THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 39, pp. 28362–28372, September 28, 2007

The CDP-ethanolamine Pathway and Phosphatidylserine Decarboxylation Generate Different Phosphatidylethanolamine Molecular Species*

Received for publication, May 8, 2007, and in revised form, July 31, 2007 Published, JBC Papers in Press, August 2, 2007, DOI 10.1074/jbc.M703786200

Onno B. Bleijerveld, Jos F. H. M. Brouwers, Arie B. Vaandrager, J. Bernd Helms, and Martin Houweling[1]

From the Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, and Institute of Biomembranes, University of Utrecht, Utrecht, The Netherlands

In mammalian cells, phosphatidylethanolamine (PtdEtn) is mainly synthesized via the CDP-ethanolamine (Kennedy) pathway and by decarboxylation of phosphatidylserine (PtdSer). However, the extent to which these two pathways contribute to overall PtdEtn synthesis both quantitatively and qualitatively is still not clear. To assess their contributions, PtdEtn species synthesized by the two routes were labeled with pathway-specific stable isotope precursors, d_3-serine and d_4-ethanolamine, and analyzed by high performance liquid chromatography-mass spectrometry. The major conclusions from this study are that (i) in both McA-RH7777 and Chinese hamster ovary K1 cells, the CDP-ethanolamine pathway was favored over PtdSer decarboxylation, and (ii) both pathways for PtdEtn synthesis are able to produce all diacyl-PtdEtn species, but most of these species were preferentially made by one pathway. For example, the CDP-ethanolamine pathway preferentially synthesized phospholipids with mono- or di-unsaturated fatty acids on the *sn*-2 position (*e.g.* (16:0-18:2)PtdEtn and (18:1-18:2)PtdEtn), whereas PtdSer decarboxylation generated species with mainly polyunsaturated fatty acids on the *sn*-2 position (*e.g.* (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn in McArdle and (18:0-20:4)PtdEtn and (18:0-22:6)PtdEtn in Chinese hamster ovary K1 cells). (iii) The main PtdEtn species newly synthesized from the Kennedy pathway in the microsomal fraction appeared to equilibrate rapidly between the endoplasmic reticulum and mitochondria. (iv) Newly synthesized PtdEtn species preferably formed in the mitochondria, which is at least in part due to the substrate specificity of the phosphatidylserine decarboxylase, seemed to be retained in this organelle. Our data suggest a potentially essential role of the PtdSer decarboxylation pathway in mitochondrial functioning.

Phosphatidylethanolamine (PtdEtn)[2] is the second most abundant phospholipid subclass in mammalian cells, comprising 15–25% of total phospholipids (1). Three pathways are present for PtdEtn biosynthesis. The majority of PtdEtn is synthesized via the CDP-ethanolamine pathway and PtdSer decarboxylation, whereas the third route, calcium stimulated base-exchange, is of little significance (2). In the CDP-ethanolamine (CDP-Etn) pathway, ethanolamine is converted to PtdEtn by the sequential actions of ethanolamine kinase, CTP: phosphoethanolamine cytidylyltransferase and finally choline/ethanolaminephosphotransferase. Choline/ethanolaminephosphotransferase has a dual specificity, as it can use both CDP-choline (CDP-Cho) and CDP-Etn as substrates for the biosynthesis of phosphatidylcholine (PtdCho) and PtdEtn, respectively (3). In addition to choline/ethanolaminephosphotransferase, a CDP-choline-specific cholinephosphotransferase is available for PtdCho biosynthesis (4).

The PtdSer decarboxylation pathway for PtdEtn biosynthesis was first described by Borkenhagen *et al.* (5). In this pathway PtdSer synthesized from PtdCho or PtdEtn by phosphatidylserine synthase-1 and -2, respectively (2, 6, 7), is decarboxylated by the enzyme phosphatidylserine decarboxylase (PSD) to generate PtdEtn. To date, only one mammalian PSD has been cloned (8), and the enzyme was shown to be located on the external aspect of the inner mitochondrial membrane (9, 10). Because PtdSer synthesis occurs in the ER and especially in ER-related membranes termed mitochondria-associated membranes (MAM) (2, 11), PtdSer decarboxylation requires transport of PtdSer from its site of synthesis to the inner mitochondrial membrane, where PSD is located (12–14).

The relative importance of the CDP-Etn and PtdSer decarboxylation pathways to overall PtdEtn biosynthesis appears to vary depending on cell type and the availability of the substrates ethanolamine and serine, respectively. From studies in Chinese hamster ovary (15, 16) and baby hamster kidney (17) cells that were cultured in medium with fetal bovine serum being the sole source of ethanolamine, it was concluded that PtdSer decarboxylation was the major pathway for PtdEtn synthesis. However, observations in hamster heart (18) and in rat liver, hepatocytes, heart, and kidney (19, 20) illustrated that the vast majority of PtdEtn is synthesized via the CDP-Etn pathway. A possible explanation for these opposite results is the availability of exogenous ethanolamine. All studies mentioned above employed incorporation of radioactive serine and ethanola-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, P. O. Box 80176, 3508 TD Utrecht, The Netherlands. Tel.: 31-30-2535379; Fax: 31-30-2535492; E-mail: M.Houweling@uu.nl.

[2] The abbreviations used are: PtdEtn, phosphatidylethanolamine; CDP-Etn, CDP-ethanolamine; CDP-Cho, CDP-choline; Cho, choline; ER, endoplasmic reticulum; Etn, ethanolamine; MAM, mitochondria-associated membrane; PSD, phosphatidylserine decarboxylase; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; Ser, serine; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; MS, mass spectroscopy.

mine into PtdEtn. Because of the presence of endogenous pools of unlabeled ethanolamine and serine, the quantitative contributions of both pathways to overall PtdEtn synthesis are still unclear.

An intriguing question is why two pathways for PtdEtn biosynthesis exist, whereas only one pathway is available for the *de novo* synthesis of PtdCho in most mammalian cells. First, a second biosynthetic pathway could serve as a backup pathway under conditions where one of the two pathways is not able to function properly. Second, the two pathways could serve mainly to "locally" supply certain organelles with PtdEtn for maintaining specific molecular species profiles within these organelles. Finally, it is possible that the two pathways yield different molecular species profiles as was shown for the synthesis of PtdCho (CDP-Cho pathway *versus* PtdEtn methylation) in rat hepatocytes (21). The large molecular diversity of PtdEtn and other phospholipid subclasses is dictated by the combination of different lengths, number of unsaturations, and types of linkages of the hydrocarbon chains. An ester linkage at the *sn*-1 position defines a diacyl molecular subspecies, whereas an ether linkage at this position defines a plasmanyl subspecies, and a plasmalogen subspecies is defined by a vinyl ether bond at the *sn*-1 position, as in all three subspecies the hydrocarbon chain at the *sn*-2 position is known to be linked to the glycerol backbone via an ester bond (22).

The development and refinement of mass spectrometry in combination with the availability of deuterated pathway-specific precursors has opened the possibility of specifically displaying the PtdEtn species synthesized via the CDP-Etn or PtdSer decarboxylation pathways. We report here that McA-RH7777 cells, when cultured at equimolar concentrations of ethanolamine and serine, prefer the CDP-Etn pathway over PtdSer decarboxylation in a ratio of ~2:1, with the decarboxylation route having a preference for the synthesis of long chain, polyunsaturated species.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, fetal bovine serum, and horse serum were from Invitrogen. d_9-choline ($HO(CH_2)_2N^+(CD_3)_3$), L-(2,3,3-d_3)-serine, and d_4-ethanolamine ($HOCD_2CD_2NH_2$) were from Cambridge Isotope Laboratories, Andover, MA. Tissue culture flasks were from Corning Inc., Acton, MA.

Cell Culture—McArdle (McA-RH7777, ATCC CRL-1601) and Chinese hamster ovary cells (CHO-K1, ATCC CRL-9618) were cultured in Dulbecco's modified Eagle's medium supplemented with 6% fetal bovine serum and 6% horse serum and Ham's F-12 containing 10% fetal bovine serum, respectively. The cells were maintained in 80-cm^2 culture flasks at 37 °C, 5% CO_2, and 90% humidity.

Incorporation of Deuterium-labeled Precursors into PtdEtn, PtdCho, and PtdSer—Cells were grown in "full" Dulbecco's modified Eagle's medium to 60–80% confluency in 175-cm^2 culture flasks. For deuterium-label studies, cells were washed twice with phosphate-buffered saline and incubated in serine- and choline-free Dulbecco's modified Eagle's medium (supplemented with serum as mentioned earlier) for 6, 24, or 72 h in the presence of 400 μM d_9-choline (d_9-Cho) and either 400 μM d_4-ethanolamine (d_4-Etn) or d_3-serine (d_3-Ser). To maintain similar substrate concentrations during incubation, 400 μM unlabeled serine was supplemented to the culture medium of the d_4-ethanolamine incubations and vice versa. Incubations were stopped by washing the cells three times with ice-cold phosphate-buffered saline, and cells were scraped into methanol. Total lipids were extracted, and phospholipid classes were isolated by normal-phase high performance liquid chromatography (HPLC) and analyzed as described in one of the sections below.

