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Do the long fatty acid chains of sphingolipids interdigitate across the center of a bilayer of shorter chain symmetric phospholipids?

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Abstract

Novel cerebroside sulfate (CBS) spin labels containing long chain C24 or C26 fatty acids with a nitroxide spin label on the 22nd carbon were synthesized and used to investigate the ability of the long fatty acid chains of glycosphingolipids to interdigitate across the center of a non-interdigitated bilayer of phospholipids formed of symmetric saturated or unsaturated shorter fatty acid chain species, in the presence or absence of cholesterol. The motion of these long chain spin labels incorporated at 1 mole% in dimyristoylphosphatidylcholine (diC14-PC), dipalmitoylphosphatidylcholine (diC16-PC), distearoylphosphatidylcholine (diC18-PC), dibehenoylphosphatidylcholine (diC22-PC), sphingomyelin (SM), 1-stearoyl-2-oleoylphosphatidylcholine (18:0,18:1-PC), and dimyristoylphosphatidylethanolamine (diC14-PE) was compared to that of CBS spin labels containing stearic acid spin labeled at the 5th carbon and at the 16th carbon. The results indicated that the C26 chain is interdigitated in the gel phase of diC14-PC, diC16-PC, SM, and possibly diC18-PC, but not diC14-PE, and the C24 chain may interdigitate in diC14-PC but not in the other phospholipids. Thus in order to interdigitate across the center of gel phase bilayers, the long acyl chain of the sphingolipid probably must be long enough to nearly span the phospholipid bilayer. The inability to interdigitate in diC14-PE is likely due to the close packing of this lipid in the gel phase. The C26 chain may also be interdigitated in these lipids in the presence of cholesterol at low temperatures. However, at physiological temperatures in the presence of cholesterol and in the liquid-crystalline phase of all the lipids, the results indicate that the long acyl chain of the glycosphingolipid is not interdigitated, but rather must terminate at the bilayer center. This may force the carbohydrate headgroup of the glycosphingolipid farther above the bilayer surface, allowing it to be recognized better by various carbohydrate binding ligands and proteins.

Key words: Glycosphingolipid; Interdigitation; Lipid bilayer; EPR; Spin label

1. Introduction

Sphingolipids in cell membranes frequently have an asymmetric hydrocarbon structure. The fatty acid chains acylated to the sphingosine base can vary in length from 14 to 26 carbons with 24 carbon chains predominating in many tissues [1]. The sphingosine chain is thought to penetrate into the bilayer by only 14 carbons [2]. Pure long chain 24:0 and 26:0 species of these lipids form bilayers of the triple chain mixed interdigitated and the double chain partially interdigitated types in the gel phase [3–7], and possibly also in the liquid-crystalline phase [8], as do asymmetric species of phospholipids [9–11]. In the mixed interdigitated bilayer, the short sphingosine chains of two molecules pack end-to-end while the long fatty acid chains span the bilayer. In the partially interdigitated bilayer, the sphingosine chain of one molecule packs end-to-end with the long acyl chain of another molecule on the other side of the bilayer.

However, these lipids are generally present at low concentrations in cell membranes, particularly in the

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Abbreviations: diC14-PC, dimyristoylphosphatidylcholine; diC16-PC, dipalmitoylphosphatidylcholine; diC18-PC, distearoylphosphatidylcholine; diC22-PC, dibehenoylphosphatidylcholine; diC14-PE, dimyristoylphosphatidylethanolamine; 18:0,18:1-PC, 1-stearoyl-2-oleoylphosphatidylcholine; bSM, bovine brain sphingomyelin; eSM, egg sphingomyelin; rSM, bovine erythrocyte sphingomyelin; CBS, cerebroside sulfate; CBS-*n*-*X*-SL, cerebroside sulfate spin label containing a fatty acid *X*, labeled on the *n*th carbon with a nitroxide group, where *X* = S for stearic acid, L for lignoceric acid, or H for hexacosanoic acid; EPR, electron paramagnetic resonance; DSC, differential scanning calorimetry; θ, reduced temperature.

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case of glycosphingolipids (GSL), and they may not cluster in GSL domains of sufficient size to form these types of interdigitated bilayer. Phospholipids, which usually make up the bulk of cell membrane lipids, generally have shorter fatty acids and a more symmetric hydrocarbon structure. The way in which single molecules of asymmetric sphingolipids pack together with other more symmetric phospholipid molecules in the bilayer is still not known. The long fatty acid chain may (i) interdigitate into the other side of the non-interdigitated phospholipid bilayer, (ii) kink up in the same side of the bilayer due to increased gauche isomerization, thus shortening its effective length, or (iii) terminate at the bilayer center but remain relatively extended, thus forcing the carbohydrate headgroup farther above the bilayer surface.

