Lipid Chain Dynamics and Molecular Location of Diacylglycerol in Hydrated Binary Mixtures with Phosphatidylcholine: Spin Label ESR Studies†

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Received November 10, 1995; Revised Manuscript Received January 25, 1996⊗

ABSTRACT: The lipid chain motions in hydrated binary mixtures of dimyristoylglycerol (DMG) with dimyristoylphosphatidylcholine (DMPC) have been studied by using ESR spectroscopy of analogues of both components that are spin-labeled at one of eight different positions along the *sn*-2 chain. The phase diagram of the binary mixtures divides into three separate regions along the composition axis, corresponding to the formation of isothermally melting compounds with DMPC/DMG stoichiometries of approximately 1:1 and 1:2 mol/mol in the gel phase [Heimburg, T., Würz, U., & Marsh, D. (1992) *Biophys. J. 63*, 1369−1378]. In the first region (up to 50 mol % DMG), comparison of the chain flexibility profiles, and the chain profiles of the polarity-dependent isotropic hyperfine coupling constant, of the two different spin-labeled components indicates that DMG is incorporated in the fluid lipid bilayer in a manner similar to that of the host DMPC but is situated approximately two CH_2 groups deeper into the hydrophobic interior. At lower contents of DMG, the chain packing is increased by the addition of DMG, whereas at higher DMG contents the lipid chain order decreases rapidly, on reaching the inverted hexagonal phase of the second region of the phase diagram. In the second region of the phase diagram (50−67 mol % DMG), the DMG fits better into the fluid inverted hexagonal phase than into the fluid lamellar phase of the first region and is located only approximately one CH_2 group deeper than the corresponding DMPC. In these first two regions of the phase diagram, the ESR spectra of both spin-labeled components display an axial anisotropy that evidences the increasing angular amplitude of motion with position down the chain that is characteristic of liquid crystalline fluid phases. In the third region of the phase diagram (above 67 mol % DMG), the fluid phase consists of isotropically tumbling DMG molecules in which the DMPC molecules are incorporated as inverted micelles as indicated by the residual anisotropic motion of the spin-labeled phosphatidylcholine analogues.

Diacylglycerol is the endogeneous lipid activator of the membrane-bound regulatory protein, protein kinase C (Nishizuka, 1986). In the cell, diacylglycerol is generated in the plasma membrane as a second messenger in the downstream signaling cascade that involves a G-protein activated phospholipase C (Berridge, 1984). Activation by diacylglycerol specifically requires the natural *sn*-1,2 stereoisomer, but the acyl chain composition is relatively unimportant (Boni & Rando, 1985). Additionally, diacylglycerol has been implicated in cell fusion (Baker, 1988) and may similarly be involved in exocytosis mediated by triggering with phospholipase C [cf., e.g., Neher (1988)]. In these processes, both the mode of integration and location of diacylglycerol in the membrane, and the effect of diacylglycerol on the lipid structure and chain packing, are likely to be of crucial importance. One approach to studying this problem is by using electron spin resonance (ESR)[1] spectroscopy of diacylglycerols and phospholipids that are selectively spin-labeled at different positions throughout the lipid chain. In this way, the environment of the diacylglycerol can be deduced from the anisotropy of its motion and the dependence on position of labeling in the chain and from the isotropic hyperfine coupling constant that is dependent on environmental polarity [see, e.g., Marsh (1981)]. The location of the diacylglycerol in the lipid assembly can then be determined from the chain flexibility profile and the dependence of environmental polarity on chain position, by comparison with similar measurements using spin-labeled phospholipids [cf. Rama Krishna and Marsh (1990)].

