Plasmenylethanolamine Facilitates Rapid Membrane Fusion: A Stopped-Flow Kinetic Investigation Correlating the Propensity of a Major Plasma Membrane Constituent To Adopt an H_{II} Phase with Its Ability To Promote Membrane Fusion†

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ABSTRACT: A critical step in membrane fusion involves the formation of a lipid intermediate which shares a conformational similarity with an inverted hexagonal phase (H_{II}). Since plasmenylethanolamines possess a marked propensity for hexagonal phase formation and represent a major lipid constituent of several membrane systems which undergo rapid membrane fusion (e.g., plasma membranes and synaptic vesicle membranes), we compared the relative fusogenicity of lipid vesicles containing plasmenylethanolamine to that of vesicles containing their diacyl phospholipid counterpart (i.e., phosphatidylethanolamine). Vesicles comprised of equimolar mixtures of phosphatidylcholine and phosphatidylethanolamine fused slowly with phosphatidylserine vesicles in the presence of 10 mM $CaCl_2$, as assessed either by lipid mixing (dequenching of octadecyl rhodamine fluorescence, $7.4F_{max\%}$ s^{-1}) or internal contents mixing (fluorescence enhancement from the resultant Tb/dipicolinic acid charge transfer complex, $8.7F_{max\%}$ s^{-1}). In stark contrast, vesicles comprised of equimolar mixtures of phosphatidylcholine and plasmenylethanolamine fused three times more rapidly, as assessed by both lipid mixing ($22.1F_{max\%}$ s^{-1}) and internal contents mixing ($21.4F_{max\%}$ s^{-1}) assays. The importance of an H_{II}-like intermediate in membrane fusion was further substantiated by demonstration that plasmenylethanolamines containing arachidonic acid at the *sn*-2 position (which demonstrate a greater propensity for H_{II} phase formation) exhibited the most rapid rate of membrane fusion (five times greater than phosphatidylethanolamine containing oleic acid at the *sn*-2 position). Furthermore, vesicles containing plasmenylethanolamines in physiologic ratios with other phospholipids (i.e., PC/PE/PS, 45:45:10, mol/mol) underwent fusion six times more rapidly ($4.4F_{max\%}$ min^{-1}) than corresponding vesicles in which plasmenylethanolamine was replaced with phosphatidylethanolamine ($0.7F_{max\%}$ min^{-1}). Collectively, these studies demonstrate the importance of plasmalogens containing arachidonic acid in facilitating membrane fusion and further substantiate the importance of an H_{II}-like intermediate in membrane fusion events.

Although membrane fusion plays a central role in a multiplicity of critical biologic processes such as synaptic transmission (von Wedel et al., 1981; Almers & Tse, 1990; Sollner et al., 1993), hormone release (Poste & Allison, 1973; Pollard et al., 1992), and membrane trafficking (Wilson et al., 1989; Diaz et al., 1989; Stamnes & Rothman, 1993), the detailed biochemical mechanisms responsible for mediating the fusion of membrane bilayers have not yet been elucidated (e.g., Duzgunes, 1985; Papahadjopoulos et al., 1990; Vogel et al., 1993). It is generally accepted that both the phospholipids destined for membrane fusion as well as the polypeptides catalyzing the fusion event are important kinetic determinants of the rate of membrane fusion *in vivo* (e.g., Meers et al., 1988; Yeagle, 1989; Wilson et al., 1991; Vogel et al., 1993; Zimmerberg et al., 1993). Moreover, substantial evidence has now been accrued which documents the importance of phospholipids predisposed to adopting an inverted hexagonal phase (H_{II}) (e.g., PS, DAG)[1] in lowering the activation energy for the formation of the putative fusion intermediate (e.g., Rupert et al., 1987; Bentz & Ellens, 1988; Siegel et al., 1989; Cullis & Hope, 1991). However, since many of the alterations in lipid composition which predispose to an H_{II} phase (e.g., alterations in both PS and DAG content) concomitantly introduce substantial changes in surface charge density and lipid packing (e.g., Bentz et al., 1987; Sundler & Papahadjopoulos, 1981), the unambiguous separation of changes in membrane physical properties from the effect of these perturbations on the propensity of forming an H_{II}-like intermediate has posed a formidable challenge. Furthermore, in some cases it seems unlikely that these moieties reach levels *in vivo* which have been shown to facilitate the fusion processes *in vitro*. In this regard, we note that plasmenylethanolamines are major phospholipid constituents of several subcellular membranes which undergo rapid membrane fusion (e.g., synaptic vesicles and plasma membranes) (Breckenridge et al., 1973; Westhead, 1987; Cullis & Hope, 1991). Since

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; 16:0–18:1 (plasmal), 1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-; 16:0–18:1 (diacyl), 1-*O*-hexadecanoyl-2-octadec-9′-enoyl-*sn*-; 18:0–20:4 (plasmal), 1-*O*-octadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-; 18:0–20:4 (diacyl), 1-*O*-octadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-; R18, octadecyl rhodamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; Fmoc, *N*-9-fluorenylmethoxylcarbonyl; LPE, lysoplasmenylethanolamine; SUV, small unilamellar vesicle; TLC, thin layer chromatography; HPLC, high-pressure liquid chromatography; DPA, dipicolinic acid; HEPES, 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DAG, diacylglycerol.

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plasmenylethanolamines have a substantially lower bilayer to hexagonal II phase transition temperature than their corresponding phosphatidylethanolamine counterparts (Lohner et al., 1984; Han & Gross, 1992), we hypothesized that plasmenylethanolamines may represent the major endogenous lipid constituent facilitating the formation of the critical fusion intermediate (i.e., the H_{II}-like phase) *in vivo*. Due to the difficulties inherent in the synthesis of plasmenylethanolamine, no prior studies investigated the relative fusogenicity of plasmenylethanolamines compared to phosphatidylethanolamines. Since plasmenylethanolamine and phosphatidylethanolamine subclasses contain identical polar head groups, surface charge densities, and *sn*-2 fatty acyl constituents and differ only in the presence of a single vinyl ether linkage in the proximal portion of their *sn*-1 constituents, comparisons between the rate of membrane fusion utilizing vesicles comprised of each subclass could provide detailed information on critical structure–activity relationships modulating the kinetics of the fusion process. To determine the effects of the vinyl ether linkage on initial rates of membrane fusion, we synthesized plasmenylethanolamines and utilized stopped-flow kinetics in conjunction with three independent assays of membrane fusion (i.e., two lipid mixing and one internal contents mixing assays). We now report that the presence of a vinyl ether linkage at the *sn*-1 position in ethanolamine glycerophospholipids (i.e., plasmenylethanolamines) substantially facilitates (as much as six-fold) calcium-dependent membrane fusion.

MATERIALS AND METHODS

Bovine brain ethanolamine glycerophospholipids, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL). Octadecyl rhodamine (R_{18}), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE), *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE), $Tb(Cl)_3$, and dipicolinic acid (DPA) were purchased from Molecular Probes (Eugene, OR). Fluorenylmethoxylcarbodiimide succinimide was purchased from Fluka (Buchs, Switzerland). Acyl chlorides, acyl anhydrides, and cholesterol were purchased from Nu Chek Prep (Elysian, MN). [^{14}C]Inulin was purchased from DuPont–New England Nuclear (Boston, MA). HPLC-grade solvents were purchased from Baxter Scientific (McGaw Park, IL). All other chemicals were obtained from Sigma (St. Louis, MO).

