Effect of Hydrostatic Pressure on Water Penetration and Rotational Dynamics in Phospholipid-Cholesterol Bilayers

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ABSTRACT The effect of high hydrostatic pressure on the lipid bilayer hydration, the mean order parameter, and rotational dynamics of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) cholesterol vesicles has been studied by time-resolved fluorescence spectroscopy up to 1500 bar. Whereas the degree of hydration in the lipid headgroup and interfacial region was assessed from fluorescence lifetime data using the probe 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), the corresponding information in the upper acyl chain region was estimated from its effect on the fluorescence lifetime of and 3-(diphenylhexatrienyl)propyl-trimethylammonium (TMAP-DPH). The lifetime data indicate a greater level of interfacial hydration for DPPC bilayers than for POPC bilayers, but there is no marked difference in interchain hydration of the two bilayer systems. The addition of cholesterol at levels from 30 to 50 mol% to DPPC has a greater effect on the increase of hydrophobicity in the interfacial region of the bilayer than the application of hydrostatic pressure of several hundred to 1000 bar. Although the same trend is observed in the corresponding system, POPC/30 mol% cholesterol, the observed effects are markedly less pronounced. Whereas the rotational correlation times of the fluorophores decrease in passing the pressure-induced liquid-crystalline to gel phase transition of DPPC, the wobbling diffusion coefficient remains essentially unchanged. The wobbling diffusion constant of the two fluorophores changes markedly upon incorporation of 30 mol% cholesterol, and increases at higher pressures, also in the case of POPC/30 mol% cholesterol. The observed effects are discussed in terms of changes in the rotational characteristics of the fluorophores and the phase-state of the lipid mixture. The results demonstrate the ability of cholesterol to adjust the structural and dynamic properties of membranes composed of different phospholipid components, and to efficiently regulate the motional freedom and hydrophobicity of membranes, so that they can withstand even drastic changes in environmental conditions, such as high external hydrostatic pressure.

INTRODUCTION

Structural and dynamic properties of cell membranes are significant for their fundamental physiological functions. One of the common constituents of natural membranes is the steroid cholesterol. It is the main sterol of animal organisms and plays a central role in modulating chemical and physical properties (Bloom and Mouritsen, 1988; Demel and de Kruyff, 1976; Yeagle, 1985, 1992). Its relative content is variable and dependent on the source of the membrane. It is equimolar with phospholipids in membranes of liver cells, erythrocytes, and myelin, whereas in the human stratum corneum (the outer layer of the epidermis), for example, it only represents about 20 wt% of the lipid amount. The cholesterol backbone (see Fig. 1) is the rigid planar transfused tetracyclic ring structure of a steroid that reaches up to 9 or 10 carbons of extended alkyl chains and to a somewhat deeper level into the hydrophobic region of a lipid bilayer in the fluid-like phase. It is well known that alterations of the phospholipid—saturated as well as unsaturated—and cholesterol composition in natural membranes lead to significant changes in the conformation and rate and amplitude of the motions of the bilayer components (see, e.g., Cevc and Marsh, 1987). However, the causative details at a molecular level are still poorly understood, particularly for unsaturated phospholipid systems and lipid mixtures.

In addition to the role of the composition of membrane system, the surrounding medium water also has a primary role in the formation and maintainance of cell membrane architecture. Water binds directly to phospholipid headgroups and participates in the lipid bilayer structure. Water molecules also reach deeper into the lipid bilayer, fitting between acyl chain packing defects, such as *trans-gauche* kinks, thus contributing to interchain hydration. The water molecules move between these defects by a kind of hopping mechanism (Deamer and Bramhall, 1986; McIntosh and Simon, 1986; Prats et al., 1987; Ho et al., 1995).

One of the variables used most frequently to study thermodynamic, structural, and dynamic properties of biological membranes is temperature. Changing the temperature of a system results in alterations of the thermal energy as well as the density. In contrast, a pressure change under isothermal conditions introduces only a change in the density of the system. Therefore, the use of pressure can provide valuable information about density-dependent processes without having to consider thermally activated processes associated with temperature-dependent studies (Böttner and Winter, 1993; Winter et al., 1994; Winter and Jonas, 1993).