The binary phase diagram for hydrated mixtures of dimyristoylglycerol (DMG) with dimyristoyl phosphatidylcholine (DMPC) has been determined previously by using differential scanning calorimetry (Heimburg et al., 1992). The phase diagram is divided into three parts along the composition axis that correspond to the formation of stoichiometric compounds with approximate DMPC/DMG compositions of 1:1 and 1:2 mol/mol. This is illustrated in Figure 1, where the phase boundaries are those established by spin-label ESR methods in the present work (see later) and are in agreement with those published earlier. Particularly interesting are the structures of the fluid phases in the three different regions of the phase diagram. In region I the structure is of the lamellar (L_α) type, in region II the structure consists of inverted hexagonally packed cylinders (H_{II}), and in region III the fluid phase is isotropic (I). Of

† This work was supported in part by the Deutsche Forschungsgemeinschaft.

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1996.

[1] Abbreviations: DMG, 1,2-dimyristoyl-*sn*-glycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; *n*-DGSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycerol; *n*-PCSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; L_α, fluid lamellar phase; H_{II}, inverted hexagonal phase; I, isotropic phase.

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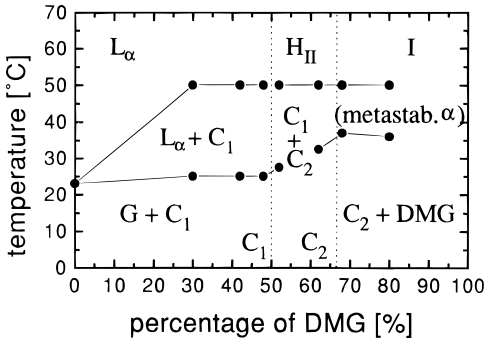

FIGURE 1: Binary phase diagram of hydrated mixtures of DMG and DMPC. Phase boundaries are established from the temperature dependence of the ESR spectra from spin-labeled analogues of the two lipid components (see later). The three regions characterized by the stoichiometric complexes, C_1 and C_2, in the gel phase are: region I (0−50 mol % DMG), region II (50−67 mol % DMG), and region III (67−100 mol % DMG). See Heimburg et al. (1992) for phase assignments.

these, the lamellar phase is representative of a fluid membrane environment. The formation of inverted and isotropic phases, on the other hand, is of direct relevance to the topological changes that may occur in membrane fusion induced by high local concentrations of diacylglycerol [cf. Siegel (1986)].

In the present work, we have investigated the lipid chain dynamics and molecular location of diacylglycerol in the three different regions of the phase diagram of hydrated DMG/DMPC mixtures by using ESR spectroscopy of spin-labeled analogues of both lipid components that bear the nitroxide reporter group on one of eight different positions in the *sn*-2 chain. Flexibility and polarity profiles with position of chain labeling have been established for both components as well as the dependence of the lipid chain packing on diacylglycerol content. The results reflect very directly the molecular structures of the different phases and their interconversion.

MATERIALS AND METHODS

Materials. DMPC was obtained from Fluka (Buchs, Switzerland). DMG was produced from DMPC by enzymatic cleavage with phospholipase C from *Bacillus cereus* (Boehringer-Mannheim, Mannheim, Germany), in ether/water (1:1, v/v) at 0 °C. DMG was extracted from the ether phase after the reaction had gone to completion, and purity was checked by thin layer chromatography. Spin-labeled phosphatidylcholines bearing the nitroxide group on the C-*n* atom of the *sn*-2 chain (*n*-PCSL) were synthesized as described by Marsh and Watts (1982). The corresponding spin-labeled diacylglycerols (*n*-DGSL) were synthesized from *n*-PCSLs by using the same enzymatic method as for DMG.

Sample Preparation. The required amounts of DMPC and DMG (ca. 2 mg in total) were dissolved in dichloromethane, together with 1 mol % of the desired spin label (*n*-PCSL or *n*-DGSL, with $n = 5, 6, 7, 8, 9, 10, 12$, or 14). The organic solvent was evaporated with a stream of dry nitrogen and the sample then dried under vacuum for at least 3 h. The dry lipid was dispersed in 100 μL of buffer (10 mM Hepes, 1 mM EDTA, pH 7.4) with vortexing. The dispersion was warmed above the chain-melting transition temperature to ensure complete hydration. The sample was then transferred to a 100 μL glass capillary (1 mm i.d.) that was sealed at one end and pelleted at room temperature by centrifugation in a bench-top centrifuge at 8000−10 000 rpm. The supernatant was removed to yield a hydrated pellet of 5 mm length and the capillary was flame-sealed.

