Disparate Molecular Dynamics of Plasmenylcholine and Phosphatidylcholine Bilayers†

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Received November 19, 1986; Revised Manuscript Received March 23, 1987

ABSTRACT: The molecular dynamics of binary dispersions of plasmenylcholine/cholesterol and phosphatidylcholine/cholesterol were quantified by electron spin resonance (ESR) and deuterium magnetic resonance (^{2}H NMR) spectroscopy. The order parameter of both 5-doxylstearate (5DS) and 16-doxylstearate (16DS) was larger in vesicles comprised of plasmenylcholine in comparison to phosphatidylcholine at all temperatures studied (e.g., S = 0.592 vs. 0.487 for 5DS and 0.107 vs. 0.099 for 16DS, respectively, at 38 °C). Similarly, the order parameter of plasmenylcholine vesicles was larger than that of phosphatidylcholine vesicles utilizing either spin-labeled phosphatidylcholine or spin-labeled plasmenylcholine as probes of molecular motion. The ratio of the low-field to the midfield peak height in ESR spectra of 16-doxylstearate containing moieties (i.e., spin-labeled plasmenylcholine and phosphatidylcholine) was lower in plasmenylcholine vesicles (0.93 ± 0.01) in comparison to phosphatidylcholine vesicles (1.03 ± 0.01). ^{2}H NMR spectroscopy demonstrated that the order parameter of plasmenylcholine was greater than that of phosphatidylcholine for one of the two diastereotopic deuterons located at the C-2 carbon of the *sn*-2 fatty acyl chain. The spin–lattice relaxation times for deuteriated plasmenylcholine and phosphatidylcholine in binary mixtures containing 0–50 mol % cholesterol varied nonmonotonically as a function of cholesterol concentration and were different for each phospholipid subclass. Taken together, the results indicate that the vinyl ether linkage in the proximal portion of the *sn*-1 aliphatic chain of plasmenylcholine has substantial effects on the molecular dynamics of membrane bilayers both locally and at sites spatially distant from the covalent alteration.

Although the chemical existence of plasmalogens in mammalian tissues has been known for over 60 years (Feulgen, 1924), the delineation of their biological function has remained elusive. Recently, we have identified plasmalogens as the predominant phospholipid constituents of canine myocardial sarcolemma and sarcoplasmic reticulum (Gross, 1984, 1985). The predominance of plasmalogens in electrically responsive membranes has suggested a possible role for these moieties in facilitating transmembrane ion flux (Gross, 1984). Plasmalogens differ from conventional diacyl phospholipids by the presence of a vinyl ether linkage in the proximal portion of the *sn*-1 aliphatic chain that alters molecular conformation and, therefore, likely results in alterations of molecular dynamics in plasmalogen vesicles in comparison to their diacyl phospholipid counterparts.

Although the phase-transition temperatures and molecular dynamics of plasmenylethanolamine have been examined by differential scanning calorimetry (Boggs et al., 1981), ESR[1] (Boggs et al., 1981; Demediuk et al., 1983), and ^{31}P NMR (Lohner et al., 1984), the molecular dynamics of plasmenylcholine have not been directly investigated. To directly interrogate the molecular dynamics of the major myocardial sarcolemmal phospholipid (i.e., plasmenylcholine), spin-labeled and specifically deuteriated plasmenylcholines were synthesized and the molecular dynamics of binary dispersions of plasmenylcholine/cholesterol were compared with those of phosphatidylcholine/cholesterol vesicles by utilizing ESR and ^{2}H NMR spectroscopy. The results of this study demonstrate that plasmenylcholine is immobilized compared to phosphatidylcholine and that this immobilization is separate and distinct from the immobilization of membrane phospholipids produced by cholesterol. These results suggest that membrane domains enriched in plasmalogen molecular species provide a unique molecular environment that can potentially facilitate the function of specific transmembrane proteins.

Materials and Methods

Preparation of Spin-Labeled Plasmenylcholine and Phosphatidylcholine. Lysoplasmenylcholine was prepared by alkaline methanolysis of beef heart choline glycerophospholipids and subsequently purified by silicic acid column chromatography by minor modifications of a previously described method (Bergelson, 1980). Briefly, to 5 g of bovine heart choline glycerophospholipids (Avanti) was added 200 mL of 0.05 M KOH in 1:1 methanol/benzene (v/v), and the solution was stirred for 40 min at 40 °C under an atmosphere of N_2. After >98% of choline glycerophospholipid had been hydrolyzed (ascertained by TLC), 50 mL of ethyl formate was slowly

†Supported in part by NIH Grant HL17646 SCOR in Ischemic Heart Disease, Grant HL34839, and an NIH BRSG award.

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[1] Abbreviations: ^{2}H NMR, deuterium magnetic resonance; ESR, electron spin resonance; 5DS, 5-doxylstearate; 16DS, 16-doxylstearate; 5DS-PhosCho, 1-palmitoyl-2-[5-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphocholine; 5DS-PlasCho, 1-*O*-(*Z*)-hexadecenyl-2-[5-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; 16DS-PhosCho, 1-palmitoyl-2-[16-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; 16DS-PlasCho, 1-*O*-(*Z*)-hexadecenyl-2-[16-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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added, and the solution was stirred at 40 °C for an additional 15 min. The reaction mixture was evaporated under vacuum, dried with 3 g of $MgSO_4$, and filtered. Lysoplasmenylcholine was purified on a 5 × 20 cm column of silicic acid by sequential step gradients of $CHCl_3$/MeOH (lysoplasmenylcholine typically eluted at 30% $CHCl_3$/70% MeOH). Typical yields of lysoplasmenylcholine ranged from 50% to 70% of theoretical yield. Purified lysoplasmenylcholine was a single intense spot after TLC in two different solvent systems (Gross & Sobel, 1982). The different molecular species of lysoplasmenylcholine were separated by isocratic reverse-phase HPLC utilizing an Altex C_{18} column as previously described (Creer & Gross, 1985) to obtain homogeneous 1-*O*-(*Z*)-hexadecenyl-*sn*-glycero-3-phosphocholine.

