Biosynthesis of plasmenylethanolamine (1-O-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine) in the guinea pig heart

Fred Y. Xu, Karmin O,[1] and Patrick C. Choy[2]

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E OW3

Abstract In this study, the isolated guinea pig heart was pulse-labeled with a precursor of ethanolamine glycerophospholipid, and then chased with the non-radioactive compound for 0–8 h. Labeling with hexadecanol revealed that plasmanylethanolamine was the immediate precursor of plasmenylethanolamine, but a substantial portion of the label was also found in phosphatidylethanolamine. When ethanolamine was used as the precursor, the labeling of plasmenylethanolamine was between 50–65% of the labeling of phosphatidylethanolamine, and this ratio was maintained throughout the perfusion. The ratio of labeling is similar to the ratio of pool sizes of these ethanolamine glycerophospholipid in the heart, which implies that the CDP-ethanolamine pathway is also important for plasmenylethanolamine biosynthesis. The role of diradylglycerol in the synthesis of each ethanolamine glycerophospholipid was also investigated. The ratio of 1-alkenyl-2-acyl glycerol to total diradylglycerol content was 7% in the homogenate and 32% in the microsomes. However, ethanolamine phosphotransferase displayed a distinct selectivity towards 1-alkenyl-2-acyl glycerol. Kinetic studies revealed that the synthesis of phosphatidylethanolamine was inhibited by 1-alkenyl-2-acyl glycerol, but the formation of plasmenylethanolamine was not affected by 1,2-diacylglycerol. In addition, the inhibition of ethanolamine phosphotransferase by 1-alkyl-2-acyl glycerol appears to be an important mechanism for the coordination of plasmenylethanolamine biosynthesis via the desaturase reaction and the CDP-ethanolamine pathway.—**Xu, F. Y., K. O and P. C. Choy.** Biosynthesis of plasmenylethanolamine (1-O-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine) in the guinea pig heart. *J. Lipid Res.* 1997. **38:** 670–679.

Supplementary key words plasmenylethanolamine • phosphatidylethanolamine • diradylglycerol • biosynthesis • ethanolamine phosphotransferase

Ethanolamine glycerophospholipid is a major phospholipid group in mammalian tissues (1). It consists of a mixture of 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (phosphatidylethanolamine), 1-O-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine), and 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmanylethanolamine) (2). Although phosphatidylethanolamine is the most abundant type of ethanolamine glycerophospholipid in mammalian tissues, up to 30–40% of ethanolamine glycerophospholipid is present as plasmenylethanolamine in neutrophils (3, 4) erythrocytes (5), vascular smooth muscles (6), and heart (7). Plasmenylethanolamine is the major form of ethanolamine glycerophospholipid in the sarcoplasmic reticulum of certain cardiac tissues (8). Alternatively, plasmanylethanolamine is a minor phospholipid group in most tissues and generally accounts for no more than 5% of the total ethanolamine glycerophospholipid content (2).

Glycerophospholipids containing the 1-O-alk-1′-enyl group are known collectively as plasmalogens. At present, the biological role of plasmalogens and the rationale for their selective distribution in tissues have not been well defined. Plasmalogens have been postulated to be stabilizers of the membrane bilayer in bacteria (9) and facilitators of calcium translocation in sarcoplasmic reticulum (8). The presence of the vinyl ether group in the plasmalogen may protect animal cells from oxidative stress (10). The high arachidonoyl content found in plasmenylethanolamine may serve as specific reservoirs of the polyunsaturated fatty acid for eicosanoid formation (11, 12).

The only known pathway for the introduction of the vinyl ether group into cellular lipids is mediated by the action of a NADH (or NADPH)-dependent alkyl ether desaturase. The enzyme catalyzes the oxidation of the 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine substrate for the production of the vinyl ether group in plasmenylethanolamine (13, 14). The desaturase reaction is highly specific and does not catalyze the forma-

[1]Present address: Department of Pharmacology, University of Hong Kong.

[2]To whom correspondence should be addressed.

tion of vinyl ether groups from 1-O-alkyl-2-acyl-*sn*-3-phosphocholine or 1-O-alkyl-2-acyl-glycerol (14). However, a significant amount of 1-O-alkenyl-2-acyl-glycerol is present in mammalian tissues and this diradylglycerol may originate from plasmenylethanolamine via the hydrolytic action of a phospholipase C or, to a lesser extent, the reversal of the CDP-ethanolamine phosphotransferase reaction (15, 16). The condensation of 1-O-alkenyl-2-acyl-glycerol with CDP-choline appears to be the sole pathway for plasmenylcholine formation (15). 1-O-alkenyl-2-acyl-glycerol also participates in the direct formation of plasmenylethanolamine via the CDP-ethanolamine pathway. We have shown earlier that the synthesis of phosphatidylcholine and plasmenylcholine in the liver and heart of the guinea pig is catalyzed by the cholinephosphotransferase, which is a bifunctional enzyme for the condensation of CDP-choline with the appropriate diradylglycerol (17). The rate of plasmenylcholine biosynthesis appears to be dependent on the availability of 1-O-alkenyl-2-acyl-glycerol and the reaction is subjected to modulation by the 1,2-diacylglycerol content of the tissue. Although the condensation of 1-O-alkenyl-2-acyl-glycerol with CDP-ethanolamine for the formation of plasmenylethanolamine has been documented (18), it was not clear whether the reaction was catalyzed by the same ethanolaminephosphotransferase for the formation of phosphatidylethanolamine. The mechanism for the regulation of plasmenylethanolamine biosynthesis via the CDP-ethanolamine pathway in the guinea pig heart has not been explored.

METHODS

Materials

CDP-choline, diacylglycerol (pig liver), and other lipid standards were purchased from Serdary Research Laboratory (London, Ontario). [1,2-^{3}H]ethanolamine chloride and CDP-[1,2-^{14}C]ethanolamine were the product of Amersham International Limited (Oakville, Ontario). Thin-layer chromatographic plates (Sil-G25) were obtained from Fisher Scientific (Edmonton, Alberta). Palmityl alcohol (hexadecanol), [1-^{14}C]palmityl alcohol, phosphalipase C (*B. cereus*), Tween-20, and alkyl-phosphatidylcholine were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were of the highest available grade and were acquired from the Canlab Division of Travenol Canada Inc. (Winnipeg, Manitoba).

Ethanolamine phosphoglycerides were isolated from the porcine heart lipid extract by silica gel chromatography as previously described (19). Phosphatidylethanolamine in the ethanolamine glycerophospholipids was preferentially hydrolyzed by 0.35 N NaOH, and the plasmenylethanolamine in the fraction was further purified by silica gel chromatography (20). 1-Alk-1′-enyl-2-acyl glycerol was obtained from plasmenylethanolamine by the hydrolytic action of phospholipase C. 1-Alkyl-2-acyl glycerol was obtained from 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by the hydrolytic action of phospholipase C. The 1-alk-1′-enyl-2-acyl glycerol or 1-alkyl-2-acyl glycerol obtained from phospholipase C hydrolysis was purified by thin-layer chromatography using a two-solvent system as described under diradylglycerol contents.