ESR Spectroscopy. ESR spectra were recorded with a Varian E-Line 9 GHz spectrometer with nitrogen gas flow temperature regulation. The glass capillary was accommodated within a standard 4 mm diameter quartz tube that contained light silicone oil for thermal stability. The temperature was regulated to ±0.1 °C by using a thermocouple that was placed in the silicone oil. Spectra were recorded with a field modulation frequency of 100 kHz and modulation amplitude of 1.25 G. The apparent order parameter, S^{app}, and the apparent isotropic hyperfine coupling constant, a_0, were calculated from the following expressions [see Marsh (1981)]:

$$S^{app} = (A_{||} - A_\perp)/\left[A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})\right](a_0'/a_0) \quad (1)$$

$$a_0 = (1/3)(A_{||} + 2A_\perp) \quad (2)$$

where $A_{||}$ is A_{max}, half of the outer hyperfine splitting, and $A_\perp$ is obtained from A_{min}, which is half of the inner hyperfine splitting according to (Hubbell & McConnell, 1971; Griffith & Jost, 1976)

$$A_\perp(G) = A_{min}(G) + 0.85 \quad (S^{app} < 0.45)$$

$$A_\perp(G) = A_{min}(G) + 1.32 + 1.86 \log (1 - S^{app}) \quad (S^{app} > 0.45) \quad (3)$$

A_{xx}, A_{yy}, and A_{zz} are the principal values of the hyperfine tensor in the nitroxide frame of axes, obtained from measurements in a single crystal environment (Jost et al., 1971), and

$$a_0' = (1/3)(A_{xx} + A_{yy} + A_{zz}) \quad (4)$$

At moderate temperatures, the ESR spectra of chain-labeled lipids contain contributions from slow long axis motions (Moser et al., 1989). Therefore, the order parameters calculated from eq 2 are best considered as apparent values, which can be used to compare the dynamic properties of the diacylglycerol and phosphatidylcholine components in the fluid phase.

RESULTS AND DISCUSSION

ESR Spectra in the Different Regions of the Phase Diagram. The ESR spectra of diacylglycerol and phosphatidylcholine spin-labeled on the sixth C-atom of the *sn*-2 chain (6-DGSL and 6-PCSL, respectively) in hydrated mixtures composed of 70:30 mol/mol DMPC/DMG are given as a function of temperature in Figure 2. These samples correspond to a lipid mixture in region I of the binary phase diagram. At low temperature, the ESR spectra consist of a single component that approaches the rigid limit of sensitivity of conventional spin label ESR spectroscopy, corresponding to the lipid gel phase. With increasing temperature, a second component of smaller outer hyperfine splitting grows progressively into the spectra. This coexistence of two spectral components corresponding to different states of mobility is

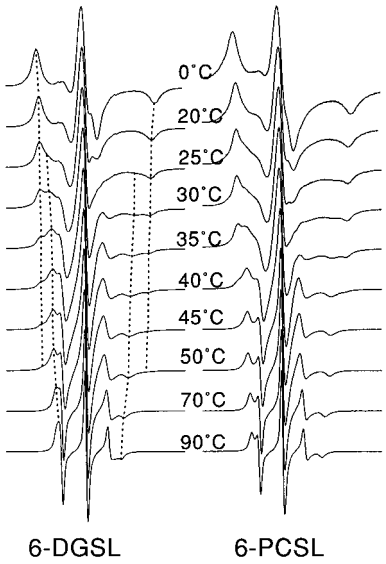

FIGURE 2: Temperature dependence of the ESR spectra of 6-DGSL (left) and 6-PCSL (right) spin-labeled analogues of diacylglycerol and phosphatidylcholine, respectively, in a hydrated 70:30 mol/mol DMPC/DMG mixture. The outer hyperfine splitting of the two spectral components for 6-DGSL is indicated by dashed lines. Total scan width = 100 G.