Spin label studies of a number of GSL's containing spin labeled fatty acids led to the suggestion that the long chain interdigitated across the center of a phosphatidylcholine (PC) bilayer. This conclusion was reached because a C24 fatty acid chain, spin labeled at the 16th carbon and bound to the glycolipid, was more ordered or motionally restricted in a shorter fatty acid PC bilayer than a C18 chain spin labeled at the same position [12,13]. However, this result could also be consistent with the other packing mechanisms mentioned above, particularly since the spin label group is on the 16th carbon in both cases and thus is not at the end of the C24 chain. NMR studies of galactosylceramide containing a deuterated long fatty acid chain in PC bilayers [14,15] did not resolve this question since the motion at the end of the acyl chain was greater than would be expected if it were interdigitated. Studies with specific carbohydrate binding proteins such as antibodies and galactose oxidase have shown that the carbohydrate headgroups of GSL's with long fatty acid chains in PC or PC/CHOL bilayers are more exposed at the bilayer surface than those with short fatty acid chains [16–21]. An increase in the chain length of the PC surrounding the GSL decreases exposure of the carbohydrate. This suggests that rather than interdigitating across the PC bilayer center, the long fatty acid chain of the GSL terminates at the bilayer center forcing the carbohydrate headgroup to protrude farther out of the bilayer at least part of the time.

In the present study we have examined this question further using the GSL cerebroside sulfate (CBS) containing C18, C24, and C26 fatty acid spin labels in which the nitroxide group is close to the end of the acyl chain in all cases. Thus it is located on the 16th carbon for the C18 chain and on the 22nd carbon for the C24 and C26 chains. The CBS spin labels are incorporated at a concentration of 1 mole% into bilayers of saturated species of PC of varying chain length in order to vary the bilayer thickness. We also compared the behavior of these CBS spin labels in PC/CHOL and SM/CHOL bilayers. In cell membranes, sphingomyelin (SM) with the same phosphorylcholine headgroup as PC, often substitutes for PC [22]. GSL's may pack differently in SM than in PC since antibody and galactose oxidase studies indicated that an increase in the fatty acid chain length of the GSL did not cause increased exposure of the carbohydrate in SM/CHOL bilayers, in contrast to its effect in PC/CHOL bilayers [20,21].

We have shown previously [4,23–25] that C18 spin labels with the nitroxide group at the 16th carbon from the carboxyl group can detect the interdigitated gel phase shown by X-ray diffraction to occur for a number of lipids under different conditions, if the fatty acid chain length of the lipid is relatively similar to the length of the spin labeled fatty acid. The novel long chain CBS spin labels can also detect the mixed interdigitated phase of pure 24:0- and 26:0-CBS [26]. Because the thickness of an interdigitated bilayer is similar to the length of the spin labeled fatty acid, the nitroxide group at the end of the fatty acid chain is located in this type of bilayer near the ordered polar headgroup region. Thus it is considerably more motionally restricted than in a non-interdigitated bilayer where it locates in the more disordered center of the bilayer. These studies show that fatty acid spin labels give accurate information about the structure of the interdigitated gel phase bilayer. Thus a GSL spin label should be able to insert into a non-interdigitated gel phase PC bilayer similarly to a non-spin labeled fatty acid. Because the disorder and motion of a non-interdigitated gel phase or liquid-crystalline phase bilayer increases on moving from the polar headgroup region to the bilayer center [27,28], the order and/or motion of a long chain spin label incorporated into a non-interdigitated bilayer should provide information about the location of the nitroxide and hence, of the terminal methyl of the GSL long fatty acid chain, in the PC bilayer.

2. Materials and methods

Dimyristoylphosphatidylcholine (diC14-PC), dipalmitoylphosphatidylcholine (diC16-PC), egg sphingomyelin (eSM) and bovine erythrocyte sphingomyelin (rSM) were purchased from Sigma (St. Louis, MO). Distearoylphosphatidylcholine (diC18-PC), dibehenoylphosphatidylcholine (diC22-PC), dimyristoylphosphatidylethanolamine (diC14-PE), 1-stearoyl-2-oleoylphosphatidylcholine (18:0,18:1-PC), and bovine brain sphingomyelin (bSM) were purchased from Avanti Polar Lipids (Birmingham, AL). Bovine brain SM contains mainly C18 and C24 chains, saturated and monounsaturated, eSM contains mainly C16 chains, and rSM contains mainly C24 chains (including some

monounsaturated in both cases) [29]. Cholesterol (CHOL) was purchased from Fluka. All lipids gave single spots by TLC.

2.1. Synthesis of spin labels

22-Doxyllignoceric acid was synthesized from docasanedioic acid following the general method of Hubbell and McConnell [30] for doxylstearic acids with minor modifications. Docasanedioic acid (Aldrich, Milwaukee, WI) was purified by recrystallization from acetone/THF (2:1, v/v) until it gave a sharp melting point. The dimethyl ester of the acid was prepared by standard methods, and the monomethyl ester by the procedure of Jones [31]. The latter was converted to the acid chloride by reaction with oxalyl chloride [32]. The acid chloride obtained was used without further purification. The methyl 21-(chloroformyl)heneicosanoate thus obtained was converted to methyl 22-ketotetracosanoate by reaction with ethyl cadmium compound prepared from ethyl magnesium bromide (Aldrich) and cadmium chloride. The keto ester was converted to the doxyl derivative by published procedures [30]. After purification by silicic acid chromatography, the methyl ester of 22-doxyllignoceric acid was hydrolysed with KOH and further purified by silicic acid chromatography to give a bright yellow solid melting at 77°C. After reduction of the *N*-oxyl group with ascorbic acid, the structure of the compound was verified by FAB MS and high resolution NMR spectroscopy at the Carbohydrate Research Center, University of Toronto.

