Interdigitation Does Not Affect Translational Diffusion of Lipids in Liquid Crystalline Bilayers

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ABSTRACT Asymmetric phosphatidylcholine molecules with one acyl chain twice as long as the other, below their phase transition temperature, form a mixed interdigitated phase in which the longer acyl chain spans the entire bilayer. Experimental evidence in the literature suggests that, above their phase transition temperature, these molecules may still exhibit partial interdigitation, with the longer acyl chain extending partially into the opposite leaflet, and are packed more tightly than equivalent symmetric phosphatidylcholines. Using the fluorescence recovery after photobleaching technique, we have investigated the translational diffusion in multilayers of a liquid crystalline phase, asymmetric phosphatidylcholine, 1-stearoyl-2-capryl-phosphatidylcholine (C18C10PC). We used as a fluorescent probe either a phospholipid analog of the same acyl chain composition, NBD-C18C10PE, or the symmetric equivalent of the same molecular weight, *N*-(7-nitrobenzoxa-2,3-diazol-4-yl)-dimyristoyl-phosphatidylethanolamine (NBD-DMPE). Translational diffusion coefficients were also determined by using both probes in multilayers of dimyristoyl-phosphatidylcholine (DMPC) and in the eutectic mixture DMPC/C18C10PC (40/60 mol). We found that in a given host lipid, NBD-C18C10PE and NBD-DMPE diffuse at the same rate, which suggests that their bilayer free area is almost identical. This result can be explained by considering that in the liquid crystalline state, the increase in molecular packing is compensated by an increase in acyl chain dynamics. This view, which is supported by literature data, clearly suggests that the acyl chain interdigitation occurring in the liquid crystalline phase is highly dynamic.

INTRODUCTION

The translational diffusion of biomembrane components plays an important role in cell function and metabolism. One of the most direct methods for measuring the long-range translational diffusion coefficient, D, is fluorescence recovery after photobleaching (FRAP). FRAP investigations on model membranes yield important insights into the molecular organization of the lipid bilayer, in particular the free molecular area (Vaz et al., 1985a). Although all such studies thus far have involved phospholipids whose acyl chain lengths are the same in the 1 and 2 glycerol positions, most of the lipids found in biological membranes are asymmetric with one acyl chain longer than the other: lengths as short as 14 carbons and as long as 24 carbons are widely distributed. This difference in length is currently described by the chain length asymmetry parameter, $\Delta C/C_L$, where ΔC is the difference in length between both chains and C_L is the length of the longer chain (Slater and Huang, 1988). This difference may be accommodated in two ways: 1) either by a bendback of the longer chain, a situation that is energetically unfavorable, or 2) by penetration of the longer acyl chain into the opposite leaflet, to create an interdigitated phase.

These interdigitated systems are best documented for the gel phase asymmetric phosphatidylcholines (for a review, see Slater and Huang, 1988). Below their phase transition temperature, asymmetric phosphatidylcholines pack in an interdigitated fashion that depends on their chain length asymmetry in the following way: 1) At moderate chain length asymmetry ($\Delta C/C_L < 0.42$), the longer acyl chain extends partially into the opposite leaflet, forming a partially interdigitated phase. 2) When the acyl chain asymmetry is close to 0.5, that is, when one acyl chain is about twice as long as the other, the gel phase bilayer adopts a mixed interdigitated configuration, in which the longer acyl chain spans the entire bilayer, resulting in three acyl chain cross-sectional areas per headgroup cross section (Huang et al., 1983; McIntosh et al., 1984; Slater et Huang, 1988).

Does interdigitation persist in the liquid crystalline phase? This point is of fundamental interest, as it addresses the question of the biological relevance of interdigitation. The interdigitation of glycosphingolipid molecules has been proposed to modulate their function as receptor and structural elements (Florio et al., 1990). Interdigitation of lipid molecules can in principle couple together the two opposing leaflets of a bilayer, and thus may have important physiological consequences. Moreover, as the difference in the bilayer hydrophobic thickness between gel and liquid crystalline phase can be extreme, interdigitation is potentially a factor in membrane microdomain formation and organization. The most difficult molecules to pack in a non-interdigitated fashion are clearly those with one acyl chain about twice as long as the other. Several lines of experimental evidence suggest that asymmetric phosphatidylcholines with a chain length asymmetry close to 0.5 remain partially interdigitated in liquid crystalline phases (Lewis and al., 1994; Zhu and Caffrey, 1993).