Lysoplasmenylcholine [1-*O*-(*Z*)-hexadecenyl-*sn*-glycero-3-phosphocholine], spin-labeled fatty acid (5DS or 16DS), and 4-(*N*,*N*-dimethylamino)pyridine [recrystallized from $CHCl_3/Et_2O$ (1:1)] were evacuated at 50 mTorr for 20 h in a desiccator containing P_2O_5. Chloroform was distilled over P_2O_5 and utilized immediately. To approximately 20 mg of 5- or 16-doxylstearate was added 15 mg of dicyclohexylcarbodiimide. The reaction mixture was stirred for 1 h, and the precipitated dicyclohexylurea was removed by filtration. The solvent was evaporated with dried nitrogen gas, and the anhydride was resuspended in freshly distilled $CHCl_3$. Spin-labeled anhydride was immediately added to a $CHCl_3$ solution containing 15 mg of lysoplasmenylcholine or lysophosphatidylcholine. Subsequently 5 mg of recrystallized 4-(*N*,*N*-dimethylamino)pyridine was added, and the reaction mixture was stirred for 24 h at room temperature. Spin-labeled plasmenylcholine or phosphatidylcholine was purified initially by preparative TLC utilizing silica OF plates that were developed with a mobile phase of $CHCl_3$/MeOH/NH_4OH (65:35:5). The regions corresponding to spin-labeled phospholipids (R_f = 0.35) were scraped into a test tube and eluted by three sequential washes with chloroform/methanol (1:1). The extract was filtered by utilizing a Millex 0.2-μm filter, evaporated to 100 μL, and further purified by isocratic HPLC utilizing a Whatman SCX column as the stationary phase and acetonitrile/MeOH/H_2O (400:100:21) as the mobile phase (Gross & Sobel, 1980). Spin-labeled plasmenylcholine and phosphatidylcholine each eluted as a single peak on HPLC (retention time = 8 min) and migrated as a single spot after TLC and iodine staining (Gross & Sobel, 1982).

Preparation of Deuteriated Plasmenylcholine and Phosphatidylcholine. Diradylphospholipids specifically deuteriated at the C-2 carbon of the *sn*-2 fatty acyl chain were prepared similarly. However, due to the larger amounts of lysoplasmenylcholine necessary to obtain sufficient quantities of phospholipid for ^2H NMR, the use of preparative reverse-phase HPLC to obtain homogeneous plasmalogens was not feasible. Gas chromatographic analysis of the *sn*-1 constituents of the semisynthetic lysoplasmenylcholine demonstrated that the aliphatic constituents at the *sn*-1 chain were comprised largely of 16:0 (72%) and 18:0 (12%) as well as smaller amounts of 15:0, 17:0, 19:0, and 18:1 aliphatic chains. Therefore, this fatty acyl chain composition was approximated in the lysophosphatidylcholine starting material by mixing 85% 1-palmitoyl-*sn*-glycero-3-phosphocholine (Avanti) and 15% 1-stearoyl-*sn*-glycero-3-phosphocholine (Avanti). The anhydride of [2,2-$^2H_2$]palmitic acid was prepared with 60 mg of dicyclohexylcarbodiimide and 70 mg of deuteriated palmitate as described above. The tetradeuteriated anhydride was added to 150 mg of lysoplasmenylcholine or lysophosphatidylcholine dissolved in $CHCl_3$. Ten milligrams of recrystallized 4-(*N*,*N*-dimethylamino)pyridine was added, and the reaction mixture was stirred for 24 h. Reaction products were extracted by the Bligh and Dyer procedure and chromatographed on 175 g of silicic acid with the use of a step gradient as described above. Deuteriated plasmenylcholine and phosphatidylcholine were a single spot on TLC after intense iodine staining.

Preparation of Samples for Electron Spin Resonance Spectroscopy. Aliquots of stock solutions of spin-labeled plasmalogen or diacyl phospholipid, unlabeled plasmalogen or diacyl phospholipid, and cholesterol were combined in the indicated amounts to comprise solutions containing 670 nmol of total lipid in $CHCl_3$. The aliphatic constituents of unlabeled phospholipids utilized in these experiments were selected to facilitate comparisons at physiological temperatures between the major myocardial plasmenylcholine (which contains oleic acid at the *sn*-2 position) and their diacyl phospholipid counterparts. Thus, stock solutions of 85% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 15% 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (each from Avanti) were prepared for comparisons with semisynthetic plasmenylcholines. Samples containing each lipid in appropriate concentrations were evaporated to dryness by N_2 gas and then exhaustively evacuated under high vacuum for 1 h. Lipids were resuspended in 50 μL of buffer (0.02 M tris(hydroxymethyl)aminomethane hydrochloride, pH 7.0) and sonicated in a Branson water-bath sonicator for 30 s 5 times. The molar ratio of lipid to spin-label was kept constant at 100:1, and the final concentration of lipid was 13.4 mM.