Psychosine sulfate was prepared from bovine brain sulfatide as described previously [33] and acylated with the spin labeled fatty acid via the *N*-hydroxysuccinimide ester of the acid by the approach of Marchesini et al. [34] with modifications. For conversion to the *N*-hydroxysuccinimide ester, 10 mg of the spin-labeled fatty acid and 3 mg of *N*-hydroxysuccinimide (Aldrich, purified by recrystallization from ethyl acetate/ethanol) were dissolved in 0.75 ml of dry pyridine in a test tube with a tight fitting screw cap and 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added. After flushing with dry nitrogen the test tube was tightly capped and heated at 60°C for 3 h. Most of the pyridine was then evaporated under a stream of nitrogen and the residue was dried under vacuum. 1 ml of water was then added followed by 4 ml of diethyl ether. After mixing thoroughly, the lower aqueous layer was removed. The ether layer was washed three times with 1 ml portions of water. The ether layer was then evaporated under a stream of nitrogen and finally under vacuum. The residue of the *N*-hydroxysuccinimide ester of 22-doxyllignoceric acid was used for the synthesis of 22-doxyllignoceroyl-cerebroside sulfate (CBS-22-L-SL) without further purification.

The reaction of *N*-hydroxysuccinimidyl 22-doxyllignocerate with psychosine sulfate was carried out under similar conditions used for the synthesis of molecular species of cerebroside sulfate using fatty acid chlorides [35,36] with the difference that an equimolar ratio of the two reagents was used and the reaction time was increased to 24 h. The product was purified by silicic acid chromatography using chloroform/methanol/ammonium hydroxide (65:25:4, v/v/v) as the eluting solvent.

The longer 22-doxylhexacosanoic acid was synthesized following the same general procedure with appropriate modifications. Methyl 21-(chloroformyl) heneicosanoate obtained as above was converted to 22-keto hexacosanoic acid methyl ester by reaction with butyl cadmium (prepared from butyl magnesium bromide and cadmium chloride). The procedure for the conversion of the keto ester to 22-doxylhexacosanoic acid and subsequent synthesis of CBS-22-H-SL was identical to that used for CBS-22-L-SL. The stearic acid spin labels 5-doxylstearic acid (5-S-SL) and 16-doxylstearic acid (16-S-SL) were purchased from Molecular Probes (Eugene, OR) and were acylated to psychosine sulfate to give the CBS spin labels, CBS-16-S-SL and CBS-5-S-SL.

2.2. Preparation of samples for EPR spectroscopy

Solutions of the CBS spin label and the PC (or SM) were prepared in chloroform/methanol (2:1, v/v) and a mole ratio of spin label to lipid of 1:100. When used, cholesterol (CHOL) was also dissolved in the organic solvent at a mole ratio of PC (or SM) to CHOL of 1:0.75. The solvent was removed by evaporation under a stream of nitrogen, followed by evacuation in a lyophilizer at 0.1 Torr for at least 2 h. To the dry lipid (1–2 mg) was added 0.2 ml of buffer (10 mM Hepes) at pH 7.4 containing 0.1 M KCl and 2 mM EDTA. The lipid was dispersed at a temperature above the CBS phase transition temperature using a vortex mixer by alternately dipping the sample into a 90–95 °C water bath and vortexing for 10 min. After centrifugation of the lipid dispersions in an Eppendorf bench centrifuge, all but about 50 μl of supernatant were removed, the lipid pellet was mixed in the remaining 50 μl of supernatant and taken up into a 50 μl glass capillary tube which was sealed at one end and centrifuged at 2000 rpm. The pellet was positioned in the cavity of the EPR spectrometer. Spectra were measured on a Varian E-104B EPR spectrometer equipped with a Varian temperature controller and a DEC LSI-11 based microcomputer system. The maximum hyperfine splitting T_{max} value and motional parameter τ_0 were measured as described earlier [23,37] and were used as relative

degrees of motion of the spin label. The maximum value of T_{max} which occurs for nitroxide spin labels is 32–34 G. Spin labels with some degree of anisotropic motion in a fluid bilayer can have T_{max} values down to about 17–18 G. If the motion becomes more isotropic, a 3 line spectrum with a T_{max} value of about 15–16 G is obtained. The T_{max} value is insensitive to further increase in motion and changes in motion are monitored from the motional parameter τ_0 instead.

Spectra were measured at 7.5°C for all samples and also at similar reduced temperatures in the gel and liquid-crystalline phases, $\theta = -0.055$ and $+0.0152$, where $\theta = (T - T_m)/T_m$, T_m is the lipid phase transition temperature in K, and T is the temperature of measurement in K [38]. Phase transition temperatures were determined as the temperature of maximum heat absorption by DSC and were 297, 315, 328.3, and 346.1 K for diC14-PC, diC16-PC, diC18-PC, and diC22-PC, respectively, as reported earlier [24]. T_m values were determined similarly for diC14-PE, bSM, eSM, and rSM and were 321.7, 314, 307, and 307 K, respectively. Spectra were thus measured in the gel phase at 7.5°C for diC14-PC, 25°C for diC16-PC, 37.2°C for diC18-PC, 54°C for diC22-PC, 33.5°C for diC14-PE, 24°C for bSM, and 19.5°C for eSM and rSM, respectively, and in the liquid-crystalline phase at 28.5°C for diC14-PC, 47°C for diC16-PC, 60.5°C for diC18-PC, 78.5°C for diC22-PC, 56.5°C for diC14-PE, 46°C for bSM, and 40.5°C for eSM and rSM, respectively. 18:0,18:1-PC was measured at 7.5°C and 28.5°C. Lipids in the presence of cholesterol were measured at the same temperatures given above and in addition were also all measured at 37°C.