Among these molecules, 1-stearoyl-2-capryl-phosphatidylcholine (C18C10PC) is probably the most extensively

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studied. Its symmetric molecular weight equivalent is 1–2-dimyristoyl-phosphatidylcholine (DMPC). The liquid crystalline phase of C18C10PC has been investigated by a variety of experimental approaches. The intramolecular and interchain disorder viewed by Raman spectroscopy in melted C18C10PC is slightly higher than in liquid crystalline DSPC (Huang et al., 1983). Relaxation NMR showed that fluid C18C10PC experiences unusual restrictions on segmental reorientation at megahertz frequencies as compared with symmetric chain species, and the NOESY cross-peak between the ω-CH3 of the C18 chain and the N(CH3)3 of the choline head group has been interpreted in terms of molecular interpenetration into opposite leaflets (Halladay et al., 1990). The molecular area of liquid crystalline C18C10PC deduced from x-ray diffraction measurement is slightly smaller (55 $Å^2$) than current values for fluid DMPC (McIntosh et al., 1984). Moreover, fluorescence anisotropy of (9-anthroyl-oxy)phospholipid derivatives has suggested that the dynamic motions of C18C10PC acyl chains are influenced by the interpenetration of the chains of the opposing leaflet (Mason, 1994).

Taken together, these data indicate that liquid crystalline C18C10PC molecules are packed more tightly than DMPC molecules. Thus, the free area in a fluid C18C10PC bilayer must be smaller than in its symmetric equivalent, DMPC. As current free area theories predict a steep dependence of the diffusion coefficient on the bilayer free area (Vaz et al., 1984), the lateral diffusion coefficient in liquid crystalline C18C10PC should be smaller than in DMPC. In addition, it seems possible that fluid phase interdigitation could cause a coupling of molecules across the bilayer midplane and thus reduce the diffusion coefficient by as much as two-thirds, as found for a transbilayer lipid (Vaz et al., 1985b). Using the FRAP technique, we have investigated the long-range translational diffusion in multilayers of fluid-phase C18C10PC. We used as the fluorescent probe either a phospholipid analogue of the same acyl-chain composition, *N*-(7-nitrobenzoxa-2, 3-diazol-4-yl)-1-stearoyl-2-capryl-phosphatidyl-ethanol-amine (NBD-C18C10PE), or the symmetric molecular weight equivalent, *N*-(7-nitrobenzoxa-2,3-diazol-4-yl)-dimyristoyl-phosphatidylethanolamine (NBD-DMPE). Results are compared with the diffusion of these probes in the symmetric equivalent lipid matrix, DMPC. Unexpectedly, whatever the fluorescent probe, the *D* values in fluid C18C10PC are essentially identical to those in fluid DMPC, which strongly suggests that the free areas in the two lipid matrices are almost identical.

MATERIALS AND METHODS

Chemicals

DMPC, C18C10PC, NBD-DMPE, and NBD-C18C10PE were obtained from Avanti Polar Lipids (Eugene, OR). All other chemicals were of analytical grade.

Multilayers

Supported multilayers were formed following the standard protocol of Vaz et al. (1985a), with a molar ratio fluorescent probe/phosphatidylcholine of 2/1000. The standard buffer was NaCl 10 mM, Na_2HPO_4 5 mM, NaH_2PO_4 5 mM, EDTA 10^{-5} M, pH = 7. The samples were incubated for 2 days at 10°C above their phase transition temperature before measurement.

FRAP experiments

FRAP experiments were carried out on an ACAS 470 workstation (Meridian Instruments, Okemos, MI). The 488 nm line of an argon ion laser (Coherent 90 SUV) operated at 200 mW is split by an acoustic-optic modulator. The resultant first-order beam is used as a bleaching (100% power) or monitoring (<10% power) beam in an inverted microscope equipped with a 40× air objective and a motorized stage. After the photobleaching phase, the fluorescence intensity profile is scanned repeatedly through the bleaching area by computer-driven translations of the microscope stage. The laser power supply, acoustic optic modulator, and motorized stage are interfaced with an IBM-compatible microcomputer and operated by the software provided by Meridian. Note that between two consecutive scans (typically 90% of the time), the beam is "parked" far away from the observation area and thus does not bleach the sample, as it would with steady illumination.

Recovery scans are analyzed with the diffusion function of Koppel (1979). The diffusion coefficient *D*, the mobile fraction *M*, and three parameters of the shape of the initial gaussian profile of the fluorophore concentration (maximum bleaching amplitude α_0, width at half-high ω_0 and *x*-coordinate of α_0) are adjusted to obtain the best fit between the experimental points and the diffusional function.