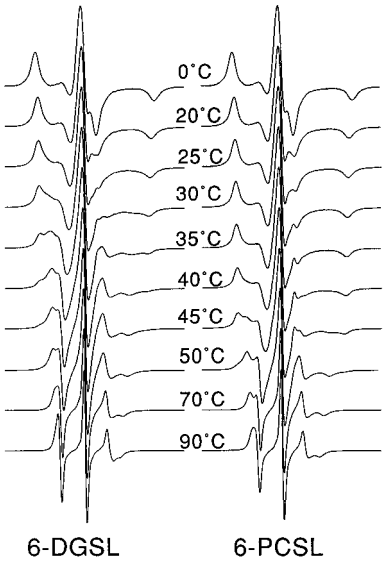

FIGURE 3: Temperature dependence of the ESR spectra of 6-DGSL (left) and 6-PCSL (right) in a hydrated 58:42 mol/mol DMPC/DMG mixture. Total scan width = 100 G.

characteristic of a region of gel−fluid lateral lipid phase separation. Finally, at higher temperatures, the spectra consist of a single, axially anisotropic component that is characteristic of a fluid liquid crystalline lipid phase. The region of coexistence of the two spectral components allows definition of the boundaries of the region of lateral phase separation which extend from 25 to 50 °C (cf. Figure 1, above). There is no evidence for phase separation in the fluid phase; the ESR spectra of both lipid species consist of a single anisotropic component.

The ESR spectra of the 6-DGSL and 6-PCSL diacylglycerol and phosphatidylcholine spin labels in hydrated mixtures composed of 58:42 mol/mol DMPC/DMG are given as a function of temperature in Figure 3. These samples correspond to region II of the binary phase diagram. The ESR spectra recorded at low and high temperatures are again characteristic of a lipid gel phase and a fluid liquid crystalline

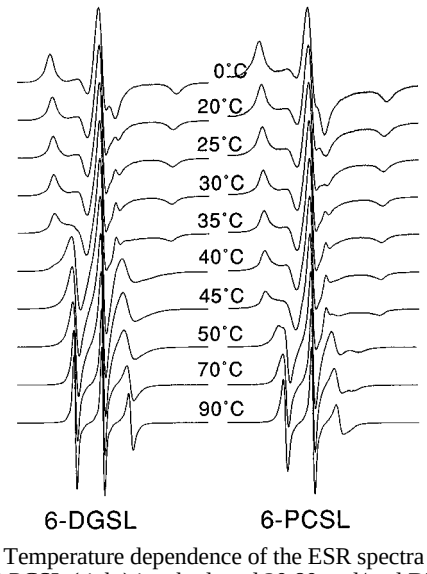

FIGURE 4: Temperature dependence of the ESR spectra of 6-DGSL (left) and 6-PCSL (right) in a hydrated 20:80 mol/mol DMPC/DMG mixture. Total scan width = 100 G.

lipid phase, respectively. The region of lateral phase separation defined by the existence of two-component spectra is, however, smaller than that found in region I of the phase diagram.

The ESR spectra of the 6-DGSL and 6-PCSL diacylglycerol and phosphatidylcholine spin labels in hydrated mixtures composed of 20:80 mol/mol DMPC/DMG are given as a function of temperature in Figure 4. These samples correspond to region III of the binary phase diagram. The spectra of the diacylglycerol spin label, 6-DGSL, at higher temperatures do not exhibit axial anisotropy but rather are of the isotropic type. This indicates that the fluid phase is an isotropic melt for the high contents of diacylglycerol that obtain in region III of the phase diagram. The spectra of the phosphatidylcholine spin label, 6-PCSL, however, do exhibit some axial anisotropy at temperatures in the fluid phase, although this is smaller than that in the liquid crystalline fluid phases that are found for the other two regions of the phase diagram. This residual motional anisotropy indicates that, in region III of the phase diagram, the phosphatidylcholine component of the hydrated mixtures is present as (inverted) micelles in the isotropic melt of diacylglycerol. Two-component spectra are observed only over a small temperature range for the 20:80 mol/mol DMPC/DMG samples, showing that the region of lateral phase separation is rather narrow in this part of the binary phase diagram.