3. Results

The motion of four CBS spin labels was compared in saturated species of PC with chain length from 14 to 22 carbons, and in 18:0,18:1-PC and SM. These spin labels included two with C18 chains. One of these was labeled near the apolar/polar interface, at the 5th carbon (CBS-5-S-SL), and the other was labeled near the terminal methyl at the 16th carbon (CBS-16-S-SL). The other two spin labels had novel C24 and C26 chains, both labeled near the terminal methyl at the 22nd carbon (CBS-22-L-SL and CBS-22-H-SL). The motion was compared in the gel and liquid-crystalline phases of these lipids at similar reduced temperatures relative to their phase transition temperatures. Spectra of CBS-16-S-SL and CBS-22-H-SL at a similar reduced temperature in the gel phases of these lipids are shown in Fig. 1. However, since temperature may affect the motion of the spin label independently of the surrounding lipid, the gel phases were also compared at the same temperature, 7.5°C. The C18 spin labels were

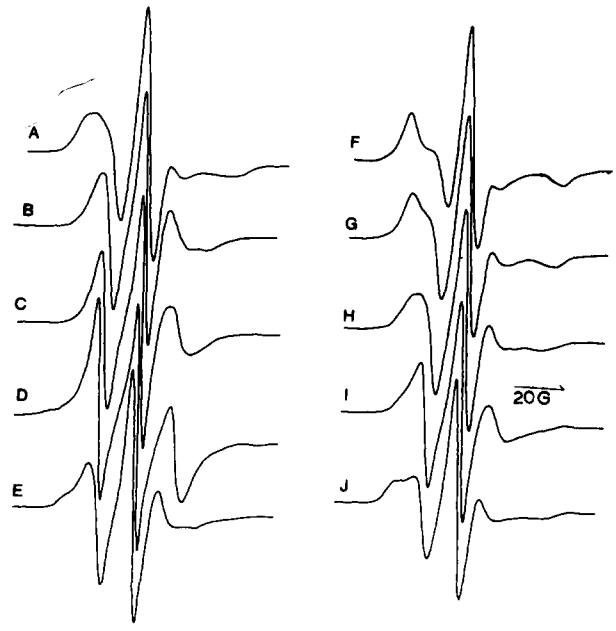

Fig. 1. EPR spectra at a similar reduced temperature $\theta = -0.055$, of (A–E) CBS-16-S-SL and (F–J) CBS-22-H-SL in the gel phase of (A,F) diC14-PC, (B,G) diC16-PC, (C,H) diC18-PC, (D,I) diC22-PC, and (E,J) bSM.

not very soluble in the gel phase of diC22-PC due to the large mismatch between the chain lengths, and gave exchange broadened spectra (Fig. 1 D). However, the longer chain spin labels were soluble (Fig. 1 I). All spin labels were soluble in the other lipids studied (Fig. 1).

At 7.5°C, CBS-5-S-SL was very motionally restricted in all the lipids with a T_{max} value of 31.2–32.5 G (Table 1). It was restricted the most in diC14-PE indicating a high degree of order for this lipid near the polar headgroup region. CBS-16-S-SL was much less motionally restricted than CBS-5-S-SL in the glycerolipids with T_{max} values of 24–25.2 G, due to the well known fluidity gradient of a non-interdigitated bilayer [27,28]. The longer chain length CBS-22-H-SL was more motionally restricted than CBS-16-S-SL with T_{max} values

Table 1
T_{max} values of CBS spin labels at 7.5°C in gel phase of phospholipids

Lipid	T_{max} (G)			
	CBS-5-S-SL	CBS-16-S-SL	CBS-22-L-SL	CBS-22-H-SL
diC14-PC	31.6	25.2	24.4	28.4
diC16-PC	31.4	24.9	25.6	29.1
diC18-PC	31.2	24.0	27.6	29.7
diC22-PC	31.3 [a]	24.2 [a]	27.2	29.4
bSM	31.6	~22, ~29 [b]	~19, ~29 [b]	30.2
eSM	32.1	30.4	~20, ~27 [b]	29.7
rSM	–	~22.4	~18, ~27 [b]	~27.8
diC14-PE	32.5	23.6	22.0	27.8

[a] Exchange broadened.
[b] Two components in spectrum, major one is underlined.