Subcellular Fractionation of McA-RH7777 Cells—Mitochondrial and microsomal fractions were isolated from labeled McA-RH7777 cells essentially as described for rat liver by Shiao *et al.* (12). Briefly, cells were scraped into phosphate-buffered saline, pelleted, and homogenized in ice-cold isolation medium (250 mM mannitol, 5 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% bovine serum albumin). After removal of nuclei and cell debris, the supernatant was centrifuged at 10,000 × *g* for 10 min to pellet crude mitochondria. The resulting supernatant was centrifuged at 100,000 × *g* for 1 h to pellet microsomes. The mitochondrial pellet was further purified from MAM by hand homogenization in isolation medium and layering of the homogenate on top of Percoll medium (225 mM mannitol, 25 mM HEPES (pH 7.4), 1 mM EGTA, and 0.1% bovine serum albumin and 30% (v/v) Percoll) followed by centrifugation for 30 min at 95,000 × *g*. Purity of mitochondria and characterization of microsomes was assessed by assaying for succinate dehydrogenase (mitochondrial marker enzyme) and aryl esterase (ER marker) and by Western blotting for cytochrome *c* (mitochondrial marker) and calnexin (ER marker).

Analysis of Phosphatidylethanolamine Molecular Species—Total lipids were extracted from cells and mitochondrial and microsomal fractions according to the method of Bligh and Dyer (23). The obtained total lipid extract was dissolved in hexane/isopropanol/acetone (82:17:1, v/v/v). Lipid classes were separated on a normal-phase HPLC column as described (24), and the PtdEtn fraction was collected manually from the column effluent using a flow splitter, dried under nitrogen, and stored at −20 °C till analysis. The fraction was dissolved in chloroform/methanol (1:1, v/v), and PtdEtn species were separated on two Synergi 4 μm MAX-RP 18A columns (250 × 3 mm) (Phenomenex, CA) in series as described (25) with a slightly modified mobile phase of acetonitrile/methanol (2:3, v/v). Identification of molecular species was performed by on-line tandem mass spectrometry in the negative-ion mode on an API 4000 Q Trap mass spectrometer fitted with an Atmospheric Pressure Chemical Ionization source (Sciex, Ontario, Canada). Analysis of PtdEtn molecular species compositions and deuterium labeling was performed by on-line single quadrupole mass spectrometry in the negative-ion mode on an API 3000 triple stage quadrupole mass spectrometer fitted with an Atmospheric Pressure Chemical Ionization source (Sciex). Nitrogen was used as nebulizer gas and curtain gas. PtdEtn molecular species compositions were determined by extracting the (labeled) molecular ions and isotope peaks of the various species from the negative Q1 chromatogram representing total PtdEtn, which was verified by detection with a Varex MKIII evaporative light scattering detector (Alltech, Deerfield, IL)

operated at 100 °C at a gas flow of 1.8 liters/min. Determination of the position of the ester linkage of fatty acids to glycerophosphoethanolamine was performed according to Brouwers *et al.* (25). Evaporative light scattering detector data were analyzed using EZChrom software (Scientific Software, San Ramon, Canada), and mass spectrometric data were analyzed using Analyst1.4 software (Sciex).

Analysis of Phosphatidylcholine Molecular Species—The PtdCho fraction of cells and mitochondrial and microsomal fractions was isolated from total lipid extracts by normal-phase HPLC as described in the previous section. The fraction was dissolved in chloroform/methanol (1:1, v/v), and PtdCho molecular species were separated on two LiChrospher 100 RP18-e columns (5 μm, 250 × 4.6 mm; Merck) in series as previously (24), with a slightly modified mobile phase of acetonitrile/methanol/triethylamine (25:24:1, v/v/v). Identification of molecular species was performed by on-line tandem mass spectrometry in the positive-ion mode on an API 4000 Q Trap mass spectrometer fitted with an electrospray ionization source (Sciex). Deuterium labeling of the various molecular species was determined by on-line mass spectrometry in the positive-ion mode on an API 2000 Q Trap mass spectrometer operated in enhanced (trapping) mode fitted with an electrospray ionization source (Sciex). PtdCho molecular species compositions were determined by evaporative light scattering detector detection as described above. Determination of the position of the ester linkage of fatty acids to glycerophosphocholine was performed according to Brouwers *et al.* (25). Evaporative light scattering detector and mass spectrometric data were analyzed using software as described above.

Analysis of Phosphatidylserine Molecular Species—The PtdSer fraction of cells and mitochondrial and microsomal fractions was isolated from total lipid extracts by normal-phase HPLC as described above. The fraction was dissolved in chloroform/methanol (1:1, v/v), and PtdSer molecular species were separated on a Synergi 4 μm MAX-RP 18A column (250 × 3 mm) (Phenomenex, CA) with a mobile phase of acetonitrile/methanol/H_2O (15:22.5:12.5, v/v/v) containing 1 μM serine and 2.5 mM ammonium acetate. Analysis and identification of molecular species was performed by on-line (tandem) mass spectrometry in the negative enhanced mass spectrometry ion mode on an API 4000 Q Trap mass spectrometer fitted with an electrospray ionization source. PtdSer species compositions were determined in PtdSer fractions obtained from unlabeled cells by extracting the molecular ions and isotope peaks of various species from the negative neutral loss 87-chromatogram representing total PtdSer.

Interpretation of Mass Spectra—The incorporation of d_4-Etn into PtdEtn and d_9-Cho into PtdCho was calculated for all molecular species by determining the intensities of the unlabeled and labeled molecular ion peak in the negative or positive Q1 mass spectrum and expressing the intensity of the labeled molecular ion as the percentage of the sum of the unlabeled and labeled molecular ion. d_3-Ser incorporation into PtdEtn and PtdSer was calculated similarly from (enhanced) negative Q1 spectra. Because d_3-Ser labeling also, unexpectedly, yielded significant d_2-Ser labeling (see "Discussion"), this d_2-Ser incorporation had to be taken into account for determination of the total d_3-Ser incorporation into the various PtdEtn and PtdSer species. All PtdCho, PtdEtn, and PtdSer molecular ions display isotope peaks in Q1 mass spectra (Fig. 1, *C* and *D*), originating from the natural presence of ^{13}C in these large biomolecules. To determine total d_3-Ser incorporation into PtdEtn and PtdSer molecular species, the natural contribution of ^{13}C to the intensities of the isotope peaks of each molecular ion was subtracted from the actual, measured intensities of the isotope peaks in the Q1 mass spectra obtained, the remaining "isotope peak" intensities (exemplified for PtdEtn in Fig. 1*D*) representing total d_3-Ser incorporation into the various PtdEtn and PtdSer species.

Measuring the Intracellular Deuterated to "Cold" Serine and Ethanolamine Ratio—Cells were labeled with 400 μM d_3-Ser or d_4-Etn for various times up to 6 h and washed twice with ice-cold phosphate-buffered saline before extracting the water-soluble components (23). The water-methanol phase was collected and evaporated to dryness, and the primary amines Ser and Etn were subsequently derivatized with fluorescamine exactly as described (26). The deuterated to cold ratio was determined using mass spectrometry.

Determination of the Substrate Specificity of Phosphatidylserine Decarboxylase—The substrate (d_3-labeled egg-PtdSer and various PtdSer molecular species) required to determine the substrate specificity of PSD by using a mass spectrometry approach was synthesized from their respective PtdCho species. Briefly, 2.5 mg of PtdCho was dissolved in 1 ml of chloroform, after which 25 mg of silica (kieselgel 60 for column chromatography) was added. The mixture was stirred for 30 min and carefully dried under a gentle stream of nitrogen. Subsequently, 250 μl of 100 mM acetate buffer (pH 5.6) containing 100 mM $CaCl_2$, 50 mg/ml d_3-serine, and 10 units of phospholipase D (*Streptomycin* species) was added to the silica, and the suspension was incubated for 36–48 h at 30 °C while shaking continuously. The reaction was stopped by adding 470 μl of H_2O and 80 μl of 6 M HCl, and phospholipids were extracted (23, 27). The amount of d_3-PtdEtn formed was quantified using the phosphorus assay (28).

PSD activity was measured in mitochondria, prepared as described in the subcellular fractionation section, as the formation of d_3-PtdEtn from d_3-PtdSer (29). The reaction mixture (final volume 0.4 ml) consisted of 100 mM KH_2PO_4 (pH 6.8), 10 mM EDTA, 0.5 mg of Triton X-100/ml of assay mixture, 50 μM PtdSer (d_3-egg PtdSer or a mixture of equal amounts (10 μM) of d_3-(16:0-16:0) PtdSer, d_3-(16:0-20:4) PtdSer, d_3-(18:0-18:1) PtdSer, d_3-(18:0-18:2) PtdSer, and d_3-(18:0-20:4) PtdSer) and enzyme (~250 μg of mitochondrial protein). Assays were carried out for 45 min at 37 °C and were terminated by adding 3.4 ml of chloroform/methanol/H_2O (15:29:6, by volume) followed by lipid extraction. After removing the Triton X-100 using small silica (kieselgel 60) columns, the amount of d_3-PtdSer (substrate) and d_3-PtdEtn (product) was quantified as described above.