ESR Spectra with Different Positions of Chain Labeling. The ESR spectra from different positional isomers of the diacylglycerol and phosphatidylcholine spin labels, *n*-DGSL and *n*-PCSL, in hydrated mixtures composed of 70:30 mol/mol DMPC/DMG in the fluid phase are compared in Figure 5. The spectra are all axially anisotropic and display a decreasing degree of anisotropy with position of labeling, *n*, down the chain toward the terminal methyl group. The latter is characteristic of the flexibility gradient in fluid liquid crystalline phases. Although the chain flexibility profiles are similar for both diacylglycerol and phosphatidylcholine spin labels, the degree of motional anisotropy is less for diacylglycerol than for phosphatidylcholine at most positions of chain labeling. This suggests that the diacylglycerol component is located deeper in the hydrophobic core of the

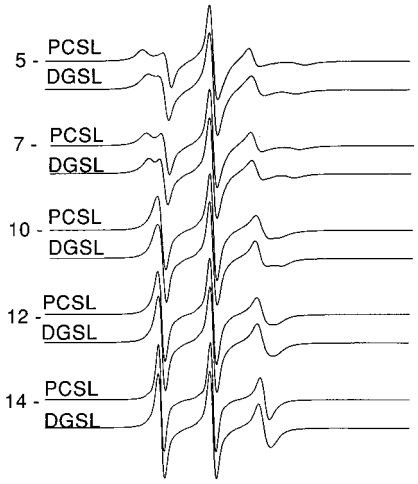

FIGURE 5: ESR spectra of the *n*-DGSL diacylglycerol and *n*-PCSL phosphatidylcholine spin labels with different positions of labeling in the *sn*-2 chain, n = 5, 7, 10, 12, and 14, in hydrated 70:30 mol/mol DMPC/DMG mixtures at 60 °C. The upper spectrum from each pair is from *n*-PCSL and the lower spectrum of each pair is from *n*-DGSL. Total scan width = 100 G.

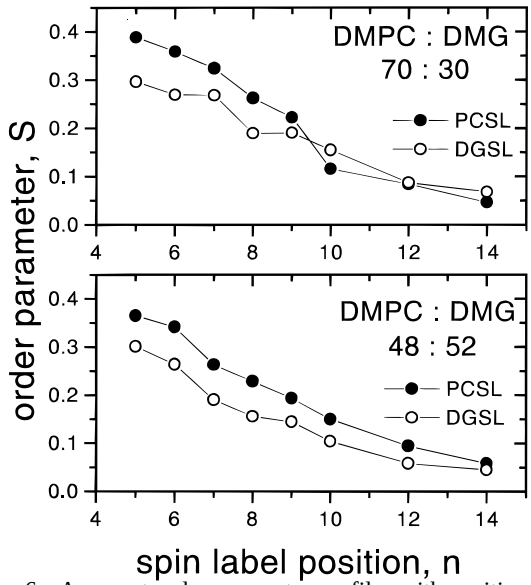

FIGURE 6: Apparent order parameter profiles with position, n, of chain labeling for *n*-DGSL (○) and *n*-PCSL (●) spin labels in hydrated DMPC/DMG mixtures of compositions 70:30 (upper) and 48:52 (lower) mol/mol, at 60 °C.

lipid bilayer than is the phosphatidylcholine component of the mixtures.

