Lateral Diffusion of Cholesterol and Dimyristoylphosphatidylcholine in a Lipid Bilayer Measured by Pulsed Field Gradient NMR Spectroscopy

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ABSTRACT The pulsed field gradient NMR method for measuring self-diffusion has been used for a direct determination of the lateral diffusion coefficient of cholesterol, fluorine labeled at the 6-position, for an oriented lamellar liquid-crystalline phase of dimyristoylphosphatidylcholine (DMPC)/cholesterol/water. It is found that the diffusion coefficients of DMPC and cholesterol are equal over a large temperature interval. The apparent energy of activation for the diffusion process (58 kJ/mol) is about the same as for a lamellar phase of DMPC/water, whereas the phospholipid lateral diffusion coefficient is approximately four times smaller in the presence of cholesterol.

INTRODUCTION

There is still no consensus, despite over 70 years of study, of the role played by cholesterol in biological membranes. The more recent suggestions propose that cholesterol is involved in the formation of domains in the membrane, usually referred to as lipid rafts (Simons and Ikonen, 1997), and that it contributes to the formation of caveolae (Andersson, 1998; Gustavsson et al., 1999). In these latter flask-like invaginations in the plasma membranes, various signaling functions of the cell are initiated such as the signal transduction pathways activated by insulin binding to its receptor. These receptors are localized in caveolae microdomains (Gustavsson et al., 1999; Parpal et al., 2001), which require the presence of cholesterol in the membrane. Depletion of cholesterol results in the disappearance of the caveolae structure (Gustavsson et al., 1999). Another very interesting idea propounded by McConnell (Radhakrishnan and McConnell, 2000) is that cholesterol may form condensed complexes with phospholipids (Anderson and McConnell, 2001) in the plasma membrane that serve a regulatory function with respect to intracellular cholesterol transport and biosynthesis. With the aim of further probing the functions of cholesterol in lipid bilayers we have started a NMR spectroscopic investigation of the lateral diffusion of both the cholesterol and phospholipid components in macroscopically aligned bilayers. In this brief communication we present, for the first time, a direct determination of the lateral diffusion coefficient (D_L) of cholesterol in a lipid bilayer. The great advantage with the pulsed field gradient NMR method (Lindblom and Orädd, 1994) is that it is nonperturbing and in most cases does not need any labeling, although for this study of cholesterol diffusion we have used a simple monofluorinated derivative.

Submitted May 13, 2002, and accepted for publication June 27, 2002.

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0006-3495/02/11/2702/03 $2.00

MATERIALS AND METHODS

To obtain a resolvable NMR signal from cholesterol we have used a singly fluorinated cholesterol (6FCH) in which the proton at the 6-position of the ring system has been replaced by a ^{19}F (Kauffman et al., 2000). The oriented samples were prepared according to a method similar to the one described in Kurtze et al. (2000). Weighed dry powders of dimyristoylphosphatidylcholine (DMPC) (Larodan, Malmö, Sweden) and 6FCH, giving a composition of 38 mol % 6FCH, was dissolved in methanol, and 1-propanol was then added to a final solution composition of 1:4 in methanol:1-propanol. The total concentration of DMPC and 6FCH was 10 mg/ml. This choice of solution provides suitable conditions for good wetting of the glass plates in the sample preparation. A 13-μl volume of the solution was applied to each of 35 glass plates (2.5×14 mm^2), and the solvent was allowed to evaporate. The last traces of solvent were removed under high vacuum for at least 8 h. This procedure resulted in a thin film covering the whole area of the glass plates. The glass plates were then stacked on top of each other and placed in a glass tube with a square cross section. The tube was placed in humid atmosphere at 30°C for 5 days. During this time the glass plates became transparent as the hydrated lamellar phase was formed. To ensure the presence of excess water, some extra water were added and the sample tube was then sealed with wax in both ends and kept in a humid atmosphere until measurement. A sample with only DMPC/water was produced in the same way.

The orientation was checked by inspection of the samples in crossed polarizers where extensive dark areas indicated that large portions of the samples were oriented with the bilayers along the glass plates. The orientation was also checked by ^{31}P-NMR from which it was estimated that more than 85% of the samples was oriented along the glass plates (Fig. 1).