Synthesis of Plasmenylethanolamine. Individual molecular species of plasmenylethanolamine were synthesized by a process involving the sequential protection, acylation, and deprotection of lysoplasmenylethanolamine. Bovine brain ethanolamine glycerophospholipids (1 g in 100 mL of chloroform) were subjected to alkaline methanolysis by mixing with 0.5 N methanolic NaOH (100 mL) for 45 min at 25 °C. The reaction was stopped by addition of 50 mL of 1 N acetic acid and placed in a separatory funnel. After gentle mixing, the lower phase was collected and dried under vacuum at room temperature. The resultant yellow paste was dissolved in 6 mL of chloroform, filtered, and injected onto a Dynamax Preparatory Si HPLC column (21.4 mm × 25 cm) previously equilibrated with chloroform. After 20 min of elution with chloroform at a flow rate of 10 mL/min, a linear gradient to 100% methanol over 100 min was initiated. Lysoplasmenylethanolamine eluted at 60% methanol was detected after TLC (Whatman LKG plates) utilizing a neutral mobile phase (system A, 95:35:6 chloroform/methanol/water, v/v) by subsequent iodine staining (lysoplasmenylethanolamine R_f = 0.17). Lysoplasmenylethanolamine-containing fractions were pooled, dried under nitrogen, and resuspended in chloroform (yield = 80% of theoretical). Next, the ethanolamine head group was blocked utilizing an *N*-9-fluorenylmethoxylcarbonyl (Fmoc) protection group. Protection of the primary amine was effected by stirring 170 mg of lysoplasmenylethanolamine (0.37 mmol) in distilled $CHCl_3$ (distilled over P_2O_5) with a 5-fold molar excess of succinimidylfluorenylmethoxylcarbodiimide (600 mg, 1.85 mmol) and 240 mg (3.7 mmol) of imidazole for 4 h at room temperature. Next, a second addition of 240 mg of succinimidyl-Fmoc (0.74 mmol) and 100 mg of imidazole (1.48 mmol) was performed, and the reaction was stirred overnight at 25 °C prior to the extraction of the reaction products by the method of Bligh and Dyer (1959). Purification of Fmoc-lysoplasmenylethanolamine was accomplished utilizing straight phase HPLC employing a chloroform/methanol linear gradient as described above, with Fmoc-LPE eluting at 20% methanol. Fractions containing Fmoc-LPE (R_f = 0.29 in TLC neutral system A) were pooled, dried under vacuum, resuspended in chloroform for storage, and stored under an atmosphere of nitrogen (yield = 81%). Next, 50 mg (0.072 mmol) of Fmoc-lysoplasmenylethanolamine was dried under nitrogen and resuspended in distilled chloroform (2 mL) under anhydrous conditions, and, after addition of recrystallized DMAP (10 mg, 0.082 mmol), the appropriate fatty acyl anhydride or acyl chloride was added [usually in 4–10-fold molar excess (100–200 mg)] and stirred for 6 h at 35 °C. Dicyclohexylcarbodiimide (in 1-mg iterative additions) was added to reactions to regenerate the anhydride every 2 h. The reaction was quenched by addition of one-quarter volume of methanol (1 mL), and deprotection of the Fmoc group was accomplished by addition of one-quarter volume of diethylamine and subsequent stirring for 8 h at room temperature. The resultant plasmenylethanolamine was purified after Bligh and Dyer extraction utilizing a Dynamax Preparatory Si HPLC column (21.4 mm × 25 cm) previously equilibrated with hexane/isopropanol/water/ammonium hydroxide (48:48:4:0.005,v/v) as the mobile phase employing a 4–8.5% H_2O gradient (Blank & Snyder, 1983). Fractions containing plasmenylethanolamine were pooled and further purified on an Altex Ultrasphere-Si column (4.6 mm × 25 cm) utilizing a mobile phase of hexane/isopropanol (50:50, v/v) with a 1–7% H_2O gradient (Geurts van Kessel et al., 1977). A total of 27 mg of arachidonoylated plasmenylethanolamine was obtained (yield = 32%, with respect to Fmoc-LPE). Individual molecular species of plasmenylethanolamine (either 16:0, 18:0, or 18:1 at the *sn*-1 position) were resolved on a Beckman C18 reverse-phase HPLC column (4.6 mm × 25 cm) utilizing a mobile phase comprised of methanol/acetonitrile/water (90.5:2.5:7,v/v) containing 20 mM choline chloride as described previously (Gross, 1984). The purity of plasmenylethanolamine was assessed by TLC employing neutral system A, a base system (65:25:5, chloroform/methanol/ammonium hydroxide), and an acid system (6:8:2:2:1 chloroform/acetone/methanol/acetic acid/water). The concentration of phospholipids was quantified by capillary gas chromatography following derivatization by acid methanolysis as described previously by comparisons with internal standard (Gross, 1984).

Phospholipid Purification. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, and 1-palmitoyl-2-oleoyl-*sn*-glycero-

Table 1: Fatty Acid and Aldehyde Composition of Ethanolamine Glycerophospholipids[a]

carbon chain	bovine brain ethanolamine glycerophospholipids	plasmalogen-depleted bovine brain ethanolamine glycerophospholipids
plasmal 16:0	6.5 ± 0.8[b]	0.5 ± 0.3
acyl 16:0	3.5 ± 0.3	6.8 ± 0.7
plasmal 18:0	11.1 ± 0.5	1.3 ± 0.2
acyl 18:0	13.0 ± 0.4	33.1 ± 2.0
plasmal 18:1	15.6 ± 1.0	0.5 ± 0.7
acyl 18:1	17.6 ± 0.9	22.6 ± 0.6
acyl 20:4	13.4 ± 0.7	12.5 ± 0.6
acyl 22:4	6.6 ± 1.3	5.7 ± 0.1
acyl 22:5	4.1 ± 0.7	5.1 ± 1.3
acyl 22:6	8.4 ± 0.4	10.3 ± 2.0

[a] Comparison of the fatty acid compositions of native and plasmalogen-depleted ethanolamine glycerophospholipids. Plasmalogen-depleted ethanolamine glycerophospholipids were prepared by acid treatment of bovine brain ethanolamine glycerophospholipids and subsequent straight-phase HPLC purification as described under Materials and Methods. The fatty acid and vinyl ether content of either native or plasmalogen-depleted ethanolamine glycerophospholipids were quantified by treatment of an aliquot with 1 N methanolic HCl at 90 °C for 90 min. Fatty acid methyl esters and dimethyl acetals were extracted into petroleum ether and subsequently quantified on capillary gas chromatography as previously described (Gross, 1984). [b] Percentage of total fatty acid and aldehyde ± standard deviation for four independent preparations.

3-phosphoserine were purified on an Altex Ultrasphere-Si column (4.6 mm × 25 cm) utilizing a mobile phase of hexane/isopropanol (50:50, v/v) with a 1–7% H_2O gradient to remove trace contaminants (Geurts van Kessel et al., 1977). All lipids were stored in chloroform under an atmosphere of nitrogen at −20 °C.