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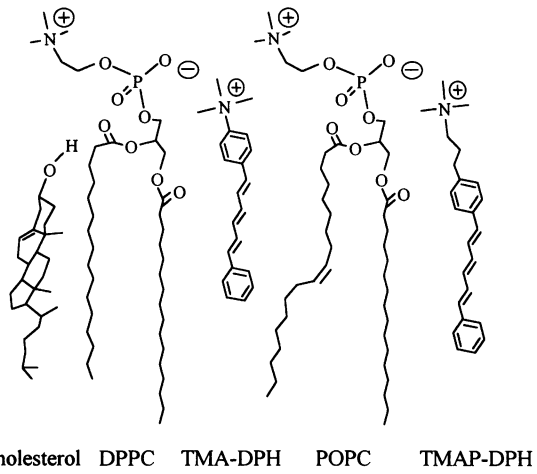

FIGURE 1 Schematic structure and location of cholesterol and the cationic fluorescence probes TMA-DPH and TMAP-DPH in the DPPC lipid bilayer.

Furthermore, studies of pressure effects on biochemical systems are also of considerable physiological and biotechnological relevance (see, e.g., Winter and Jonas, 1993; Balny et al., 1992). The effect of hydrostatic pressure on lipid bilayers and cellular membranes has been examined, for example, by fluorescence probe techniques (Chong and Weber, 1983; Lakowicz and Thompson, 1983; Paladini and Weber, 1981; Mateo et al., 1993; Scarlata, 1991; Bernsdorff et al., 1996), other spectroscopical techniques, such as NMR (Jonas et al., 1988, 1990; Peng and Jonas, 1992; Bonev and Morrow, 1995), electron paramagnetic resonance (Trudell et al., 1974), Fourier transform infrared spectroscopy (Wong et al., 1988; Ley and Drickamer, 1990; Reis et al., 1996), and diffraction methods (Braganza and Worcester, 1986a,b; Winter and Pilgrim, 1989; Czeslik et al., 1995, 1996).

Fluorescence techniques, steady-state and time-resolved, allow the study of pressure-induced membrane structural and dynamic changes at the same time. They are, in general, dependent on the use of selectively labeled molecules within the bilayer. The assumption is made that the order and dynamics of these probe molecules reflect the behavior of the surrounding lipid molecules themselves (Engel and Prendergast, 1981; Wolber and Hudson, 1981).

In the present work we have carried out time-resolved fluorescence spectroscopy studies to investigate the order parameter and dynamic behavior of two amphiphilic derivatives of the rod-shaped membrane probe DPH, namely 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and 3-(diphenylhexatrienyl)propyltrimethylammonium (TMAP-DPH) (see Fig. 1), embedded in phospholipid/cholesterol vesicles, as a function of pressure. TMAP-DPH is a homolog of the probe TMA-DPH with a spacer of three carbon atoms between the DPH moiety and the positively charged trimethylammonium group. Thus the two probes can be considered as fluorophores, probing the membrane bilayer at different depths from the bilayer-water interface (Beck et al., 1993).

One of the most important photophysical parameters of a fluorophore is its fluorescence lifetime. Determination of the fluorescence lifetime of the DPH derivatives can provide valuable information about water penetration into the membrane, depending on the position of the fluorescent probe in the bilayer (Shinitzky and Barenholz, 1978; Cranney et al., 1983; Barrow and Lentz, 1985; Lentz, 1989; Pap et al., 1994). The basic idea is that the fluorescence lifetime of DPH is strongly influenced by the dielectric permittivity of the surrounding medium water and thus the level of hydration of the lipid bilayer (Fiorini et al., 1987, 1988; Toptygin et al., 1992; Gratton and Parasassi, 1995; Stubbs et al., 1995). The excited-state lifetime of DPH and its derivatives is found to be shorter for an increased content of water in the excited-state solvent cage.

