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# Effects of cholesterol or gramicidin on slow and fast motions of phospholipids in oriented bilayers

($^{19}$F NMR/nuclear spin-lattice relaxation/rotating-frame relaxation/molecular motions/difluorodeuterated phospholipid)

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**ABSTRACT** **Nuclear spin-lattice relaxation both in the rotating frame and in the laboratory frame is used to investigate the slow and fast molecular motions of phospholipids in oriented bilayers in the liquid crystalline phase. The bilayers are prepared from a perdeuterated phospholipid labeled with a pair of $^{19}$F atoms at the 7 position of the 2-*sn* acyl chain. Phospholipid–cholesterol or phospholipid–gramicidin interactions are characterized by measuring the relaxation rates as a function of the bilayer orientation, the locking field, and the temperature. Our studies show that cholesterol or gramicidin can specifically enhance the relaxation due to slow motions in phospholipid bilayers with correlation times $\tau_s$ longer than $10^{-8}$ sec. The perturbations of the geometry of the slow motions induced by cholesterol are qualitatively different from those induced by gramicidin. In contrast, the presence of cholesterol or gramicidin slightly suppresses the fast motions with correlation times $\tau_f = 10^{-9}$ to $10^{-10}$ sec without significantly affecting their geometry. Weak locking-field and temperature dependences are observed for both pure lipid bilayers and bilayers containing either cholesterol or gramicidin, suggesting that the motions of phospholipid acyl chains may have dispersed correlation times.**

To establish a relationship among the structure, dynamics, and function of biological membranes requires knowledge of (*i*) the packing and motion of phospholipid molecules in a membrane and (*ii*) the mechanisms for lipid–sterol and lipid–protein interactions. During the last three decades, considerable efforts have been devoted to the biophysical studies of phospholipid bilayers, which are often viewed as a simplified model of natural membranes (1). Although the static properties of a lipid bilayer, such as the conformation and order of phospholipids, have been carefully characterized (2, 3), the dynamic behavior of phospholipids in a bilayer environment is less well understood (4).

The physical state of a phospholipid bilayer under physiological conditions is that of a lyotropic liquid crystal surrounded by water molecules. Due to the amorphous packing and prevalence of molecular motions, high-resolution x-ray diffraction cannot be applied to lipid bilayers in a liquid crystalline phase. Hence, magnetic resonance, which can give both static and dynamic information, is of vital importance in the field of membrane biophysics. In particular, when combined with isotopic labeling, nuclear relaxation becomes a unique technique which can monitor the microscopic dynamics of a specific site within a macromolecule or a supermolecular assembly. Normally, the spin-lattice relaxation in the laboratory frame ($T_1$) is sensitive to molecular motions on the time scale of $10^{-10}$ to $10^{-8}$ sec, while the spin-lattice relaxation in the rotating frame ($T_{1\rho}$) is sensitive to motions on the time scale of $10^{-6}$ to $10^{-4}$ sec (5).

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A number of nuclear magnetic resonance (NMR) investigations have focused on the molecular motions of phospholipids in a pure lipid bilayer, and various theoretical models have been proposed (6–22). Aside from those studies emphasizing lateral diffusion (9, 19), most existing models of the dynamics of phospholipids belong to one of two classes: (*i*) one or more noncollective anisotropic rotations of a lipid molecule (or a segment of the molecule) with well-defined correlation times (6–8, 10, 11, 16–18, 22); or (*ii*) collective bilayer disturbances with small angular modulations and a broad distribution of correlation times (13–15, 20). Multiple motions coexist in a bilayer, even though different motions may have quite different geometries and time scales (20, 21).

Since most biochemical and biophysical processes occur on a microsecond or slower time scale, we believe that the slow motions of phospholipids (i.e., correlation time, $\tau \geq 10^{-6}$ sec) are more relevant to the function of biological membranes than their fast motions. Several studies have suggested that slow motions play an important role in lipid–protein interactions or lipid-mediated protein–protein interactions (4, 7, 14, 23).