Electron Spin Resonance Spectroscopy. Electron spin resonance spectra were obtained by utilizing an IBM–Bruker 200D electron spin resonance spectrometer operating at a centerfield strength of 3435 G. The scan width was set at 50 or 100 G for 16-doxyl- and 5-doxylstearoyl moieties, respectively. The time constant was set at 0.5 s, and a modulation amplitude of 1 G was utilized. Ten milliwatts of microwave power at 9.1 GHz were employed. These settings were empirically found to be free of significant saturation effects. Spectra were recorded by utilizing an Aspect 2000 data system and subsequently transferred to an IBM 9000 computer for data analysis.

ESR spectra were analyzed by utilizing an interactive program we developed. Each individual peak and trough of the ESR spectrum was expanded to full screen width. Next, a cursor was placed on the "apparent" maximum or minimum of the peak, and an iterative curve fitting procedure was initiated that fit all data points within 0.01% of the assigned field value to a quadratic equation utilizing a χ^2 test as the criterion of "best fit". It was empirically found that purposefully poor choices in initial operator-assigned values did not result in substantial alterations of the computer-generated χ^2 best fits and subsequent determinations of peak maxima and minima. Although the computer-generated best fit did not differ significantly from operator-assigned values of each experimental spectra, it was utilized to provide an objective assignment of relevant spectral parameters. After individual fitting of each of the six maxima and minima, then the peak splittings, peak widths, and relative peak heights (normalized to a midfield peak height of 1.0) were directly calculated. The apparent order parameter (S) was calculated according to the formula of Hubbell and McConnell (1972)

$$S = [(T_{\parallel} - T_{\perp})/(T_{zz} - T_{xx})]\ (a_N/a'_N)$$

where $T_{\parallel}$ and $T_{\perp}$ are the parallel and perpendicular components of the hyperfine tensor and a_N and a'_N are one-third the trace of the static and dynamic hyperfine tensors, respectively. The apparent rotational correlation time (T) for 16-doxyl-

stearate or spin-labeled phospholipids labeled at the C-16 carbon of the *sn*-2 fatty acyl chain was approximated according to the formalism initially given by Kivelson (1964) and subsequently modified by Henry and Keith (1971) for relative comparisons of correlation times in membrane bilayers:

$$T = K(h_0)(\sqrt{H_0/H_{-1}} - 1)$$

where $K = 6.5 \times 10^{-10}$ s, h_0 is the midfield peak width, and H_0/H_{-1} is the ratio of the midfield and high-field peak heights.

Preparation of Samples for Deuterium Magnetic Resonance Spectroscopy. Synthetic deuteriated plasmenylcholine or phosphatidylcholine in chloroform was placed in a glass ampule, the appropriate amount of cholesterol was added (0–50 mol %), and the solution was evaporated to dryness with N_2 gas. Each sample was subsequently exhaustively evacuated under high vacuum for 1 h. Deuterium-depleted H_2O was added at a ratio of 3:2 (H_2O:lipid) by weight. After each solution was thoroughly stirred, the tube was sealed under vacuum and at least five freeze–thaw cycles were performed prior to ^2H NMR spectroscopy. The solution was placed in a Bruker probe that had previously been modified to accommodate high-power transients.

Deuterium Magnetic Resonance Spectroscopy. ^2H NMR transients were obtained at 30.7 MHz with a Bruker CXP-200 spectrometer equipped with a high-power probe. A quadrupolar echo sequence (Davis, 1983) with $\pi/2$ pulses of 4-μs duration and a delay between pulses of 40 μs were utilized for all experiments. All data were accumulated by utilizing quadrature detection with a spectral width of 200 kHz. Sample temperature was regulated to within 0.5 K with a Bruker VT-1000 temperature control unit. All spectra were obtained with a recycle delay time of at least $3T_1$. No differences in spectral features could be detected with a wide range of recycle delay times. All data were collected and analyzed with an Aspect-2000 computer system.

Spin–lattice relaxation measurements were performed by the saturation recovery technique (Fukishima & Roeder, 1981). The spin–lattice relaxation times were calculated from the amplitude of the echo since the echo maximum was more clearly resolved than individual peak heights in the corresponding Fourier transformations. T_1 data obtained from the echo maximum and from the Fourier transformation are expected to be nearly identical since the membranes studied herein were specifically deuteriated in only one position of the acyl side chain and T_1 does not vary significantly as a function of line shape (Brown & Davis, 1981).

RESULTS

Molecular Dynamics of Binary Dispersions of Plasmenylcholine/Cholesterol and Phosphatidylcholine/Cholesterol Ascertained by Electron Spin Resonance Spectroscopy Utilizing 5- or 16-Doxylstearic Acid as Probes of Molecular Motion. Electron spin resonance spectra of plasmenylcholine or phosphatidylcholine vesicles containing 5DS or 16DS (I and II, Figure 1) as substitutional impurities (i.e., 1 mol %) demonstrated substantial and reproducible differences in the hyperfine extrema, peak widths, and peak height ratios at physiological temperature (Figure 2). Similar differences were present at each of five separate temperatures (14–38 °C) in multiple independent preparations. Accordingly, the apparent order parameter for both 5- and 16DS-labeled vesicles was higher for vesicles comprised of plasmenylcholine in comparison to phosphatidylcholine ($S = 0.592 \pm 0.002$ vs. 0.487 ± 0.001 for 5DS and 0.107 ± 0.002 vs. 0.099 ± 0.001 for 16DS, respectively at 38 °C). The addition of incremental