Table 2
T_{max} value of CBS spin labels at same reduced temperature in gel phase of phospholipids [a]

Lipid	T_{max} (G)			
	CBS-5-S-SL	CBS-16-S-SL	CBS-22-L-SL	CBS-22-H-SL
diC14-PC	31.6	25.2	24.4	28.4
diC16-PC	29.5	19.2	19.2	26.5
diC18-PC	27.4	16.8	18.5	23.2
diC22-PC	25.3 [b]	~16 [b]	19.2	~16
bSM	30.5	~19, ~28 [c]	~18, ~26 [c]	~22, ~28 [c]
eSM	31.4	~21, ~29 [c]	~19, ~22 [c]	27.7
rSM	–	~19	~18, ~27 [c]	~18, ~27
diC14-PE	31.1	20.2	17.6	23.7

[a] Measured at a similar reduced temperature, $\theta = -0.055$.
[b] Exchange broadened.
[c] Two components in spectrum, major component is underlined.

Table 3
Spectral parameters of CBS spin labels at same temperature in liquid-crystalline phase of phospholipids [a]

Lipid	CBS-5-S-SL T_{max} (G)	CBS-16-S-SL τ_0 (ns)	CBS-22-L-SL τ_0 (ns)	CBS-22-H-SL τ_0 (ns)
diC14-PC	26.7	1.34	1.52	1.60
diC16-PC	24.7	0.76	0.65	0.57
diC18-PC	23.8	0.52	0.47	0.65
diC22-PC	22.6	0.36	0.40	0.55
18:0,18:1-PC	–	1.10	–	1.15
bSM	26.7	1.14	0.82	0.97
eSM	27.4	1.27	1.02	1.51
rSM	–	1.31	0.87	1.17
diC14-PE	24.7	0.83	0.39	0.45

[a] Measured at a similar reduced temperature, $\theta = +0.015$.

of 28–30 G. However, CBS-22-L-SL was no more immobilized in diC14-PC and diC16-PC than the shorter chain CBS-16-S-SL. The T_{max} values of CBS-22-L-SL and CBS-22-H-SL increased with increase in chain length of the PC. This may be due to the different transition temperatures of these lipids.

Therefore, the T_{max} values were also compared in the gel phases of these lipids at similar reduced temperatures (Table 2). CBS-22-H-SL was more immobilized than CBS-16-S-SL in diC14-PC, diC16-PC and diC18-PC, but not in diC22-PC. CBS-16-S-SL was more immobilized in diC14-PC than in the longer chain PC's and the mobility of CBS-22-H-SL increased with increase in fatty acid chain length of PC in contrast to its behavior at 7.5°C. The immobilization of CBS-22-H-SL approached that of CBS-5-S-SL, especially in diC14-PC and diC16-PC, and less so in diC18-PC, but not in diC14-PE. CBS-22-L-SL behaved differently from CBS-22-H-SL even though its spin labeled fatty acid is only two carbons shorter than that of the latter. Although CBS-22-L-SL was more immobilized in diC14-PC than in the longer chain PC's, it was less immobilized in diC14-PC than the shorter chain length CBS-16-S-SL, in contrast to the behavior of CBS-22-H-SL. Indeed, CBS-22-L-SL had similar mobility in diC16-PC and diC18-PC as in diC22-PC. It was much more mobile in all lipids than CBS-5-S-SL.

In the gel phase of all three types of the sphingolipid SM, the spectra of CBS-16-S-SL, CBS-22-L-SL, and CBS-22-H-SL had two components, one of which was relatively immobilized (Fig. 1 E,J). This immobilized component, with T_{max} value of about 28–29 G, predominated for CBS-22-H-SL but the more mobile component, with T_{max} value of 18–21 G, predominated for the shorter chain length spin labels (Table 2).

In the liquid-crystalline phase of these lipids as well as of 18:0,18:1-PC, all three spin labels with the

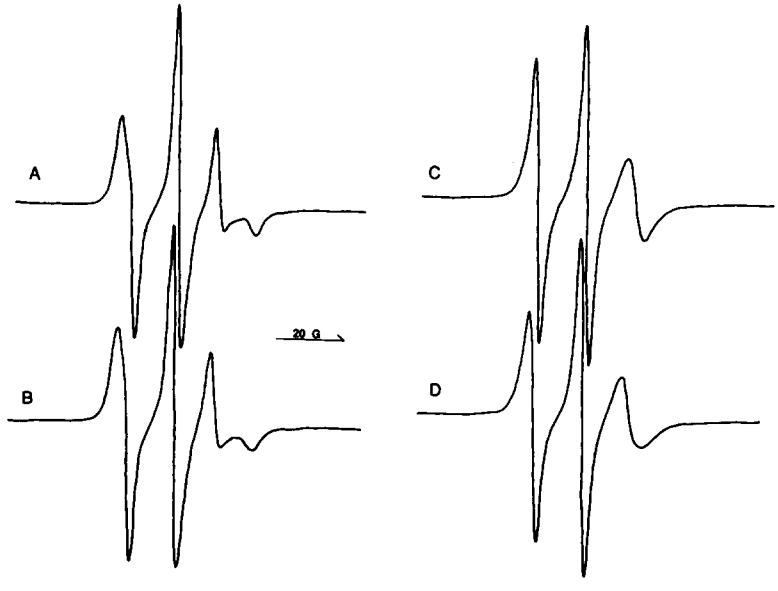

Fig. 2. EPR spectra at 37°C of (A,B) CBS-16-S-SL and (C,D) CBS-22-H-SL in (A,C) diC14-PC/CHOL and (B,D) diC18-PC/CHOL.