Preparation of Plasmalogen-Depleted Bovine Brain Ethanolamine Glycerophospholipids. Plasmenylethanolamine was selectively removed from total bovine brain ethanolamine glycerophospholipids by exploiting the acid lability of the vinyl ether linkage. Briefly, bovine brain ethanolamine glycerophospholipids (15 mg) were dried under nitrogen and subsequently exposed to HCl fumes for 25 min. After 2 min of flushing with nitrogen, the sample was resuspended in chloroform, and plasmalogen-depleted PE was isolated on an Altex Ultrasphere-Si column (4.6 mm × 25 cm) utilizing a mobile phase of hexane/isopropanol (50:50, v/v) with a 1–7% H_2O gradient (Geurts van Kessel et al., 1977). The fatty acid compositions of bovine brain PE and plasmalogen-depleted bovine brain PE were quantified by capillary gas chromatography after acid methanolysis (Table 1).

Reverse-Phase HPLC of Canine Myocardial Ethanolamine Glycerophospholipids. Canine myocardium was minced with scissors and disrupted by Potter-Elvehjem homogenization. After centrifugation at $10000g_{max}$ for 20 min, the pellet was extracted using the method of Bligh and Dyer (1959), and lipid classes were subsequently purified on an Altex Ultrasphere-Si column (4.6 mm × 25 cm) utilizing a mobile phase comprised of hexane/isopropanol (50:50, v/v) with a 1–7% H_2O gradient (Geurts van Kessel et al., 1977). The ethanolamine glycerophospholipid peak was enriched in both phosphatidylethanolamine and plasmenylethanolamine molecular species containing 18:0 and 20:4 aliphatic chains at the *sn*-1 and *sn*-2 positions, respectively. These species were resolved utilizing a Beckman C18 reverse-phase HPLC column (4.6 mm × 25 cm) employing a mobile phase comprised of methanol/acetonitrile/water (90.5:2.5:7,v/v) containing 20 mM choline chloride as described previously (Gross, 1984).

Liposome Preparation and Fusion Assays. Phospholipids utilized for fusion assays were first codissolved with lipid fluorescent probes in chloroform, evaporated under nitrogen, and subsequently evacuated for 1 h at 100 mTorr. Multilamellar liposomes were formed by resuspension in liposome buffer A (100 mM NaCl, 5 mM Na HEPES, 0.1 mM EGTA, pH 7.4) and vigorous vortexing. Next, small unilamellar vesicles (SUVs) were formed by sonicating the multilamellar liposomes for 5 min at 46 °C utilizing a 40% duty cycle at a power level of 1.5 with a Vibra Cell sonicator equipped with a medium tip. All liposomes were used immediately after preparation and were maintained under a nitrogen atmosphere during all steps in the procedure. The octadecyl (R_{18}) fusion assay was performed as previously described (Hoekstra et al., 1984) with the following modifications. Phosphatidylserine vesicles were prepared with 4% R_{18} and were mixed with an equimolar amount of unlabeled vesicles comprised of 50% 16:0–18:1 phosphatidylcholine and 50% ethanolamine glycerophospholipid. This mixture was loaded into one chamber of an SLM-Aminco 4800 spectrofluorometer equipped with an SLM stopped-flow apparatus (model no. FP-052). The other chamber was loaded with liposome buffer alone or with liposome buffer containing, in addition, 20 mM $CaCl_2$. The contents of the chambers were rapidly mixed (dead time = 7 ms) in a 1:1 (v/v) ratio. Fusion was monitored by the temporal dependence of R_{18} dequenching observed at 590 nm after excitation at 560 nm. The final total lipid concentration in these assays was approximately 200 μM. The 0% fusion level was assessed by monitoring fluorescence when the vesicles were mixed at 0 mM $CaCl_2$. The 100% fusion level was assessed by preparing liposomes comprised of the mixture which would result if all vesicles fused (e.g., 48% PS, 25% PC, 25% PE, 2% R_{18}) and subsequently quantifying the resultant R_{18} fluorescence. Fluorescence tracings were normalized utilizing the 0% and 100% fusion levels and were expressed as a percentage of maximum fusion ($F_{max\%}$). The reported initial rates (expressed in terms of $F_{max\%}$ s^{-1}) represent predominately fusion rates under the conditions employed since the high Ca^{2+} concentration utilized in conjunction with the use of SUVs each predispose to membrane fusion (Wilschut et al., 1980). This was confirmed in experiments examining internal contents mixing (*vide infra*). The NBD-PE/Rh-PE assay was performed by preparing phosphatidylserine SUVs containing 1.8% Rh-PE and 1.2% NBD-PE and adding an equimolar amount of unlabeled PC/PE vesicles (Struck et al., 1981; Hoekstra, 1982). Fusion was monitored by NBD-PE fluorescence at 530 nm after excitation at 464 nm. The 100% fusion level was assessed by preparing liposomes composed of 48.5% PS, 25% PC, 25% PE, 0.9% Rh-PE, and 0.6% NBD-PE and subsequently quantifying fluorescence intensity.

Reagents for fusion assays examining contents mixing were prepared by first resuspending phospholipids in either 20 mM NaCl, 50 mM DPA, and 5 mM Na HEPES (pH 7.4) or 2.5 mM $Tb(Cl)_3$, 50 mM sodium citrate, and 5 mM Na HEPES (pH 7.4) (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980; Duzgunes et al., 1987). Next, SUVs containing entrapped $Tb(Cl)_3$ or DPA were separated from unencapsulated probe by gel filtration chromatography employing a Sepharose 6B column equilibrated with 100 mM NaCl, 5 mM Na HEPES, and 1.0 mM EDTA, pH 7.4. At this juncture, an aliquot of the vesicle-containing fraction was subjected to Bligh and Dyer extraction, acid methanolysis, and capillary gas chromatography to quantify the liposome lipid concentration for subsequent fluorescence assays. Contents-mixing assays were performed by incorporating the DPA probe into PS SUVs and the Tb probe into PC/PE SUVs.

After mixing in equimolar concentrations and loading into the stopped-flow apparatus, fusion was monitored through the formation of the fluorescent Tb/DPA complex measured at >470 nm after excitation at 276 nm. Final total lipid concentrations in the assay were approximately 200 μM. The 100% fusion level was assessed by measuring the fluorescence of vesicles prepared in a buffer of 10 mM NaCl, 25 mM sodium citrate, 1.25 mM $Tb(Cl)_3$, 25 mM DPA, and 5 mM Na HEPES (pH 7.4) and processed as described above. All fusion assays were performed at 37 °C.

To verify that differences in observed fusion rates were not due to selective breakdown of one subclass of ethanolamine glycerophospholipid, aliquots of liposomes employed in the fusion studies were extracted by the method of Bligh and Dyer (1959) and analyzed by TLC and straight-phase HPLC. No lysophospholipids or other contaminants were detected within the time frame of the assay. For higher sensitivity detection, 16:0–18:1 plasmenylethanolamine was synthesized with a ^3H label on the *sn*-2 oleoyl group. This label was added to a liposome preparation containing unlabeled PC and PE. No significant (<1%) generation of radiolabeled fatty acid or lysophospholipid was observed during the procedures employed.

Vesicle Sizing. PS and PC/PE vesicles were sized through comparisons of their surface/volume ratios utilizing [^{14}C]-inulin and [^3H]phosphatidylcholine. SUVs were prepared by sonication in liposome buffer A containing 2.2×10^7 dpm (~0.4 μM) [^{14}C]inulin. Both PS and PC/PE vesicles were prepared incorporating [^3H]phosphatidylcholine as a substitutional impurity (<0.1% of total lipid). Following sonication, vesicles containing encapsulated [^{14}C]inulin were separated from unencapsulated material by Sepharose 6B chromatography. Aliquots were removed from the vesicle fraction, and the relative amounts of ^3H and ^{14}C radiolabel present in each type of vesicle were quantified by scintillation spectrometry.