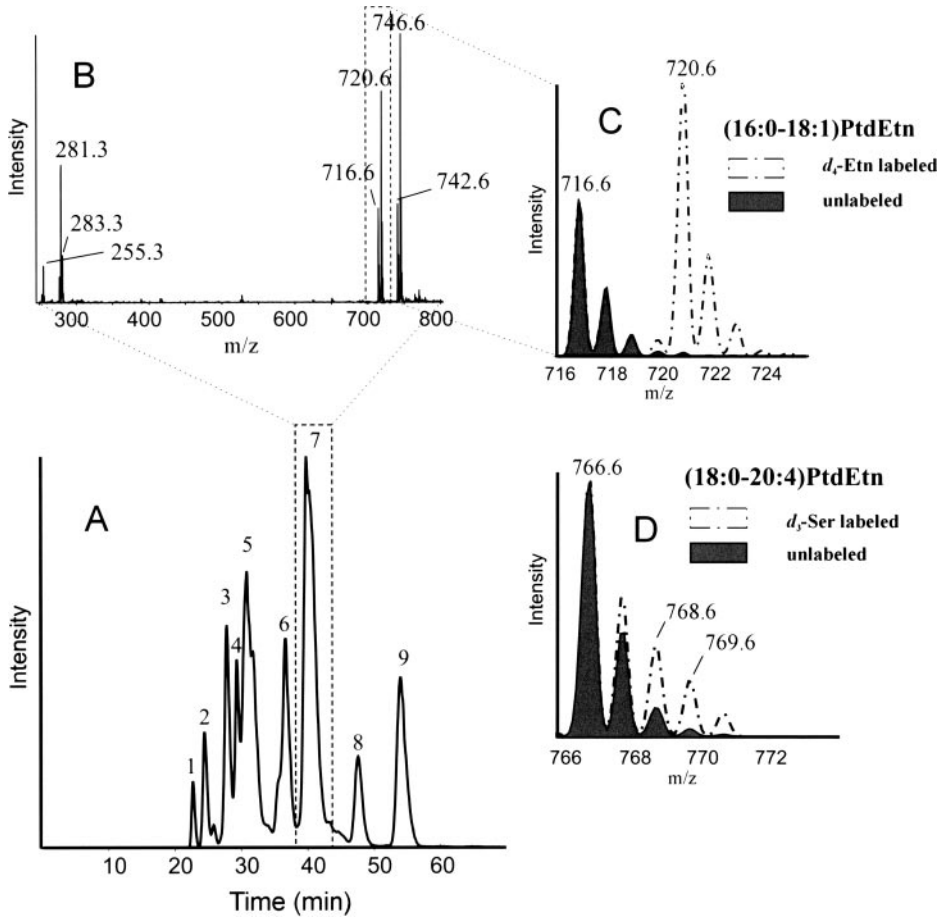

FIGURE 1. **HPLC-MS analysis of McA-RH7777 phosphatidylethanolamine molecular species.** McA-RH7777 cells were labeled with d_4-Etn or d_3-Ser as described under "Experimental Procedures." The PtdEtn fraction was isolated from total lipid extracts and analyzed for its molecular species composition and deuterium labeling by HPLC-MS. Representative figures are shown in *panels A–D*. *A*, negative-ion Q1 (*−Q1*) chromatogram of PtdEtn molecular species (peak identification is given in Table 1). *B*, typical −Q1 mass spectrum showing fatty acids originating from the eluted PtdEtn molecular species in the region *m/z* 200–300 and d_4-Etn-labeled PtdEtn molecular species eluting in peak 7 of the chromatogram in the region *m/z* 650–850. *C*, close-up view of d_4-Etn labeled (16:0-18:1)PtdEtn, showing the molecular ions of the labeled ("new") and unlabeled ("old") species and isotope peaks originating from the natural presence of ^{13}C in (large) biomolecules. *D*, the example is a close-up view of d_3-Ser-labeled (18:0-20:4)PtdEtn, showing the total d_3-Ser labeling (both d_2-Ser and d_3-Ser labeling) of this molecular species. *amu*, atomic mass unit.

RESULTS

Pathway-specific Monitoring of Phosphatidylethanolamine Biosynthesis in McA-RH7777 Cells—To get insight into the qualitative and quantitative contributions of the CDP-Etn pathway and PtdSer decarboxylation to overall PtdEtn synthesis in mammalian cells, McA-RH7777 (McArdle) cells were incubated in the presence of deuterated, pathway-specific precursors. After various times, total lipids were extracted and subfractionated into phospholipid subclasses, and the PtdEtn fraction was analyzed by HPLC-mass spectrometry (MS). A typical chromatogram of the HPLC separation of McArdle PtdEtn molecular species is shown in Fig. 1*A*, with the peak identification given in Table 1. The major advantage of on-line HPLC separation before MS is that isobaric molecular species, *i.e.* species having the same mass but different radyl groups in their diacylglycerol backbones, are largely separated, thus allowing individual analysis.

Labeling of cells with d_4-Etn or d_3-Ser in combination with mass spectrometric phospholipid analysis allowed us to distinguish between PtdEtn species synthesized via the CDP-Etn pathway and species formed by PtdSer decarboxylation. d_4-Etn-labeled PtdEtn species *de novo* synthesized via the CDP-Etn pathway could be easily discriminated from unlabeled PtdEtn species because of a 4-Da mass difference (Fig. 1*C*) between the molecular ions in the (negative) Q1 spectrum (Fig. 1*B*). In d_3-Ser, three carbon-bound protons of serine are replaced by deuterium atoms. Once the d_3-Ser label is incorporated into the head group of PtdSer species, the 3-Da mass difference between labeled PtdSer species and their non-labeled counterparts is retained upon decarboxylation to PtdEtn (Fig. 1*D*; the unexpected appearance of d_2-PtdSer will be explained under "Discussion" and is corrected for, see "Experimental Procedures"). In addition to PtdSer decarboxylation, (labeled) serine can be incorporated into PtdEtn via two other routes; (i) it can enter the CDP-Etn pathway as (labeled) phosphoethanolamine, generated as an intermediate of sphingomyelin metabolism (30), and (ii) it can be incorporated into the diacylglycerol moiety (31). However, experiments with β-chloro-L-alanine, a potent inhibitor of sphingomyelin synthesis (32), revealed that phosphoethanolamine liberated from sphingomyelin breakdown only marginally (<2%) contributed to serine labeling of PtdEtn in our experimental system (data not shown). Furthermore, only a slight amount of deuterium label was detected in the diacylglycerol moiety of PtdEtn and PtdCho in McArdle cells labeled with d_3-Ser for 24 h, which was clear from the fact that less than 4% of the PtdCho molecules were detected with a mass up to 3 mass units heavier than the parental molecular species. Therefore, it was concluded that in McArdle cells deuterated Ser-labeled PtdEtn species were derived from PtdSer decarboxylation (>95%).

Qualitative and Quantitative Contributions of the CDP-ethanolamine Pathway and PtdSer Decarboxylation to Overall Phosphatidylethanolamine Synthesis—We first determined a suitable labeling time to study the contribution of the various pathways to phospholipid biosynthesis by labeling McArdle cells with 400 μM d_9-Cho and 400 μM d_4-Etn for 6, 24, and 72 h. Because McArdle cells have only one route for *de novo* PtdCho synthesis, namely the CDP-Cho pathway, it was expected that all PtdCho species would be labeled to the same extent when remodeling was completed. Fig. 2*A* shows that already after 6 h

TABLE 1

Phosphatidylethanolamine molecular species composition in McA-RH7777 cells, mitochondria and microsomes

The PtdEtn fraction was collected from total lipid extracts of McA-RH7777 cells, mitochondria and microsomes, and the molecular species compositions were determined as described under "Experimental Procedures." Shown in this table is the PtdEtn molecular species composition (first row) of the total cell homogenate (third row), the mitochondrial fraction (fourth row), and the microsomal fraction (fifth row). The second row indicates the chromatogram peak in which the various molecular species elute in the HPLC-MS chromatogram displayed in Fig. 1*A*. Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

PtdEtn species	Peak (Fig.1*A*)	Whole cell	Mitochondria	Microsomes
		% of total PtdEtn (average ± S.D.)		
16:0-18:1	7	18.2 ± 0.1	14.4 ± 1.0	19.5 ± 3.7
16:0-18:2	5	6.1 ± 0.4	5.7 ± 0.6	6.2 ± 1.6
16:0-20:4	3	1.7 ± 0.8	2.6 ± 1.0	0.6 ± 0.4
16:0-20:5	1	0.4 ± 1	1.4 ± 0.7	0.3 ± 0.3
16:0-22:6	2	0.8 ± 0.2	1.2 ± 0.2	1.3 ± 0.5
18:0-18:1	9	6.1 ± 2.0	5.5 ± 2.6	7.8 ± 1.4
18:0-18:2/18:1-18:1	7	20.9 ± 1.4	15.4 ± 0.9	27.5 ± 3.6
18:0-20:3	8	0.5 ± 0.1	0.5 ± 0.2	0.2 ± 0.1
18:0-20:4	6	5.1 ± 0.5	12.3 ± 3.3	0.9 ± 0.8
18:0-20:5	4	8.7 ± 1.3	15.3 ± 3.5	1.6 ± 1.3
18:0-22:5/20:1-20:4	6	0.8 ± 0.2	1.3 ± 0.1	0.4 ± 0.3
18:0-22:6	5	2.1 ± 0.1	2.3 ± 0.1	1.3 ± 0.4
18:1-18:2	5	5.8 ± 0.3	4.6 ± 0.2	5.7 ± 1.6
18:1-18:3	2	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
18:1-20:1/18:0-20:2	9	1.0 ± 0.3	0.5 ± 0.2	1.5 ± 0.2
18:1-20:4	3	1.7 ± 0.6	1.4 ± 0.5	1.0 ± 0.6
18:1-20:5	1	0.3 ± 0.1	1.1 ± 0.5	0.3 ± 0.2
18:1-22:6	2	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
Plas(16:0-18:1)	8	0.5 ± 0.2	0.2 ± 0.1	0.8 ± 0.3
Plas(16:0-22:6)	3	1.8 ± 0.51	1.3 ± 0.1	1.9 ± 0.9