One goal of this work is to investigate the degree of water penetration into phospholipid/cholesterol bilayers as a function of pressure. The pressure range covered was from 1 bar to 1500 bar. In addition to the disaturated phospholipid DPPC, we also studied the monounsaturated phospholipid system POPC. We examined the fluorescence lifetime of TMA-DPH and TMAP-DPH to yield information about the water penetration into different depths of the lipid bilayers. Because of the conformational complexity of the lipid bilayer assembly, we assumed a distribution of lifetimes of the fluorophore encountering different environments during its excited-state lifetime (Fiorini et al., 1987; Zolese et al., 1990). The width of the fluorescence lifetime distributions yields information on the "heterogeneity" of the membrane (Ho and Stubbs, 1992; Ho et al., 1992, 1995; Kalb et al., 1989). We have fitted the lifetime data by an unimodal Lorentzian distribution with a lifetime center τ_C and a width w. We have also chosen cholesterol concentrations in which Tang and Chong (1992) found dips in the ratio of excimer to monomer fluorescence (E/M) of the fluorophore PyrPC in dimyristoylphosphatidylcholine when plotting the E/M ratio versus PyrPC mole fraction. This phenomenon has been interpreted in terms of a regular distribution of cholesterol in a hexagonal superlattice of the lipid bilayer. Similar results where obtained for other fluorophores (Chong, 1994; Parasassi et al., 1994a,b, 1995; Tang et al., 1995).

Additional time-resolved fluorescence polarization measurements were carried out to determine the rotational diffusion rate as well as the second rank-order parameter of the fluorophore as a function of pressure and cholesterol concentration. The motion of the probe molecules TMA-DPH and TMAP-DPH was described by the wobbling-in-cone model. It is one of the simplest models that accounts for the anisotropic motion of a linear probe in a membrane bilayer. It views the probe as undergoing free rotation in a cone-shaped volume symmetrically distributed about an axis normal to the plane of the membrane bilayer (Engel and Prendergast, 1981; Stubbs et al., 1981; Schroeder et al., 1987; Lakowicz et al., 1979; Straume and Litman, 1987a,b; Lentz,

1989). The cone angle is directly correlated to the "degree of orientational constraint," i.e., to the square of the second rank-order parameter of the acyl chains in the bilayer. In these studies of time-resolved anisotropy, 30 mol% cholesterol has been incorporated into DPPC and POPC vesicles to characterize its influence on lipid conformational and dynamic properties at a cholesterol concentration at which the main phase transition of the pure phospholipid bilayer is largely abolished.

MATERIALS AND METHODS

Materials

1,2-Dipalmitoyl-sn-glycero-phosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidylcholine (POPC), from Avanti Polar Lipids (Alabaster, AL), and the cholesterol, from Sigma Chemical Co. (St. Louis, MO), were used without further purification. 1-(4-Trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and 3-(diphenylhexatrienyl)propyl-trimethylammonium (TMAP-DPH) as toluene-sulfonate salts were purchased from Molecular Probes (Eugene, OR). Lipid and cholesterol stock solutions were prepared in chloroform at concentrations of 10 mmol/liter. TMA-DPH and TMAP-DPH were dissolved in ethanol at a concentration of 1 mmol/liter.

Preparation of unilamellar vesicles

Large DPPC and POPC vesicles containing the desired amount of cholesterol (0–50 mol%) were prepared by cosonication. The solvent chloroform was removed by a flow of nitrogen gas. The remaining film was then resuspended in Tris buffer (50 mmol/liter, pH 7.4), vortexed, and sonicated for 5 min in a bath-type sonicator (Brown).

The fluorescent probes TMA-DPH and TMAP-DPH were not cosonicated with the vesicle solutions, because DPH derivatives bind rapidly to phospholipid membranes. The probe stock solution was added to the final vesicle dispersions to yield a 1:500 fluorophore-to-lipid mixture on a molecular basis. The final concentration of the lipid vesicles in the samples used for the fluorescence measurements was 0.3 mmol/liter, and that of the fluorescent probe was 0.6 μmol/liter.