RESULTS

To compare the relative propensity of vesicles containing plasmenylethanolamine or phosphatidylethanolamine to facilitate membrane fusion, we utilized small unilamellar vesicles (SUVs) comprised of an equimolar ratio of choline and ethanolamine glycerophospholipids which were coinjected in equal parts with PS SUVs containing R_{18}. The rate of liposome fusion was quantified by stopped-flow kinetics through measurement of the increase in R_{18} fluorescence intensity which reflects its attenuated quenching as the effective membrane surface area of probe distribution increases after fusion with an unlabeled vesicle. A 3-fold higher rate of membrane fusion was observed in vesicles containing 16:0–18:1 plasmenylethanolamine in comparison to vesicles containing 16:0–18:1 phosphatidylethanolamine (i.e., $22.1F_{max\%}$ s^{-1} vs $7.4F_{max\%}$ s^{-1}, respectively, Figure 1). The rate of membrane fusion in vesicles containing arachidonoylated plasmenylethanolamine was also greater than that manifest by vesicles containing arachidonoylated phosphatidylethanolamine (i.e., $39.3F_{max\%}$ s^{-1} vs $19.0F_{max\%}$ s^{-1}, respectively). Comparisons of vesicles containing 18:0–20:4 plasmenylethanolamine to those containing 16:0–18:1 plasmenylethanolamine demonstrated a 2-fold increase in membrane fusion rates in vesicles containing arachidonic acid, corresponding to the increased volume of distribution of the *sn*-2 aliphatic chain and hence an increased propensity for formation of an H_{II}-like intermediate. The presence of a vinyl ether linkage was not an obligatory requirement for fusion utilizing this system since 18:0–20:4 phosphatidylethanolamine also underwent rapid membrane fusion albeit at rates slower than its plasmalogen counterpart (Figure 1). The increase in fusion rate resulting from the presence of the vinyl ether linkage was approximately similar to that induced by the presence of an arachidonoyl fatty acyl group at the *sn*-2 position ($22.1F_{max\%}$ s^{-1} vs $19.0F_{max\%}$ s^{-1}, respectively). It should be noted that vesicles containing plasmenylethanolamine did not induce alterations in the calcium profile or threshold (approximately 1 mM $CaCl_2$ for each subclass, data not shown) of liposome fusion. Both semisynthetic 18:0–20:4 plasmenylethanolamine (synthesized as described under Materials and Methods) and 18:0–20:4 plasmenylethanolamine purified from canine myocardium gave identical fusion results. Additionally, measured fusion rates of PC/PE vesicles containing the R_{18} label to unlabeled PS SUVs were identical to those obtained in systems containing the R_{18} label in the PS SUVs.

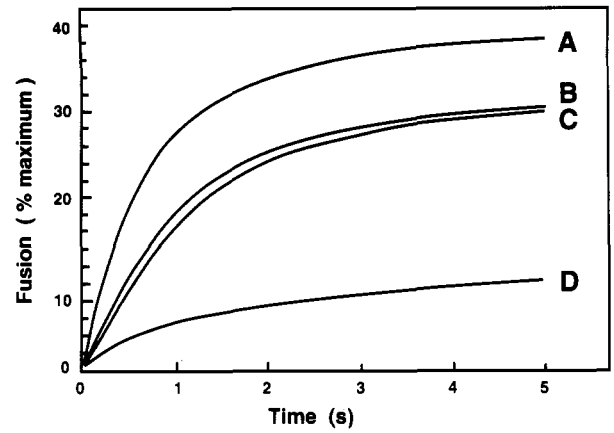

FIGURE 1: Calcium-induced fusion of phosphatidylserine liposomes with liposomes comprised of equimolar mixtures of phosphatidylcholine and ethanolamine glycerophospholipids. Phospholipids were first codissolved with lipid fluorescent probes in chloroform, the solvent was evaporated, and multilamellar liposomes were formed by vigorous vortexing in 100 mM NaCl, 5 mM Na HEPES, and 0.1 mM EGTA (pH 7.4). Next, small unilamellar vesicles (SUVs) were formed by sonicating the multilamellar liposomes as described under Materials and Methods. Phosphatidylserine SUVs containing 4% octadecyl rhodamine (R_{18}) were mixed with an equimolar amount of unlabeled vesicles comprised of 50% phosphatidylcholine and 50% ethanolamine glycerophospholipid and loaded into one chamber of an SLM-Aminco spectrofluorometer equipped with a stopped-flow apparatus (the other chamber contained either buffer alone or buffer containing 20 mM $CaCl_2$), and the contents of the chambers were rapidly mixed (dead time = 7 ms) in a 1:1 (v/v) ratio (final concentration of total lipid = 200 μM). Fusion was monitored by the temporal dependence of R_{18} dequenching observed at 590 nm after excitation at 560 nm. Fluorescence profiles represent the fusion of 16:0–18:1 phosphatidylserine SUVs with vesicles containing equimolar mixtures of POPC/18:0–20:4 plasmenylethanolamine (A), POPC/16:0–18:1 plasmenylethanolamine (B), POPC/18:0–20:4 phosphatidylethanolamine (C), or POPC/16:0–18:1 phosphatidylethanolamine (D). Fluorescence tracings from four independent preparations, all performed in triplicate, were averaged after normalization to the maximum fluorescence increase which would occur after all vesicles fused as described under Materials and Methods.

To further substantiate the importance of the vinyl ether linkage in membrane fusion, plasmalogen-depleted bovine brain PE was prepared (i.e., >90% of its endogenous plasmenylethanolamines were removed by exposure to acid fumes as described under Materials and Methods resulting in an ethanolamine glycerophospholipid fraction enriched in phosphatidylethanolamine, Table 1). Importantly, no significant changes were present in the percentages of polyunsaturated fatty acids at the *sn*-2 position in this fraction, thus facilitating direct assessment of the importance of the vinyl ether linkage in membrane fusion. Comparison of control