Experimental animals

Male albino guinea pigs, weighing 250–300 g, were obtained from High Oak, Ontario. The animals were maintained on Purina chow and tap water, ad libitum, in a light- and temperature-controlled room.

Perfusion of the isolated guinea pig heart

The animal was decapitated under light ether anesthesia. The heart was excised and placed in Krebs-Henseleit buffer (pH 7.4) that was saturated with 95% oxygen–5% carbon dioxide. The aorta was cannulated and the isolated heart was perfused at 37°C in the Langendorff mode at a constant pressure of 80–100 mm Hg with a coronary flow rate of 4 ml/min (21). Each heart was initially perfused with Krebs-Henseleit buffer for 15 min in order to restore the regular cardiac rhythm. After the stabilization period, the heart was perfused with the same buffer containing 0.5 μM radioactive ethanolamine or hexadecanol for 20 min, followed by perfusion with 5.0 μM of the same non-radioactive compound for the prescribed period of time. Subsequent to perfusion, 10 ml of air was forced through the cannula to remove the buffer from the vascular space of the organ. The heart was cut open and blotted dry for wet weight determination prior to homogenization.

Analyses of ethanolamine-containing metabolites

Immediately after perfusion, the guinea pig heart was homogenized in chloroform–methanol 1:1 (v/v) to yield a 15% (w/v) homogenate. The homogenate was centrifuged at 1,000 *g* for 10 min and the resultant pellet was washed twice with the same solution and re-centrifuged under the same conditions. The supernatants were pooled and phase separation was achieved by the addition of chloroform and water. The ethanolamine glycerophospholipid (containing phosphatidylethanolamine, plasmenylethanolamine, and plasmanylethanolamine) in the organic phase was separated from other phospholipids by thin-layer chromatography with solvent A, which was composed of chloroform–methanol–acetic acid–water 35:15:1:2 (v/v) (19, 22). The etha-

nolamine glycerophospholipid fraction was eluted from the silica gel by three washes with chloroform–methanol–acetic acid–water 50:39:1:10 (v/v) and an aliquot of the eluant was used for radioactivity determination. For the quantitative determination of plasmenylethanolamine, the ethanolamine glycerophospholipid fraction eluted from the chromatogram was applied to another thin-layer chromatographic plate. The plate was exposed to HCl vapors for 15 min and then developed in solvent A for the separation of the ethanolamine glycerophospholipid fraction (containing phosphatidylethanolamine and plasmanylethanolamine) and the 2-acylglycerophosphoethanolamine fraction (containing the hydrolytic product of plasmenylethanolamine) (11). The exposure of the ethanolamine glycerophospholipid fraction to HCl vapor in this manner resulted in the hydrolysis of 95% of the vinyl ether group in plasmenylethanolamine but less than 1% of the acyl groups in phosphatidylethanolamine. In some experiments, the radioactivity in plasmenylethanolamine was confirmed by determining the labeling of the fatty aldehyde released by HCl vapor treatment. The ethanolamine glycerophospholipid fraction was eluted and the hydrolysis of the acyl groups in phosphatidylethanolamine and plasmanylethanolamine was achieved by incubation with 0.35 N NaOH for 60 min (23). The NaOH treatment resulted in the hydrolysis of over 99% of the acyl groups. The sample after NaOH treatment was reapplied to a thin-layer chromatographic plate that was developed in solvent A in order to separate the lysoplasmanylethanolamine fraction (from the hydrolysis of plasmanylethanolamine) and free acyl groups. The phosphatidylethanolamine content was calculated by subtracting the amount of lysoplasmanylethanolamine from the total amount of ethanolamine glycerophospholipid before thin-layer chromatography.

Determination of ethanolaminephosphotransferase activity

Immediately after excision, the guinea pig heart was homogenized in a buffer containing 0.25 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4). The homogenate was centrifuged at 10,000 *g* for 10 min in order to pellet the nuclei and mitochondrial fractions. The microsomal fraction was prepared from the resultant supernatant by centrifugation at 100,000 *g* for 60 min. The microsomal pellet was washed once with the same buffer, and then resuspended in the 0.25 M sucrose buffer. Enzyme activity was determined in an assay mixture (1 ml) containing 40 mM Tris-HCl (pH 8), 10 mM magnesium chloride, 1 mM EDTA, 0.2 mM CDP-[1,2-^{14}C]ethanolamine (2 μCi/μmol), 0.6 mM diacylglycerol, alkylacylglycerol, or alkenylacylglycerol (in 0.015% Tween 20) and 0.05–0.10 mg of enzyme protein (24). The reaction was initiated by the addition of the enzyme preparation and the mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 6 ml of chloroform–methanol 2:1 (v/v). Water was added to the mixture to cause phase separation. The organic phase was removed and washed three times with 3 ml 40% methanol. The radioactivity in phosphatidylethanolamine, plasmenylethanolamine, or plasmanylethanolamine was confirmed by thin-layer chromatographic analysis.

Enzyme activity was also assayed using endogenous diradylglycerol in the microsomes in the absence or presence of exogenous diradylglycerol. The exogenous diradylglycerol was added to the reaction mixture after dispersion by sonication without detergent. As all three types of ethanolamine glycerophospholipid would be produced from this reaction, it was important to determine the labeling in each product. After the reaction, the ethanolamine glycerophospholipid in the organic phase was isolated by thin-layer chromatography and an aliquot of the sample was applied to another thin-layer chromatographic plate. The plate was then exposed to HCl vapor and subsequently developed in solvent A. After development, the labelings in the ethanolamine glycerophospholipid and ethanolamine lysoglycerophospholipid fractions were determined. The labeling in plasmenylethanolamine was estimated from the ethanolamine lysoglycerophospholipid fraction, whereas the total labeling of phosphatidylethanolamine and plasmanylethanolamine was estimated from the ethanolamine glycerophospholipid fraction. The ethanolamine glycerophospholipid was eluted from the silica gel and treated with 0.35 N NaOH for 60 min in order to hydrolyze the acyl groups. The sample after NaOH treatment was reapplied to a thin-layer chromatographic plate that was developed in solvent A. The labeling of plasmanylethanolamine was estimated from the lysoplasmanylethanolamine fraction, whereas the labeling of phosphatidylethanolamine was calculated from the total amount of radioactivity in the sample before NaOH treatment.