Cholesterol and gramicidin are commonly chosen as prototypes for investigating interactions with phospholipids (24, 25). Cholesterol can be found in many biological membranes in ratios that range from 10 to 40 mol % (26). In the liquid crystalline phase, cholesterol increases the order of the phospholipid acyl chains and decreases the membrane permeability; at low temperatures, it prevents the acyl chains from forming a highly ordered gel phase (26–28). Gramicidin is a pentadecapeptide which can be considered as a model of membrane proteins; it folds into a helical structure and forms channels in a lipid bilayer (29, 30). In general, membrane peptides and proteins have little or no effect on the chain ordering of phospholipids (24, 31–34). For recent reviews on using NMR to study lipid bilayers containing cholesterol or gramicidin, see refs. 4 and 35.

To investigate the effects of membrane-intercalating molecules on the dynamics of phospholipids, Cornell and coworkers (23, 36) have determined the ratios of averaged $T_{1\rho}^{-1}$ to $T_1^{-1}$ for methylene protons in dispersions of pure phospholipids and phospholipid mixed with cholesterol or gramicidin, as well as for erythrocyte membranes. They have found that both additives cause the $T_{1\rho}$ to shorten significantly, which indicates that the intensity of the slow motions of the phospholipid has increased. On the other hand, only small effects on the fast motion of the acyl chain have been observed. Since the ratio of the relaxation rates observed for

Abbreviations: $T_1$, spin-lattice relaxation in the laboratory frame; $T_{1\rho}$, spin-lattice relaxation in the rotating frame; 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$, 1-(myristoyl-$d_{27}$)-2-(7,7-difluoromyristoyl-$d_{25}$)-*sn*-glycero-3-phosphocholine; $H_0$, static magnetic field; $H_1$, locking field; CSA, chemical shift anisotropy.

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phospholipid dispersions with cholesterol or gramicidin is closer to that found for natural membranes, they conclude that biological membranes are rich in slow motions, and such motions can be induced or enhanced by adding foreign molecules to pure phospholipid bilayers.

Using macroscopically oriented bilayers prepared from a phospholipid with perdeuterated acyl chains in which a single $CF_2$ group occurs at the 7 position of the *sn*-2 acyl chain, 1-(myristoyl-$d_{27}$)-2-(7,7-difluoromyristoyl-$d_{25}$)-*sn*-glycero-3-phosphocholine (2-[7,7-$^{19}F_2$]DMPC-$d_{52}$), we have investigated both slow and fast motions by measuring $^{19}F$ $T_{1\rho}^{-1}$ and $T_1^{-1}$ as a function of the bilayer orientation, the locking field, and the temperature. The advantages of using a $^{19}F$ label include the following: the NMR sensitivity is high and there is no natural background (37); the relaxation of $^{19}F$ is dominated by intramolecular interactions (21); and the cross-polarization between $^{19}F$ and $^2H$ is negligible in the range of locking fields employed in this study. We have found that adding cholesterol to bilayers of the perdeuterated fluorolipid can dramatically alter the orientation dependence of $T_{1\rho}^{-1}$, indicating that, besides increasing the intensity of the slow motions, a large perturbation of the geometry of these motions is induced compared to the pure phospholipid bilayers. The addition of gramicidin produces a smaller change in the geometry of the slow motions, although there is still a significant increase in the relaxation rate. For all cases, the geometry of the fast motions remains basically the same. Several motional models have been tested against our experimental results. The systematic differences observed between the effects produced by cholesterol and gramicidin can lead to new insight into the nature of the structure–function relationship of various membrane components.

## MATERIALS AND METHODS

The synthesis of 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ will be described elsewhere. Cholesterol and gramicidin (Dubos) were purchased from Sigma and used without further purification.

Oriented bilayers of phospholipid were prepared by dissolving about 30 mg of 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ with or without 12 wt % cholesterol (22 mol %) or 14 wt % gramicidin (6.2 mol %) in 0.6 ml of 3:1 (vol/vol) chloroform/methanol. Aliquots (20 $\mu$l) were deposited on microscope coverslips (7.8 × 20 mm) and dried under reduced pressure. The slips were then stacked and hydrated in a closed chamber in the presence of 1 M $MgCl_2$ at 32°C (relative humidity 90%) for 5–15 days, during which the lipids gradually oriented.