From the spectral hyperfine anisotropy it is possible to obtain the apparent order parameters, S^{app}, of the spin-labeled chain segments, and from the mean value of the hyperfine splittings to obtain the isotropic hyperfine constants, a_0 [see, e.g., Marsh (1981)]. The order parameter is related to the angular amplitude of motion of the chain segment, and the isotropic hyperfine constant to the polarity of the environment of the spin-labeled chain segment. For ESR spectra that contain contributions from slow molecular motions, the apparent hyperfine splittings depend on the rates as well as on the amplitudes of motion. For the spectra recorded at 60 °C, these slow motional contributions are likely to be small.

The apparent order parameters of both the *n*-DGSL and the *n*-PCSL spin labels at 60 °C are given in Figure 6 as a function of the position of chain labeling, for samples of

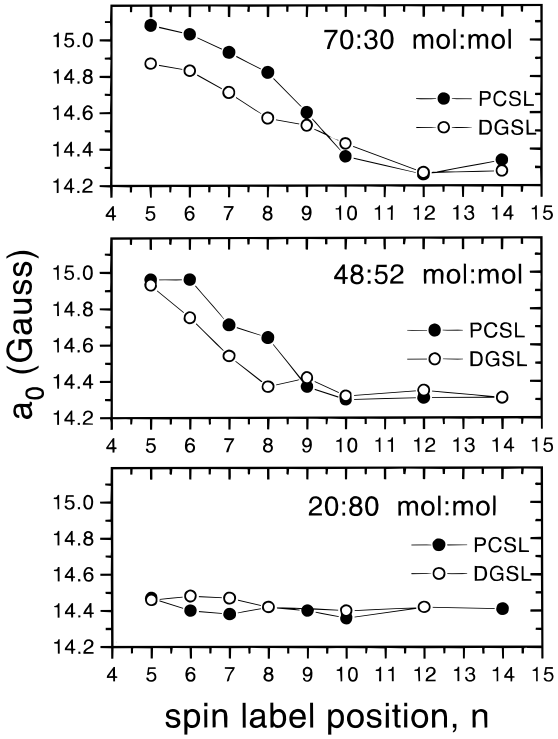

FIGURE 7: Dependence on position of chain labeling, n, of the isotropic hyperfine coupling constants for *n*-DGSL (○) and *n*-PCSL (●) spin labels in hydrated DMPC/DMG mixtures of compositions 70:30 (top), 48:52 (middle), and 20:80 (bottom) mol/mol, in the fluid phase.

compositions corresponding to regions I and II of the binary phase diagram. For region III, the order parameters of the *n*-DGSL labels are zero, because the spectra are isotropic, and those of the *n*-PCSL labels are rather small, because they are in inverted micelles (data not shown). The apparent order parameter profiles for both components reveal a steadily increasing segmental motion down the acyl chain, in both regions I and II of the binary phase diagram. The apparent order parameters are, in the main, smaller for the *n*-DGSL than for the *n*-PCSL spin labels. Because the overall profiles are rather similar for the two sets of spin labels, the relative locations of the two lipid components can be determined from the alignments of the respective profiles [cf. Rama Krishna and Marsh (1990)]. It is found that diacylglycerol is located approximately two CH_2 groups deeper toward the terminal methyl group than is phosphatidylcholine in the fluid bilayer phase of region I and only one CH_2 group deeper in the inverted hexagonal phase of region II.

The positional profiles of the isotropic hyperfine coupling constants of the *n*-DGSL and *n*-PCSL spin labels in mixtures with compositions corresponding to regions I, II, and III of the phase diagram are given in Figure 7. In determining these values for regions I and II by using Figures 2 and 3, it was checked that measurements were made at sufficiently high temperature that constant values were obtained, indicating the absence of slow motional effects. For region III, values were obtained from truly isotropic spectra at sufficiently high temperature. Well defined polarity profiles are obtained for regions I and II that are characteristic of the extent of water penetration in lyotropic structures, whereas for region III a constant rather low polarity is obtained that is consistent with an isotropic apolar phase. In the fluid bilayer phase of region I, the polarity decreases