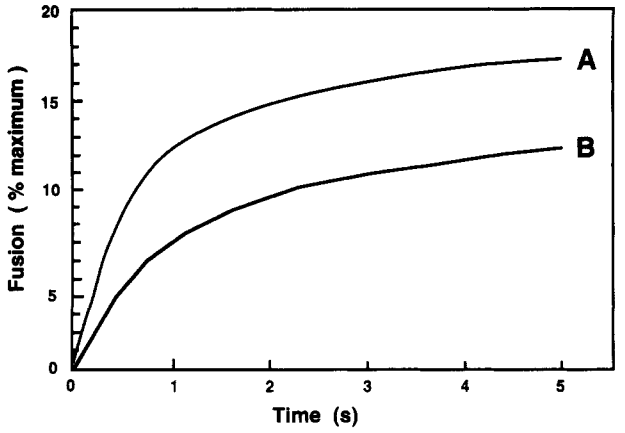

FIGURE 2: Comparison of the effect of endogenous or plasmalogen-depleted bovine brain ethanolamine glycerophospholipids on calcium-induced fusion of phosphatidylserine liposomes with phosphatidylcholine/ethanolamine glycerophospholipid (1:1) liposomes. Plasmalogen-depleted ethanolamine glycerophospholipids were prepared by acid treatment of bovine brain ethanolamine glycerophospholipids and subsequent HPLC purification as described under Materials and Methods. The purified acid-stable ethanolamine glycerophospholipids were dissolved in chloroform and mixed with phosphatidylcholine to form SUVs containing equimolar mixtures of phosphatidylcholine and ethanolamine glycerophospholipids as described in the legend to Figure 1. Phosphatidylserine SUVs containing 4% R_{18} were mixed with an equimolar amount of unlabeled vesicles comprised of 50% phosphatidylcholine and 50% ethanolamine glycerophospholipid (either native or plasmalogen-depleted) and loaded into one chamber of an SLM-Aminco spectrofluorometer equipped with a stopped-flow apparatus (the other chamber contained either buffer alone or buffer containing 20 mM $CaCl_2$), and the contents of the chambers were rapidly mixed in a 1:1 (v/v) ratio (final concentration of total lipid = 200 μM). Fluorescence profiles represent the fusion of 16:0–18:1 phosphatidylserine SUVs with vesicles containing equimolar mixtures of POPC/bovine brain PE (A), or POPC/plasmalogen-depleted bovine brain PE (B). Fluorescence tracings from four independent preparations, all performed in triplicate, were averaged after normalization as described under Materials and Methods.

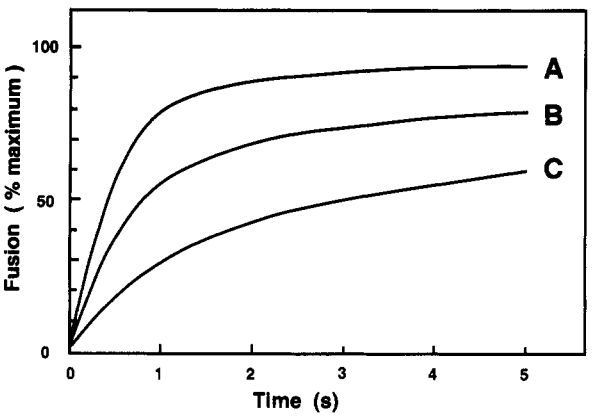

FIGURE 3: *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine/*N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE/Rh-PE) fusion assay of calcium-induced fusion of phosphatidylserine liposomes with liposomes comprised of equimolar mixtures of phosphatidylcholine and ethanolamine glycerophospholipids. Small unilamellar vesicles were prepared as described in the legend to Figure 1. Phosphatidylserine SUVs containing both 1.8 mol % NBD-PE and 1.2 mol % Rh-PE were prepared and were mixed with an equimolar amount of vesicles comprised of 50% phosphatidylcholine and 50% ethanolamine glycerophospholipid. This mixture was loaded into one chamber of an SLM-Aminco spectrofluorometer equipped with a stopped-flow apparatus (the other chamber contained either buffer alone or buffer containing 20 mM $CaCl_2$), and the contents of the chambers were rapidly mixed in a 1:1 (v/v) ratio (final concentration of total lipid = 200 μM). Fusion was monitored by NBD-PE fluorescence at 530 nm after excitation at 464 nm. Fluorescence profiles represent the fusion of 16:0–18:1 phosphatidylserine SUVs with vesicles containing equimolar mixtures of POPC/16:0–18:1 plasmenylethanolamine (A), POPC/bovine brain PE (B), or POPC/16:0–18:1 phosphatidylethanolamine (C). Fluorescence tracings were obtained from two independent preparations, performed in quadruplicate, which were averaged after normalization as described under Materials and Methods.

and plasmalogen-depleted bovine brain ethanolamine glycerophospholipids in a PC/PE and PS vesicle fusion system demonstrated that vesicles containing native bovine brain ethanolamine glycerophospholipids were twice as fusogenic as those containing plasmalogen-depleted ethanolamine glycerophospholipids (i.e., $18.3F_{max\%}$ s^{-1} vs $9.6F_{max\%}$ s^{-1}, respectively, Figure 2). Bovine brain PE, which contains a mixture of both plasmalogen and diacyl-PE, supports a fusion rate that falls between that of mixed vesicles containing only plasmalogen PE and those containing only diacyl PE (i.e., compare Figures 1 and 2). Plasmalogen-depleted bovine brain PE supported vesicle fusion rates similar to those manifest by phosphatidylethanolamine containing oleic acid at the *sn*-2 position.

To verify the results obtained with the R_{18} fusion assay, a second lipid-mixing assay utilizing NBD-PE and Rh-PE was employed (Figure 3). Since Rh-PE partially quenches NBD-PE fluorescence by resonance energy transfer at the starting concentrations used in these experiments (Struck et al., 1981), membrane fusion results in an increase in NBD-PE fluorescence as available membrane surface area increases. Vesicles comprised of equimolar mixtures of PC and 16:0–18:1 plasmenylethanolamine fused to PS SUVs three times more rapidly than vesicles comprised of equimolar mixtures of PC and 16:0–18:1 phosphatidylethanolamine as determined by the dequenching of NBD-PE fluorescence by Rh-PE as the surface area increased after vesicle fusion. Although detailed full time course kinetic analyses of vesicle fusion processes demonstrate substantial complexity (Nir et al., 1980), the results herein largely reflect differences in the initial rates of membrane fusion where contributions from second order processes are minimized.

Since neither of the aforementioned fusion assays discriminate entirely between membrane apposition and *bona fide* membrane fusion, the conclusion that plasmenylethanolamine molecular species facilitate membrane fusion was further substantiated by quantification of internal contents mixing. To assess membrane fusion rates, the interior of POPS and PC/PE SUVs were loaded with dipicolinic acid or Tb^{3+}, respectively, and the rates of mixing of internal contents were quantified on the basis of the increase in fluorescence intensity resulting from the formation of the Tb/DPA complex (Wilschut et al., 1980). The fusion of vesicles comprised of PS to vesicles comprised of equimolar mixtures of PC/PE for both 16:0–18:1 plasmenylethanolamine ($21.4F_{max\%}$ s^{-1}) and 16:0–18:1 phosphatidylethanolamine ($8.7F_{max\%}$ s^{-1}) exhibits a time course, initial rate, and extent similar to that observed in the R_{18} fusion assay (compare Figure 4 to Figure 1). The positive correlation of rate constants derived from contents-mixing and lipid-mixing fusion assays substantiates the fact that the lipid-mixing assays reflect, in large part, *bona fide* membrane fusion and do not reflect vesicle apposition and lipid transfer.