Time-resolved fluorescence measurements and data analysis

Time-resolved fluorescence measurements under pressure were recorded with a high-pressure optical cell mounted in a multifrequency cross-correlation phase fluorometer. The high-pressure system, including the optical cell, inner cuvette, and pressure-generating system, was essentially identical to that described by Paladini and Weber (1981). Corrections for strain birefringence of the quartz windows of the pressure optical cell at each pressure and temperature were made by measuring the polarization of a diluted solution of glycogen in water. A mode-locked Nd-YAG laser was used as a light source (green light of 532 nm, power 1.8 W). The picosecond optical pulse train generated by this system synchronously pumped a Pyridine-1 dye laser, the output of which is cavity dumped at 3.81 MHz. For excitation in the ultraviolet, the dye laser was frequency-doubled at 345 nm. The average UV power is about 0.5 mW. For the time-resolved anisotropy measurements, Glan Thompson prisms were used to polarize the excitation and emission light. The time-resolved fluorescence intensity was recorded by orientating the emission polarizer at the magic angle of 54.7° with respect to the excitation polarizer. The operational principle of the multifrequency cross-correlation phase fluorometer has been described in detail elsewhere (Gratton and Limkemann, 1983; Gratton et al., 1984; Jameson et al., 1984; Lakowicz and Gryczynski, 1991). Phase and modulation data were collected for 9–11 modulation frequencies for both lifetime and time-resolved anisotropy measurements in the range of 4–250 MHz. All experiments were carried out at 15°C for the POPC dispersions and at 58°C for the DPPC dispersions. Data were collected using decay acquisition software from ISS (Urbana, IL) and were analyzed using Globals Unlimited software (University of Illinois at Urbana-Champaign, IL) (Alcala et al., 1987; Beechem and Gratton, 1988; Beechem, 1989; Beechem et al., 1991). The fluorescence decay of the probes was modeled using a Lorentzian distribution, and the scatter of the sample itself was described using a discrete component. The Lorentzian distribution is characterized by three parameters: the center of the distribution τ_c, the width of the distribution at half-height w, and its fractional intensity f. The discrete component was characterized by two parameters: the lifetime (fixed at 0.001 ns for the background light scattering) and its fractional intensity. For the Lorentzian distribution, the reduced χ^2 ranges from 1 to 4.

The fitting function for the time-resolved anisotropy measurements is based on the "wobble-in-cone" model, which includes a hindered rotation component (i.e., two rotational correlation times, where the second rotational correlation time is fixed at a large value (1 ms) relative to the lifetime). The wobble-in-cone angle Θ is the average amplitude of the angular distribution of the probe about an axis normal to the membrane plane (Lentz, 1989). The hindered rotation can be expressed as the residual anisotropy, r_∞, which is the lowest value of anisotropy attainable at times that are long compared to the fluorescence lifetime, and thus reflects the hindered rotational freedom of the probe in the lipid bilayer. Both r_∞ and Θ reflect the degree of constraint imposed upon the motion of the probe by its neighbouring molecules, so that the determination of the second rank-order parameter S of the fluorophore is possible (Bernsdorff et al., 1995). It has been found that the r_∞ value is very sensitive to the lipid phase state, whereas the rotational correlation time seems to be quite insensitive to lipid phase transformations. The fast decaying or kinetic component of the fluorescence anisotropy is determined by the rotational rate, R, which is calculated from the measured rotational correlation time $\phi = (6R)^{-1}$. The rotational rate is proportional to the wobbling diffusion constant of the probe in the cone. Each experiment was repeated three times on separate vesicle preparations, and the results given are the mean of three determinations. For the rotational analysis, the reduced χ^2 ranges from 2 up to maximum values of 12 for phase and modulation data with higher statistical error. The error bars of the physical quantities obtained are given in the figures. Often the error bars are within the size of the symbols.