Diradylglycerol contents in the microsomal fraction of the guinea pig heart

Diradylglycerols were extracted from the microsomal fraction of the guinea pig heart and isolated by thin-layer chromatography as previously reported (17). The diacylglycerol, alkenylacylglycerol, and alkylacylglycerol fractions were separated from each other by thin-layer chromatography developed in a first solvent system containing hexane–diethyl ether 95:5 (v/v) and then developed twice in a second solvent system containing petroleum ether–diethyl ether–acetic acid 80:20:1 (v/v) (25, 26). The R_f values of diacylglycerol, alkenylacylglyc-

erol, and alkylacylglycerol were 0.28, 0.38, and 0.35, respectively. No detectable cross-contamination of the diradylglycerol species was detected when each species was analyzed by mild acid or alkaline treatment in order to quantitate the alk-1-enyl group or the acyl group (17, 23). For pool size study, the fatty acid groups in each diradylglycerol fraction were converted into the appropriate methyl esters or dimethylketals. The contents of these methyl derivatives were estimated by gas–liquid chromatography (17).

Other determinations

Protein was determined by the method of Lowry et al. (27). Lipid phosphorus was determined by the procedure of Bartlett (28). The vinyl ether groups in plasmenylethanolamine and 1-alk-1′-enyl-2-acyl glycerol were determined by the procedure of Gottfried and Rapport (29). Student's *t*-test was used for statistical analyses and the level of significance was set at 0.05.

RESULTS

Ethanolamine glycerophospholipid contents in the guinea pig heart

Lipids were extracted from the guinea pig heart and the content of each type of ethanolamine glycerophospholipid was determined (**Table 1**). Phosphatidylethanolamine (64%) and plasmenylethanolamine (35%) were major groups whereas plasmanylethanolamine (<2%) was the minor ethanolamine glycerophospholipid in the cardiac tissue. The content of ethanolamine glycerophospholipid and its composition in the guinea pig heart were not significantly changed when the heart was perfused for 2 h in Krebs-Henseleit buffer (Table 1) or in Krebs-Henseleit buffer containing 0.5 μM palmitoyl alcohol or 0.5 μM ethanolamine.

TABLE 1. Ethanolamine glycerophospholipid contents in guinea pig heart

Ethanolamine Phosphoglyceride	Content	
	0 h	2 h
	μmol of lipid protein/g wet weight	
Phosphatidylethanolamine	6.016 ± 0.26	5.831 ± 0.47
Plasmanylethanolamine	0.141 ± 0.01	0.173 ± 0.01
Plasmenylethanolamine	3.243 ± 0.14	3.677 ± 0.29
Total ethanolamine phosphoglyceride	9.401 ± 0.77	9.690 ± 0.56

Guinea pig hearts were pulse-labeled with 0.5 μM [^{14}C]hexadecanol (10 μCi/nmol) for 20 min and chased with 5.0 μM unlabeled hexadecanol for 0 or 2 h. After perfusion, hearts were homogenized in chloroform–methanol 1:1 (v/v) and the lipid extract was analyzed for ethanolamine glycerophospholipid content. Each set of values represents the mean ± standard deviation of three separate experiments.

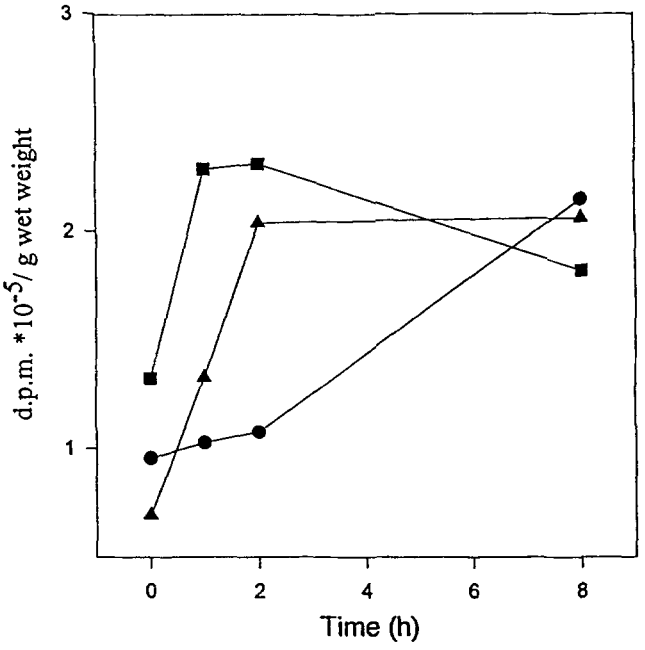

Fig. 1. Time course for the labeling of ethanolamine glycerophospholipids, by palmitoyl alcohol in the guinea pig heart. Guinea pig hearts were pulse-labeled with 0.5 μM [^{14}C]hexadecanol (10 μCi/nmol) for 20 min and chased with 5.0 μM hexadecanol for up to 8 h. The heart was homogenized after the chase, and radioactivities in the phosphatidylethanolamine (▲), plasmenylethanolamine (○), and plasmanylethanolamine (■) fractions were determined. Each point is the mean of four separate experiments.

Synthesis of plasmenylethanolamine in the isolated guinea pig heart

The synthesis of plasmenylethanolamine from a labeled long-chain alcohol in the guinea pig heart was examined. The isolated heart was perfused with 0.5 μM labeled hexadecanol (10 μCi/nmol) in Krebs-Henseleit buffer for 20 min and then chased with 5 μM unlabeled hexadecanol in the same buffer for 2–8 h. After perfusion, the heart was homogenized in chloroform–methanol 1:1 (v/v) and the radioactivity associated with phosphatidylethanolamine, plasmenylethanolamine, and plasmanylethanolamine in the lipid extract was determined (**Fig. 1**). The labeling of plasmanylethanolamine increased sharply during the first 2 h of chase, and then decreased gradually from 2–8 h. Alternatively, the labeling of plasmenylethanolamine increased gradually during the first two of chase, and the increase became more rapid between 2–8 h. A precursor–product relationship pattern between the labeling of plasmanylethanolamine and plasmenylethanolamine was observed during the pulse-chase study. Interestingly, the labeling of phosphatidylethanolamine was rapid and reached a pla-

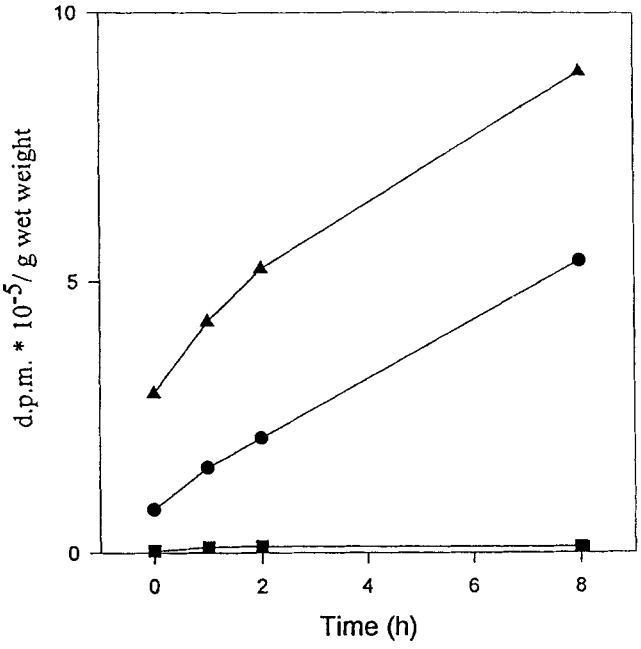

Fig. 2. Time course for the labeling of ethanolamine in the guinea pig heart. Guinea pig hearts were pulse-labeled with 0.5 μM [1,2-^{3}H]ethanolamine (10 μCi/nmol) for 20 min and chased with 5.0 μM ethanolamine for up to 8 h. The symbols are the same as in Fig. 1 and each point is the mean of three separate experiments.