It has been well established that liposomes having a smaller diameter and a high radius of curvature (and, consequently, more internal strain) exhibit faster fusion rates, in general, than those having a larger diameter, a lower radius of curvature and less internal strain (Cullis & Hope, 1991). To verify that observed differences in fusion rates were attributable to

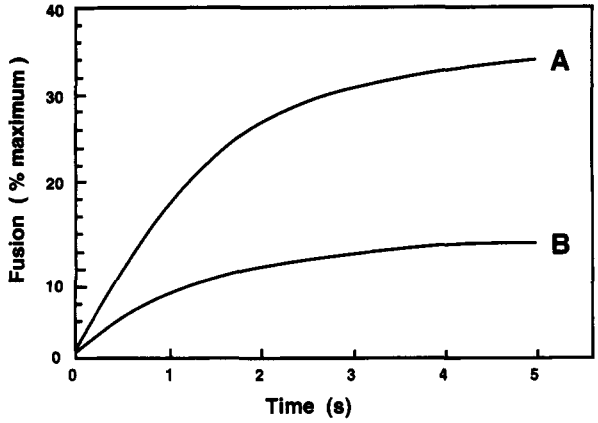

FIGURE 4: Terbium/dipicolinic acid (Tb/DPA) assay of calcium-induced fusion of phosphatidylserine liposomes with liposomes comprised of equimolar mixtures of phosphatidylcholine and ethanolamine glycerophospholipids. Phosphatidylserine SUVs were prepared by sonication in a dipicolinic acid containing buffer while phosphatidylcholine/ethanolamine glycerophospholipid SUVs were prepared in a terbium containing buffer as described under Materials and Methods. Unencapsulated probes were removed from both vesicle populations by gel filtration chromatography on Sepharose 6B resin equilibrated with 100 mM NaCl, 5 mM HEPES, and 1 mM EDTA, pH 7.4. A 1:1 ratio of DPA^{2-} containing phosphatidylserine vesicles and Tb^{3+} containing phosphatidylcholine/ethanolamine glycerophospholipid vesicles were loaded into one chamber of an SLM-Aminco spectrofluorometer equipped with a stopped-flow apparatus (the other chamber contained either buffer alone or buffer containing 20 mM $CaCl_2$), and the contents of the chambers were rapidly mixed in a 1:1 (v/v) ratio (final concentration of total lipid = 200 μM). Measurement of fluorescence emission (monitored at >470 nm after excitation at 276nm) allowed quantitation of the formation of the Tb/DPA complex representative of fusion between 16:0–18:1 phosphatidylserine SUVs and vesicles containing equimolar mixtures of POPC/16:0–18:1 plasmenylethanolamine (A) or POPC/16:0–18:1 phosphatidylethanolamine (B). Fluorescence tracings from three independent preparations, all performed in quadruplicate, were averaged after normalization to the maximum fluorescence increase which would occur after all vesicles fused as described under Materials and Methods.

Table 2: Vesicle Incorporation of [^{14}C]Inulin[a]

liposome composition	[^{14}C]inulin incorporated/μmol of lipid (dpm ± SD)
phosphatidylserine	72 500 ± 4200
POPC/16:0–18:1 plasmenylethanolamine	43 600 ± 3700
POPC/16:0–18:1 phosphatidylethanolamine	43 200 ± 3200
POPC/bovine brain PE	40 900 ● 3000

[a] Determination of the vesicle sizes of small unilamellar vesicles comprised of either 16:0–18:1 phosphatidylserine, POPC/16:0–18:1 plasmenylethanolamine (1:1), POPC/bovine brain PE (1:1), or POPC/16:0–18:1 phosphatidylethanolamine (1:1). SUVs were prepared in 100 mM NaCl, 5 mM HEPES, and 0.1 mM EGTA (pH 7.4) containing 2.2 × 10^7 dpm [^{14}C]inulin and sonicated as described under Materials and Methods. Both PS and PC/PE vesicles contained 4.4 × 10^6 dpm [^{3}H]phosphatidylcholine as a substitutional impurity (<0.1% of total lipid). Following sonication, vesicles were separated from unencapsulated material by Sepharose 6B chromatography. Aliquots were removed from the vesicle fraction and the relative amounts of ^{3}H and ^{14}C were quantified by scintillation spectrometry.

properties of the vinyl ether linkage and did not result from differences in vesicle size, we compared the diameter of diacyl- and plasmalogen-containing vesicles using [^{14}C]inulin as described under Materials and Methods. There were no significant differences in size between the various binary mixtures of PC and PE SUVs comprised of distinct ethanolamine glycerophospholipid subclasses (Table 2). The diameter of PS vesicles was approximately 1.2 times larger than

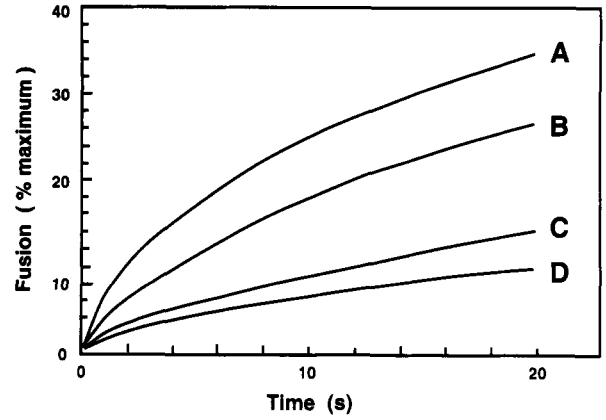

FIGURE 5: Calcium-induced fusion of vesicles comprised of equimolar ternary mixtures of serine, choline, and ethanolamine glycerophospholipids. Small unilamellar vesicles were prepared by codissolving the appropriate amounts of lipid in chloroform and drying under nitrogen and vacuum, followed by sonication as described under Materials and Methods. SUVs comprised of phosphatidylserine/phosphatidylcholine/ethanolamine glycerophospholipid (1:1:1) were mixed with an equimolar amount of identical vesicles except containing, in addition, 4% R_{18}. Vesicles were loaded into one chamber of an SLM-Aminco spectrofluorometer equipped with a stopped-flow apparatus while the other chamber contained either buffer alone or buffer containing 20 mM $CaCl_2$. The contents of the chambers were rapidly mixed in a 1:1 (v/v) ratio resulting in a final concentration of total lipid = 200 μM and a final calcium = 10 mM. Fusion was monitored by the temporal dependence of R_{18} dequenching observed at 590 nm after excitation at 560 nm. Fluorescence profiles represent the fusion of SUVs comprised of POPS/POPC/16:0–18:1 plasmenylethanolamine (1:1:1) (A), POPS/POPC/ bovine brain PE (1:1:1) (B), POPS/POPC/plasmalogen-depleted bovine brain PE (1:1:1) (C), and POPS/POPC/16:0–18:1 phosphatidylethanolamine (1:1:1) (D). Fluorescence tracings were obtained from three independent preparations, performed in quadruplicate, which were averaged after normalization as described under Materials and Methods.

SUVs comprised of equimolar mixtures of choline and ethanolamine glycerophospholipids.

Finally, to confirm that plasmenylethanolamines are more fusogenic than their diacyl counterparts in a homogeneous fusion system, we developed a fusion assay in which both labeled and unlabeled vesicles were comprised of equimolar mixtures of POPS, POPC, and various ethanolamine glycerophospholipid subclasses and molecular species (Figure 5). The presence of plasmenylethanolamines induces an even greater increase in relative membrane fusion rates in this liposome fusion system. Vesicles containing 16:0–18:1 plasmenylethanolamine ($4.9F_{max\%}$ s^{-1}) were six times more fusogenic than those containing 16:0–18:1 phosphatidylethanolamine ($0.8F_{max\%}$ s^{-1}). Similarly, vesicles containing bovine brain PE ($3.1F_{max\%}$ s^{-1}) were more than twice as fusogenic as those containing plasmalogen-depleted PE ($1.5F_{max\%}$ s^{-1}) in this system. To approach even more closely the physiologic complement of phospholipid classes in a synaptic vesicle, liposomes comprised of 45% PC/45% PE/10% PS were also employed (Figure 6). Although fusion rates were significantly slower in this system in comparison to the previous systems, vesicles containing 45% 16:0–18:1 plasmenylethanolamine demonstrated an initial rate of membrane fusion significantly greater than vesicles containing corresponding amounts of 16:0–18:1 phosphatidylethanolamine ($4.4F_{max\%}$ min^{-1} vs $0.7F_{max\%}$ min^{-1}).