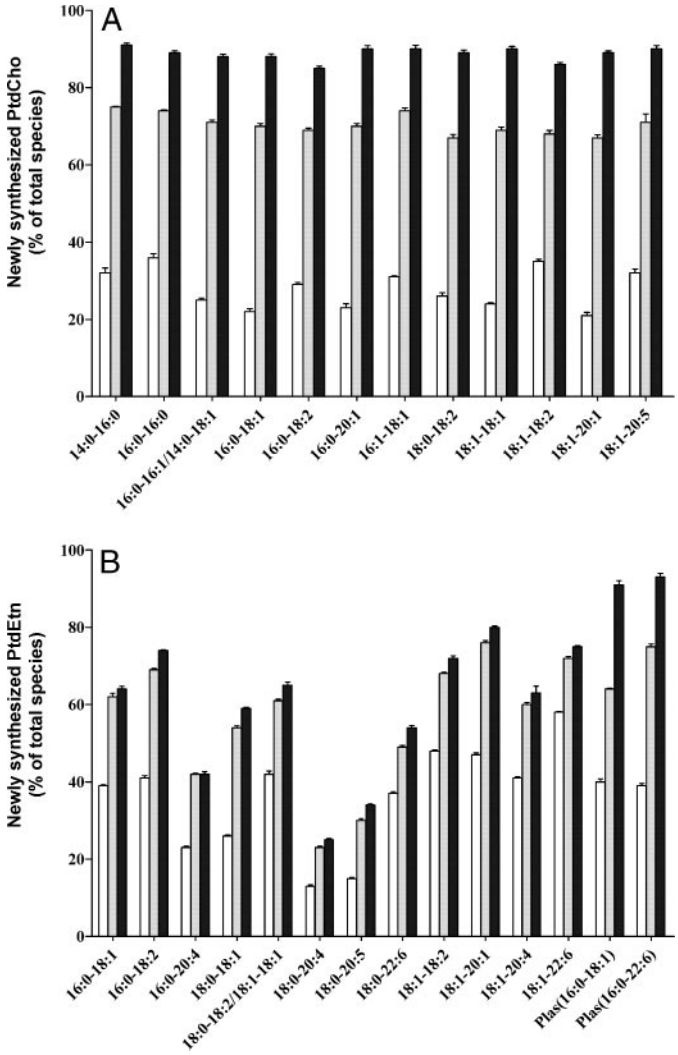

FIGURE 2. **Phosphatidylcholine and phosphatidylethanolamine synthesis in McArdle cells via their respective CDP-pathway.** McArdle cells were grown at 37 °C in 175-cm^2 flasks. When the cells were about 75% confluent, cells were incubated with d_9-Cho (*A*) or d_4-Etn (*B*), and after 6, 24, and 72 h of labeling with the deuturated precursors, cells were harvested for lipid extraction. Subsequently lipids were separated into the major phospholipid classes by HPLC followed by molecular species profiling of the various phospholipids classes by HPLC-MS as described under "Experimental Procedures." PtdCho synthesis is shown in *A*, and PtdEtn synthesis is shown in B at 6 h (*open bars*), 24 h (*gray bars*), and 72 h (*black bars*). Results (mean ± S.D. of triplicate) of a representative experiment are shown, which was repeated once with similar results.

24% of PtdCho mass in the cells is *de novo*-synthesized, but not all species are equally labeled yet (28.1 ± 7.2%). After 24 h, approximately the doubling time of the McArdle cells under our growth conditions, 69% of the PtdCho mass is newly synthesized, and all PtdCho species are equally labeled (70.4 ± 1.9), showing that remodeling was completed at this time. The observed increase, from 69% newly synthesized PtdCho at 24 h to 90% at 72 h, is in agreement with the fact that after 3 cell divisions maximal 12.5% "old" PtdCho (parental PtdCho) can still be present in the cells. In comparison with *de novo* PtdCho synthesis, the incorporation of d_4-Etn into PtdEtn shows a totally different profile as not all PtdEtn species were labeled to the same level at 24 and 72 h. Furthermore, it is clear from Fig. 2*B* that there is no or only a very modest increase in the percentage labeling of the diacyl-PtdEtn species between 24 and 72 h. Because 72 h of labeling shows exactly the same distribution profile of newly synthesized PtdEtn as the 24-h time point, this suggests that not all species are made via the CDP-Etn pathway in the same quantity and that this is not due to incomplete remodeling of some species. From here on the 24-h time point was used to study PtdEtn synthesis via CDP-Etn pathway and PtdSer decarboxylation on a subcellular level.

To study the contribution of the two routes for *de novo* PtdEtn synthesis in detail, McArdle cells were incubated for 24 h in the presence of 400 μM d_4-Etn and 400 μM unlabeled Ser or d_3-Ser and 400 μM unlabeled Etn, respectively. Under both conditions the medium was supplemented with 400 μM d_9-Cho to profile PtdCho biosynthesis. The composition of the major PtdEtn and PtdCho molecular species in McArdle cells is displayed in Tables 1 and 2 ("Whole cell"). The results clearly show that PtdEtn and PtdCho have distinct species profiles, PtdEtn containing significantly more polyunsaturated (~30% of total PtdEtn *versus* 5% in PtdCho) and plasmalogen (~10% of total PtdEtn) species than PtdCho (<5%). In line with the data presented in Fig. 2*B*, the various PtdEtn species were labeled with d_3-Ser to a different level (Figs. 3 and 4). PtdEtn species that were only modestly labeled with d_3-Ser after 24 h (*e.g.* (16:0-18:1)PtdEtn, d_3-Ser labeled for 14%) displayed a high d_4-Etn incorporation (66%) and vice versa (*e.g.* (18:0-20:5)PtdEtn was d_4-Etn-labeled for 21% and labeled with d_3-Ser for 36%). To obtain the quantities (Fig. 3) by which various phospholipid species are synthesized, label incorporation data were coupled to the molecular species compositions (Tables 1–3).

Fig. 4 displays the relative amounts by which the various PtdEtn species were *de novo* synthesized via the Kennedy pathway (*gray bars*) and PtdSer decarboxylation (*black bars*) in 24 h. The contribution of PtdSer decarboxylation to

TABLE 2

Phosphatidylcholine molecular species composition in McA-RH7777 cells, mitochondria, and microsomes

The PtdCho fraction was collected from total lipid extracts of McA-RH7777 cells, mitochondria, and microsomes, and the molecular species compositions were determined as described under "Experimental Procedures." Shown in this table is the PtdCho molecular species composition (first row) of the total cell homogenate (second row), the mitochondrial fraction (third row), and the microsomal fraction (fourth row). Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

PtdCho species	Whole cell	Mitochondria	Microsomes
	% of total PtdCho (average ± S.D.)		
14:0-16:0	1.1 ± 0.1	1.4 ± 0.2	1.4 ± 0.2
16:0-16:0	1.0 ± 0.2	0.6 ± 0.2	1.3 ± 0.4
16:0-16:1/14:0-18:1	7.6 ± 1.3	8.1 ± 0.6	8.0 ± 0.4
16:0-18:1	41.7 ± 1.9	39.6 ± 0.9	39.0 ± 1.9
16:0-18:2	5.4 ± 0.9	5.8 ± 0.4	5.7 ± 0.2
16:0-20:1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
16:0-20:2	3.2 ± 0.1	2.9 ± 0.1	3.0 ± 0.1
16:0-20:4	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
16:0-22:6	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
16:1-18:1	5.1 ± 0.5	6.5 ± 0.4	5.6 ± 1.6
18:0-18:1	1.0 ± 0.2	1.1 ± 0.1	1.1 ± 0.0
18:0-18:2	7.8 ± 0.2	8.9 ± 0.3	7.8 ± 0.6
18:1-18:1	5.8 ± 0.7	6.7 ± 0.5	6.3 ± 1.2
18:1-18:2	1.1 ± 0.1	1.4 ± 0.1	1.2 ± 0.4
18:1-20:1	1.2 ± 0.3	1.4 ± 0.1	1.3 ± 0.1
18:1-20:5	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0

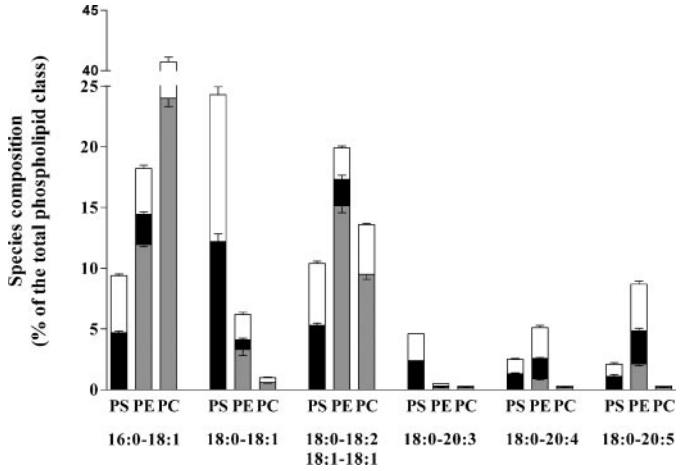

FIGURE 3. **Amount of phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine in McArdle cells and the contribution of various synthetic routes to *de novo* phospholipid synthesis.** McArdle cells were grown at 37 °C in 175-cm^2 flasks. When the cells were about 75% confluent, cells were labeled with deuterated pathway-specific precursors for 24 h to monitor phospholipid composition (*open bars*) and *de novo* synthesis (d_9-Cho, PtdCho, *gray* inset in the *PC bar*; d_3-Ser, *black inset* in the PtdSer (*PS*), and PtdEtn (*PE*) bars; d_4-Etn, *gray* in the *PE bar*. After labeling the cells were collected, and lipids were extracted and separated into the major phospholipid classes by HPLC before quantification of the molecular species composition of the various phospholipids classes by HPLC-MS as described under "Experimental Procedures." Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