RESULTS

Fluorescence lifetime analysis of DPPC and POPC as a function of pressure and cholesterol concentration

Pure DPPC and POPC lipid vesicles

The fluorescence lifetime of TMA-DPH and TMAP-DPH in vesicles of DPPC and POPC without and with cholesterol was measured at selected temperatures as a function of pressure up to 1500 bar. We have chosen temperatures at which the pure phospholipids are in the liquid-crystalline fluid-like state at atmospheric pressure. The results are shown in Figs. 2 and 3. Analysis of the data suggests a unimodal fluorescence lifetime distribution at all pressures studied.

The fluorescence lifetime distribution of TMA-DPH in DPPC vesicles at 58°C and ambient pressure (Fig. 2) is centered at 2.5 ns with a width of about 0.6 ns. The lifetime center τ_c increases slightly with increasing pressure up to 700 bar, suggesting a slight reduction of water penetration to the level of TMA-DPH. At pressures around 750, τ_c is found to increase markedly from 3.8 ns to about 6.0 ns. This

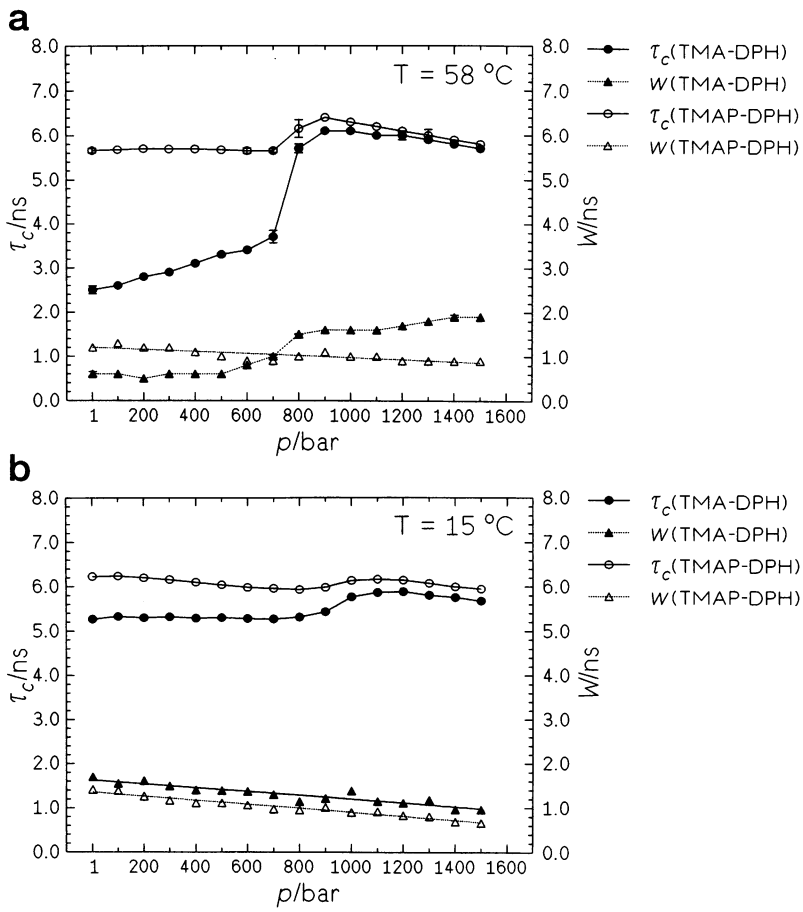

FIGURE 2 (*a*) Center τ_c and width w of the fluorescence lifetime distribution of TMA-DPH and TMAP-DPH in DPPC vesicles at T = 58°C as a function of pressure. (*b*) Center τ_c and width w of the fluorescence lifetime distribution of TMA-DPH and TMAP-DPH in POPC vesicles at T = 15°C as a function of pressure.

drastic change in the fluorescence lifetime is due to the pressure-induced fluid-to-gel phase transition in DPPC at 58°C (Winter and Pilgrim, 1989). The transition temperature at ambient pressure is T_m = 41.5°C.