DISCUSSION

The results of the present study unambiguously demonstrate that the covalent nature of the *sn*-1 aliphatic constituent in ethanolamine glycerophospholipids is an important determi-

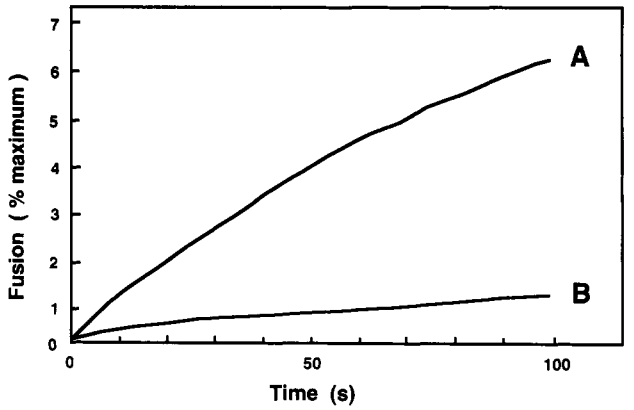

FIGURE 6: Calcium-induced fusion of small unilamellar vesicles comprised of physiologically relevant ratios of phospholipids. Small unilamellar vesicles were prepared by codissolving the appropriate amounts of lipid in chloroform and drying under nitrogen and vacuum, followed by sonication as described in the legend of Figure 1. SUVs comprised of phosphatidylcholine/ethanolamine glycerophospholipids/phosphatidylserine (45:45:10) were mixed with an equimolar amount of identical vesicles except containing, in addition, 4% R_{18}. Vesicles were loaded into one chamber of an SLM-Aminco spectrofluorometer equipped with a stopped-flow apparatus while the other chamber contained either buffer alone or buffer containing 20 mM $CaCl_2$. The contents of the chambers were rapidly mixed in a 1:1 (v/v) ratio resulting in a final concentration of total lipid = 200 μM and a final calcium = 10 mM. Fusion was monitored by the temporal dependence of R_{18} dequenching observed at 590 nm after excitation at 560 nm. Fluorescence profiles represent the fusion of SUVs comprised of POPC/16:0–18:1 plasmenylethanolamine/POPS (45:45:10) (A), and POPC/16:0–18:1 phosphatidylethanolamine/POPS (45:45:10) (B). Fluorescence tracings were obtained from two independent preparations, performed in quadruplicate, which were averaged after normalization as described under Materials and Methods.

nant of the rate of membrane fusion. These results are substantiated through fast-flow kinetic analyses utilizing multiple independent methods to quantify membrane fusion, including both lipid-mixing and internal contents mixing fusion assays. The development of a facile semisynthetic approach allowing the procurement of large quantities (~50 mg) of homogeneous plasmenylethanolamine molecular species was clearly an enabling event in these studies. Collectively, these results (1) provide the first evidence documenting the importance of plasmenylethanolamine molecular species in membrane fusion; (2) identify the chemical rationale underlying the high content of plasmenylethanolamine molecular species containing polyunsaturated fatty acyl constituents at the *sn*-2 position in membrane compartments undergoing rapid membrane fusion; and (3) illustrate a facile semisynthetic route to the procurement of homogeneous molecular species of plasmenylethanolamine.

Although the mechanisms leading to the fusion of biological membranes are not known in precise molecular detail, many studies have implicated the importance of the formation of a transient fusion intermediate comprised of phospholipids arranged in aggregates which bear a structural similarity to an H_{II}-like geometry (Cullis & de Kruijff, 1979; Verkleij, 1984; Ellens et al., 1986; Cullis & Hope, 1991). The formation of transitory nonbilayer structures represents a putative obligatory step in the energy profile of membrane fusion, where destabilization of the membrane bilayer leads to the formation of a critical metastable phospholipid aggregate which likely represents the rate-determining step in the fusion process. The subsequent reorganization of this transient intermediate along an energy-minimized reaction coordinate leads to the formation of a new fused bilayer structure from the previously apposed vesicular partners. One corollary of assigning the formation of an H_{II}-like fusion intermediate as the rate-determining step in the fusion process, and indeed the main argument utilized as proof of the importance of the inverted hexagonal phase-like intermediate in most membrane fusion events, is that lipids which have a propensity for forming the H_{II} phase accelerate the rate of membrane fusion [see Allen et al. (1990) for a discussion of membrane fusion which may not involve H_{II}-type intermediates]. In previous work, it was demonstrated that the H_{II} phase-transition temperatures for multiple plasmenylethanolamine molecular species were substantially lower than those of phosphatidylethanolamine molecular species as assessed by both NMR (Lohner et al., 1984) and fluorescence spectroscopy (Han & Gross, 1992). Since the phase transition temperature reflects the relative volume of distribution of the aliphatic groups compared to the polar head groups, we recognized that modest alterations in the covalent nature of the proximal portion of the *sn*-1 aliphatic constituent could effect large differences in the propensity of individual subclasses to facilitate membrane fusion. Accordingly, systematic examination of membrane fusion rates of vesicles containing plasmenylethanolamine in comparison to phosphatidylethanolamine would provide compelling evidence substantiating the importance of the H_{II}-like fusion intermediate, since this covalent change does not result in substantial alterations in the stereoelectronic nature of the polar head group, the surface charge distribution, or the *sn*-2 aliphatic constituents, nor does it produce dramatic changes in domain formation, lipid packing, or membrane physical properties in the context of choline glycerophospholipid bilayers. These features stand in sharp contrast to the substantial alterations in molecular packing, surface charge characteristics, and domain formation observed in vesicles whose chemical constitution is manipulated to favor an H_{II}-like phase through utilization of substitutional impurities such as diacylglycerol (which alters the polar head group conformational mobility and membrane surface charge characteristics and results in domain formation within biological membranes) or nonisosteric replacement of the polar head group (e.g., incorporation of phosphatidic acid, phosphatidylserine, or cardiolipin) which results in substantial alterations in membrane surface charge and interfacial molecular dynamics, especially with anionic phospholipids in the presence of calcium ion. The current approach, exploiting the differential phase propensity of plasmenylethanolamine in comparison to phosphatidylethanolamine, is uncomplicated by differential interactions of these constituents with calcium ion and/or the surface charge characteristics of the vesicles studied. Accordingly, the demonstration that substitution of the *sn*-1 ester linkage with a vinyl ether moiety induces 3–6-fold increases in membrane fusion rates underscores the importance of the propensity of the system for H_{II}-like phase formation as the rate-determining step in the fusion process.