The relaxation measurements were performed on a modified WH-300 spectrometer operating at 282.4 MHz for $^{19}F$. A home-built probe with a loop-gap resonator as the NMR detector was employed. The relaxation time $T_1$ was determined by inversion recovery and $T_{1\rho}$ was determined by spin locking, with the lengths of the 90° pulses being 3.7 and 3.2 $\mu$sec, respectively. For the $T_{1\rho}$ measurements, the locking-field ($H_1$) intensity was controlled by an electronic attenuator and calibrated by the corresponding 360° pulse duration. As the sample orientation was changed, the carrier frequency of the spin-locking pulse was adjusted such that the off-resonance effect caused by the chemical shift anisotropy (CSA) could be compensated. The temperature was held at 32°C for $T_{1\rho}$ measurements and varied from 28°C to 48°C for $T_1$ measurements. At all temperatures used for the NMR studies, the bilayer was in the liquid crystalline phase.

## RESULTS

The $^{19}F$-NMR spectrum for oriented bilayers of 2-[7,7-$^{19}F_2$]-DMPC-$d_{52}$ is shown in Fig. 1, where the orientation is measured as the angle between the bilayer director and the static magnetic field ($H_0$). Except for the magic angle orientation (54.7°), the spectrum consists of a well-resolved doublet which arises from the $^{19}F$–$^{19}F$ homonuclear dipolar interaction, with little broadening from the heteronuclear $^{19}F$–$^2H$ dipolar interactions. At the magic angle, a single sharp peak is observed. The center of the doublet shifts due to the large CSA of the $CF_2$ group as the orientation of the bilayer changes.

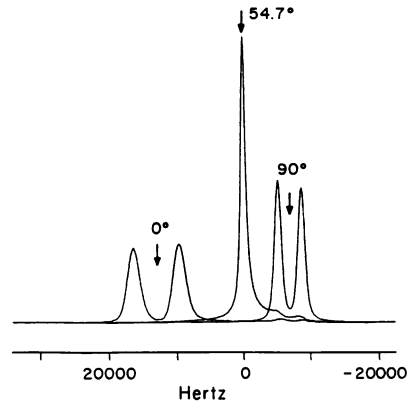


FIG. 1. The 282.4-MHz $^{19}F$-NMR spectra of oriented 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ bilayers at 32°C and 0°, 54.7°, or 90° orientation with respect to the static magnetic field. Arrows indicate the transmitter frequency for the spin-locking pulse.

Fig. 2 shows the orientation dependence of $T_{1\rho}^{-1}$ measured under a 4-gauss (4 × $10^{-4}$ tesla) locking field, for bilayers made of pure 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ (Fig. 2*A*), 2-[7,7-$^{19}F_2$]-DMPC-$d_{52}$ plus 22 mol % cholesterol (Fig. 2*B*), or 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ plus 6.2 mol % gramicidin (Fig. 2*C*). Clearly,

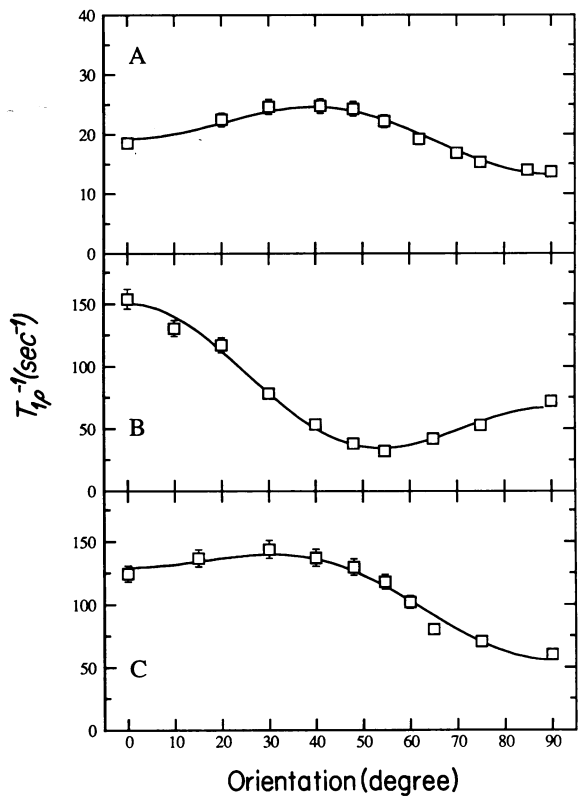


FIG. 2. Orientation dependence of $^{19}F$ $T_{1\rho}^{-1}$ for oriented phospholipid bilayers under a 4-gauss locking field at 32°C. (*A*) Pure 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$; (*B*) 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ plus 22 mol % cholesterol; and (*C*) 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ plus 6.2 mol % gramicidin. Error bars represent ±5% experimental error. The solid lines represent the best theoretical fits.