Above the phase transition pressure, a slight decrease in the fluorescence lifetime is observed up to 1500 bar (τ_c(1500 bar) ≈ 5.7 ns). This decrease might be due to a slight pressure-induced vertical displacement of the probe within the lipid bilayer toward the interfacial region combined with a change in the rotational mode (see below). It cannot be excluded, however, that such a small change in τ_c could also be due to an imperfect correction of the pressure-induced window birefringence.

In contrast to TMA-DPH, the lifetime center of TMAP-DPH in the liquid-crystalline state of DPPC vesicles has much higher values (τ_c ≈ 5.7 ns), whereas above the pressure-induced fluid-to-gel phase transition, the τ_c of both probes reached similar values. The change in τ_c(TMAP-DPH) at the main transition of DPPC is thus less pronounced than that of τ_c(TMA-DPH). τ_c of TMAP-DPH remains constant with increasing pressure in the fluid phase of DPPC, suggesting that the dielectric permittivity does not change at this depth of the lipid bilayer upon pressurization.

The width w of the fluorescence lifetime distribution of TMA-DPH also shows a detectable change at the pressure-induced phase transition. It increases from 0.6 ns to 1.8 ns, which can be interpreted in terms of an increase in the environmental heterogeneity of the probe. In contrast, the width w(TMAP-DPH) remains almost constant (w ≈ 1 ns) over the whole pressure range. The heterogeneity of the environment of this probe seems to be independent of the physical state of the lipid bilayer.

The corresponding values of the fluorescence lifetime center and width for both probes embedded in POPC vesicles are shown in Fig. 2 *b*. The marked difference with respect to the DPPC data is the resemblance between the TMA-DPH and TMAP-DPH data in both phases of POPC. Whereas τ_c(TMA-DPH) in DPPC exhibits a sharp increase at the fluid-to-gel phase transition, the change in τ_c at the corresponding pressure-induced phase transition of POPC, which is known to occur at p_m ≈ 950 bar at 15°C (see neutron diffraction results of Winter and Pilgrim, 1989), is markedly less pronounced and relatively broad. τ_c(TMA-DPH) in POPC increases from 5.3 ns in the liquid-crystalline state to 5.9 ns in the gel state, and pressure has only a minor effect on the fluorescence lifetime.

The significant difference in fluorescence lifetime of the two fluorescence probes in the liquid-crystalline state of the saturated lipid DPPC diminishes drastically in the mono-*cis*-unsaturated lipid POPC. The τ_c(TMA-DPH) value in the fluid-like phase of POPC at 15°C is much greater compared to DPPC at 58°C. This suggests a lower value for the water