The sample was placed in a diffusion probe equipped with a goniometer stage that allows the orientation of the sample to be adjusted from outside the magnet. When the angle between the bilayer normal and the main magnetic field (B_0) was close to the magic angle (54.7°), significant narrowing of the proton NMR signal from the water and DMPC was observed. This is a consequence of the reduction of the dipole-dipole interactions, and the sample then regains some of the characteristics of an isotropic sample (Lindblom and Orädd, 1994). In particular, the signal from a sample oriented at the magic angle can be refocused to produce a spin-echo, which is required in the pulsed field gradient NMR method of diffusion measurements. The refocused proton signal was used to adjust the sample to the magic angle.

The stimulated spin-echo pulse sequence (Tanner, 1970) was used for both ^{19}F and ^{1}H diffusion experiments. The 90° pulse width was 11 μs, the short delay was 5 ms, and the long delay was 20 ms. Both delays were kept at a minimum due to the rapid relaxation of the fluorine NMR signal. The gradient pulse duration was 3 ms, the gradient strength was varied between 0.45 and 8.9 T/m, and the diffusion coefficient was obtained from a nonlinear fit of the Fourier-transformed peak amplitudes according to the

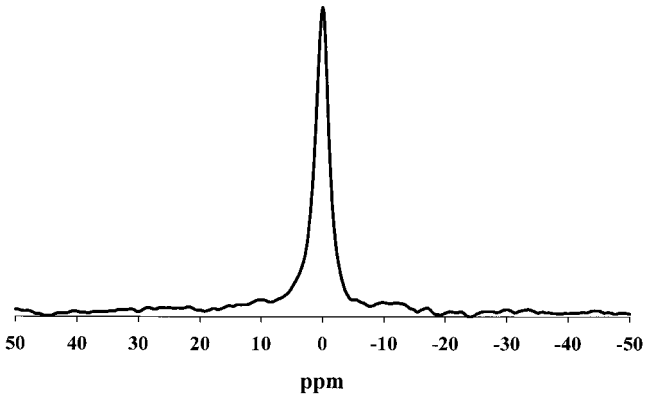

FIGURE 1 ^{31}P NMR spectrum at the magic angle of the macroscopically aligned lamellar phase of DMPC/6FCH/H_2O taken at 30°C. The amount of unoriented sample is estimated to be less than 15%.

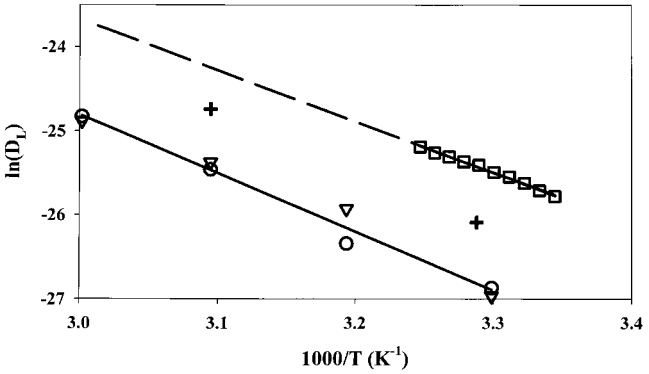

FIGURE 2 Arrhenius plots of D_L of 6FCH (○) and DMPC (▽) in the mixed DMPC/6FCH sample and D_L of DMPC in the DMPC sample (□). The obtained apparent energy of activation is 58 kJ/mol for the mixed sample and 49 kJ/mol for the DMPC sample. Also shown are values of D_L in a DMPC sample with 20 wt % water taken from Kuo and Wade, 1979 (+).

Stejskal-Tanner equation (Stejskal and Tanner, 1965). The obtained diffusion coefficients were finally multiplied by the factor 1.5 to compensate for the fact that the measured diffusion is along the *z* axis, whereas the lipid motion occurs along the bilayers oriented with their normal at 54.7° with respect to this axis (Lindblom and Orädd, 1994).