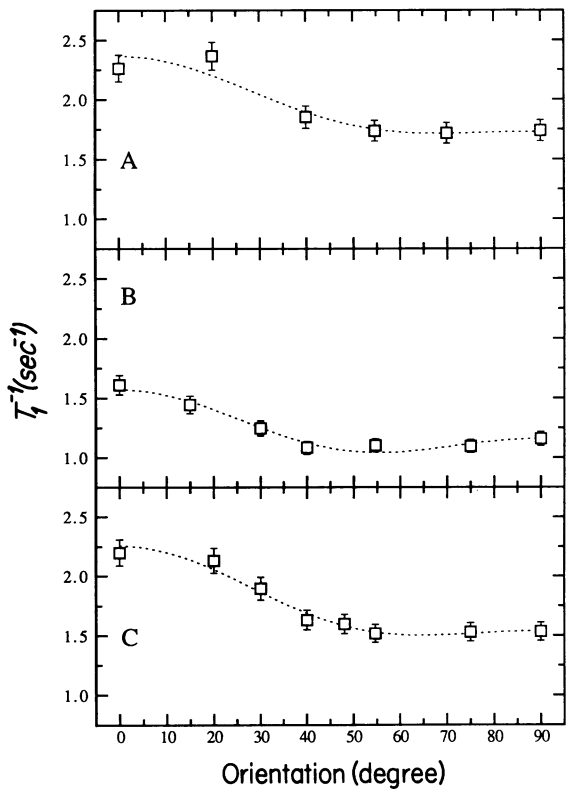


FIG. 3. Orientation dependence of $^{19}F$ $T_1^{-1}$ for oriented phospholipid bilayers in a $7 \times 10^4$ gauss static field (282.4 MHz) at 32°C. (*A*) Pure 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$; (*B*) 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ plus 22 mol % cholesterol; (*C*) 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ plus 6.2 mol % gramicidin.

the effect of cholesterol on the orientation dependence of $T_{1\rho}^{-1}$ is qualitatively different from the effect of gramicidin. Fig. 3 shows the orientation dependence of $T_1^{-1}$ measured on the same samples in a static field of $7 \times 10^4$ gauss, and only minor changes can be seen.

The locking-field dependence of $T_{1\rho}^{-1}$ and the temperature dependence of $T_1^{-1}$ for all three samples are shown in Fig. 4 *Left* and *Right*, respectively. The field dependences are always weak and display little effect when cholesterol or gramicidin is added to the bilayer. The $T_{1\rho}^{-1}$ decreases only about 50% over a 1- to 8-gauss range of locking-field intensities. For pure 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ bilayers and for bilayers containing gramicidin, there is essentially no temperature dependence of $T_1^{-1}$ between 28°C and 48°C. For bilayers containing cholesterol, $T_1^{-1}$ increases slightly with temperature.

## DISCUSSION

Detecting a differential effect caused by any membrane component on the structure and dynamics of the membrane bilayer is important because it can potentially be related to the function of that component. The order parameter has found widespread use for investigating the interactions of phospholipids with various membrane additives (38, 39). For example, the hydrocarbon chains in a lipid–cholesterol mixture have been found to be approximately twice as ordered as those in pure lipid bilayers, but no significant lipid–peptide or lipid–protein interaction has been observed by this method (4). Our results show that the geometry of the slow motions of phospholipid is at least equally sensitive to specific interactions between phospholipid and cholesterol or phospholipid and gramicidin. We have found that 22 mol % cholesterol is able to induce a drastic change in the orientation dependence of $T_{1\rho}^{-1}$. A smaller but appreciable change in the orientation dependence has been observed for 6.2 mol % gramicidin, in agreement with a 25% increase in the order parameter (31).