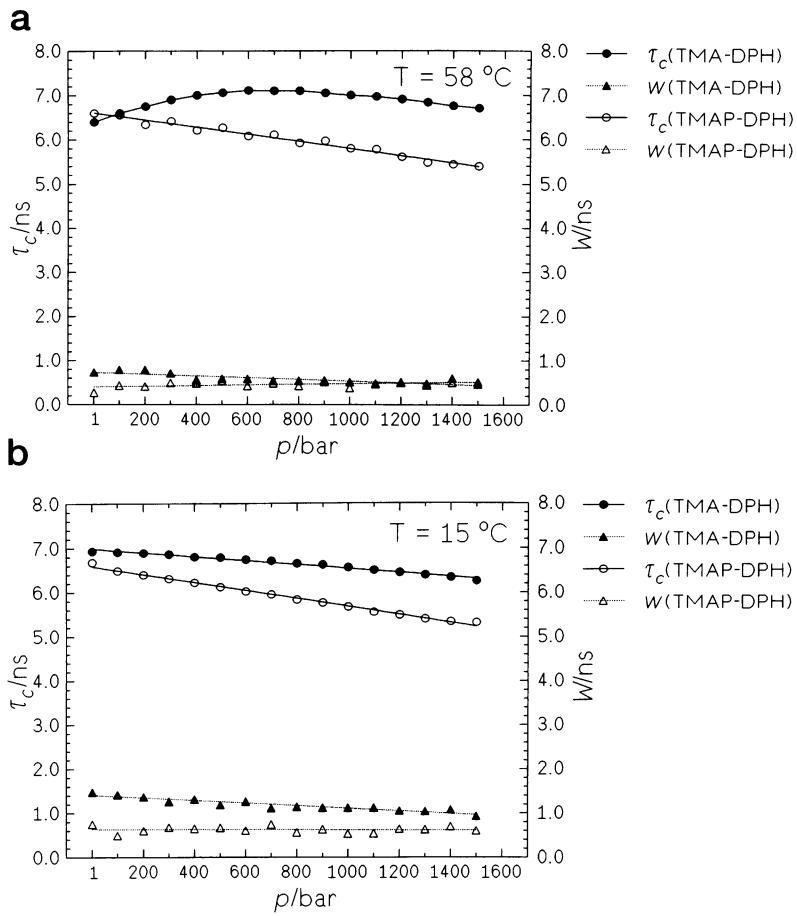

FIGURE 3 (*a*) Center τ_c and width w of the fluorescence lifetime distribution of TMA-DPH and TMAP-DPH in DPPC/30 mol% cholesterol vesicles at $T = 58°C$ as a function of pressure. (*b*) Center τ_c and width w of the fluorescence lifetime distribution of TMA-DPH and TMAP-DPH in POPC/30 mol% cholesterol vesicles at $T = 15°C$ as a function of pressure.

penetration in the fluid phase of the POPC bilayer. This is indeed confirmed by measurements of the steady-state fluorescence polarization r_{ss}, where values of 0.22 and 0.16 are found for POPC and DPPC, respectively.

The width of the fluorescence lifetime distribution of TMA-DPH is similar to that of TMAP-DPH in POPC. It decreases slightly with pressure and does not change in passing the main transition, contrary to what has been observed in the case of DPPC vesicles.

The effect of incorporating 30 mol% cholesterol into DPPC and POPC lipid vesicles

The effect of the incorporation of 30 mol% cholesterol into DPPC and POPC vesicles is displayed in Fig. 3. The lifetime of TMA-DPH in the system DPPC/30 mol% cholesterol (Fig. 3 *a*) is centered at 6.4 ns at 1 bar with a width of 0.6 ns, i.e., the incorporation of cholesterol leads to an increase in τ_c(TMA-DPH) of 4 ns, at similar width. Upon pressurization, τ_c(TMA-DPH) changes only slightly. It increases and reaches a plateau value of 7.2 ns around 600–900 bar, then decays slightly again to 6.7 ns at 1500 bar.

In contrast, τ_c of the probe TMAP-DPH increases only slightly by adding 30 mol% of the sterol, and decreases linearly from 6.6 ns at 1 bar to 5.4 ns at 1500 bar, i.e., τ_c(TMAP-DPH) is generally lower than τ_c(TMA-DPH) in the system DPPC/30 mol% cholesterol at higher pressures. The corresponding widths are similar (0.4–0.6 ns) and are almost pressure independent.

A similar behavior is observed for POPC/30 mol% cholesterol (Fig. 3 *b*): the significant enhancement of τ_c of TMA-DPH by the addition of 30 mol% cholesterol to POPC. τ_c(TMA-DPH) decreases linearly with increasing pressure and reaches τ_c values that are close to those in DPPC/30 mol% cholesterol vesicles.

In contrast to TMA-DPH, τ_c and w of TMAP-DPH change only slightly with the addition of 30 mol% cholesterol. After a slight increase in τ_c with the addition of the sterol, the τ_c value decreases from 6.6 ns at 1 bar to 5.2 ns at 1500 bar, with a constant width of about 0.6 ns.

The widths w(TMA-DPH) are slightly higher ($w \approx 1.1$ ns) in POPC/30 mol% cholesterol than in DPPC/30 mol% cholesterol ($w \approx 0.6$ ns), suggesting a more heterogeneous distribution of the fluorophore in the POPC/30 mol% cholesterol mixture.