Table 4
Spectral parameters of CBS spin labels in phospholipid/cholesterol mixtures at various temperatures [a]

Lipid	CBS-5-S-SL			CBS-16-S-SL				CBS-22-L-SL			CBS-22-H-SL			
	7.5°C	$\theta =$ −0.055	$\theta =$ +0.015	7.5°C	$\theta =$ −0.055	$\theta =$ +0.015	37°C	7.5°C	$\theta =$ −0.055	$\theta =$ +0.015	7.5°C	$\theta =$ −0.055	$\theta =$ +0.015	37°C
diC14-PC/CHOL	32.1	32.1	30.4	19.9	19.9	19.0	18.5	17.0	17.0	1.22	22.2	22.2	2.20	1.52
diC18-PC/CHOL	32.4	29.8	27.1	20.0	18.6	17.8	18.6	18.2	1.06	0.55	25.0	1.70	0.65	1.70
bSM/CHOL	32.4	31.4	29.4	20.2	19.0	18.1	18.4	17.4	1.46	0.61	22.2	2.57	1.12	1.71
eSM/CHOL	32.6	31.9	30.1	20.2	19.6	18.6	18.8	17.4	1.84	0.84	23.9	~ 20	1.67	1.84
rSM/CHOL	–	–	–	19.8	19.0	18.2	18.3	17.2	1.50	0.77	20.3	2.55	1.28	1.48

[a] T_{max} values (in G) are those values which are 17.0 or greater; τ_0 values (in nsec) are those values which are less than 3.0.

nitroxide group near the terminal methyl gave sharp three-line spectra characteristic of nearly isotropic motion resembling that shown in Fig. 2 C. The motional parameters are given in Table 3. No motionally restricted component or component characteristic of anisotropic motion was detected in any of the spectra, even for CBS-22-H-SL, even though the spin label near the polar headgroup region, CBS-5-S-SL, was still relatively immobilized in the liquid-crystalline phase (Table 3).

The motion was then examined in diC14-PC, diC18-PC, and SM in the presence of cholesterol at 7.5°C and 37°C and at the same reduced temperatures used in the absence of cholesterol. Spectra of CBS-16-S-SL and CBS-22-H-SL at 37°C in diC14-PC/CHOL and diC18-PC/CHOL are compared in Fig. 2. At 7.5°C, CBS-22-H-SL was a little more motionally restricted than CBS-16-S-SL in all of the lipids (Table 4). However, at a similar reduced temperature below the T_m value of the pure lipids, $\theta = -0.055$, CBS-22-H-SL had nearly isotropic motion in all of the lipids except diC14-PC/CHOL and eSM/CHOL even though CBS-16-S-SL had anisotropic motion in all of them. At this reduced temperature, CBS-22-H-SL still had a greater T_{max} value in diC14-PC/CHOL than CBS-16-S-SL. However, at a higher reduced temperature, $\theta = +0.015$, and at 37°C in all cases, CBS-22-H-SL and CBS-22-L-SL had isotropic motion (Fig. 2 C,D) while CBS-16-S-SL still had anisotropic motion (Fig. 2 A,B). Thus the longer chain CBS-22-H-SL and CBS-22-L-SL had greater motion in these lipids in the presence of CHOL than CBS-16-S-SL at physiological temperatures. CBS-5-S-SL was still immobilized in these lipids at this higher temperature.

4. Discussion

This study was carried out in order to determine if the long fatty acid chains of GSL's can interdigitate across the center of a non-interdigitated bilayer formed by shorter chain phospholipids. The motion and disorder of fatty acid chains is greatest in the center of the bilayer in both the gel and liquid-crystalline phases [27,28]. If the nitroxide near the terminal methyl of a fatty acid spin label is located in the center of the bilayer, it should have greater motion than if it is located closer to the polar headgroup region. Thus if it interdigitates across the bilayer center a significant distance into the other side of the bilayer, its motion should decrease. If a very long fatty acid does not interdigitate but kinks up in the same side of the bilayer in order to minimize chain length differences, the effect on its motion is less predictable. Hence a decrease in motion of the spin label would be consistent with interdigitation across the bilayer center but would not prove that it occurs. On the other hand, if the motion is similar to that expected for a nitroxide group located in the center of the bilayer, it is unlikely that the spin labeled fatty acid interdigitates across the bilayer center, although a non-spin labeled fatty acid might behave differently.