teau after 2 h of chase. Hydrolysis of the acyl group of the labeled plasmanylethanolamine and plasmenylethanolamine by phospholipase A_2 and subsequent analysis of the lysophospholipid formed revealed that 86–90% of the radioactivity was associated with the alkyl or alkenyl group at the *sn*-1 position. Analysis of the fatty acyl group released after phospholipase A_2 treatment confirmed that less than 15% of the radioactivity was associated with the group at the *sn*-2 position. Alternatively, 73% of the radioactivity in phosphatidylethanolamine was found in the acyl group at the *sn*-1 position, and 25% in the acyl group at the *sn*-2 position. The results suggest that hexadecanol alcohol was first incorporated into plasmanylethanolamine, followed by the desaturation of the ether group for the formation of plasmenylethanolamine. In addition, a considerable portion of the long-chain alcohol was converted to the corresponding fatty acid which was subsequently incorporated as an acyl group in phosphatidylethanolamine. The ratio of labeling of ethanolamine phosphoglycerides was found to be entirely different from the ratio of their natural distribution in the guinea pig heart.

The synthesis of plasmenylethanolamine from labeled ethanolamine in the guinea pig heart was examined. We showed in earlier studies that the vast majority of exogenous ethanolamine would be incorporated into the ethanolamine glycerophospholipid via the CDP-ethanolamine pathway. The isolated heart was perfused with 0.5 μM labeled ethanolamine (10 μCi/nmol) in Krebs-Henseleit buffer for 20 min and then chased with 5 μM ethanolamine in the same buffer for 2–8 h. After perfusion, the heart was homogenized in chloroform–methanol 1:1, (v/v) and the radioactivity associated with each ethanolamine glycerophospholipid group was determined (**Fig. 2**). The highest amount of labeling (65–68%) was associated with the phosphatidylethanolamine fraction, and a near linear increase in the labeling was observed throughout the chase period. A significant amount of labeling (24–29%) was associated with plasmenylethanolamine, and its labeling also increased in a near linear fashion at the same rate as phosphatidylethanolamine. The labeling in plasmanylethanolamine was low (0.4–1.0%) at all time points throughout the chase period. The ratio of labeling of ethanolamine glycerophospholipids appears to resemble the ratio of their natural distribution in the guinea pig heart.

Diradylglycerol species in the homogenate and the microsomal fraction of guinea pig heart

In the mammalian heart, the CDP-ethanolamine pathway is the major pathway for the synthesis of phosphatidylethanolamine (30, 31). In this pathway, ethanolamine is converted to phosphoethanolamine and subsequently to CDP-ethanolamine, which is condensed with 1,2-diacylglycerol for the formation of the phospholipid. The last step of the pathway is catalyzed by ethanolamine phosphotransferase which may be able to condense other diradylglycerol groups for the formation of the corresponding ethanolamine glycerophospholipid. If the condensation of diradylglycerols with CDP-ethanolamine is catalyzed by the same enzyme, the pool size of each diradylglycerol moiety as well as the selectivity of the enzyme toward each diradylglycerol group are important factors in regulating the formation of ethanolamine glycerophospholipids.

TABLE 2. Diradylglyceride contents in the homogenate and microsomal fraction of the guinea pig heart

Diradylglycerol	Homogenate	Microsome
	nmol/g wet weight	*nmol/mg protein*
Diacylglycerol	1157 ± 250	26.1 ± 3.6
Alkenylacylglycerol	85.1 ± 5.7	13.9 ± 2.5
Alkylacylglycerol	8.0 ± 0.7	3.2 ± 0.3

Guinea pig hearts were homogenized in Tris-HCl buffer (pH 7.4) and the microsomal fraction was isolated by differential centrifugation. Lipids were extracted from the microsomal fraction and analyzed for diradylglycerol contents. Each set of values represents the mean ± standard deviation of four separate experiments.

TABLE 3. Effect of exogenous diacylglycerol on the synthesis of ethanolamine glycerophospholipids

Diacylglycerol	Phosphatidyl-ethanolamine	Plasmenyl-ethanolamine	Plasmanyl-ethanolamine
μm		*pmol/mg/min*	
0	24.00 ± 1.8	31.71 ± 2.5	1.82 ± 0.08
50	31.13 ± 2.9	31.64 ± 2.5	1.77 ± 0.07
100	36.02 ± 3.9	31.78 ± 1.9	1.72 ± 0.05
200	47.21 ± 5.3	32.03 ± 2.7	1.63 ± 0.06
400	63.46 ± 7.3	33.52 ± 2.8	1.53 ± 0.04

The activity of ethanolaminephosphotransferase was assayed with CDP-[^{14}C]ethanolamine in the absence or presence (0–400 μM) of exogenous diacylglycerol. Each set of values represents the mean ± standard deviation of three separate experiments.

TABLE 4. Effect of exogenous alkylacylglycerol on the synthesis of ethanolamine glycerophospholipids

Alkylacylglycerol	Phosphatidyl-ethanolamine	Plasmenyl-ethanolamine	Plasmanyl-ethanolamine
μm		*pmol/mg/min*	
0	24.00 ± 1.8	31.71 ± 2.5	1.82 ± 0.08
50	21.56 ± 2.1	27.19 ± 2.4	2.68 ± 1.14
100	17.83 ± 1.3	18.26 ± 1.9	3.38 ± 3.26
200	12.48 ± 0.9	13.99 ± 2.7	3.79 ± 1.45
400	10.78 ± 0.8	10.37 ± 3.8	3.69 ± 2.04

The activity of ethanolaminephosphotransferase was assayed with CDP-[^{14}C]ethanolamine in the absence or presence (0–400 μM) of exogenous alkylacylglycerol. Each set of values represents the mean ± standard deviation of three separate experiments.

Hence, the pool size of each diradylglycerol group in the guinea pig heart was determined. The guinea pig heart was homogenized in 0.25 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4), and the microsomal fraction was prepared by ultracentrifugation. The content of each diradylglycerol species in the homogenate as well as in the microsomal fraction was determined (**Table 2**). It is clear that 1,2-diacylglycerol was the principal form of diradylglycerol in the homogenate as well as in the microsomal fraction and that 1-alkyl-2-acyl-glycerol was the minor form of diradylglycerol in both cases. The amount of 1-alk-1′-enyl-2-acyl-glycerol found in the homogenate was in the same order of magnitude as reported earlier by us and other investigators (16, 17). The total diradylglycerol content in the microsomal fraction of the guinea pig heart was 125 nmol/g wet weight (43 nmol/mg protein) which represents 10% of the total diradylglycerol found in the homogenate. As depicted in Table 2, only 6.5% of the total 1,2-diacylglycerol, but over 47% of the total 1-alkenyl-2-acyl glycerol in the heart were found in the microsomal fraction.