first slowly and then more rapidly with the distance of the spin label group from the polar headgroup region. The polarity then flattens off at a low constant value from C-10 to C-12 onward, corresponding to the apolar region at the centre of the bilayer. The profiles are similar for both *n*-DGSL and *n*-PCSL, but the polarity closer to the headgroup region is less for the former, to an extent that again indicates that the diacylglycerol component is located approximately two CH_2 groups deeper into the bilayer than the phosphatidylcholine component. In the inverted hexagonal phase of region II, the polarity decreases more rapidly from the headgroup regions and reaches a constant low value from C9-C10 onward. The polarity is again lower for *n*-DGSL than for *n*-PCSL in the sensitive region of the profile. The extent of this reduction indicates that the diacylglycerol component is located approximately one CH_2 group deeper into the hydrophobic interior than is phosphatidylcholine, in agreement with the conclusions based on the chain flexibility profile. A similar consistency in determining the relative locations of spin-labeled lipid components has also been found in fatty acid−phosphatidylcholine mixtures (Rama Krishna & Marsh, 1990). In the latter case, it was found that the protonated fatty acid component was located approximately one CH_2 deeper in the hydrophobic interior of fluid inverted nonlamellar phases than was the phosphatidylcholine component. This closely parallels the present findings for diacylglycerol.

Dependence of Chain Packing on DMG Content. The dependence of the apparent order parameters in the fluid phase at 60 °C on the DMG content in hydrated mixtures with DMPC is given in Figure 8, for the different positional isomers of the *n*-PCSL and *n*-DGSL spin labels. Admixture of DMG in the fluid bilayer phase of region I of the phase diagram first gives rise to an increase in the chain ordering, in agreement with the findings of ^{2}H NMR for mixtures of other chain compositions in the fluid phase (De Boeck & Zidovetzki, 1989, 1991). This increase in order corresponds to an initial increase in the chain packing density on incorporation of DMG in the hydrophobic region of the lipid bilayer. Because DMG has a higher chain-melting temperature than that of DMPC bilayers, it tends initially to increase the chain order in the bilayer configuration.

At higher DMG contents, while still in region I of the phase diagram, the chain ordering begins to decrease with increasing mole fraction of DMG in the mixtures. This can be attributed to the lack of a polar headgroup of the DMG molecule which endows it with an effective inverted conical shape in ordered fluid phases. The effective splay of the DMG chains tends to decrease the chain ordering in the mixtures. This effect competes with the increased bulk contributed by DMG to the hydrophobic regions of the mixtures. At the phase boundary with region II of the phase diagram, the chain order decreases rapidly at most positions of labeling, on transition from the fluid lamellar to the inverted hexagonal phase. At this point, the chain splay of DMG plays a decisive role and is responsible for the polymorphic phase transition. The steep drop in apparent order parameter at the phase boundary is most pronounced for labels positioned toward the center of the chain. For positions $n = 5-6$, a sharp discontinuity is not observed, suggesting that this position may correspond to the location of a neutral surface for the $L_\alpha-H_{II}$ transition [cf. Rand et al. (1990)]. Toward the terminal methyl ends of the chains, the effects are less marked because the apparent order parameters are low but are nonetheless consistent with the results from the other positions of chain labeling.