The temperature of the sample is regulated by a Peltier stage unit. Samples are preincubated at the highest temperature of the scan for 1 h, then cooled down in steps of 2°C, allowing 20 min of equilibration at each temperature before measurement.

RESULTS AND DISCUSSION

The diffusion coefficients obtained in liquid crystalline C18C10PC with the probes NBD-DMPE, NBD-C18C10PE, and *N*-(7-nitrobenzoxa-2,3-diazol-4-yl)-dioleoyl-phosphatidylethanolamine (NBD-DOPE) are shown in an Arrhenius plot in Fig. 1 *A*. The *D* values obtained for each probe are essentially identical. Diffusion coefficients obtained in liquid crystalline DMPC with NBD-DMPE and NBD-C18C10PE are plotted in Fig. 1 *B*. Once again, the differences in the *D* values obtained with the two probes are less than the experimental uncertainty. In each case, the mobile fractions are extremely close to 1 (data not shown). For each lipid matrix, C18C10PC and DMPC, we have fitted ln(*D*) versus $1/T$ with a straight line. For the sake of comparison, we show in each Arrhenius plot the result of the linear regression obtained in the other lipid matrix. The diffusion coefficients in DMPC seem slightly smaller than those in C18C10PC (compare straight lines and experimental points in Fig. 1, *A* and *B*), but the differences are at the limit of the experimental uncertainty and probably not meaningful. Note that the eutectic mixture DMPC/C18C10PC 40/60 molar (Lin and Huang, 1988) falls also on the common plot (Fig. 1 *B*), which indicates that, from the point of view of the translational diffusion, C18C10PC and DMPC mix almost ideally in the liquid crystalline phase.

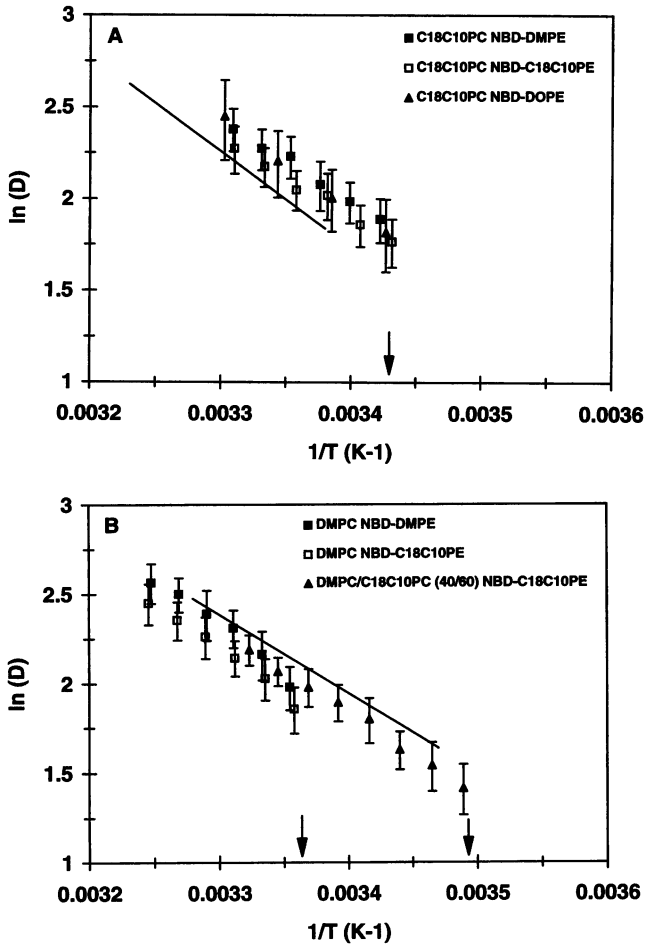

FIGURE 1 Logarithm of the diffusion coefficient of NBD-PE probes versus the inverse of the absolute temperature. (*A*) Multilayers of C18C10PC: ■, NBD-DMPE; □, NBD-C18C10PE; ▲, NBD-DOPE. (*B*) ■, NBD-DMPE; or □, NBD-C18C10PE in multilayers of DMPC; ▲, NBD-C18C10PE in multilayers of DMPC/C18C10PC (40/60 mol). Each point represents the average D value obtained with 40 recoveries on four different samples, and is shown with the standard deviation. Straight lines are the linear regressions obtained in the other lipid matrix (DMPC in (*A*), activation energy: 10.4 kcal/mol, and C18C10PC in (*B*), activation energy: 8.8 kcal/mol). The arrows indicate the respective phase transition temperatures for C18C10PC (*A*), DMPC and DMPC/C18C10PC 40/60 mol (*B*).