RESULTS AND DISCUSSION

The choice of 6FCH was made on the basis of monolayer studies, which indicated that fluorine substitution at the 6-position has a negligible effect on the physico-chemical properties of cholesterol (Kauffman et al., 2000). In addition, we have shown by deuterium NMR that the effect of 10 mol % 6-fluorocholesterol on the orientational ordering at all positions of the alkyl chains in chain-perdeuterated (d_{54}) DMPC bilayers is identical to the effect of an equivalent amount of cholesterol (P. Westerman and S. Prosser, unpublished results). However, the disadvantage of the 6-position is that the T_2 relaxation of the fluorine is fast, because the rigid ring structure gives the fluorine a highly restricted motion. Therefore, extensive signal averaging was necessary due to the weak echo signal. The low signal-to-noise level gives an estimated error in the diffusion coefficient for cholesterol of ±5%, whereas the error in D_L of DMPC is much smaller.

Fig. 2 shows Arrhenius plots of D_L for DMPC and 6FCH in the mixed DMPC/6FCH sample along with D_L for the DMPC sample. We also include earlier published values of D_L in a DMPC sample with 20 wt % water (Kuo and Wade, 1979). As seen in the figure, the temperature dependence is similar to our values, but the values of Kuo and Wade is lower than ours. This is most probably a consequence of the low water content in the sample. In the temperature interval 30–60°C, D_L for DMPC and D_L for 6FCH) are equal for the mixed sample, with an apparent energy of activation of 58 kJ/mol. This contrasts with the lateral diffusion of the phospholipid (D_L) in DMPC/water bilayers, in which the activation energy is comparable (49 kJ/mol), but D_L is approximately four times larger.

The most interesting observations in this study are that 1) cholesterol and DMPC exhibit the same lateral diffusion coefficients over the whole temperature interval investigated, 2) the addition of cholesterol to the phospholipid bilayer results in a decrease of the translational diffusion of the phospholipid, and 3) the apparent energy of activation of the diffusion process is the same for both components as well as for DMPC in a pure lipid/water system. Many workers have reported the second observation before, for saturated phospholipids in both monolayers and bilayers (Cullis, 1996; Kuo and Wade, 1979; Rubenstein et al., 1979; Vaz et al., 1979). In a previous study, more than 20 years ago, we did not observe this (or any) effect of cholesterol on unsaturated lipid diffusion (Lindblom et al., 1981). Whether this is due to the unsaturation of the acyl chains of the phospholipids or due to a much lower accuracy in the measurements with the old lower-field NMR spectrometer used at that time, needs additional investigation.

At present we can give only a qualitative explanation to the observed decrease in the lipid diffusion upon addition of cholesterol. It is well known that cholesterol has a condensing effect on the lipid packing in lipid bilayers (Leathes, 1925), and it seems reasonable to assume that a decrease in the phospholipid lateral diffusion may arise from such an effect. The fact that both components have the same diffusion coefficient indicates that most probably the translational diffusion is bestowed by some kind of cooperative mechanism. This is in line with our previous observation on studies of the effect of different additives to a cubic liquid-crystalline phase composed of monooleoylglycerol, where it was found that, depending on the additive, the diffusion coefficient could either increase or decrease (Eriksson and Lindblom, 1993). Thus, the addition of a short-chained

amphiphile, characterized by a relatively rapid diffusion, will result in an increase in the lipid diffusion, and interestingly enough the overall diffusion coefficients of both components will be the same. For a long-chained compound, the opposite will prevail. Finally, the straight line obtained in the Arrhenius plot in Fig. 2 indicates that there are no phase transitions or domain or complex formations occurring in the temperature interval investigated. If complexes or domains were present in the bilayer one would expect to see the effect of some kind of change of their number or sizes. However, recently published results indicate the presence of condensed complexes at lower temperatures (Radhakrishnan and McConnell, 2002), and we are therefore extending our future investigations toward lower temperatures as well as to several different cholesterol concentrations.

The technical support of Marcia Malmer (Northeastern Ohio Universities' College of Medicine) is gratefully acknowledged.

This work was supported by grants from The Swedish Research Council.

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