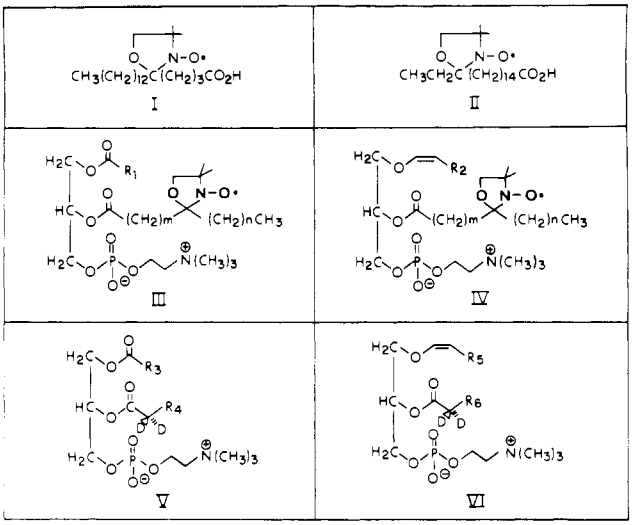

FIGURE 1: Electron spin resonance and deuterium magnetic resonance probes utilized for quantification of molecular dynamics: I, 5-doxylstearate; II, 16-doxylstearate; III, doxylphosphatidylcholine; IV, doxylplasmenylcholine; V, 1-palmitoyl-2-[2,2-$^2H_2$]palmitoyl-*sn*-glycero-3-phosphocholine; VI, 1-*O*-(*Z*)-hexadecenyl-2-[2,2-$^2H_2$]-palmitoyl-*sn*-glycero-3-phosphocholine.

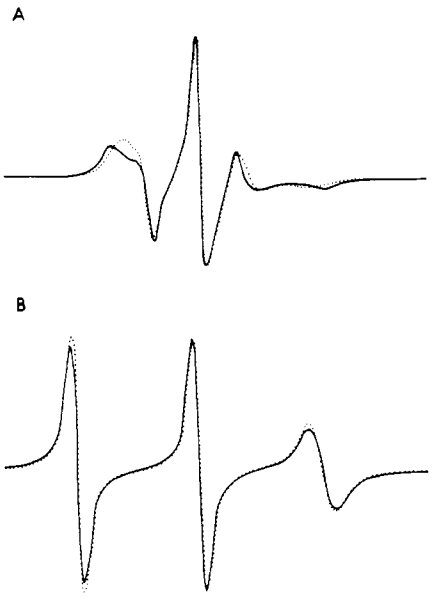

FIGURE 2: Electron spin resonance spectra of 5- and 16-doxylstearate spin-labeled plasmenylcholine and phosphatidylcholine vesicles. Phosphatidylcholine (···) or plasmenylcholine (—) vesicles were spin-labeled with 5-doxylstearic acid (A) or 16-doxylstearic acid (B), and electron spin resonance spectra were obtained at 38 °C as described under Materials and Methods. The midfield peak in each spectrum is normalized to a value of 1.

amounts of cholesterol (0–50 mol %) to either plasmenylcholine or phosphatidylcholine vesicles resulted in an increase in the order parameter and the apparent rotational correlation time for each phospholipid subclass. For example, the apparent order parameter of 5DS-spin-labeled vesicles of plasmenylcholine or phosphatidylcholine containing 30 mol % cholesterol was 0.630 ± 0.001 vs. 0.564 ± 0.005, respectively, at 38 °C. The apparent order parameter of 16DS-spin-labeled vesicles of plasmenylcholine or phosphatidylcholine containing 30 mol % cholesterol was 0.164 ± 0.001 vs. 0.155 ± 0.001, respectively, at 38 °C. Similar differences for each phospholipid subclass were present at each cholesterol concentration and temperature examined. The ratio of the low-field to the midfield peak height (H_1/H_0) for 16DS-spin-labeled vesicles

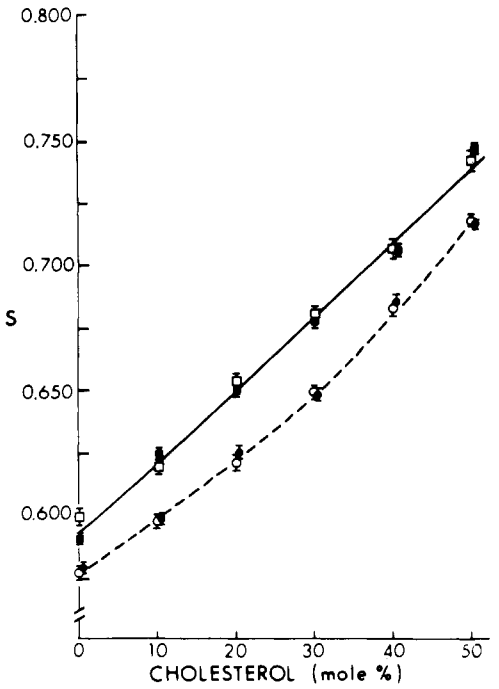

FIGURE 3: Comparison of the apparent order parameter of 5-doxylstearate spin-labeled phospholipids in binary dispersions of plasmenylcholine/cholesterol and phosphatidylcholine/cholesterol. Vesicles comprised of spin-labeled phospholipid (■ and ●, 5DS-PhosCho; □ and ○, 5DS-PlasCho) and unlabeled phospholipid present in 99 mol % (○ and ●, phosphatidylcholine; □ and ■, plasmenylcholine) containing the indicated amounts of cholesterol were prepared, and electron spin resonance spectra were acquired at 38 °C as described under Materials and Methods. The apparent order parameter (S) was calculated from the splitting of the hyperfine extrema as described under Materials and Methods. Results represent the $\bar{X} \pm$ SE of six determinations.