Effect of exogenous diradylglycerol on the formation of phosphatidylethanolamine, plasmanylethanolamine, and plasmenylethanolamine

The distribution of diradylglycerol was found to be different between the homogenate and the microsomal fraction. It was not clear, however, whether the synthesis of ethanolamine glycerophospholipid in the microsomal fraction would be affected by exogenously added diradylglycerols. In this study, the formation of labeled phosphatidylethanolamine, plasmenylethanolamine, and plasmanylethanolamine was determined in the microsomal fraction in the absence or the presence of various exogenous diradylglycerol. As depicted in **Table 3, Table 4,** and **Table 5,** phosphatidylethanolamine formation was enhanced more than 2-fold by the presence of 0.4 mM 1,2-diacylglycerol, but the reaction was inhibited by 0.4 mM of 1-alkenyl-2-acyl glycerol (55%) or 1-alkyl-2-acyl glycerol (55%). Alternatively, the formation of plasmenylethanolamine was enhanced by 0.1–0.4 mM 1-alkenyl-2-acyl glycerol, but was severely inhibited by 0.4 mM 1-alkyl-2-acyl glycerol (66%). Interestingly, up to 0.4 mM 1,2-diacylglycerol had no effect on the reaction. The formation of plasmanylethanolamine was enhanced 2-fold by 0.2–0.4 mM 1-alkyl-2-acyl glycerol, while the same concentration of 1,2-diacylglycerol or 1-alkenyl-2-acyl glycerol had limited inhibitory effect (18%) on the reaction.

Identity of CDP-ethanolamine: 1,2-diradylglycerol ethanolaminephosphotransferase

The ability of 1-alkenyl-2-acyl glycerol to inhibit the formation of phosphatidylethanolamine but not vice versa suggests that different sites might be involved in the two reactions. Hence, the identity of CDP-ethanolamine: 1,2-diradylglycerol ethanolaminephosphotransferase in the guinea pig heart microsome for the synthesis of phosphatidylethanolamine, plasmenylethanolamine, and plasmanylethanolamine was examined by kinetic studies. The enzyme activity for the formation of phosphatidylethanolamine was assayed at 0.1–0.8 mM 1,2-diacylglycerol in the absence or presence of 0.4

TABLE 5. Effect of exogenous alkenylacylglycerol on the synthesis of ethanolamine glycerophospholipids

Alkenyl-AG	Phosphatidyl-ethanolamine	Plasmenyl-ethanolamine	Plasmanyl-ethanolamine
μm		*pmol/mg/min*	
0	24.00 ± 1.8	31.71 ± 2.5	1.82 ± 0.08
50	21.74 ± 1.56	36.36 ± 3.6	1.72 ± 0.07
100	19.39 ± 1.12	46.45 ± 3.9	1.63 ± .07
200	12.53 ± 0.89	49.03 ± 4.5	1.53 ± 0.06
400	10.77 ± 0.59	48.26 ± 7.3	1.48 ± 0.06

The activity of ethanolaminephosphotransferase was assayed with CDP-[^{14}C]ethanolamine in the absence or presence (0–400 μM) of exogenous alkenylacylglycerol. Each set of values represents the mean ± standard deviation of three separate experiments.

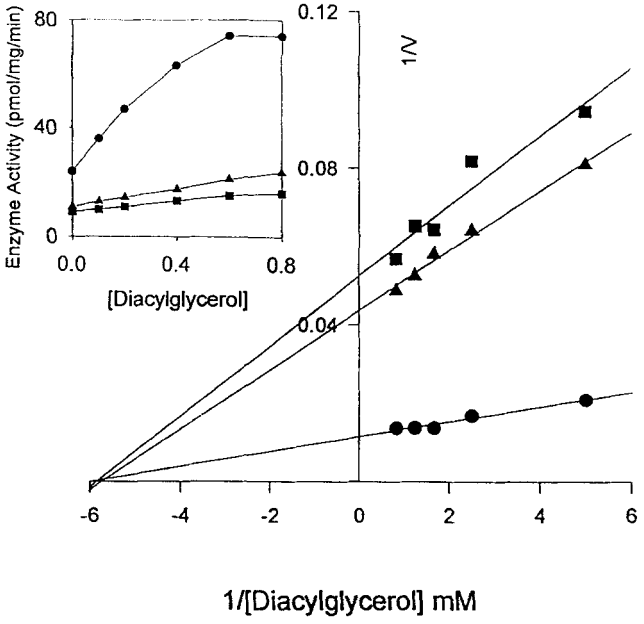

Fig. 3. Lineweaver-Burk plot of guinea pig heart ethanolaminephosphotransferase activity versus diradylglycerol concentration. The double reciprocal plot of phosphatidylethanolamine formation (pmol/mg/min) versus 1,2-diacylglycerol concentration (100–800 μM) in the absence (○) or presence (■) of 0.4 mM alkylacylglycerol or (▲) of 0.5 mM alkenylacylglycerol. The enzyme activity (pmol/mg/min) versus 1,2-diacylglycerol concentration (0–800 μM) is depicted in the inset. Each point is the mean of four separate experiments.

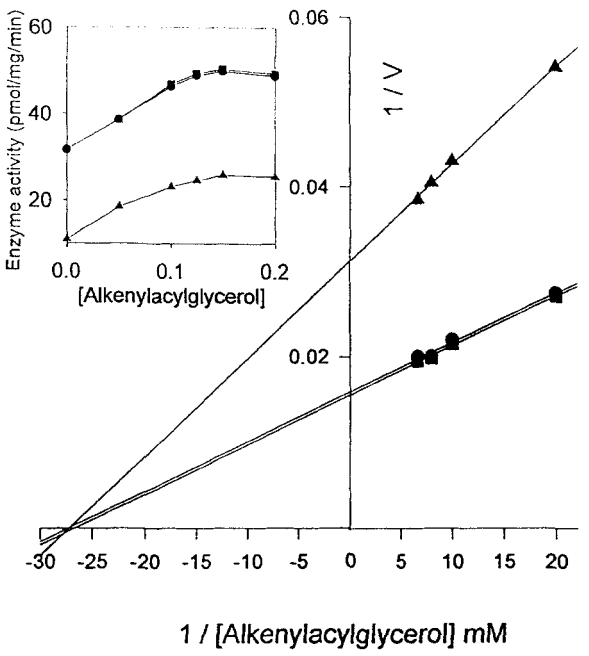

Fig. 4. Lineweaver-Burk plot of guinea pig heart ethanolaminephosphotransferase activity versus diradylglycerol concentration. The double reciprocal plot of phospatidylethanolamine formation (pmol/mg/min) versus alkenylacylglycerol concentration (50–200 μM) in the absence (○) or presence (■) of 0.4 mM alkylacylglycerol or (▲) of 0.5 mM diacylglycerol. The enzyme activity (pmol/mg/min) versus alkenylacylglycerol concentration (0–200 μM) is depicted in the inset. Each point is the mean of four separate experiments.