A further interpretation of the orientation dependence of $T_{1\rho}^{-1}$ must be based on specific motional models. The major relaxation mechanisms for $^{19}F$ in the perdeuterated phospholipid are modulations of the homonuclear $^{19}F$–$^{19}F$ dipolar interaction and modulations of the CSA; therefore, a translation of the phospholipid molecule does not cause relaxation. To study slow motions, the phospholipid molecule can be treated as an axially symmetric molecule because it rotates around its long axis at a rate much faster than the time scale defined by the $T_{1\rho}$ experiment (21). We first consider a model consisting of only one slow motion. In a uniaxial bilayer environment, the orientation dependence of $T_{1\rho}^{-1}$ induced by a general anisotropic reorientation is given by (21):

$$\frac{1}{T_{1\rho}} = R[\tfrac{1}{5}(1 - \langle P_4\rangle) + \tfrac{2}{7}P_2(\cos\beta)(\langle P_2\rangle - \langle P_4\rangle) + P_2^2(\cos\beta)(\langle P_4\rangle - \langle P_2\rangle^2)],$$

where $R$ is an orientation-independent relaxation rate and $\langle P_2\rangle$ and $\langle P_4\rangle$ are averages of second- and fourth-rank Legendre polynomials weighted by the angular distribution function of the slow motion. The geometrical information for the slow motion is encoded in $\langle P_2\rangle$ and $\langle P_4\rangle$, which determine the shape of the orientation dependence. This functional form fits well the observed orientation dependences for all three types of samples, as shown by the solid lines in Fig. 2. For pure 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ bilayers, we found that $\langle P_2\rangle \approx 0.3$, $\langle P_4\rangle \approx 0$, and $R/5 \approx 23$ sec$^{-1}$; for bilayers with cholesterol, $\langle P_2\rangle \approx \langle P_4\rangle \approx 0.6$, and $R/5 \approx 84$ sec$^{-1}$. Finally, for bilayers with gramicidin, $\langle P_2\rangle \approx 0.8$, $\langle P_4\rangle \approx 0.6$, and $R/5 \approx 260$ sec$^{-1}$ are obtained.

A second motional model consists of a superposition of two specific types of slow motions (21):

$$\frac{1}{T_{1\rho}} = R_1(1 - S)[\tfrac{1}{5} + S\,P_2^2(\cos\beta)] + R_2\tfrac{3}{2}\sin^2\beta\cos^2\beta,$$

where the first term represents an anisotropic reorientation with $\langle P_2\rangle = \langle P_4\rangle = S$, and the second term represents a director fluctuation with small angular modulations. The relative weight of the two slow motions is determined by the ratio $R_1/R_2$, optimized during the least-squares fit. The effect of adding cholesterol can be interpreted as eliminating the director fluctuation term from the orientation dependence—i.e., making $R_2 = 0$. Both additives tend to increase the parameter $S$ for the anisotropic reorientation. For gramicidin-containing bilayers, this makes the relative contribution from the anisotropic reorientation become smaller while keeping the ratio $R_1/R_2$ approximately the same as for pure 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ bilayers. These two fitting schemes lead to indistinguishable results within the experimental error, even though the two functions are not equivalent mathematically.

If there is only a single slow motion, the geometry of this motion must change when cholesterol or gramicidin is added to the bilayer, such that the orientation dependence of $T_{1\rho}^{-1}$ changes accordingly. If two or more slow motions coexist, both the geometry and the weighting between motions can respond to the dynamic perturbations caused by the additive. For instance, cholesterol may perturb one motion more or less specifically, while gramicidin may have its major effect on another motion. It should be noted that the fitting parameters, $\langle P_2\rangle$, $\langle P_4\rangle$, or $S$, cannot be directly related to static order parameters such as $S_{CD}$, since there can be a hierarchy of motions with different time scales in the phospholipid bilayer,