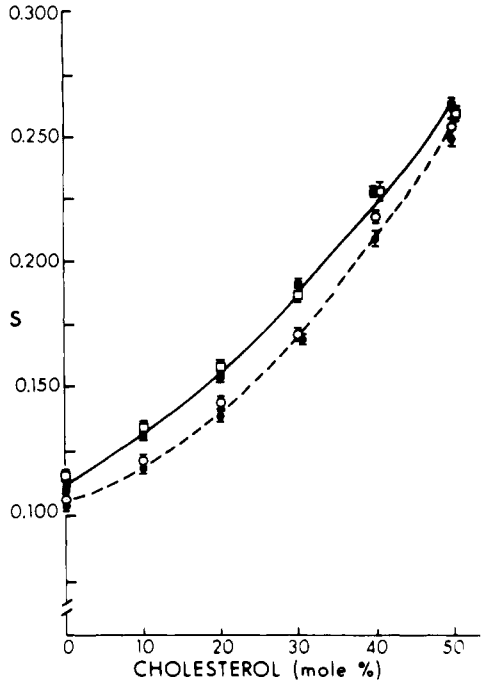

FIGURE 4: Comparison of the apparent order parameter of 16-doxylstearate spin-labeled phospholipids in binary dispersions of plasmenylcholine/cholesterol and phosphatidylcholine/cholesterol. Vesicles comprised of spin-labeled phospholipid (■ and ●, 16DS-PhosCho; □ and ○, 16DS-PlasCho) and unlabeled phospholipid present in 99 mol % (○ and ●, phosphatidylcholine; □ and ■, plasmenylcholine) containing the indicated amounts of cholesterol were prepared as described under Materials and Methods. Electron spin resonance spectra were obtained at 38 °C, and the apparent order parameter (S) was calculated as described under Materials and Methods. Results represent the $\bar{X} \pm$ SE of six determinations.

comprised of plasmenylcholine was lower than that for 16DS-spin-labeled vesicles comprised of phosphatidylcholine (0.93 ± 0.01 vs. 1.03 ± 0.01 at 38 °C). Taken together, these results demonstrate differences in the molecular dynamics of plasmenylcholine vesicles in comparison to their diacyl phospholipid counterparts, utilizing either 5- or 16-doxylstearate as probes of molecular motion.

Molecular Dynamics of Plasmenylcholine and Phosphatidylcholine Vesicles Utilizing Spin-Labeled Plasmenylcholines or Spin-Labeled Phosphatidylcholines as Probes of Molecular Motion. To exclude potential differences resulting from specific fatty acid–plasmenylcholine or fatty acid–phosphatidylcholine interactions that are not representative of membrane dynamics as a whole, spin-labeled plasmenylcholines and phosphatidylcholines were synthesized as described under Materials and Methods. These probes were utilized to interrogate molecular dynamics near the hydrophobic–hydrophilic interface [III (m = 3, n = 12) and IV (m = 3, n = 12), Figure 1] and dynamics in the membrane interior [III (m = 14, n = 1) and IV (m = 14, n = 1), Figure 1]. All four combinations of spin-label (spin-labeled plasmenylcholine or spin-labeled phosphatidylcholine) and unlabeled phospholipid (plasmenylcholine or phosphatidylcholine) were prepared in the absence or in the presence of selected concentrations of cholesterol (0–50 mol %), and motional dynamics were quantified by ESR spectroscopy as a function of temperature (14, 22, 28, 32, and 38 °C).

The dynamics of 5DS-PlasCho [IV (m = 3, n = 12), Figure 1] in vesicles comprised of unlabeled plasmenylcholine were different in comparison to vesicles comprised of unlabeled phosphatidylcholine (Figure 3). Similarly, the dynamics of 5DS-PhosCho III (m = 3, n = 12), Figure 1] in vesicles comprised of unlabeled phosphatidylcholine were different in comparison with vesicles comprised of unlabeled plasmenylcholine (Figure 3). The addition of cholesterol to each of the four vesicle populations (5DS-PlasCho and unlabeled plasmenylcholine, 5DS-PhosCho and unlabeled plasmenylcholine, 5DS-PhosCho and unlabeled phosphatidylcholine, and 5DS-PlasCho and unlabeled phosphatidylcholine) increased the order parameter for each ternary mixture of lipid (spin-labeled phospholipid, unlabeled phospholipid, and cholesterol). At each cholesterol concentration the order parameter for vesicles comprised of unlabeled plasmenylcholine was higher in comparison to vesicles containing unlabeled phosphatidylcholine. These differences were apparent at each of five different temperatures studied (14, 22, 28, 32, and 38 °C). Experiments utilizing mixed vesicles demonstrated that no significant differences in spectral parameters were observed with either spin-labeled phospholipid (Figure 3). Thus, the observed alterations in motional properties reflected the predominant (99 mol %) choline glycerophospholipid present and were independent of the subclass of the spin-labeled phospholipid employed. Accordingly, the dynamics of the doxyl reporter group reflect the molecular environment of its lipidic surroundings without significant effects produced by the phospholipid subclass of the spin-labeled moiety itself.