The independence of the diffusion coefficient in liquid crystalline C18C10PC on the acyl chain composition of the probe, symmetric or not, indicates that the translational diffusion in this lipid matrix falls in the classical frame of the surface restricted diffusion (Macedo and Litovitz, 1965). In this regime, D depends exponentially of the free area in the bilayer:

$$D = A \cdot \exp(-a_0/(a(T) - a_0)) \cdot \exp(-E_a/(kT))$$

where a_0 is the close-packed molecular area, $a(T)$ the mean molecular area at the absolute temperature T, E_a the activation energy for lateral diffusion, and k the Boltzman constant. As ln(D) was demonstrated to be a linear function of the free area ($a(T) - a_0$) (Vaz et al., 1984), the preexponential factor A seems to be independent of T. Moreover, as the molecular area of the phosphocholine headgroup is consistently larger than the molecular area of two close-packed acyl chains (Pearson and Pascher, 1979; Tardieu et al., 1973), it can reasonably be assumed that the close-packed molecular areas of C18C10PC and DMPC are identical. Finally, because the temperature dependence of the molecular areas is weak (Almeida et al., 1992), we considered them constant over the limited range of temperature sampled here (about 10°C). The activation energies can thus be assimilated to the slope of the linear regressions in Fig. 1, *A* and *B*. They are quite similar (8.8 and 10.4 kcal/mol for C18C10PC and DMPC, respectively) and are in the range of expected values for the diffusion of a phospholipid analogue in liquid crystalline bilayers. Our results thus indicate that the free areas in liquid crystalline C18C10PC and DMPC bilayers are almost the same, which seems contradictory to the increase in molecular packing in C18C10PC evidenced by various experimental approaches (Huang et al., 1983; Halladay et al., 1990; Mason, 1994).

However, the partial interdigitation occurring in a liquid crystalline phase cannot be considered as static as the situation is in the gel phase. It is more realistic to consider a highly dynamic lipid matrix, in which the hydrocarbon chains are rapidly sampling both their own leaflet and regions of the opposing monolayer. Indeed, one may think about a liquid crystalline interdigitated phase as a bilayer in which the acyl chains are at the same time more motionally restricted and more disordered than their symmetric equivalent. This duality is illustrated by the unusual motional restrictions experienced by fluid-phase C18C10PC, while chain motions at mid-kilohertz frequencies are enhanced (Halladay et al., 1990). In the same way, fluorescence anisotropy profiles of 9-anthroyl-oxy chromophores linked at different carbon positions of a phospholipid acyl chain (sampling different depths in the bilayer) in fluid C18C10PC clearly show that the bilayer does not expand to the point where a discrete bilayer midplane exists (Mason, 1994). A dynamic intermixing of the acyl chains of opposing leaflet exists in fluid C18C10PC, even if the average values of the anisotropy are about the same in liquid crystalline C18C10PC and DSPC. Note that this intermixing is not favorable to a molecular coupling across the bilayer midplane. If such a coupling exists, our results show that it has no influence on the translational diffusion.

CONCLUSION

The diffusion coefficients and activation energies measured by FRAP in multilayers of liquid crystalline C18C10PC and DMPC are almost identical, whether the probe interdigitates (NBD-C18C10PE) or not (NBD-DMPE). Thus, the interdigitation occurring in the liquid crystalline phase of C18C10PC has no measurable effect on lipid translational diffusion. This result can be explained in two ways: either 1) the effect of the fluid phase interdigitation, in terms of

reduction of the global free area or in terms of transbilayer coupling, is too weak to be detected, or 2) the tighter molecular packing of C18C10PC molecules is compensated by the faster dynamic motion of their acyl chains. This view, supported by relaxation NMR (Halladay et al., 1990) and fluorescence anisotropy (Mason, 1994) data, supposes that these liquid crystalline, asymmetric phosphatidylcholines experience a "statistical" partial interdigitation, with the longer hydrocarbon chains rapidly sampling both their own monolayer and regions of the opposing monolayer. Although this particular acyl chain dynamics may have some biological relevance (see Florio et al., 1990), our results show that fluid phase interdigitation has no measurable influence on the long-range translational diffusion as sampled by the FRAP technique in bilayer membranes. Nevertheless, the considerable difference in the bilayer thickness between the gel and liquid crystalline phase of certain asymmetric phosphatidylcholines may have an impact in terms of gel-phase domain formation and organization. This topic is currently under investigation in our laboratory.

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