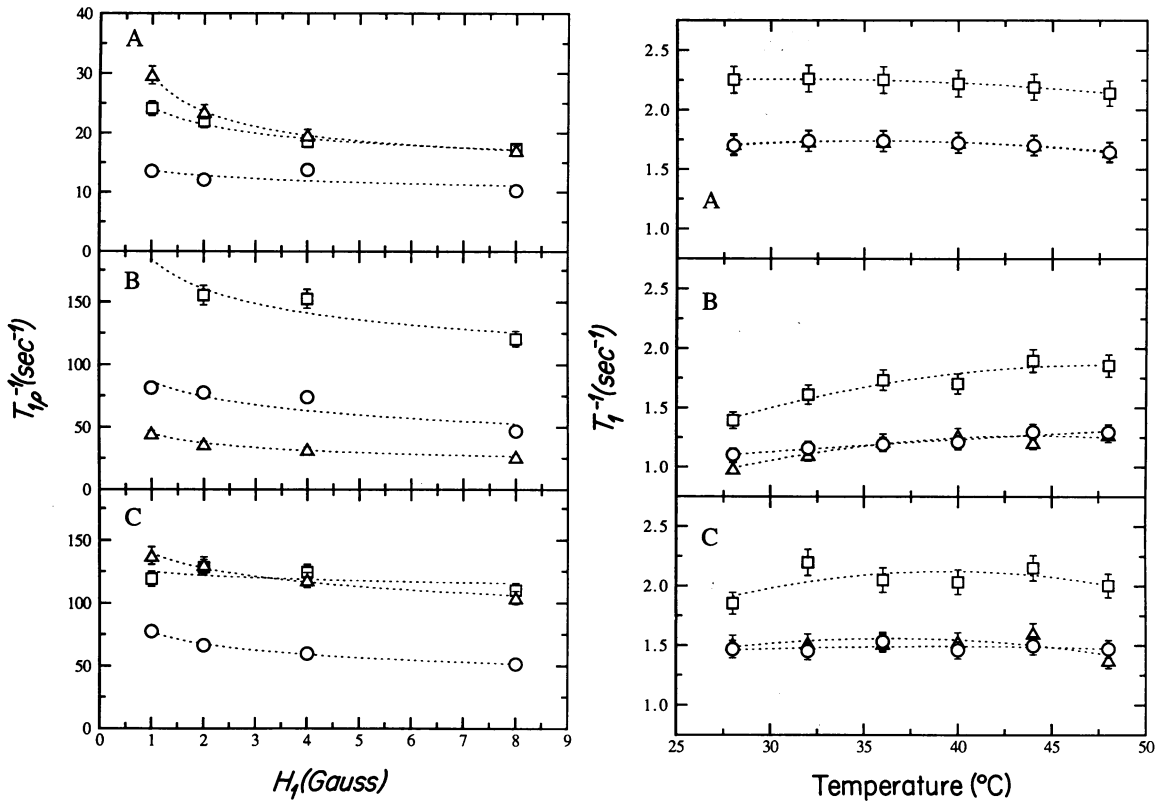


FIG. 4. (*Left*) Locking-field dependence of $^{19}F$ $T_{1\rho}^{-1}$ for oriented phospholipid bilayers at 32°C. (*Right*) Temperature dependence of $^{19}F$ $T_1^{-1}$ for oriented phospholipid bilayers. (*A*) Pure 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$; (*B*) 2-[7,7-$^{19}F_2$]DMPC-$d_{52}$ plus 22 mol % cholesterol; and (*C*) 2-[7,7-$^{19}F_2$]-DMPC-$d_{52}$ plus 6.2 mol % gramicidin. □, 0° orientation; △, 54.7° orientation; ○, 90° orientation.

with only a small portion of them contributing to the relaxation at a given frequency (40).

The weak locking-field dependence of $T_{1\rho}^{-1}$ indicates that the correlation time of the slow motion is shorter than the smallest value of $1/\omega_1$, namely 5 μsec. An alternative possibility would be the existence of a large number of collective motions with a broad distribution of correlation times on a time scale of 5–40 μsec. The addition of cholesterol or gramicidin enhances the relaxation rate without affecting the locking-field dependence significantly.