To investigate the molecular dynamics of plasmenylcholine and phosphatidylcholine in the membrane interior, we prepared vesicles comprised of all four combinations of spin-labeled [III and IV (m = 14, n = 1), Figure 1] and unlabeled plasmenylcholine or phosphatidylcholine and examined their motional characteristics by ESR spectroscopy. In agreement with the results utilizing fatty acid spin-labeled membranes, differences

Table I: Apparent Rotational Correlation Times of Spin-Labeled Plasmenylcholine and Phosphatidylcholine Vesicles[a]

cholesterol (mol %)	$T_{phosphatidylcholine}$ (ns)	$T_{plasmenylcholine}$ (ns)
0	0.86 ± 0.01	0.89 ± 0.01
10	0.97 ± 0.01	1.02 ± 0.01
20	1.06 ± 0.01	1.11 ± 0.01
30	1.17 ± 0.02	1.26 ± 0.03
40	1.23 ± 0.01	1.26 ± 0.01
50	1.24 ± 0.02	1.26 ± 0.01

[a] Vesicles comprised of spin-labeled plasmenylcholine (16DS-PlasCho) and unlabeled plasmenylcholine or spin-labeled phosphatidylcholine (16DS-PhosCho) and unlabeled phosphatidylcholine were prepared as described under Materials and Methods. Electron spin resonance spectroscopy was performed at 38 °C, and the apparent rotational correlation time (T) was calculated from the width of the midfield peak and the ratio of the midfield to high-field peak heights. Results represent the $\bar{X}$ ± SE of six determinations.

were observed in multiple spectral parameters with phospholipid spin-labeled vesicles comprised of plasmenylcholine in comparison to vesicles comprised of phosphatidylcholine (Figure 4). Experiments with mixed vesicles (e.g., 16DS-PlasCho and unlabeled phosphatidylcholine) again demonstrated that no significant differences were present with either spin-labeled phospholipid probe, but rather the observed alterations in molecular dynamics reflected the unlabeled phospholipid subclass present in large molar excess (Figure 4). The addition of cholesterol (0–50 mol %) significantly immobilized both phosphatidylcholine and plasmenylcholine vesicles. Differences between the time-averaged molecular dynamics in each phospholipid subclass were present at each cholesterol concentration examined (Figure 4 and Table I).

A major difference in ESR spectra of 16DS-PlasCho or 16DS-PhosCho spin-labeled vesicles of plasmenylcholine or phosphatidylcholine was the ratio of the low-field to the midfield peak heights (H_1/H_0). This parameter was less than unity for plasmenylcholine vesicles in the absence of cholesterol and decreased slowly with increasing mole percent cholesterol with either spin-labeled phospholipid (Figure 5). In contrast, this ratio was greater than unity in vesicles comprised of phosphatidylcholine in the absence of cholesterol and decreased dramatically upon the addition of cholesterol. No significant differences were present in this parameter with each of three different spin-labels (16DS, 16DS-PlasCho, or 16DS-PhosCho) in plasmenylcholine vesicles. Similarly, no significant differences were present in this ratio with each of these three spin-labels incorporated into phosphatidylcholine vesicles. Thus, the differences in H_1/H_0 in plasmenylcholine vesicles in comparison to phosphatidylcholine vesicles reflect intrinsic alterations in the molecular conformation and dynamics of the unlabeled phospholipid present in large molar excess and are independent of the chemical identity of the spin-labeled moiety itself.

^2H NMR Spectroscopy of Deuteriated Plasmenylcholine and Phosphatidylcholine. Since spin-label probes are potentially membrane-perturbing agents, additional experiments were performed with deuteriated plasmenylcholine and phosphatidylcholine to unequivocally establish that these two phospholipid subclasses have separate and distinct molecular dynamics independent of the reporter group employed. Plasmenylcholine and phosphatidylcholine, each deuteriated at the C-2 position of the *sn*-2 aliphatic chain (V and VI, Figure 1) were prepared as described under Materials and Methods. Typical ^2H NMR spectra of specifically deuteriated phosphatidylcholine (V, Figure 1) and plasmenylcholine (VI, Figure 1) are shown in Figure 6. Each diastereotopic deuteron gives rise to a single Pake doublet with overlapping chemical