PtdEtn synthesis is likely to be somewhat underestimated. This is due to the fact that, in contrast to Etn and Cho, the cells are able to synthesize this precursor *de novo*. The intracellular pool size of Ser is for only 80 ± 5% deuterated (d_3-Ser and d_2-Ser), whereas in the case of Etn the pool is for 94 ± 2% deuterated. It can be concluded from Fig. 4 in combination with Fig. 2*B* that (i) both routes seem capable of synthesizing all diacyl-PtdEtn species, whereas ethanolamine plasmalogens appeared to be predominantly synthesized via the Kennedy pathway and (ii) overall, the Kennedy pathway was favored for PtdEtn biosynthesis over PtdSer decarboxylation in a ratio of ~2:1. Furthermore, Fig. 4 clearly shows that the CDP-Etn pathway (*gray bars*) synthesized the largest amounts of PtdEtn in the form of (16:0-18:1)PtdEtn, (16:0-18:2)PtdEtn, (18:0-18:1) PtdEtn, (18:0-18:2/18:1-18:1)PtdEtn, and (18:1-18:2)PtdEtn, whereas PtdSer decarboxylation (*black bars*) was the predominant route to synthesize (18:0-20:4)PtdEtn, (18:0-20:5)PtdEtn, and (16:0-20:4)PtdEtn.

The d_9-Cho data show (as already mentioned before, Fig. 2*A*) that because all PtdCho species were labeled to a similar extent, the picture illustrating the amount by which the various PtdCho species were synthesized *de novo* after 24 h of incubation (Fig. 3) reflected the PtdCho molecular species composition as presented in Table 2. Thus, the bulk of PtdCho synthesized by the CDP-Cho pathway is composed of (16:0-16:1)-PtdCho, (16:0-18:1) PtdCho, (16:1-18:1)PtdCho, (18:0-18:2)PtdCho, and (18:1-18:1)PtdCho.

To determine whether the observed preference of the PtdSer dearboxylation route to synthesize PtdEtn species with polyunsaturated fatty acids at the *sn*-2 position is a more common phenomenon and not restricted to McArdle cells, a non-rat liver-derived cell line was studied. CHO cells were labeled for 24 h with d_4-Etn or d_3-Ser, and the amounts by which the various PtdEtn species were *de novo*-synthesized via the CDP-Etn pathway and PtdSer decarboxylation are shown in Fig. 5. In CHO cells the CDP-Etn pathway is favored over PtdSer decarboxylation in a ratio of 3:1. The higher contribution of the Kennedy pathway to PtdEtn synthesis in the CHO cells in comparison with McArdle cells is most likely due to the elevated amount of PtdEtn plasmalogens in CHO cells (CHO (~40%) *versus* McArdle (~10%)), as they are predominantly synthesized by the CDP-Etn pathway (Fig. 5). The only PtdEtn species synthesized for more than 50% by the PtdSer decarboxylation route are (18:0-20:3)PtdEtn, (18:0-20:4)PtdEtn, (18:0-22:6)PtdEtn, and (18:1-22:5)PtdEtn. Thus, in agreement with our observations in McArdle cells, the PtdSer decarboxylation preferentially synthesizes PtdEtn species with a polyunsaturated fatty acid.

(Sub)cellular Species Composition and Deuterium Labeling of Phosphatidylserine in McA-RH7777 Cells—To gain more insight into the origin of species selectivity in the PtdSer decarboxylation route (see Figs. 3 and 4), PtdSer was isolated from (i) McArdle cells, (ii) the mitochondrial, and (iii) the microsomal fraction after 24 h of d_3-Ser labeling, as described under "Experimental Procedures." The (sub)cellular composition of six selected PtdSer species is presented in Table 3. No significant difference in PtdSer species distribution between the whole cell, mitochondria, and microsomes was observed. The PtdSer "parent molecules" of (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn (the two PtdEtn species that are mostly synthesized by PtdSer decarboxylation) composed only 2–3% of total PtdSer in the whole cell, mitochondria, and microsomes. The PtdSer precursors of the other two main "PSD-PtdEtn species," (16:0-18:1)PtdSer and (18:0-18:2)PtdSer, were present in higher amounts. (18:0-18:1)PtdSer, precursor of a PtdEtn species predominantly synthesized via the CDP-Etn pathway, is however, the most abundant PtdSer species in McArdle cells, composing 24 ± 2% of total PtdSer in cells, mitochondria, and microsomes. All PtdSer species were labeled with d_3-Ser to a similar extent

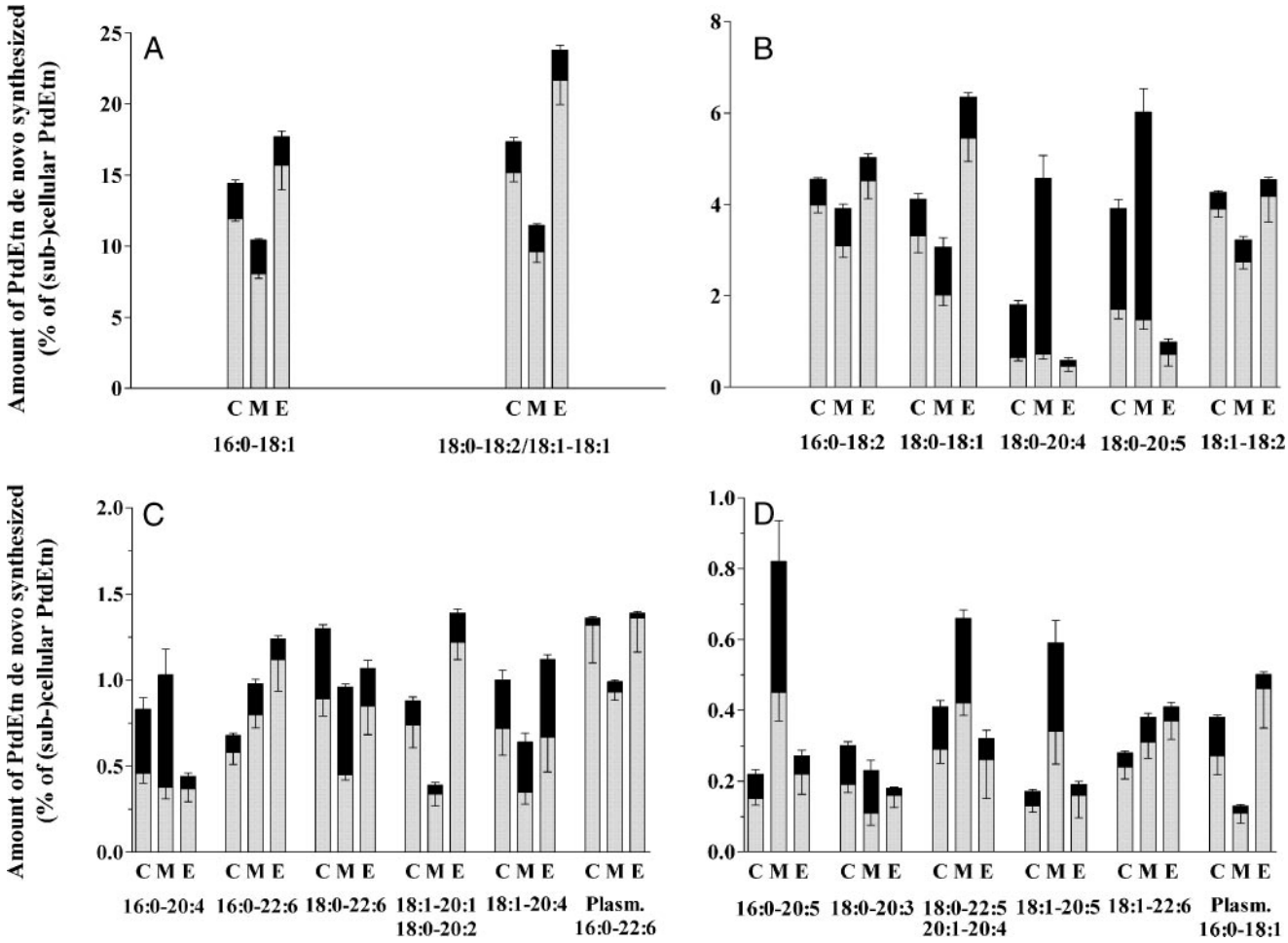

FIGURE 4. **Amount of phosphatidylethanolamine synthesized via the CDP-ethanolamine pathway and phosphatidylserine decarboxylation.** McArdle cells were grown at 37 °C in 175-cm^2 flasks. When the cells were about 75% confluent, cells were labeled with d_3-Ser or d_4-Etn for 24 h. After labeling, the cells (*C*) were collected for lipid extraction or subcellular fractions (mitochondria (*M*) and microsomes (*E*)) were prepared before lipid extraction. Subsequently lipids were separated into the major phospholipid classes by HPLC followed by molecular species profiling of the various phospholipids classes by HPLC-MS as described under "Experimental Procedures." The *black bars* represent the contribution of PtdSer decarboxylation, and the *gray bars* the contribution of the CDP-Etn pathway to newly synthesized PtdEtn. Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

TABLE 3

Composition of some phosphatidylserine molecular species in McA-RH7777 cells, mitochondria, and microsomes

The PtdSer fraction was collected from total lipid extracts of McA-RH7777 cells, mitochondria, and microsomes, and the molecular species compositions were determined as described under "Experimental Procedures." Shown in this table is the composition of six selected PtdSer molecular species (first row) that were abundant and/or interesting with respect to the research question in the total cell homogenate (second row), the mitochondrial fraction (third row), and the microsomal fraction (fourth row). Data are expressed as the mean ± S.D. of three experiments, performed in triplicate.