mM 1-alkyl-2-acyl glycerol or 0.5 mM 1-alkenyl-2-acyl glycerol. The double reciprocal plot of velocity versus substrate concentration is depicted in **Fig. 3.** The K_m for 1,2-diacylglycerol was estimated to be 0.17 mM and the reaction was inhibited by both 1-alkyl-2-acylglycerol and 1-alkenyl-2-acylglycerol in a noncompetitive manner. The activity of the enzyme for the formation of plasmenylethanolamine was assayed with 0.05–0.2 mM 1-alkenyl-2-acyl glycerol in the absence or presence of 0.4 mM 1-alkyl-2-acyl glycerol or 0.5 mM 1-2-diacylglycerol. The double reciprocal plot of velocity versus substrate concentration is depicted in **Fig. 4.** The K_m for 1-alkenyl-2-acyl glycerol was estimated to be 0.04 mM and the reaction was inhibited by 1-alkyl-2-acyl glycerol in a noncompetitive manner. In agreement with the observation in the preceding section, the reaction was not inhibited by 1,2-diacylglycerol. The activity of the enzyme for the formation of plasmanylethanolamine was assayed at 0.05–0.3 mM 1-alkyl-2-acyl glycerol in the absence or presence of 0.4 mM 1-alkenyl-2-acyl glycerol or 0.5 mM 1,2-diacylglycerol. The double reciprocal plot of velocity versus substrate concentration is depicted in **Fig. 5.** The K_m for 1-alkyl-2-acyl glycerol was estimated to be 0.04 mM and the reaction was inhibited by both 1,2-diacylglycerol and 1-alkenyl-2-acyl glycerol in a noncompetitive manner.

The identity of the enzyme(s) for the formation of phosphatidylethanolamine, plasmenylethanolamine, and plasmanylethanolamine was further examined by incubation of the microsomal fraction at 55°C for 0–10 min. An aliquot of the microsomal fraction was taken out at the prescribed time interval and assayed for the activity of the three reactions. As depicted in **Fig. 6,** the activity of the enzyme for the formation of phosphatidylethanolamine and plasmenylethanolamine was inactivated in an identical manner. However, the activity for the formation of plasmanylethanolamine appeared to be more stable under the incubating conditions.

DISCUSSION

The purpose of the study was to delineate the major pathway for the formation of plasmenylethanolamine in the adult guinea pig heart and to study the regulation of its formation. We found that hexadecanol was rapidly incorporated into the 1-O-alkyl group in plasmanylethanolamine, and there was a precursor–product relationship between the labeling of plasmanylethanolamine and plasmenylethanolamine. The rapid labeling of the

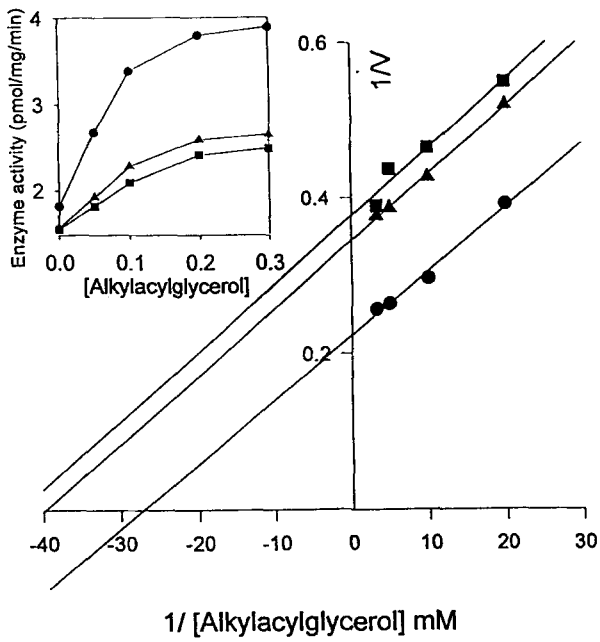

Fig. 5. Lineweaver-Burk plot of guinea pig heart ethanolaminephosphotransferase activity versus diradylglycerol concentration. The double reciprocal plot of phosphatidylethanolamine formation (pmol/mg/min) versus alkylacylglycerol concentration (50–300 μM) in the absence (○) or presence (■) of 0.5 mM diacylglycerol or (▲) of 0.5 mM alkenylacylglycerol. The enzyme activity (pmol/mg/min) versus alkylacylglycerol concentration (0–300 μM) is depicted in the inset. Each point is the mean of four separate experiments.

sn-1 aliphatic chain by hexadecanol was not surprising as the labeled compound was also incorporated into plasmanylethanolamine in the rabbit myocardium (32). The subsequent formation of the 1-O-alk-1-enyl group in plasmenylethanolamine via the desaturase reaction has been well documented (14, 33). Interestingly, a substantial amount of hexadecanol was oxidized into the corresponding fatty acid and subsequently incorporated into phosphatidylethanolamine. Only a small amount of the labeled fatty acid, however, was incorporated into plasmanylethanolamine or plasmenylethanolamine.

The presence of a significant pool of 1-alkenyl-2-acylglycerol in the homogenate and the microsomal fraction suggests that the 1-O-alk-1′-enyl group is conserved during the metabolism of plasmenylethanolamine (18). The conservation of the 1-O-alk-1′-enyl group is especially important for the formation of plasmenylcholine as no desaturase enzyme has been found to convert plasmanylcholine into plasmenylcholine (15, 16). Indeed, a pathway for the recycling of 1-alkenyl-2-acyl glycerol has been proposed (18). In addition, the vinyl ether linkage in choline glycerophospholipids has been shown to originate from plasmenylethanolamine (34). It is interesting to note that the value of 1-alkenyl-2-acyl glycerol obtained in this study is slightly higher than that reported in an earlier study (17). The reason for the difference is not defined, but could be attributed to some variations among animal groups.