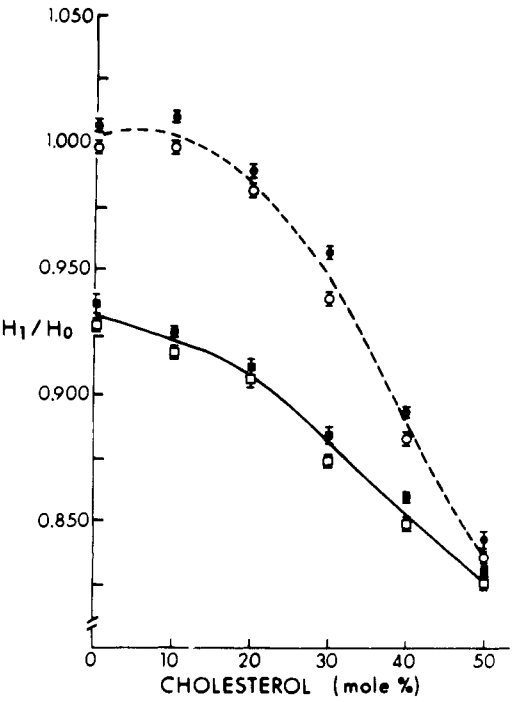

FIGURE 5: Comparison of low-field to midfield peak height ratios of vesicles comprised of binary mixtures of phosphatidylcholine/cholesterol and plasmenylcholine/cholesterol. Spin-labeled phospholipid (■ and ●, 16DS-PhosCho; □ and ○, 16DS-PlasCho) and unlabeled phospholipid (○ and ●, phosphatidylcholine; □ and ■, plasmenylcholine) with the indicated amounts of cholesterol were prepared as described under Materials and Methods. Electron spin resonance spectra were obtained at 38 °C, and the relative ratio of the low-field to midfield peak height was calculated. Results are the $\bar{X}$ ± SE of at least six determinations.

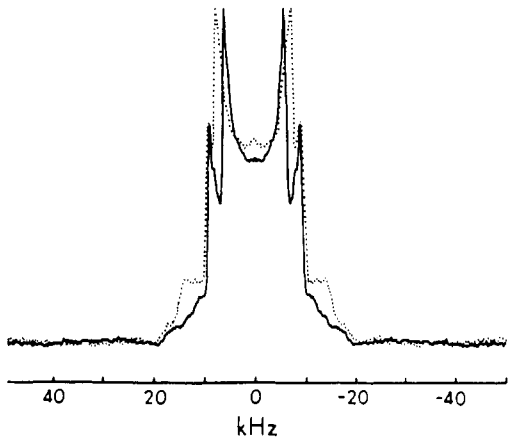

FIGURE 6: Comparison of deuterium magnetic resonance spectra of specifically deuteriated phosphatidylcholine and plasmenylcholine. Deuteriated phospholipids (probes V and VI in Figure 1) were prepared and hydrated as described under Materials and Methods. Deuterium magnetic resonance spectra were recorded at 30.7 MHz by utilizing a quadrupolar echo sequence with $\pi/2$ pulses of 4-μs duration and a delay of 40 μs between pulses at 41 °C. Spectra of plasmenylcholine (···) and phosphatidylcholine (—) reproducibly demonstrated differences in the splitting of the inner pair of doublets and alterations in the spectral intensity of the wings of the observed resonances.

shifts resulting in the superposition of two quadrupolar patterns. The inner splitting in spectra of deuteriated plasmenylcholine vesicles was markedly greater in comparison with phosphatidylcholine vesicles although the outer splitting of each phospholipid subclass was identical (Figure 6). Furthermore, the spectral intensity in the wings of plasmenylcholine ^2H NMR spectra was greater than the intensity in ^2H NMR spectra of phosphatidylcholine vesicles (Figure 6). These

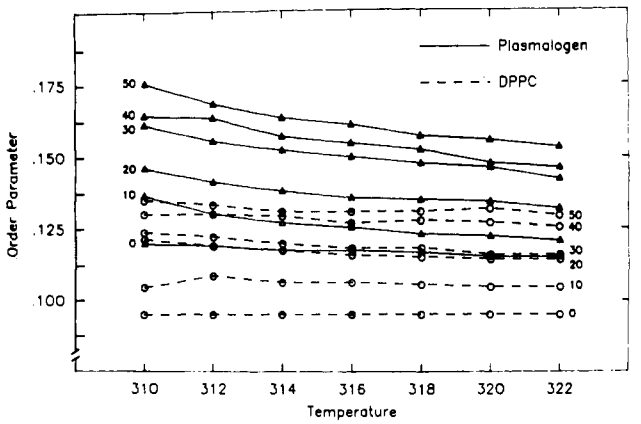

FIGURE 7: Comparison of the apparent order parameter of hydrated dispersions of plasmenylcholine/cholesterol and phosphatidylcholine/cholesterol by utilizing deuterium magnetic resonance spectroscopy. Deuteriated plasmenylcholine (△) or phosphatidylcholine (○) was hydrated in the presence of the indicated amounts of cholesterol, and deuterium magnetic resonance spectra were recorded at the indicated temperatures as described under Materials and Methods. The apparent order parameter was directly calculated from the splitting of the inner resonances as previously described by Seelig and Seelig (1974).

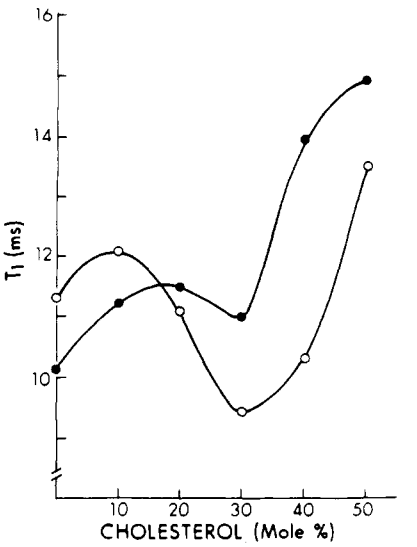

FIGURE 8: Comparison of spin–lattice relaxation times of deuteriated plasmenylcholine and phosphatidylcholine. Hydrated binary dispersions of deuteriated plasmenylcholine (●) or phosphatidylcholine (○) were prepared, and their spin–lattice relaxation times were determined at 43 °C according to the saturation recovery method as described under Materials and Methods.

results were reproducibly present in several independent preparations of deuteriated plasmenylcholine and phosphatidylcholine that were >99% pure as ascertained by both TLC and HPLC. As expected, the addition of cholesterol to deuteriated plasmenylcholine or phosphatidylcholine increased the splitting of both the inner and outer resonances in both phospholipid subclasses. Significant differences in the splitting of the inner peaks of the ^{2}H NMR spectra obtained from both phospholipid subclasses and the resultant calculated order parameter were present at each of five cholesterol concentrations tested at seven different temperatures (Figure 7). The order parameter of the inner quadrupolar splitting for deuteriated plasmenylcholine in the absence of cholesterol corresponded to that of phosphatidylcholine in the presence of 20–30 mol % cholesterol. In contrast, only minor differences in the outer splitting of both phospholipid subclasses were present at all temperatures and cholesterol concentrations examined.