The C18 spin labels might be long enough to interdigitate a short distance across the bilayer center of diC14-PC but not that of the other longer chain saturated PC's used. The C24 and C26 spin labels are long enough to interdigitate a significant distance across the bilayer center of diC14-PC, diC16-PC, diC18-PC and 18:0,18:1-PC, but probably not that of diC22-PC. Therefore, the nitroxide of CBS-16-S-SL should be located near the bilayer center of diC16-PC and diC18-PC and that of CBS-22-L-SL, and possibly also CBS-22-H-SL, should be near the bilayer center of diC22-PC. Thus the motion in these lipids should be typical of the motion in the center of the bilayer. In order to conclude that the long spin labeled chain of CBS interdigitates across the PC (or SM) bilayer center from its spectral behavior, the following criteria should be fulfilled: (i) A decrease in mobility with increase in acyl chain length of the CBS spin label should occur in all lipids except diC22-PC. For the latter an increase in mobility might be expected instead as the terminal methyl of the fatty acid approaches the bilayer center. (ii) CBS-16-S-SL should be more immobilized in diC14-PC than in the other lipids and CBS-22-L-SL and CBS-22-H-SL should be more immobilized in diC14-PC, diC16-PC, diC18-PC than in diC22-PC. (iii) For a significant degree of interdigitation across the

bilayer center, the nitroxide on CBS-22-H-SL should approach the region on the other side of the bilayer where the nitroxide on CBS-5-S-SL is located. Therefore, CBS-22-H-SL should be immobilized almost as much as CBS-5-S-SL, at least in diC14-PC and diC16-PC.

At 7.5°C in the gel phase, the motion of CBS-22-L-SL and CBS-22-H-SL decreased with increase in chain length of the PC, contrary to the expectation if the long chains were interdigitated. On the other hand, CBS-22-H-SL was more motionally restricted than CBS-16-S-SL, consistent with interdigitation of the long chain. However, CBS-22-L-SL was no more immobilized in diC14-PC and diC16-PC than the shorter chain CBS-16-S-SL.

However, at similar reduced temperatures in the gel phase, the three criteria for interdigitation were fulfilled for CBS-22-H-SL. It was more immobilized than the shorter chain spin labels in diC14-PC, diC16-PC and diC18-PC. CBS-16-S-SL was more immobilized in diC14-PC than in the longer chain PC's and CBS-22-H-SL was more immobilized in diC14-PC, diC16-PC, and diC18-PC, than in diC22-PC. The immobilization of CBS-22-H-SL approached that of CBS-5-S-SL, especially in diC14-PC and diC16-PC, suggesting that the nitroxide group on the 22nd carbon of a molecule of CBS-22-H-SL in one side of the bilayer was relatively close to the location of the nitroxide group on the 5th carbon of CBS-5-S-SL in the other side of the bilayer. These results suggested that CBS-22-H-SL interdigitated across the bilayer center in the gel phase of diC14-PC and diC16-PC, and possibly diC18-PC. In diC14-PE, however, the difference between the T_{max} values of CBS-5-S-SL and CBS-22-H-SL was 7.4 G, not consistent with a significant degree of interdigitation. The predominance of an immobilized component in the spectrum of CBS-22-H-SL in SM suggested that this spin label was also interdigitated across the bilayer center in the gel phase of SM. CBS-16-S-SL may also interdigitate to some extent in diC14-PC but not diC14-PE or the longer chain PC's. It is unlikely that these results, especially the large difference in behavior of CBS-22-H-SL in diC14-PC and diC14-PE can all be accounted for by kinking up of the long chain in the same side of the gel phase bilayer.

The long fatty acid chain of CBS may not be able to interdigitate a significant distance across the bilayer center of diC14-PE, even though it has a similar bilayer thickness to diC14-PC, because of the close packing of diC14-PE due to intermolecular hydrogen bonding [39]. This is reflected in the high degree of order experienced by a nitroxide located on the 5th carbon of CBS-5-S-SL.

The similar mobility of CBS-22-L-SL in diC16-PC, diC18-PC, and diC22-PC as of CBS-16-S-SL in diC16-PC, suggested that the C24 chain terminated near the bilayer center in those lipids. Thus CBS-22-L-SL did not interdigitate in diC16-PC and diC18-PC but may have in diC14-PC. This difference in behavior from CBS-22-H-SL may be due to its shorter length or to the fact that the spin label group is closer to the end of the chain than for the C26 label. The C26 label has a 4 carbon tail past the nitroxide-labeled carbon which may allow it to interact with the surrounding lipid fatty acids by van der Waals interactions and compensate for any perturbing effect of the nitroxide group, while the C24 label has only a 2 carbon tail. However, the nitroxide of 16-S-SL fatty acid and lipid spin labels, which also have only a 2 carbon tail past the nitroxide group, is able to localize near the polar/apolar interface, where the nitroxide of 5-S-SL resides, in fully interdigitated and mixed interdigitated bilayers formed by a variety of lipids [4,23–25] indicating that the nitroxide group does not have too great a perturbing effect for this purpose. CBS-22-L-SL and CBS-22-H-SL behave similarly in the mixed interdigitated bilayer of pure C24-and C26-CBS [26]. Thus the C24 chain is probably less able to interdigitate in the non-interdigitated phospholipid bilayers than the C26 chain because of its shorter length. A long acyl chain of an asymmetric lipid may be able to interdigitate across the center of a PC bilayer only if it can span or nearly span the bilayer. Otherwise, it would severely disrupt van der Waals interactions between the PC fatty acids.