The contribution of plasmenylethanolamine biosynthesis via the CDP-ethanolamine pathway was also explored. This pathway has been shown to be the principal pathway for the biosynthesis of phosphatidylethanolamine in mammalian hearts (26, 30, 31). The fact that the labeling of plasmenylethanolamine was 55–60% of phosphatidylethanolamine throughout the perfusion suggests that a considerable amount of plasmenylethanolamine could be synthesized via this pathway. The resemblance between the ratio of labeling phosphatidylethanolamine and of plasmenylethanolamine and the ratio of their pool size is another indica-

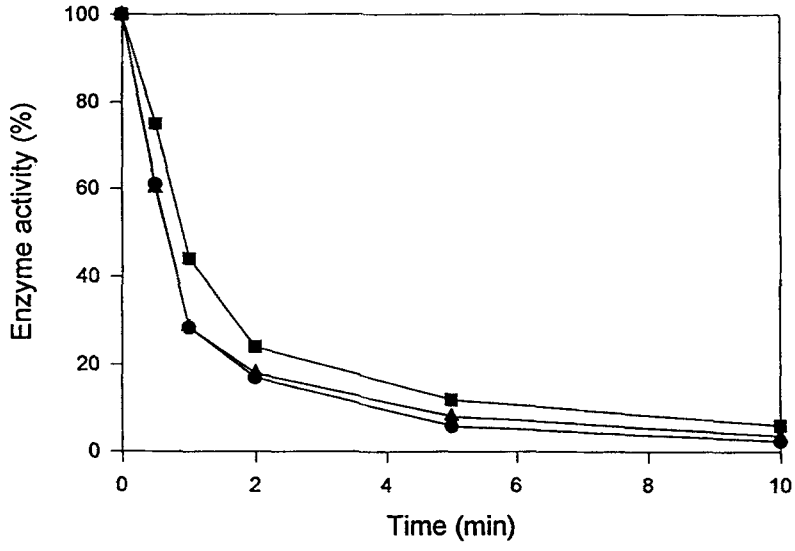

Fig. 6. Effect of heat treatment on guinea pig heart ethanolaminephosphotransferase activities. The enzyme in the microsomal fraction was incubated at 55°C for 1–10 min. The activity of the enzyme after incubation was assayed with diradylglycerol as substrate. Enzyme activity obtained without incubation is regarded as 100%. The symbols are the same as in Fig. 1 and each point is the mean of two separate experiments.

tion of the importance of this pathway for the synthesis of plasmenylethanolamine. The results of this study do not eliminate the possible synthesis of plasmenylethanolamine from plasmanylethanolamine via the desaturation reaction. The lack of accumulation of labeled plasmanylethanolamine could be attributed to a highly efficient and rapid desaturation reaction.

In the rabbit heart, ethanolaminephosphotransferase was found to display a higher degree of selectivity for the 1-alkenyl-2-acyl glycerol than for 1,2-diacylglycerol under optimal assay conditions (18). Selectivity of diradylglycerol for plasmenylethanolamine formation was also observed in platelet membrane (35). In the presence of exogenous diradylglycerol, the enzyme in the guinea pig heart did not exhibit a high degree of substrate selectivity. The enzyme became highly selective towards 1-alkenyl-2-acyl glycerol when endogenous diradylglycerol was used as substrate. The formation of plasmenylethanolamine was substantially higher than that of phosphatidylethanolamine even when the level of endogenous 1-alkenyl-2-acyl glycerol was substantially lower than that of 1,2-diacylglycerol.

In the mammalian heart, the diradylglycerol pool is modulated by a number of factors including external stimuli (36, 37). Hence, effects of other diradylglycerol on the synthesis of ethanolamine glycerophospholipids were examined. The rate of plasmenylethanolamine synthesis appears to be independent of 1,2-diacylglycerol concentrations but inhibited by 1-alkyl-2-acyl glycerol. The inhibition of plasmenylethanolamine by high concentrations of 1-alkyl-2-acyl glycerol may represent a coordinated mechanism to balance the contribution of the desaturase and the CDP-ethanolamine pathways for the formation of plasmenylethanolamine. For example, a high level of 1-alkyl-2-acyl glycerol would enhance the formation of plasmenylethanolamine via the desaturase reaction, with a corresponding reduction of plasmenylethanolamine synthesis via the CDP-ethanolamine pathway by inhibiting the ethanolaminephosphotransferase reaction. The inhibition of phosphatidylethanolamine formation by 1-alk-1′-enyl-2-acyl glycerol may also be regarded as another mechanism for the coordinated synthesis of different groups of ethanolamine glycerophospholipids, but the rationale for such inhibition remains to be explained. The inhibition of plasmenylethanolamine and phosphatidylethanolamine formations by high levels of 1-alkyl-2-acyl glycerol is interesting. However, it is unlikely that the level of 1-alkyl-2-acyl glycerol in the cardiac cell might become high enough to produce the observed effect. The synthesis of plasmanylethanolamine was marginally affected by 1,2-diacylglycerol and 1-alk-1′-enyl-2-acyl glycerol.

The identities of the ethanolaminephosphotransferase for the formation of various ethanolamine glycerophospholipids have not been defined. Based on kinetic studies, it appears that the active sites for 1,2-diacylglycerol, 1-alkenyl-2-acyl glycerol, and 1-alkyl-2-acyl glycerol are distinct from each other. The inhibition of phosphatidylethanolamine formation by 1-alkenyl-2-acyl glycerol and 1-alkyl-2-acyl glycerol, as well as the inhibition of plasmenylethanolamine formation by 1-alkyl-2-acyl glycerol can be explained by the relative size of the diradylglycerol species. As the size of the diradylglycerol is dependent solely on the size of the *sn*-1 group, where 1-acyl (ester bond) > 1-alkenyl (vinyl ether bond) > 1-alkyl (ether bond), it is plausible that the active site for the larger molecule could partially accommodate the smaller size molecule through hydrophobic association to produce the noncompetitive inhibition. We speculate from the heat treatment study that the ethanolaminephosphotransferase for both phosphatidylethanolamine and plasmenylethanolamine might reside in the same protein but different from the one for the formation of plasmenylethanolamine. Although the formation of plasmanylethanolamine was inhibited by 1,2-diacylglycerol or 1-alkenyl-2-acyl glycerol, the maximum inhibition obtained was less than 20%. Hence, these diradylglycerols may have limited ability to regulate the biosynthesis of plasmanylethanolamine in the guinea pig heart.

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REFERENCES

1. White, D. A. 1973. The phospholipid composition of mammalian tissues. *In* Form and Function of Phospholipids. G. B. Ansell, J. N. Hawthorne, and R. M. C. Dawson, editors. Elsevier Press, Amsterdam. 444–458.
2. Horrocks, L. A., and M. Sharma. 1982. Plasmalogens and O-alkylglycerophospholipids. *In* Phospholipids. J. N. Hawthorne and G. B. Ansell, editors. Elsevier Biomedical Press, Amsterdam. 51–93.
3. Mueller, H. W., J. T. O'Flaherty, and R. L. Wykle. 1982. Ether lipid content and fatty acid distribution in rabbit polymorphonuclear neutrophil phospholipids. *Lipids.* **17:** 72–77.
4. Careaga-Houck, M., and H. Sprecher. 1989. Effect of a fish oil diet on the composition of rat neutrophil lipids and the molecular species of choline and ethanolamine glycerophospholipids. *J. Lipid Res.* **30:** 77–87.
5. Myher, J. J., A. Kuksis, and S. Pind. 1989. Molecular species of glycerophospholipids and sphingomyelins of human erythrocytes: improved method of analysis. *Lipids.* **24:** 396–407.
6. Ford, D. A., and R. W. Gross. 1989. Plasmenylethanolamine is the major storage depot for arachidonic acid in

