Semi-synthetic approach for the preparation of homogeneous plasmenylethanolamine utilizing phospholipase D from *Streptomyces chromofuscus*

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Abstract A simple method for the preparation of homogeneous molecular species of plasmenylcholine and plasmenylethanolamine was developed. The method utilized reverse phase high performance liquid chromatography to isolate homogeneous molecular species of plasmenylcholine prepared by acylation of lysoplasmenylcholine. Plasmenylcholine was directly converted to plasmenylethanolamine by transphosphatidylation utilizing phospholipase D from *Streptomyces chromofuscus*. This method permits the facile labeling of homogeneous molecular species of plasmalogens in the polar head group, the *sn*-2 acyl chain, or both, for the first time. —**Wolf, R. A., and R. W. Gross.** Semi-synthetic approach for the preparation of homogeneous plasmenylethanolamine utilizing phospholipase D from *Streptomyces chromofuscus*. *J. Lipid Res.* 1985. **26:** 629–633.

Supplementary key words plasmalogens · phospholipase D · transphosphatidylation

Plasmalogens have recently been recognized as major phospholipid constituents of plasma membranes in several mammalian tissues. The striking predominance of plasmalogens in myocardial sarcolemma (1), brain (2), and peripheral nerve (3) suggests an important function of plasmalogens in electrically active tissues. Although plasmalogens are present in specific tissues and subcellular compartments, delineation of their biologic function remains elusive (see 4, 5 for reviews).

Direct investigation of the metabolism and physical-chemical properties of plasmalogens has been impeded by the difficulty in obtaining homogeneous chemical probes. Plasmenylethanolamine is particularly difficult to prepare due to the presence of a reactive nucleophilic group (primary amine) which precludes direct acylation of lysoplasmenylethanolamine. A previous scheme for the preparation of plasmenylethanolamine utilized protection and deprotection of the amine group but the product from this previous method contained a mixture of molecular species (6). This study reports a new scheme (**Fig. 1**) for the rapid preparation and isolation of homogeneous molecular species of plasmenylcholine and plasmenylethanolamine. This method permits, for the first time, the facile incorporation of radiolabels or physical probes into the polar head group and/or the *sn*-2 fatty acyl group of homogeneous molecular species of plasmenylcholine or plasmenylethanolamine.

MATERIALS

Oleic acid, arachidonic acid, ethanolamine, Triton X-100, bovine serum albumin, and N,N'-dicylohexylcarbodiimide were purchased from Sigma Chemical Co. (St. Louis, MO). N,N'-dimethyl-4-aminopyridine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Derivatized rape seed oil (methyl ester) was obtained from Supelco, Inc. (Bellefonte, PA). Bovine heart lecithin was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Anasil-OF thin-layer chromatography plates were purchased from Analabs (North Haven, CT). Silicic acid (400 mesh) was obtained from Bio-Rad (Richmond, CA). Radiolabeled [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]arachidonic acid (87.4 Ci/mmol), [1,2-^{14}C]ethanolamine hydrochloride (4.0 mCi/mmol), and [9,10-^{3}H]oleic acid (13.3 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Phospholipase D from *Streptomyces chromofuscus* was purchased from Calbiochem-Behring (San Diego, CA). Aquasol-2 was obtained from New England Nuclear (Boston, MA).

METHODS

Phospholipid separation and analysis

All synthetic products were stored under nitrogen and protected from light throughout the synthesis. Thin-layer

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography.

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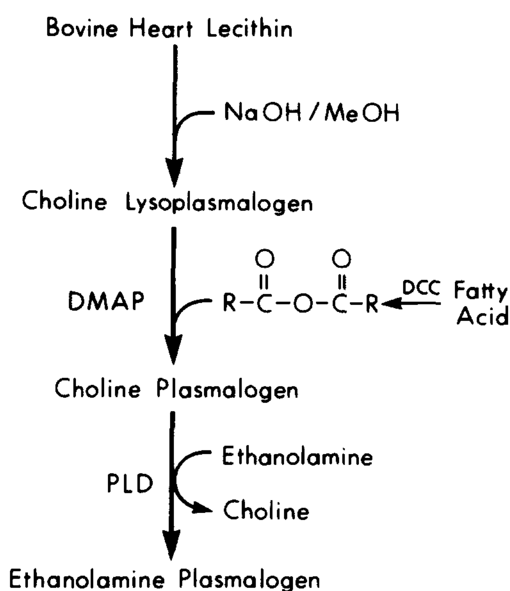