Assuming that the slow motion has an effective correlation time $\tau_s$, it can be shown that the orientation-independent relaxation rate should be (21):

$$\frac{R}{5} = \omega_{eff}^2 \frac{2\tau_s}{1 + \omega_1^2\tau_s^2},$$

with $\omega_{eff} = S_f \cdot \omega_{static}$. Here, $\omega_{static}$ is the rigid-lattice interaction and $S_f$ describes the reduction of the ordering as a result of the fast motions. The lower limit of $\tau_s$ can be estimated by taking $\omega_{eff} \approx 20$ kHz and $R/5 \approx 20$ sec$^{-1}$, which gives $\tau_s \geq 25$ nsec. It appears that cholesterol and gramicidin enhance $T_{1\rho}^{-1}$ through different mechanisms. Since the presence of cholesterol increases the fast-motion order parameter $S_f$, the elevated magnitude of $T_{1\rho}^{-1}$ can be easily explained. But the effect of gramicidin must depend on a shift of the average correlation time, as $S_f$ is much less affected.

In contrast to the slow motions, cholesterol and gramicidin have rather similar effects on the fast motions of the phospholipid acyl chains (Fig. 3). $T_1^{-1}$ decreases by 15–35% in the presence of the additives, suggesting a restriction of the motional mobility. Such a spatial restriction would cause a decrease in the amplitude of the fast motions, and, if the kinetic energy is constant, an increase in their frequency would occur. Both factors may lower the observed relaxation rate, if the motions are relatively fast on the time scale of the Larmor frequency. As expected, the effect is more visible for the rigid, disk-like cholesterol molecule than for gramicidin.

The orientation dependence of $T_1^{-1}$ does not change significantly upon the addition of cholesterol or gramicidin (Fig. 3). This is consistent with the geometry of the fast axial rotation of the phospholipid molecule and of the internal bond isomerization being rather insensitive to the presence of an additive. The lack of a clear temperature dependence for $T_1^{-1}$ indicates that the correlation times for the fast motions are likely to be dispersed so that there can be a broad $T_1$ minimum near our experimental time scale ($5\times10^{-10}$sec). Distributed correlation times are not uncommon in a system where many different local configurations are present (41). The results of a Brownian dynamics simulation (22) suggest that at least three correlation times are needed to describe the decay of the auto-correlation function caused by the internal bond (*trans–gauche*) isomerization. Although it is generally believed that *trans–gauche* isomerization is faster than the time scale of our $T_1$ experiments (22), the axial rotation of the entire phospholipid is expected to be on the slow side of that time scale. Thus, changing the temperature may have no net effect on the relaxation rates. As distinct from the slow motions, adding cholesterol to the bilayer seems to affect the correlation times for fast motions to a small extent, while gramicidin has very little effect.

The possible coexistence of two-fluid phases in the phospholipid–cholesterol and phospholipid–gramicidin systems should also be discussed. It has been reported that a phase separation can be observed in binary mixtures of DMPC [or dipalmitoyl phosphatidylcholine (DPPC)] with 10–20 mol % cholesterol (42–44). In our experiments, since $T_{1\rho}$ and $T_1$ are very long compared to the exchange lifetime of lipid molecules between domains, we can monitor only the averaged properties of the phospholipid molecule in both phases. In practice, since our samples contain 22 mol % cholesterol,

most of the lipid molecules should be in the cholesterol-rich phase. Only a single exponential decay of the magnetization is observed in our measurements. Gramicidin does not cause a phase separation at low concentrations above the main phase transition temperature (45). It should be mentioned that at high concentrations the bilayer tends to reorganize into hexagonal phases (46). This situation has been avoided in our experiments.

## CONCLUDING REMARKS

The dynamic behavior of a phospholipid in oriented bilayers monitored at the 7 position of the *sn*-2-acyl chain can be summarized as follows. Since $T_{1\rho} >> T_1$, at least two classes of correlation times must exist and satisfy the condition, $\tau_f \leq \omega_0^{-1} << \tau_s \leq \omega_1^{-1}$. Thus, the correlation time of the slow motions ($\tau_s$) is much longer than that of the fast motions ($\tau_f$). The lack of a strong-field dependence in $T_{1\rho}^{-1}$ further implies that either $\omega_1\tau_s << 1$ or there is a broad distribution of $\tau_s$. The addition of cholesterol (or gramicidin) to the bilayer can induce slow motions that have a geometry different from those found in the absence of cholesterol (or gramicidin), as illustrated by the strikingly different orientation dependence of $T_{1\rho}^{-1}$. For fast motions, adding cholesterol or gramicidin has no effect on the geometry of the motion. Cholesterol exerts a slightly more perturbing effect than gramicidin on both the correlation time and the relaxation rate.

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