The reasons underlying the wide diversity in phospholipid classes, subclasses, and molecular species in mammalian subcellular membrane compartments is largely unknown. However, the evolutionary conservation of complex regulatory processes which preserve the precise complement of phospholipid classes, subclasses, and individual molecular species in each membrane compartment underscores the biological importance of phospholipid diversity, even if the physiologic reasons underlying this diversity have not yet been precisely defined. Although the predominance of ethanolamine plasmalogens in both the synaptosomal and plasma membranes of several cell types is well known (Breckenridge et al., 1973; Westhead, 1987; Cullis & Hope, 1991), the reasons underlying

their predominance in membrane compartments undergoing rapid membrane fusion and their role in the fusion process have remained enigmatic. This study integrates a biophysical finding (the facilitation of membrane fusion by plasmenylethanolamines) with biochemical fact (the enrichment of membranes participating in fusion events in plasmenylethanolamine molecular species) to provide an explanation underlying the abundance of plasmalogen molecular species containing polyunsaturated *sn*-2 fatty acids in membrane compartments destined for fusion. Thus, previously established arguments extolling the virtues of natural selection of specific domains of amino acid sequences which encode critical protein functional characteristics (i.e., "form follows function") also appear to be applicable to phospholipid structure and function, where the unique covalent structure present in the proximal portion of the *sn*-1 aliphatic constituent facilitates membrane fusion.

While it seems clear that more than one family of proteins may facilitate the appropriate apposition of specific cellular vesicular constituents (e.g., trafficking of vesicles by cytoskeletal components) to allow the targeted docking of fusion partners, it also seems likely that evolutionary selection has exploited the minimized free energy present in a fusion intermediate containing plasmenylethanolamines with polyunsaturated fatty acids to facilitate the formation of the H_{II}-like intermediate. Thus, the subclass and individual molecular species distribution of phospholipids in membranes undergoing biological fusion have likely been selected to optimally exploit their intrinsic capacity to form an inverted hexagonal phase-like intermediate and promote membrane fusion. Accordingly, we anticipate that a new family of fusion proteins will be identified which exploit the unique properties inherent in the structure of plasmenylethanolamine molecular species to allow the intrinsic fusogenic capacity present in the covalent structure of the vinyl ether linkage to facilitate intracellular membrane fusion.

REFERENCES

Allen, T. M., Hong, K., & Papahadjopoulos, D. (1990) *Biochemistry 29*, 2976–2985.

Almers, W., & Tse, F. W. (1990) *Neuron 4*, 813–818.

Bentz, J., & Ellens, H. (1988) *Colloids Surf. 30*, 65–112.

Bentz, J., Ellens, H., & Szoka, F. C. (1987) *Biochemistry 26*, 2105–2116.

Blank, M. L., & Snyder, F. (1983) *J. Chromatogr. 273*, 415–420.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Phys. 37*, 911–917.

Breckenridge, W. C., Morgan, I. G., Zanetta, J. P., & Vincendon, G. (1973) *Biochim. Biophys. Acta 320*, 681–686.

Chernomordik, L. V., Vogel, S. S., Sokoloff, A., Onaran, H. O., Leikina, E. A., & Zimmerberg, J. (1993) *FEBS Lett. 318*, 71–76.

Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Cullis, P. R., & Hope, M. J. (1991) in *Biochemistry of Lipids, Lipoproteins, and Membranes*, Elsevier.

Diaz, R., Mayorga, L. S., Weidman, P. J., Rothman, J. E., & Stahl, P. D. (1989) *Nature 339*, 398–400.

Duzgunes, N. (1985) *Subcell. Biochem. 11*, 195–286.

Duzgunes, N., Allen, T. M., Fedor, J., & Papahadjopoulos, D. (1987) *Biochemistry 26*, 8435–8442.

Ellens, H., Bentz, J., & Szoka, F. C. (1986) *Biochemistry 25*, 285–294.

Ellens, H., Siegel, D. P., Alford, D., Yeagle, P. L., Boni, L., Lis, L. J., Quinn, P. J., & Bentz, J. (1989) *Biochemistry 28*, 3692–3703.

Fraley, R., Wilschut, J., Duzgunes, N., Smith, C., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6021–6029.

Geurts van Kessel, W. S. M., Hax, W. M. A., Demel, R. A., & de Gier, J. (1977) *Biochim. Biophys. Acta 486*, 524–530.

Gross, R. W. (1984) *Biochemistry 23*, 158–165.

Han, X., & Gross, R. W. (1990) *Biochemistry 29*, 4992–4996.

Han, X., & Gross, R. W. (1992) *Biophys. J. 63*, 309–316.

Han, X., Zupan, L. A., Hazen, S. L., & Gross, R. W. (1992) *Anal. Biochem 200*, 119–124.

Hoekstra, D. (1982) *Biochemistry 21*, 2833–2840.

Hoekstra, D., de Boer, T., Klappe, K., & Wilschut, J. (1984) *Biochemistry 23*, 5675–5681.

Lockband, D. E., Helm, C. A., & Israelachvili, J. (1993) *Biochemistry 32*, 1127–1140.

Lohner, K., Hermetter, A., & Paltauf, F. (1984) *Chem. Phys. Lipids 34*, 163–170.

Meers, P., Hong, K., & Papahadjopoulos, D. (1988) *Biochemistry 27*, 6784–6794.

Nir, S., Bentz, J., & Wilschut, J. (1980) *Biochemistry 19*, 6030–6036.

Papahadjopoulos, D., Vail, W. J., Jacobson, K., & Poste, G. (1975) *Biochim. Biophys. Acta 394*, 483–491.

Papahadjopoulos, D., Nir, S., & Duzgunes, N. (1990) *J. Bioenerg. Biomembr. 22*, 157–179.

Pollard, H. B., Rojas, E., & Burns, A. L. (1992) *Prog. Brain Res. 92*, 247–255.

Poste, G., & Allison, A. C. (1973) *Biochim. Biophys. Acta 300*, 421–465.

Rupert, L. A. M., Van Breemen, J. F. L., Van Bruggen, E. F. J., Engberts, J. B. F. N., & Hoekstra, D. (1987) *Membr. Biol. 95*, 255–263.

Siegel, D. P., Banschbach, J., Alford, D., Ellens, M., Jis, L. J., Quinn, P. J., Yeagle, P. L., & Bentz, J. (1989) *Biochemistry 28*, 3703–3709.

Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, M., Geromanos, S., Tempst, P., & Rothman, J. E. (1993) *Nature 362*, 318–324.

Stamnes, M. A., & Rothman, J. E. (1993) *Cell 73*, 999–1005.

Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.

Sundler, R., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 649*, 743–750.

Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43–63.

Vogel, S. S., Leikina, E. A., & Chernomordik, L. V. (1993) *J. Biol. Chem. 268*, 25764–25768.

von Wedel, R. J., Carlson, S. S., & Kelly, R. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1014–1018.

Westhead, E. W. (1987) *Ann. N.Y. Acad. Sci. 493*, 92–100.

Wilschut, J., & Papahadjopoulos, D. (1979) *Nature (London) 281*, 690–692.

Wilschut, J., & Hoekstra, D. (1986) *Chem. Phys. Lipids 40*, 145–166.

Wilschut, J., Duzgunes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.

Wilson, D. W., Wilcox, C. A., Flynn, G. C., Chen, E., Kuang, W. J., Henzel, W. J., Block, M. R., Ullrich, A., & Rothman, J. E. (1989) *Nature 339*, 355–359.

Wilson, D. W., Whiteheart, S. W., Orci, L., & Rothman, J. E. (1991) *Trends Biochem. Sci. 16*, 334–337.

Yeagle, P. L. (1989) *FASEB J. 3*, 1833–1842.

Zimmerberg, J., Vogel, S. S., & Chernomordik, L. V. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 433–466.