Fig. 1 The synthetic scheme for plasmenylcholine and plasmenylethanolamine synthesis. PLD, phospholipase D; DCC, N,N'-dicyclohexylcarbodiimide; DMAP, N,N'-dimethyl-4-aminopyridine.

chromatography (TLC) plates were developed with chloroform–methanol–ammonium hydroxide 65:35:5. Plasmenylcholine and plasmenylethanolamine had R_f values of 0.31 and 0.48, respectively, which were similar to their diacyl analogues. Lysoplasmenylcholine had an R_f value of 0.10. Silicic acid column chromatography was performed on a 2.5 × 30 cm column initially equilibrated with chloroform, and stepwise gradients of $CHCl_3$–MeOH were used to elute reaction products as described previously (7). Phospholipids purified by silicic acid chromatography appeared as a single intense spot on TLC after staining with iodine overnight.

High performance liquid chromatography (HPLC) was performed as previously described (8). Homogeneous molecular species of plasmenylcholine or plasmenylethanolamine were isolated by reverse phase HPLC. Phospholipid was applied to a Beckman Ultrasphere ODS C-18 column (4.6 mm × 25 cm, 0.5–1.5 μmol of phospholipid) or for larger scale syntheses to an Altech C-18 column (16 mm × 25 cm, 20–50 μmol of phospholipid) and eluted isocratically at 2 or 10 ml/min, respectively.

Phospholipids were derivatized by acid methanolysis (1) and analyzed by capillary gas–liquid chromatography (GLC) as previously described (9). Peak assignments were determined by comparison with derivatized standards and GLC–mass spectrometry (1). Fast-atom bombardment mass spectrometry was performed as previously described (1).

1-O-(Z)-1'-alkenyl-*sn*-glycero-3-phosphocholine (lysoplasmenylcholine)

Lysoplasmenylcholine was prepared by the method of Wheeldon, Schumert, and Turner (10) and purified by silicic acid column chromatography. Thin-layer chromatography of this material demonstrated a single spot (R_f = 0.10) after intense iodine staining.

1-O-(Z)-1'-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine (plasmenylcholine)

Lysoplasmenylcholine was acylated by a method adapted from Gupta, Radhakrishnan, and Khorana (11). Oleic anhydride or arachidonoyl anhydride were synthesized (Fig. 1) as described by Selinger and Lapidot (12). For preparation of radiolabeled plasmenylcholine, [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]arachidonic acid (sp act 0.3 μCi/μmol) or [9, 10-^{3}H]oleic acid (sp act 280 μCi/μmol) were used in the anhydride synthesis.

Prior to the acylation reaction, lysoplasmenylcholine was repeatedly azeotroped with benzene. The fatty acid anhydride and lysoplasmenylcholine were dried over phosphorus pentoxide in vacuo overnight, and chloroform was freshly distilled from phosphorus pentoxide. All reagents were transferred in a dry nitrogen atmosphere into a reaction vessel containing (final concentration) lysoplasmenylcholine (50 μmol/ml of chloroform); anhydride (75 μmol/ml); and N-N'-dimethyl-4-aminopyridine (50 μmol/ml) and stirred at room temperature for 24–36 hr. Reaction products were identified by TLC and synthetic plasmenylcholine (R_f = 0.31) was purified by preparative TLC or silicic acid chromatography. The phospholipid class purity was established by TLC and HPLC.