To further substantiate the observed alterations in the dynamics of deuteriated plasmenylcholine and phosphatidylcholine vesicles, the spin–lattice relaxation time was determined at 316 K in the absence or in the presence of five different concentrations of cholesterol (0–50 mol %) (Figure 8). The T_1 for phosphatidylcholine (V, Figure 1) was 11.4 ms, in close agreement with other values previously reported in the literature (Seelig & Seelig, 1980). The T_1 for plasmenylcholine was similar (10.2 ms). The addition of physiological concentrations of cholesterol nonmonotonically altered the spin–lattice relaxation times of both plasmenylcholine and phosphatidylcholine. Differences in the spin–lattice relaxation time of plasmenylcholine and phosphatidylcholine were present at physiological concentrations of cholesterol (i.e., 30–40 mol %). Since quadrupolar relaxation dominates the spin–lattice relaxation time, these results demonstrate that the rotational correlation time of the proximal portion of the *sn*-2 acyl chain in plasmalogens is likely different from their diacyl phospholipid counterparts at physiological concentrations of cholesterol.

Discussion

The results of this study demonstrated that vesicles comprised of plasmenylcholine have different molecular dynamics in comparison with phosphatidylcholine vesicles by utilizing multiple probes of molecular motion employing independent techniques. ^{2}H NMR spectroscopy indicated that the observed differences in plasmenylcholine and phosphatidylcholine molecular dynamics ascertained by ESR spectroscopy were not exclusively due to the perturbing influence exerted by the sterically bulky spin-label but rather were bona fide indicators of differences in the molecular dynamics of these two phospholipid subclasses.

Although the multiple canonical forms of specific conformations of phospholipid constituents, their residence times, and interconversion rates have not as yet been quantitatively defined in membrane bilayers in general, several qualitative conclusions regarding the characteristics of the time-averaged motion manifest in vesicles comprised of these two phospholipid subclasses can be made. First, plasmenylcholine vesicles are more ordered than phosphatidylcholine vesicles both in the interior of the membrane and in regions near the hydrophobic–hydrophilic interface. This conclusion is substantiated by the higher order parameter and apparent rotational correlation time present in vesicles comprised of plasmenylcholine compared to phosphatidylcholine. Second, the presence of cholesterol in binary mixtures with each phospholipid subclass results in immobilization of both phospholipid subclasses. Third, although molecular motion for both phospholipid subclasses is highly anisotropic, the specific axis through which some types of molecular motion occur differs for each phospholipid subclass. This result is substantiated by the similar quadrupolar splitting for one but not the other of the two diastereotopic deuterons located at the C-2 position of the *sn*-2 fatty acyl chain. Furthermore, the increased intensity in the wings of the plasmenylcholine ^{2}H NMR spectra (in comparison to phosphatidylcholine ^{2}H NMR spectra) likely indicates either a lower mobility of the proximal portion of the *sn*-2 fatty acyl chain of plasmenylcholine or a change in the anisotropy of its motion. Fourth, the interaction of plasmenylcholine with cholesterol likely results in macromolecular organization as has previously been demonstrated in phosphatidylcholine/cholesterol binary dispersions. These macromolecular domains are likely present in plasmalogen bilayers since the spin–lattice relaxation times for each phospholipid

subclass are nonmonotonic and contain a node at 30 mol % cholesterol similar to previously reported studies utilizing phosphatidylcholine/cholesterol dispersions interrogated by ESR (Rubenstein et al., 1980).

Collectively, these results demonstrate that membrane domains enriched in plasmalogen molecular species provide a unique molecular environment that differs from that produced by diacyl phospholipids. Given the abundance of plasmalogens in electrically active tissues, it is tempting to speculate that these moieties may facilitate the function of transmembrane proteins in electrically active tissues by providing a suitable membrane microenvironment for their optimal function. Recently we have purified two phospholipases A_2 that are highly selective for plasmalogen substrate (Wolf & Gross, 1985; Loeb & Gross, 1986). Since phospholipase A_2 activity is highly dependent upon the physical state of the membrane [e.g., Thuren (1984)], the observed plasmenylcholine substrate selectivities may be the result of different membrane microenvironments in plasmenylcholine and phosphatidylcholine bilayers.

The results of this study demonstrate that separate and distinct membrane molecular dynamics are present in plasmenylcholine bilayers in comparison to phosphatidylcholine bilayers. Although identification of the biological significance of plasmalogens has remained elusive, the demonstration of disparate molecular dynamics present in plasmenylcholine and phosphatidylcholine bilayers will hopefully facilitate the understanding of the functional role of plasmalogens in biological membranes.

Registry No. I, 108795-72-8; II, 108795-73-9; cholesterol, 57-88-5.

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