The results on gel phase lipids presented in this study are consistent with results of an investigation of the thermotropic phase behavior of mixtures of 24:0-CBS or 26:0-CBS with diC14-PC, diC16-PC, diC18-PC, or diC14-PE where low concentrations of only 26:0-CBS stabilized the gel phase of only diC14-PC, while all other combinations of C24- and C26-CBS with phospholipid had a destabilizing effect [26]. These results on gel phase lipids agree with results obtained by Grant and colleagues using different C24 long chain spin labeled glycolipids with the nitroxide group on the 16th carbon indicating that the long chain was interdigitated across the bilayer center in the gel phase and at low temperatures in diC16-PC/CHOL [12,13].

However, our results on liquid-crystalline phase lipids do not agree with the results of this group. They found that the C24 chain labeled at the 16th carbon was also more immobilized than a C18 chain in the liquid-crystalline phase and concluded that the long acyl chains of GSLs were interdigitated across the bilayer center in this phase also [12,13]. Our results with C24 and C26 chains labeled at the 22nd carbon in the liquid-crystalline phase indicate a much higher degree of motion at the end of the chain than would be consistent with interdigitation. Although the motional parameter of the spin labels with the nitroxide group near the terminal methyl was greater in diC14-PC than in the other lipids, and although it increased with chain

length of the spin label in diC14-PC, the differences were not great enough to be consistent with interdigitation. If the long chain spin labels were interdigitated across the bilayer center, they should be ordered or motionally restricted enough that isotropic averaging of the tensor components would not occur and they would have anisotropic spectra more like that of CBS-5-S-SL. Thus our results indicate that the long chain spin labels do not interdigitate across the bilayer center in the liquid-crystalline phase.

NMR results on glycolipids with deuterated C24 chains also indicated a higher degree of motion at the end of the chain than would be expected if they interdigitated across the bilayer center [14,15]. The high degree of motion of C24 and C26 chains spin labeled at the 22nd carbon in the liquid-crystalline phase was similar to that of a C18 chain labeled at the 16th carbon in diC16-PC or diC18-PC suggesting that these long chains terminate at the bilayer center. This could then force the rest of the chain closer to the polar headgroup region. Indeed, this might account for the decrease in motion experienced by the long chain probes labeled at the 16th carbon [12,13].

The motion of the long chain glycolipids was also examined in these lipids in the presence of cholesterol since vesicles containing cholesterol were used to examine recognition of glycolipids by antibody and galactose oxidase [19–21]. At 7.5°C, CBS-22-H-SL was a little more motionally restricted than CBS-16-S-SL in all of the lipids, possibly consistent with interdigitation. At a similar reduced temperature below the T_m value of the pure lipids, CBS-22-H-SL had a greater T_{max} value in diC14-PC/CHOL than CBS-16-S-SL which may indicate that it was interdigitated in diC14-PC/CHOL but not in the other lipids. However, our results indicate that at 37°C the long chains of glycolipids are not interdigitated across the bilayer center in the presence of cholesterol. If they were, CBS-22-H-SL would be expected to have anisotropic motion and a T_{max} value closer to that of CBS-5-S-SL, which is 27–30 G even at these higher temperatures. Indeed, the nitroxide group near the end of the C24 and C26 chains had more motion in PC/CHOL than that near the end of a C18 chain. This may be caused by increased *gauche* isomerization of the long acyl chain of the glycolipid in order to decrease the mismatch in length of the glycolipid and PC fatty acid chains.

However, the fact that the carbohydrate headgroup of long fatty acid chain species of glycolipids in the liquid-crystalline phase of PC or PC/CHOL is better recognized by carbohydrate binding proteins, than that of short fatty acid chain species [16–21] indicates that the carbohydrate of long chain species protrudes farther above the plane formed by the PC headgroups. Thus in PC, the acyl chains of the glycolipid and the PC apparently do not achieve an equal length by this mechanism. Exposure of the lipid carbohydrate at the bilayer surface may be a dynamic phenomenon with the glycolipid headgroup bobbing up and down out of the bilayer due to rapid *gauche-trans* isomerization of the fatty acid chain and consequent fluctuations in its effective length. This may explain why increased acyl chain length of the GSL had no detectable effect on the motion or orientation of the galactose of galactosylceramide in PC bilayers, determined by ^{2}H-NMR spectroscopy of the lipid labeled with deuterium at the C-6 of the galactose residue [40].

The spin label results indicate that interdigitation of the glycolipid fatty acid chain also does not occur in SM/CHOL bilayers at higher temperatures. However, an increase in chain length of the glycolipid does not cause increased recognition of the carbohydrate in bSM/CHOL indicating that increased protrusion does not occur either, in contrast to PC/CHOL [20,21]. This has been attributed to intermolecular hydrogen bonding of the amide moiety and/or sphingosine hydroxyl of the glycolipid with similar groups in sphingomyelin, thus anchoring the glycolipid in the bilayer. This would require minimization of chain length mismatch which might be accomplished in bSM by another mechanism in addition to increased *gauche* isomerization of the long fatty acid chain of the glycolipid in SM. Longer chain length species of SM present in the heterogeneous natural bSM mixture may preferentially associate with the glycolipid in order to achieve closer matching of the chain lengths of the glycolipid and the surrounding SM molecules. A small effect of fatty acid chain length of the glycolipid on its recognition was observed in the more homogeneous eSM and rSM than in bSM [20], consistent with this explanation, although this needs to be confirmed with synthetic species of SM.

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