rabbit vascular smooth muscle and is rapidly hydrolyzed after angiotensin II stimulation. *Proc. Natl. Acad. Sci. USA.* **86:** 3479–3483.
7. Arthur, G., T. Mock, C. Zaborniak, and P. C. Choy. 1985. The distribution and fatty acid composition of plasmalogens in guinea pig heart microsomes and mitochondria. *Lipids.* **20:** 693–698.
8. Gross, R. W. 1985. Identification of plasmalogen as the major phospholipid constituent of cardiac sarcoplasmic reticulum. *Biochemistry.* **24:** 1662–1668.
9. Goldfine, H., and T. A. Langworthy. 1988. A growing interest in bacterial ether lipids. *Trends Biochem. Sci.* **13:** 217–221.
10. Zoeller, R. A., O. H. Morand, and G. R. H. Raetz. 1988. A possible role for plasmalogens in protecting animal cells against photosensitized killing. *J. Biol. Chem.* **263:** 11590–11596.
11. Thomas, S. E., D. M. Byers, F. B. St.C. Palmer, M. W. Spence, and H. W. Cook. 1990. Incorporation of polyunsaturated fatty acids into plasmalogens, compared to other phospholipids of cultured glioma cells, is more dependent on chain length than on selectivity between (n–3) and (n–6) families. *Biochim. Biophys. Acta.* **1044:** 349–356.
12. Van der Vusse, G. J., J. F. C. Glatz, H. C. G. Stam, and R. S. Reneman. 1992. Fatty acid homeostasis in the normoxic and ischemic heart. *Physiol. Rev.* **72:** 881–940.
13. Wykle, R. L., M. L. Blank, B. Malone, and F. Snyder. 1972. Evidence for a mixed function oxidase in the biosynthesis of ethanolamine plasmalogens from 1-alkyl-2-acyl-*sn*-glycero-3-phosphorylethanolamine. *J. Biol. Chem.* **247:** 5442–5447.
14. Paltauf, F. 1983. Biosynthesis of 1-*O*-(1′alkenyl)glycerolipids (plasmalogens) *In* Ether Lipids. H. K. Mangold and F. Paltauf, editors. Academic Press, New York. 107–128.
15. Wientzek, M., R. Y. K. Man, and P. C. Choy. 1987. Choline glycerophospholipid biosynthesis in the guinea pig heart. *Biochem. Cell Biol.* **65:** 860–868.
16. Ford, D. A., and R. W. Gross. 1988. Identification of endogenous 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol in myocardium and its effective utilization by choline phosphotransferase. *J. Biol. Chem.* **263:** 2644–2650.
17. Xu, Y.-F., K.-M. O, and P. C. Choy. 1994. Plasmenylcholine (1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine) biosynthesis in guinea-pig heart and liver: cholinephosphotransferase is a bifunctional enzyme for the synthesis of phosphatidylcholine and plasmenylcholine. *Biochem. J.* **301:** 131–137.
18. Ford, D. A., K. B. Rosenbloom, and R. W. Gross. 1992. The primary determinant of rabbit myocardial ethanolamine phosphotransferase substrate selectivity is the covalent nature of the *sn*-1 aliphatic group of diradyl glycerol acceptors. *J. Biol. Chem.* **267:** 11222–11228.
19. McMaster, C. R., C-Q. Lu, and P. C. Choy. 1992. The existence of a soluble plasmalogenase in guinea pig tissues. *Lipids,* **27:** 945–949.
20. Arthur, G., L. Kovac, M. Wientzek, and P. C. Choy. 1985. Plasmalogenase in hamster heart. *Biochim. Biophys. Acta.* **833:** 189–195.
21. Zelinski, T. A., J. D. Savard, R. Y. K. Man, and P. C. Choy. 1980. Phosphatidylcholine biosynthesis in isolated hamster heart. *J. Biol. Chem.* **255:** 11423–11428.
22. Arthur, G., L. L. Page, C. L. Zaborniak, and P. C. Choy. 1987. The acylation of choline lysophosphoglycerides in guinea pig heart mitochondria. *Biochem. J.* **242:** 171–175.
23. Arthur, G., L. Page, and P. C. Choy. 1987. Acylation of 1-alkenyl-glycero-phosphoethanolamine in guinea pig heart microsomes. *Biochim. Biophys. Acta.* **921:** 259–265.
24. McMaster, C. R., and P. C. Choy. 1992. Serine regulates phosphatidylethanolamine biosynthesis in the hamster heart. *J. Biol. Chem.* **267:** 14586–14591.
25. Schmid, H. H. O., P. C. Bandl, and K. L. Su. 1975. Analysis and quantification of ether lipids by chromatographic methods. *J. Chromatogr. Sci.* **13:** 478–486.
26. Ford, D. A., and R. W. Gross. 1990. Differential metabolism of diradyl glycerol molecular subclasses and molecular species by rabbit brain diglyceride kinase. *J. Biol. Chem.* **265:** 12280–12286.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
28. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
29. Gottfried, E. L., and M. M. Rapport. 1962. The biochemistry of plasmalogens. *J. Biol. Chem.* **237:** 329–333.
30. Zelinski, T. A., and P. C. Choy. 1982. Phosphatidylethanolamine biosythesis in isolated hamster heart. *Can. J. Biochem.* **60:** 817–823.
31. Zelinski, T. A., and P. C. Choy. 1982. Choline regulates phosphatidylethanolamine biosynthesis in isolated hamster heart. *J. Biol. Chem.* **257:** 13201–13204.
32. Ford, D. A., and R. W. Gross. 1994. The discordant rates of *sn*-1 aliphatic chain and polar head group incorporation into plasmalogen molecular species demonstrate the fundamental importance of polar head group remodeling in plasmalogen metabolism in rabbit myocardium. *Biochemistry.* **33:** 1216–1222.
33. Strum, J. C., A. Emilsson, R. L. Wykle, and L. W. Daniel. 1992. Conversion of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine to 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine. *J. Biol. Chem.* **267:** 1576–1583.
34. Strum, J. C., and L. W. Daniel. 1993. Identification of a lysophospholipase C that may be responsible for the biosynthesis of choline plasmalogens by Madin-Darby canine kidney cells. *J. Biol. Chem.* **268:** 25500–25508.
35. Morikawa, S., S. Taniguchi, K. Fujii, H. Mori, K. Kumada, M. Fujiwara, and M. Fujiwara. 1987. Preferential synthesis of diacyl and alkenylacyl ethanolamine and choline glycerophospholipids in rabbit platelet membranes. *J. Biol. Chem.* **262:** 1213–1217.
36. Tardi, P. G., R. Y. K. Man, and P. C. Choy. 1992. The effect of methyl-lidocaine on the de novo biosynthesis of phospholipids in the isolated hamster heart. *Biochem. J.* **285:** 161–166.
37. Wong, J. T., R. Y. K. Man, and P. C. Choy. 1994. The effect of lidocaine on de novo phospholipid biosynthesis on the isolated hamster heart. *Lipids.* **29:** 391–396.