucleus, and peroxisomes (9, 10, 18, 51, 52, 62–65). It is an intriguing possibility that organelles harbor different sets of remodeling enzymes, which could contribute to their specific phospholipid molecular species profiles. Evidence for such organelle-specific remodeling has been obtained for nuclear phosphatidylcholine in mammalian cells (66) and for yeast phospholipids in general (67). Thus the observed differences in remodeling of aminophospholipid molecular species might, at least in part, result from their different intracellular trafficking and distribution (68). Conversely, the remodeling process might contribute to interorganelle trafficking of phospholipids, since the rather hydrophilic lysolipid formed upon a PLA reaction, unless immediately reacylated, should be able to rapidly diffuse to another organelle wherein it might be reacylated to reform an intact phospholipid molecule.

Are exogenous aminophospholipids remodeled similarly to those synthesized endogenously? It seems very likely that their remodeling is qualitatively similar to that of the endogenous ones, because studies with labeled ethanolamine showed that the newly formed species were, like the exogenous ones, exten-
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evisely remodeled eventually yielding a species pattern identical to the unlabeled ones (Supplemental Fig. S3). However, the kinetics of remodeling are likely to differ somewhat, because it takes time before the exogenous species fully mixes with the endogenous ones, despite the fact that intracellular trafficking of lipids is thought to be a relatively rapid process (69).

Albeit not studied here, it is very likely the exogenous species can be used to study remodeling of other glycerolipids as well. This, however, may require that the rate of transbilayer movement of the exogenous lipid to be studied is significant so that it will be readily accessible for the remodeling enzymes. While PS and PE are known to be rapidly translocated to the inner leaflet of the plasma membrane of all mammalian cells studied, flipping of e.g. PC seems to depend on the cell type (70).

Many crucial questions regarding glycerophospholipid acyl chain remodeling remain to be unanswered. First of all, it will be essential to identify the particular phospholipases and acyl transferases/transacylases involved in aminophospholipid remodeling. RNA interference (RNAi) or cells derived from knock-out animals should be very useful here. However, major efforts are required due to a large number of potential remodeling enzymes, off-target effects, and compensatory phenomena (71). Second, once the enzymes involved in remodeling have been identified, studies with model membranes with systematically varied compositions are needed to define the factors responsible for the high specificity of the remodeling process. Third, the subcellular site(s) of phospholipid remodeling needs to be established, and this probably requires in vitro studies with purified organelles. Fourth, remodeling of other glycerophospholipids such as PC, PI, and PA needs to be studied in various cell types to complete the picture. Yet, it is important to establish the relationship between the phospholipases involved in remodeling and those maintaining phospholipid class homeostasis. The approach described here should be very useful when studying many of these issues.

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