PtdSer species	Whole cell	Mitochondria	Microsomes
	% of total PtdSer (average ± S.D.)		
16:0-18:1	9.4 ± 0.4	9.4 ± 0.7	9.1 ± 0.5
18:0-18:1	24.3 ± 2.1	23.8 ± 2.5	23.5 ± 3.7
18:0-18:2/18:1-18:1	10.4 ± 0.6	10.0 ± 0.6	10.1 ± 0.8
18:0-20:3	4.6 ± 0.1	4.7 ± 0.4	5.1 ± 0.4
18:0-20:4	2.5 ± 0.3	2.5 ± 0.2	2.7 ± 0.5
18:0-20:5	2.1 ± 0.5	2.0 ± 0.3	2.1 ± 0.4

(50%) (Fig. 3), which is lower than the amount of newly synthesized PtdCho (see Fig. 2*A*) due to the lower specific activity of deuterated Ser. The similar extent of labeling of all PtdSer species implicates that the relative amounts of the various PtdSer species, synthesized in 24 h, reflected the PtdSer molecular species composition shown in Table 3.

Contribution of CDP-Etn Pathway and PtdSer Decarboxylation to Newly Synthesized PtdEtn in McA-RH7777 Mitochondria and Microsomes—PtdEtn synthesized via the CDP-Etn pathway originates from the ER (1, 10, 33, 34), whereas PtdSer decarboxylation occurs in the mitochondria (1, 2, 11). Shiao *et al.* (12) concluded from radioactive labeling studies that in Chinese hamster ovary cells the majority of mitochondrial PtdEtn was derived from PtdSer decarboxylation and that essentially no PtdEtn was imported from the ER. To get a picture of "local" PtdEtn and PtdCho synthesis in our cell system, a similar experiment as described in the previous section was performed using McArdle cells labeled with the three pathway-specific deuterated precursors (d_4-Etn, d_9-Cho, and d_3-Ser) for 24 h.

No significant difference was detected in the PtdCho species profile between the whole cell and the mitochondrial and microsomal fractions (Table 2), nor were any significant differences found in subcellular d_9-Cho incorporation. As already described for the whole cell, PtdCho species in the mitochondria and microsomes were labeled to the same degree (70%) (data not shown). PtdCho is synthesized in the Golgi via CPT and in the ER via choline/ethanolaminephos-

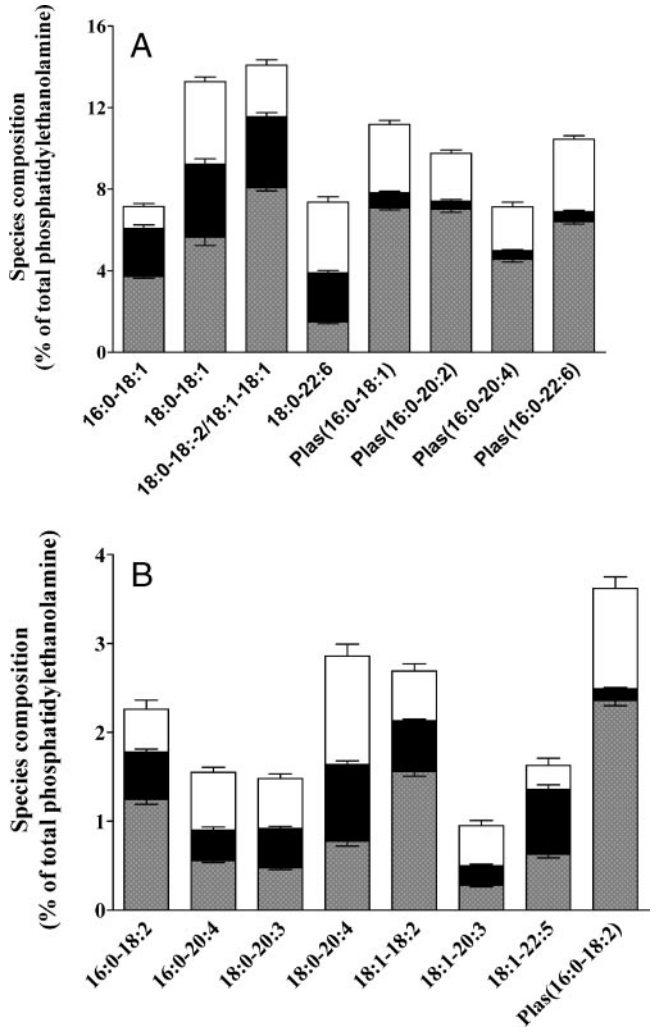

FIGURE 5. **Amount of phosphatidylethanolamine in CHO-K1 cells and contribution of the CDP-ethanolamine pathway and phoshatidylserine decarboxylation to its *de novo* synthesis.** CHO-K1 cells were grown at 37 °C in 100-mm dishes. When the cells were ~75% confluent, cells were labeled with deuterated pathway-specific precursors for 24 h to monitor the PtdEtn composition (*open bars*) and *de novo* PtdEtn synthesis from d_3-Ser (*black inset*) and d_4-Etn (*gray inset*). After labeling, the cells were collected, and lipids were extracted, and the major PtdEtn species were separated and quantified using HPLC-MS as described under "Experimental Procedures." Data are expressed as the mean ± S.D. of three experiments performed in triplicate.

photransferase (33); therefore, these data suggest a tendency of newly synthesized PtdCho to equilibrate relative quickly between its place of synthesis, mitochondria, and presumably other cellular membranes.

In contrast to what was observed for PtdCho, the PtdEtn species distribution over the mitochondria and microsomes was very distinct (Table 1). Whereas some species were more or less equally distributed over both fractions, (16:0-18:1)PtdEtn, (18:0-18:2/18:1-18:1)PtdEtn, and (18:1-20:1)PtdEtn were 1.5–3 times more abundant in the ER compared with the mitochondria. On the contrary, (16:0-20:4)-PtdEtn, (16:0-20:5)PtdEtn, (18:0-20:3)PtdEtn, (18:0-22:6)PtdEtn, and (18:1-20:5)PtdEtn were enriched in the mitochondria. The most striking difference, however, was the distribution of (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn; these species were 14- and 10-fold more abundant in the mitochondria compared with the microsomes, respectively.

The preferential synthesis of (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn via PtdSer decarboxylation, as observed in whole cells (Fig. 4, *cf.* Fig. 2*B*), was even more pronounced in mitochondria (Fig. 4); ~80% of the newly synthesized molecules of these PtdEtn species were derived from PtdSer decarboxylation (*black bars*). In addition, newly synthesized PtdEtn of several other species was found to be preferentially derived from PtdSer decarboxylation in the mitochondria, including (16:0-20:4)PtdEtn, (18:0-20:3)PtdEtn, and (18:0-22:6)PtdEtn, whereas almost half of newly synthesized (16:0-20:5)PtdEtn, (16:0-22:5)PtdEtn, and (18:1-20:5)PtdEtn in the mitochondria was derived from this route. For all other newly synthesized PtdEtn species (except the ethanolamine plasmalogens) in the mitochondria, the balance was shifted toward PtdSer decarboxylation as well but only to a modest extent; the vast majority of *de novo* synthesis for these species had taken place via the CDP-Etn pathway. In mitochondria the ratio CDP-Etn *versus* PtdSer decarboxylation-derived PtdEtn was shifted toward the decarboxylation route in comparison to the whole cell. The balance Kennedy pathway/PtdSer decarboxylation observed in the microsomal fraction (Fig. 4) was the opposite of what was found in the mitochondrial fraction; all newly synthesized PtdEtn species in the microsomes, including (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn, were almost exclusively derived from the CDP-Etn pathway.

The PtdSer decarboxylation pathway generated preferentially PtdEtn species with a polyunsaturated (*e.g.* 20:4 or 20:5) fatty acid at the *sn*-2 position. The preference of PSD for various PtdSer species was determined to get insight whether the substrate specificity of the enzyme contributes to this observation. Using egg-d_3-PtdSer containing different concentrations of various PtdSer molecular species (Fig. 6*A*) and equal amounts of five d_3-labeled PtdSer species (Fig. 6*C*) as substrates, it was shown that the PtdSer species with a 20:4 fatty acid on the *sn*-2 position were preferentially decarboxylated by mitochondrial PSD *in vitro* (Figs. 6, *B* and *D*).