1-O-(Z)-1'-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine)

Plasmenylethanolamine was prepared directly from the choline analogue by transphosphatidylation catalyzed by phospholipase D from *Streptomyces chromofuscus*. Plasmenylcholine (5 μmol/ml of distilled water) was sonicated at 4°C with three bursts of 2 min duration using a Branson ultrasonicator (Branson Corporation, Danbury, CT) at 30 watts output. Plasmenylcholine vesicles were transferred to a reaction vessel containing (final concentration) 40 mM Tris; 4 mM calcium chloride; 0.25% Triton X-100 (v/v); 0.05% bovine serum albumin (w/v); 15% ethanolamine (v/v), and 25 units/ml phospholipase D at pH 8.5. This reaction mixture was incubated at room temperature for 20 min, and the reaction was stopped by the addition of 0.5 ml of 200 mM EDTA. The reaction products were then extracted (13) and the organic phase was filtered (1 μm filter) prior to evaporation under nitrogen. Reaction products were analyzed by TLC, HPLC, and GLC. Synthetic plasmenylethanolamine was purified by preparative TLC and subsequent reverse phase HPLC.

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RESULTS

Characterization of 1-O-(Z)-1'-alkenyl-*sn*-glycero-3-phosphocholine (lysoplasmenylcholine)

Approximately 2.0 g (4.2 mmol) of lysoplasmenylcholine was routinely isolated by silicic acid column chromatography following alkaline methanolysis of 10.0 g (13.4 mmol) of bovine heart lecithin. Fast-atom bombardment mass spectrometry of purified lysoplasmenylcholine demonstrated a mixture of molecular species, with the major component at *m/z* 480. These molecular species were quantified by GLC (**Table 1**) and were corroborated by fast-atom bombardment mass spectrometry. Thus the major component of lysoplasmenylcholine utilized in the acylation reaction was 1-0-(Z)-(1'-hexadecenyl)-*sn*-glycero-3-phosphocholine.

Preparation and characterization of 1-O-(Z)-1'-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine (plasmenylcholine)

Approximately 75 mg (156 μmol) of lysoplasmenylcholine was acylated with 100 mg (170 μmol) of arachidonoyl anhydride as described in Methods. Following silicic acid column chromatography, 61 mg (79 μmol) of plasmenylcholine was isolated (yield = 49%).

As expected, mass spectrometry and GLC of plasmenylcholine revealed a mixture of products resulting from the acylation of heterogeneous lysoplasmenylcholine. Homogeneous 1-O-(Z)-(1'-hexadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine was isolated by reverse phase HPLC (**Fig. 2**). Of note, several of the minor peaks eluted by reverse phase HPLC contained a large molar excess of fatty acid methyl ester to dimethyl acetal after derivatization by acid methanolysis and analysis by GLC. In contrast, 1-O-(Z)-(1'-hexadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine isolated by reverse phase HPLC contained stoichiometric amounts (± 2%) of the dimethyl acetal of palmitate and the fatty acid methyl ester of arachidonic acid. Acid methanolysis and capillary GLC revealed that this purified product was essentially free of contamination by other molecular species. Fast-atom bombardment mass spectrometry revealed a single

TABLE 1. Characterization of 1-O-(Z)-1'-alkenyl-*sn*-glycero-3-phosphocholine

sn-1	% of Total
14:0	6 ± 2
15:0	7 ± 2
16:0	68 ± 3
17:0	4 ± 1
18:0	10 ± 1
18:1	4 ± 1

Lysoplasmenylcholine prepared from bovine heart lecithin was isolated by silicic acid column chromatography and aldehydes were quantified by capillary GLC after acid methanolysis.