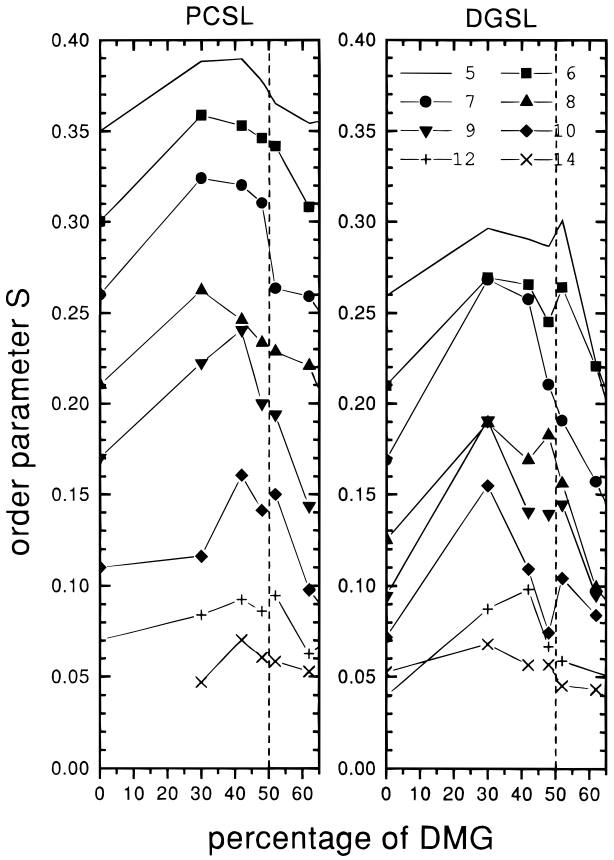

FIGURE 8: Dependence of the apparent order parameters at 60 °C of the *n*-PCSL (left) and *n*-DGSL (right) spin labels on the composition of hydrated mixtures of DMG with DMPC. The different positions of chain labeling are: $n = 5$ (−), 6 (■), 7 (●), 8 (▲), 9 (▼), 10 (◆), 12 (+), and 14 (×). The vertical dashed line indicates the boundary between regions I and II of the binary phase diagram.

Interestingly, in contrast to the behavior of the *n*-PCSL labels, the apparent order parameters of the *n*-DGSL labels increase slightly on entering the H_{II} phase. This can be attributed to the different location of diacylglycerol relative to the polar−apolar interface in the L_α and H_{II} phases. It was found above that diacylglycerol is positioned approximately one CH_2 group closer to the phosphatidylcholine headgroups in the H_{II} phase than it is in the L_α phase.

In the inverted hexagonal phase of region II of the phase diagram, the apparent order parameters continue to decrease with increasing DMG content in the mixtures. This is a further manifestation of a dominating contribution from the effective inverted cone shape of the DMG molecule in fluid phases and is most prominent for the *n*-DGSL spin labels. Nevertheless, the apparent order parameters in the initial portions of region II are still larger than those for bilayers of DMPC alone in the fluid phase at the same temperature. This indicates that the increased bulk of the hydrophobic region that is contributed by DMG is still playing a significant role in the H_{II} phase. Close to the upper boundary of region II, the apparent order parameters in the H_{II} phase are reduced to values equal to or less than those in the L_α phase of DMPC. On transition to region III of the phase diagram, the order parameters are further reduced, becoming zero in the isotropic phase for the *n*-DGSL spin labels.

Conclusions. In the fluid L_α phase at DMG contents up to 50 mol %, the diacylglycerol molecules are incorporated with their acyl chains parallel to those of phosphatidylcholine but are located approximately two CH_2 groups deeper into the hydrophobic region. Initially, the chain order is increased by addition of DMG. In the H_{II} phase at DMG contents from 50 to 66 mol %, the diacylglycerol and phosphatidylcholine molecules are oriented with their chains splayed radially outward, and the diacylglycerol is located approximately one CH_2 group deeper into the hydrophobic milieu. The chain order decreases with increasing DMG content in the H_{II} phase. In the isotropic phase at DMG contents of greater than 66 mol %, the phosphatidylcholine molecules are incorporated as inverted micelles dissolved in liquid DMG.

These results demonstrate the way in which diacylglycerol incorporates in phospholipid membranes and destabilizes their structure. High concentrations of diacylglycerol can be integrated in phospholipid membranes and may be produced locally *in vivo* by the action of phospholipase C. High local concentrations may facilitate the interaction with protein kinase C, as evidenced by the higher vertical location of diacylglycerol found in region II of the phase diagram.

ACKNOWLEDGMENT

We thank Frau B. Angerstein for synthesis of spin labels and lipids.

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BI952688B