DISCUSSION

The CDP-Etn pathway and PtdSer decarboxylation are present in all mammalian cells. Here, we described the detailed analysis of the qualitative and quantitative contributions of the various pathways responsible for PtdEtn, PtdSer, and PtdCho biosynthesis in McA-RH7777 cells by pathway-specific deuterium labeling coupled to HPLC-MS analysis. At the 24-h labeling time point, the amount of newly synthesized PtdSer is somewhat lower than that of PtdCho and PtdEtn via their respective CDP pathways (Figs. 2, *A* and *B*). This can be explained as, in contrast to Cho and Etn, McArdle cells synthesize Ser *de novo*. As a result, the intracellular pool-size of Ser, which is composed of d_3-Ser, d_2-Ser, and Ser is only for 80 ± 5% deuterated (ratio d_3-Ser to d_2-Ser is 1:1), whereas in the case of Etn the pool is for 94 ± 2 deuterated. Therefore, it can be concluded that in the Figs. 3 and 4 the contribution of the PtdSer decarboxylation pathway to PtdEtn synthesis is somewhat underestimated. However, this lower specific activity of the deuterated intracellular Ser pool does not influence the major conclusions drawn from Figs. 3 and 4. Furthermore, Fig. 2*B*

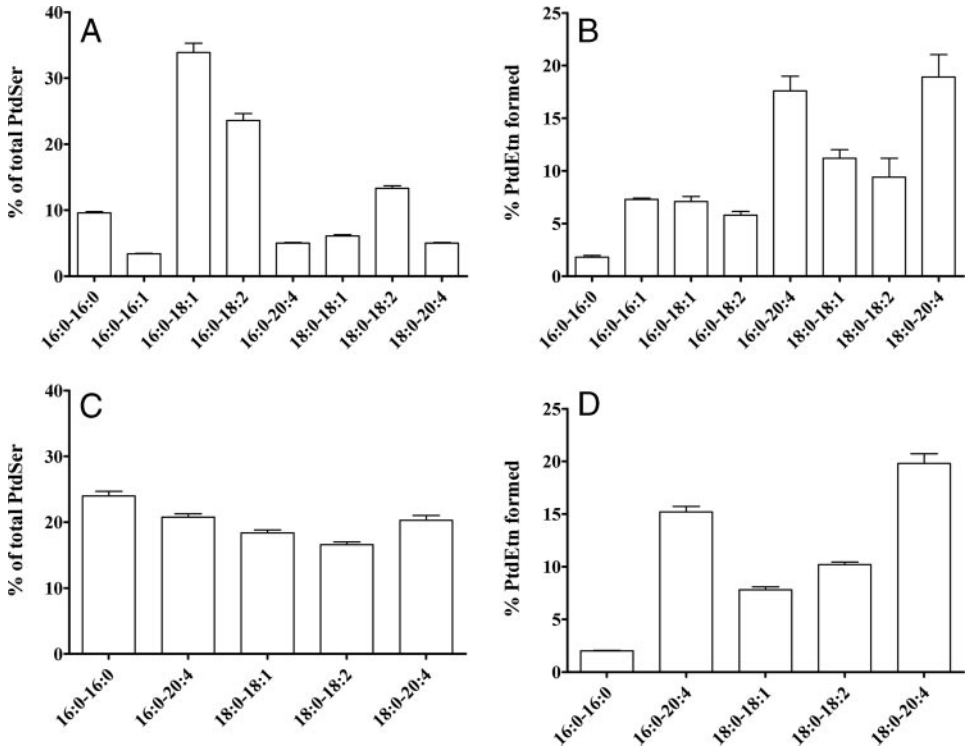

FIGURE 6. **Substrate specificity of phosphatidylserine decarboxylase.** McArdle cells were grown at 37 °C in 175-cm^2 flasks. When the cells were about 75% confluent, cells were incubated in the presence of 400 μM ethanolamine and serine for 24 h. Cells were harvested, and mitochondria were prepared and used in the PSD assay. Lipids were extracted and quantified as described under "Experimental Procedures." *B*, d_3-PtdEtn formed in 45 min from its substrate d_3-egg PtdSer (*A*). *D*, d_3-PtdEtn formed from its substrate five synthesized d_3-PtdSer species (*C*). The amount of PtdEtn formed is expressed as the percentage of its corresponding PtdSer species. Data are expressed as the mean ± S.D. of one representative experiment, which was repeated twice with similar results.

shows the quantitative contribution of the CDP-Etn route to the synthesis of the various PtdEtn species independent of the d_3-Ser labeling experiments. We expected the observation that not all PtdEtn species were labeled to the same extent at 24 and 72 h to be due to the fact that not all PtdEtn species were made via the CDP-Etn pathway in the same quantity and not due to incomplete remodeling of some species. This was supported by the observations that (i) the two plasmalogen PtdEtn species show a similar profile as PtdCho (see Figs. 2, *A* and *B*), which is in agreement with the fact that plasmalogens are exclusively synthesized via the CDP-Etn pathway and (ii) the PtdEtn species with a relative low labeling percentage from the CDP-Etn pathway were rapidly synthesized via the decarboxylation route, as was confirmed by the d_3-Ser incorporation studies (Figs. 3 and 4).

Preferential Synthesis of Polyunsaturated PtdEtn Species via the PtdSer Decarboxylation Route—Although the PtdSer decarboxylation pathway is capable of synthesizing all diacyl species analyzed in CHO-K1 and McArdle cells, it generated preferentially PtdEtn species with a polyunsaturated fatty acid at the *sn*-2 position (CHO-K1, 20:3, 20:4, and 22:6; McArdle, 20:4 and 20:5). Where does the distinct species profile synthesized by PtdSer decarboxylation in McArdle cells originate from? Three possibilities were considered. (i) Certain PtdSer species are preferentially translocated from the site of PtdSer biosynthesis (ER/MAM) to the organelle where PtdSer decarboxylation takes place (mitochondria) or equally imported into mitochondria and subjected to remodeling before decarboxylation; (ii) all PtdSer species are transported from ER/MAM to mitochondria with equal efficiency, and selectivity is introduced by substrate specificity of the PtdSer decarboxylase (PSD); (iii) PSD is indiscriminative toward imported PtdSer species, and selectivity is introduced by remodeling of PtdEtn synthesized by PSD in the mitochondria.

To assess the possibility that PtdSer species were preferentially imported into the mitochondria (i), the PtdSer species composition and d_3-Ser incorporation into this phospholipid class was determined in cells, mitochondria, and microsomes. There was no difference in PtdSer species composition between the whole cell, mitochondria, and microsomes, and more importantly, all PtdSer species were labeled with d_3-Ser with equal efficiency in all three fractions. Based on these results, it seems that PtdSer, once synthesized in the ER/MAM, readily equilibrates over the cell. Therefore, we consider it unlikely that the species selectivity observed in the PSD pathway originates from selectivity in PtdSer import into mitochondria or specific remodeling of PtdSer in the mitochondria.

The second possibility (ii), *i.e.* that the species selectivity originates from substrate specificity of the PSD, seems a likely explanation as an *in vitro* PSD assay, using isolated mitochondria as source for the decarboxylase, showed a clear substrate preference for PtdSer species with a 20:4 fatty acyl chain at the *sn*-2 position (see Fig. 6). So the substrate specificity of the decarboxylase explains at least in part the preferential synthesis of PtdEtn species with long-chain polyunsaturated fatty acids at the *sn-2* position. This observation is in contrast to earlier reports, where no clear preference of PSD for polyunsaturated fatty acids was detected in *in vitro* studies (35, 36). We have no ready explanation for this discrepancy except that different tissue, cell lines, and methodologies were used.

Alternatively, selectivity is introduced by rapid remodeling of PtdEtn generated by decarboxylation of PtdSer (iii). Although it has been shown that the *de novo*-synthesized phospholipids are extensively remodeled by deacylation-reacylation (37–39), studies on phospholipid remodeling have yielded conflicting results. Because the enzymes involved in remodeling are reported to reside in several subcellular compartments (40–43), including microsomes and mitochondria, remodeling of PtdEtn "freshly" synthesized by PtdSer decarboxylation in the mitochondria is a possible explanation for the preferential syn-

thesis of (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn via this route that cannot be excluded by our data.

The Molecular Species Profiles Synthesized by the Kennedy Pathways Are Similar—The final reaction of the Kennedy pathways for PtdEtn and PtdCho synthesis is catalyzed by a phosphotransferase. Mammalian cells express a phosphotransferase with a dual specificity for CDP-Etn and CDP-Cho. Because the phosphotransferase reaction supplies the diacylglycerol part of the PtdEtn and PtdCho molecules, one would expect, when remodeling is of minor contribution, the species profiles of PtdCho and PtdEtn made by the respective Kennedy pathways to be similar. As shown in Fig. 3, this seems to be largely the case, as the CDP-Etn/Cho pathways synthesize predominantly saturated and mono- and di-unsaturated phospholipid species. Thus, the large variety of PtdEtn species (*versus* PtdCho) (Tables 1 and 2) is mainly caused by the presence of an additional pathway, the PtdSer decarboxylation route.