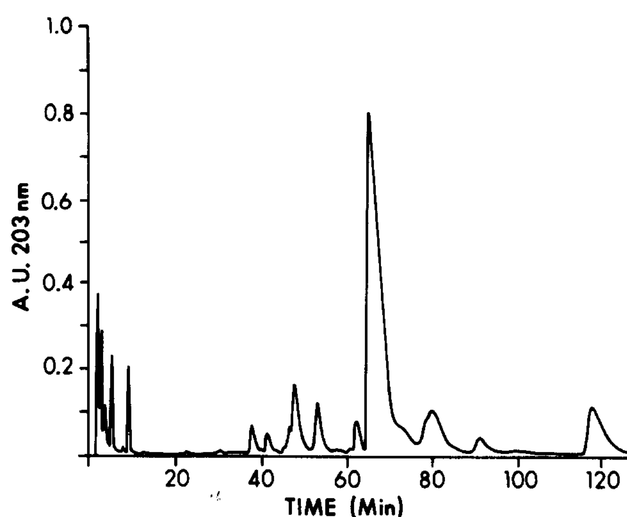

Fig. 2 Reverse phase HPLC of semi-synthetic plasmenylcholine. Lysoplasmenylcholine was acylated with arachidonic acid, purified by TLC, and injected onto a C-18 column with methanol-acetonitrile-water as the mobile phase as described in Methods. 1-O-(Z)-(1'-hexadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine was eluted at 65 min. The other major reaction product, 1-O-(Z)-(1'-octadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine eluted at 116 min.

parent ion at *m/z* 766 (**Fig. 3**), with the major fragmentation peak at *m/z* 480 due to the loss of the *sn*-2 acyl group. The [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]arachidonoyl plasmenylcholine (sp act 0.3 μCi/μmol) was prepared by an identical scheme.

In additional experiments, 100 mg (208 μmol) of lysoplasmenylcholine was acylated with 176 mg (322 μmol) of oleic anhydride, yielding 92 mg (124 μmol) of plasmenylcholine (yield = 60%). 1-O-(Z)-(1'-hexadecenyl)-2-oleoyl-*sn*-glycero-3-phosphocholine was isolated by reverse phase HPLC and was a single molecular species as ascertained by fast-atom bombardment mass spectrometry (Fig. 3) and GLC.

Preparation and characterization of 1-O-(Z)-1'-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine)

1-O-(Z)-(1'-hexadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine was directly converted to the ethanolamine derivative by transphosphatidylation catalyzed by phospholipase D from *Streptomyces chromofuscus*. Initial experiments demonstrated that this enzyme could hydrolyze the phosphoester linkage in plasmenylcholine to yield the corresponding phosphatidic acid. Inclusion of a primary alcohol such as ethanolamine in the reaction mixture resulted in the transphosphatidylation of plasmenylcholine to plasmenylethanolamine. Synthesis of plasmenylethanolamine was confirmed by incorporation of ^{14}C-labeled ethanolamine (0.12 μCi/μmol) into the reaction product. The purified plasmenylethanolamine was a single peak that comigrated with authentic phosphatidylethanolamine on straight phase HPLC (8). The

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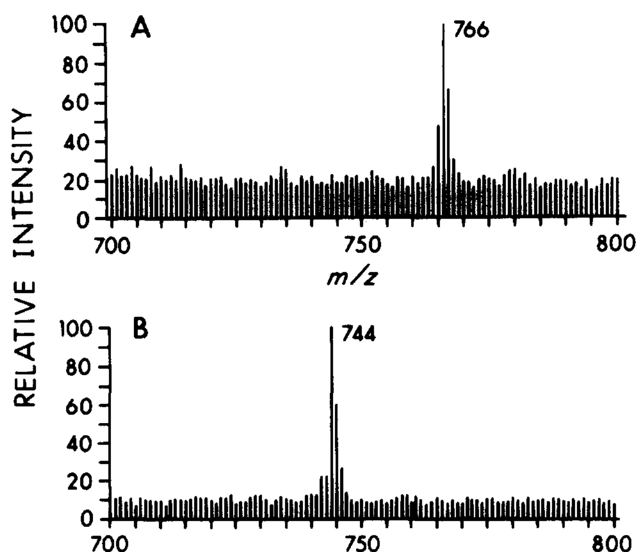

Fig. 3 Fast-atom bombardment mass spectrometry of plasmenylcholine. Reverse phase HPLC purified plasmenylcholine was analyzed by fast-atom bombardment mass spectrometry. The protonated parent ion peaks occurred at *m/z* 766 for 1-O-(Z)-(1'-hexadecenyl)-2-arachidonoyl-*sn*-glyero-3-phosphocholine (panel A) and at *m/z* 744 for 1-O-(Z)-(1'-hexadecenyl)-2-oleoyl-*sn*-glycero-3-phosphocholine (panel B).