Involvement of a Serine Racemase Explains the Synthesis of d_2-PtdSer from d_3-serine—In mammalian cells PtdSer is synthesized by a calcium-dependent base-exchange reaction in which the head group of pre-existing PtdCho and PtdEtn is replaced with L-serine, catalyzed by PtdSer synthase 1 and 2, respectively (44). The molecular species profile of PtdSer agrees with that of its substrate, microsomal PtdCho and PtdEtn, except for the enrichment in (18:0-18:1)PtdSer (Tables 1–3). Whether this is caused by a substrate preference of the PtdSer synthases or by the existence of specific PtdSer pools between endoplasmic reticulum and MAM, cannot be concluded from our data. The analysis of d_3-Ser incorporation into PtdSer and PtdEtn showed an unexpected finding; in addition to endogenous, unlabeled phospholipid species and d_3-Ser labeled species, significant d_2-Ser incorporation in all PtdEtn and PtdSer species was detected (Fig. 1*D*). This phenomenon could not be correlated to impurity of the d_3-Ser (L-[2,3,3-d_3]-serine) label used nor to a-specific, non-enzymatic exchange of deuterium atoms from d_3-Ser with protons from water molecules during incubation (not shown). One possible explanation is that during the exchange of the head group from PtdCho or PtdEtn with serine, a deuterium atom is lost. This seems unlikely, as the hydroxyl group participating in the PtdSer synthase-catalyzed reaction is not deuterated in the precursor (L-[2,3,3-d_3]-serine). More likely may be the involvement of a serine racemase activity. This enzyme, which is present in various tissues like brain, heart, kidney, and liver (45), catalyzes the conversion of L-serine to D-serine and vice versa. The reaction mechanism of amino acid racemases, such as alanine and serine racemase (46, 47), shows that the hydrogen attached to the C2 (α-atom) of the amino acid gets detached from the molecule, resulting in the conversion (2,3,3-d_3-labeled) L-serine to (3,3-d_2-labeled) D-serine. The presence of a substantial amount of d_2-Ser-labeled PtdSer and PtdEtn suggests that one or both PtdSer synthases may possibly use D-serine as substrate provided that a racemase is indeed the cause of the d_2-Ser formation. However, Kuksis and Itabashi recently showed that in rat brain, a tissue with a relatively high serine racemase activity, the serine in PtdSer was exclusively in the L-configuration (48). This observation suggests that (3,3-d_2-labeled) D-serine is most likely converted back to (3,3-d_2-labeled) L-serine despite a 10× higher k_m for the D- to L-serine than for the L- to D-serine conversion before it is used by the PtdSer synthase.

Local Phosphatidylethanolamine Synthesis Suggests an Essential Role of the PtdSer Decarboxylation Route in Mitochondria—The main PtdEtn species newly synthesized from the Kennedy pathway in the ER/microsomes appeared to rapidly equilibrate between ER and mitochondria and seemed to be actively imported into mitochondria. The rapid equilibration of PtdEtn *de novo* synthesized by the CDP-Etn pathway but also of the newly formed PtdSer and PtdCho observed in our study is in agreement with previous reports (13). Because the Kennedy pathway was found to be responsible for the bulk of the PtdEtn synthesis, rapid equilibration over the whole cell is probably required to keep up with the cellular demand for this phospholipid. In contrast, (18:0-20:4)PtdEtn and (18:0-20:5)PtdEtn, mainly formed by PtdSer decarboxylation in the mitochondria, appeared to be actively retained within this organelle, as these two species are notably enriched in the mitochondria but are hardly present in the microsomal fraction. Although the biochemical significance of this observation is not known yet, Steenbergen *et al.* (49) recently showed that embryos of mice lacking PSD activity die in the uterus and have fragmented and misshapen mitochondria, an abnormality that likely contributes to the embryonic lethality of the PSD knock-out.

REFERENCES

1. Vance, D. E. (1996) *Biochemistry of Lipids, Lipoproteins and Membranes*, pp. 1–33 Elsevier Science Publishers B.V., Amsterdam, The Netherlands
2. Vance, J. E., and Vance, D. E. (2004) *Biochem. Cell Biol.* **82,** 113–128
3. Henneberry, A. L., and McMaster, C. R. (1999) *Biochem. J.* **339,** 291–298
4. Henneberry, A. L., Wistow, G., and McMaster, C. R. (2000) *J. Biol. Chem.* **275,** 29808–29815
5. Borkenhagen, L. F., Kennedy, E. P., and Fielding, L. (1961) *J. Biol. Chem.* **236,** PC28–PC30
6. Kuge, O., Nishijima, M., and Akamatsu, Y. (1991) *J. Biol. Chem.* **266,** 24184–24189
7. Kuge, O., Saito, K., and Nishijima, M. (1997) *J. Biol. Chem.* **272,** 19133–19139
8. Kuge, O., Nishijima, M., and Akamatsu, Y. (1991) *J. Biol. Chem.* **266,** 6370–6376
9. Zborowski, J., Dygas, A., and Wojtczak, L. (1983) *FEBS Lett.* **157,** 179–182
10. van Golde, L. M., Raben, J., Batenburg, J. J., Fleischer, B., Zambrano, F., and Fleischer, S. (1974) *Biochim. Biophys. Acta* **360,** 179–192
11. Vance, J. E. (1990) *J. Biol. Chem.* **265,** 7248–7256
12. Shiao, Y. J., Lupo, G., and Vance, J. E. (1995) *J. Biol. Chem.* **270,** 11190–11198
13. Vance, J. E. (1991) *J. Biol. Chem.* **266,** 89–97
14. Voelker, D. R. (1990) *J. Biol. Chem.* **265,** 14340–14346
15. Kuge, O., Nishijima, M., and Akamatsu, Y. (1986) *J. Biol. Chem.* **261,** 5790–5794
16. Miller, M. A., and Kent, C. (1986) *J. Biol. Chem.* **261,** 9753–9761
17. Voelker, D. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 2669–2673
18. Zelinski, T. A., and Choy, P. C. (1982) *Can. J. Biochem.* **60,** 817–823
19. Arthur, G., and Page, L. (1991) *Biochem. J.* **273,** 121–125
20. Tijburg, L. B., Geelen, M. J., and van Golde, L. M. (1989) *Biochem. Biophys. Res. Commun.* **160,** 1275–1280
21. DeLong, C. J., Shen, Y. J., Thomas, M. J., and Cui, Z. (1999) *J. Biol. Chem.* **274,** 29683–29688
22. Hsu, F. F., Turk, J., Thukkani, A. K., Messner, M. C., Wildsmith, K. R., and Ford, D. A. (2003) *J. Mass Spectrom.* **38,** 752–763
23. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
24. Brouwers, J. F., Gadella, B. M., van Golde, L. M., and Tielens, A. G. (1998) *J. Lipid Res.* **39,** 344–353
25. Brouwers, J. F., Vernooij, E. A., Tielens, A. G., and van Golde, L. M. (1999)

J. Lipid Res. **40,** 164–169
26. De Bernardo, S., Weigele, M., Toome, V., Manhart, K., Leimgruber, W., Bohlen, P., Stein, S., and Udenfriend, S. (1974) *Arch. Biochem. Biophys.* **163,** 390–399
27. Yamane, T., Iwasaki, Y., Mizumoto, Y., Kasai, M., and Okada, T. (December 9, 2003) U.S. Patent 6,660,504
28. Rouser, G., Siakotos, A. N., and Fleischer, S. (1966) *Lipids* **1,** 85–86
29. Voelker, D. R. (1985) *J. Biol. Chem.* **260,** 14671–14676
30. Merrill, A. H., Jr., and Jones, D. D. (1990) *Biochim. Biophys. Acta* **1044,** 1–12
31. Steenbergen, R., Nanowski, T. S., Nelson, R., Young, S. G., and Vance, J. E. (2006) *Biochim. Biophys. Acta* **1761,** 313–323
32. Medlock, K. A., and Merrill, A. H., Jr. (1988) *Biochemistry* **27,** 7079–7084
33. Henneberry, A. L., Wright, M. M., and McMaster, C. R. (2002) *Mol. Biol. Cell* **13,** 3148–3161
34. Vance, J. E., and Vance, D. E. (1988) *J. Biol. Chem.* **263,** 5898–5909
35. Heikinheimo, L., and Somerharju, P. (2002) *Traffic* **3,** 367–377
36. Kevala, J. H., and Kim, H. Y. (2001) *Anal. Biochem.* **292,** 130–138
37. Samborski, R. W., Ridgway, N. D., and Vance, D. E. (1990) *J. Biol. Chem.* **265,** 18322–18329
38. Schmid, P. C., Johnson, S. B., and Schmid, H. H. (1991) *J. Biol. Chem.* **266,** 13690–13697
39. Schmid, P. C., Spimrova, I., and Schmid, H. H. (1997) *Lipids* **32,** 1181–1187
40. Xu, Y., Kelley, R. I., Blanck, T. J., and Schlame, M. (2003) *J. Biol. Chem.* **278,** 51380–51385
41. Dircks, L., and Sul, H. S. (1999) *Prog. Lipid Res.* **38,** 461–479
42. Dennis, E. A. (1994) *J. Biol. Chem.* **269,** 13057–13060
43. Arthur, G., Zaborniak, C. L., and Choy, P. C. (1987) *Biochem. Cell Biol.* **65,** 1016–1021
44. Vance, J. E., and Steenbergen, R. (2005) *Prog. Lipid Res.* **44,** 207–234
45. Xia, M., Liu, Y., Figueroa, D. J., Chiu, C. S., Wei, N., Lawlor, A. M., Lu, P., Sur, C., Koblan, K. S., and Connolly, T. M. (2004) *Brain Res. Mol. Brain Res.* **125,** 96–104
46. Faraci, W. S., and Walsh, C. T. (1988) *Biochemistry* **27,** 3267–3276
47. Foltyn, V. N., Bendikov, I., De, M. J., Panizzutti, R., Dumin, E., Shleper, M., Li, P., Toney, M. D., Kartvelishvily, E., and Wolosker, H. (2005) *J. Biol. Chem.* **280,** 1754–1763
48. Kuksis, A., and Itabashi, Y. (2005) *Methods* **36,** 172–185
49. Steenbergen, R., Nanowski, T. S., Beigneux, A., Kulinski, A., Young, S. G., and Vance, J. E. (2005) *J. Biol. Chem.* **280,** 40032–40040