purified reaction product was a single spot on TLC that comigrated with phosphatidylethanolamine. Acid methanolysis and subsequent analysis by GLC revealed that the *sn*-1 vinyl ether group and the *sn*-2 acyl group were present in stoichiometric amounts (± 2%) and were not altered during the transphosphatidylation reaction. In a typical small scale reaction, 2.7 mg (3.5 μmol) of 1-O-(Z)-(1'-hexadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine was converted to 1.3 mg (1.8 μmol) of 1-O-(Z)-(1'-hexadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (50% yield). In a larger scale reaction, 10 mg (13.4 μmol) of 1-O-(Z)-1'-alkenyl-2-oleoyl-*sn*-glycero-3-phosphocholine was converted to 4.9 mg (6.8 μmol) of 1-O-(Z)-1'-alkenyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (51% yield) with generation of 0.7 mg (1.1 μmol) of 1-O-(Z)-1'-alkenyl-2-oleoyl-*sn*-glycero-3-phosphate. 1-O-(Z)-(1'-hexadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine was subsequently purified by reverse phase HPLC.

DISCUSSION

Investigation of the structure and function of plasmalogens in biologic membranes has been hampered by difficulty in preparing this class of phospholipids. This report described a new semi-synthetic route to homogeneous molecular species of plasmenylcholine and plasmenylethanolamine. This scheme obviates the need for protection and deprotection of the primary amine group in plasmenylethanolamine and facilitates the synthesis of homogeneous plasmenylcholine and plasmenylethanolamine from a common intermediate. Furthermore, it permits the introduction of specific probes into both the fatty acyl group as well as the polar head group, thus facilitating the study of both the hydrophobic and hydrophilic domains of plasmalogens.

The critical steps in the semi-synthetic scheme were the separation of multiple plasmenylcholine molecular species and the enzymatic conversion of plasmenylcholine to plasmenylethanolamine. A reverse phase HPLC separation of diacyl phospholipid species has been described by Patton, Fasulo, and Robins (14) and, as this study demonstrates, identical chromatographic conditions are also useful for the separation of plasmalogen molecular species. Plasmalogens were retained longer by the reverse phase column than their diacyl counterparts, presumably reflecting their decreased polarity due to the absence of a carbonyl at the *sn*-1 acyl chain. Although base exchange reactions have been extensively utilized in the synthesis of diacyl glycerophospholipids employing phospholipase D from Savoy cabbage (15), plasmalogen substrates are almost completely resistant to hydrolysis or base exchange under identical conditions (16, 17). The identification of rapid transphosphatidylation of plasmenylcholine catalyzed by phospholipase D from *Streptomyces chromofuscus* allows access to many different plasmalogen molecular classes and facilitates the placement of specific probes in the polar head group.

Lysoplasmenylcholine prepared by alkaline methanolysis of bovine heart lecithin is contaminated by small amounts (5-7%) of alkyl ether lipid. Alkyl ether contamination of purified plasmenylcholine after reverse phase HPLC was less than 2% as evidenced by the stoichiometric ratio of dimethyl acetal to fatty acid methyl ester in derivatized plasmenylcholine. The high ratio of fatty acid methyl ester to dimethyl acetal present in minor peaks after reverse phase HPLC suggests that alkyl ether lipids were resolved by this technique. Resolution of other molecular species of plasmalogens from alkyl ether lipids by reverse phase HPLC awaits further studies.

The synthetic and chromatographic methods described in this study allow the rapid preparation of specifically labeled homogeneous molecular species of plasmenylcholine or plasmenylethanolamine from a common intermediate (lysoplasmenylcholine). The application of these methods to the preparation of specific metabolic and physical probes should facilitate the identification of the role of plasmalogens in biologic membranes.

The authors with to thank Ms. Karen Brennan for expert technical assistance and Ms. Helen Nikolaisen for her help in preparation of this manuscript. Supported in part by NIH Grant HL 17646 in Ischemic Heart Disease, an American Heart Association Grant-in-Aid, and a Searle Scholar Award.

Manuscript received 27 November 1984.

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