Fluorescence lifetime of TMA-DPH in DPPC and POPC vesicles at 1 and 1000 bar as a function of cholesterol concentration (up to 50 mol%)

In Fig. 4 the center of the Lorentzian distribution of the fluorescence lifetime of TMA-DPH in DPPC vesicles at $T = 58°C$ is plotted as a function of cholesterol concentra-

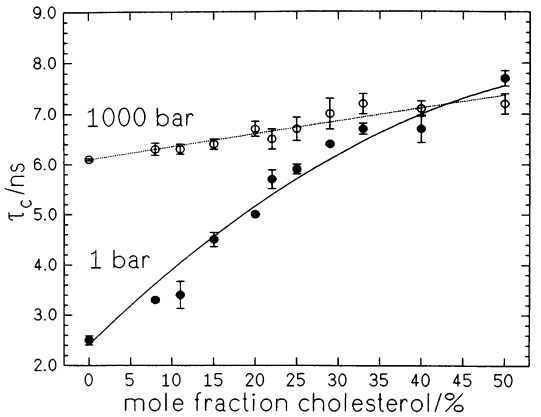

FIGURE 4 Fluorescence lifetime center τ_c of TMA-DPH in DPPC vesicles as a function of cholesterol concentration at 1 bar and 1000 bar, respectively (T = 58°C).

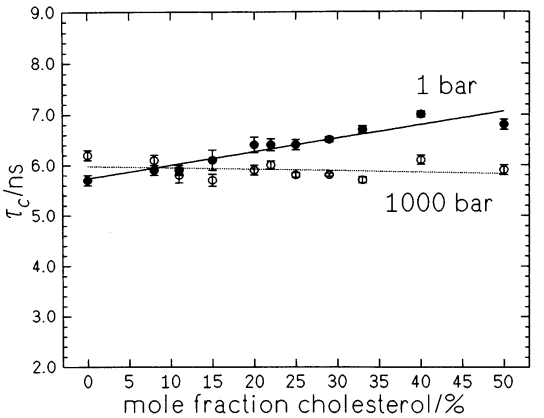

FIGURE 5 Fluorescence lifetime center τ_c of TMAP-DPH in DPPC vesicles as a function of cholesterol concentration at 1 bar and 1000 bar, respectively (T = 58°C).

tion, at 1 bar and 1000 bar, respectively. Whereas at ambient pressure DPPC is in the liquid-crystalline state, DPPC at 1000 bar is in the pressure-induced gel state (the transition pressure of pure DPPC at 58°C is p_m(58°C) ≈ 750 bar).

The slope $\tau_c(x_{chol})$ is different for both physical states of the lipid bilayer. The τ_c values are relatively low at 1 bar in pure DPPC vesicles (τ_c = 2.5 ns) and increase up to 7.7 ns for a cholesterol concentration of 50 mol% in DPPC. In the pressure-induced gel state of DPPC, at 1000 bar, the fluorescence lifetime depends only modestly on cholesterol concentration. A small linear increase in $\tau_c(x_{chol})$ is observed at 1000 bar only. In pure DPPC vesicles, τ_c at 1000 bar is 6.1 ns, i.e., τ_c is 3.6 ns higher than the corresponding value at 1 bar. Increasing cholesterol concentration leads to τ_c values that are similar for both pressures. Cholesterol concentrations greater than about 30 mol% lead to similar effects on the rise of τ_c as the transformation to the gel state of the lipid bilayer, and even higher τ_c values can be found at higher cholesterol levels.

In Fig. 5 the corresponding $\tau_c(x_{chol})$ values of TMAP-DPH are given. For 1 bar, the addition of cholesterol leads to a slight increase in τ_c only. It increases from 5.7 ns for pure DPPC to about 7 ns at 50 mol% cholesterol. At 1000 bar, τ_c values remain independent of cholesterol concentration within the accuracy of the experiment.

Figs. 6 and 7 exhibit the corresponding graphs for the two fluorophores in the system POPC/cholesterol at T = 15°C. The pressure-induced liquid-crystalline/gel state transition of pure POPC at 15°C occurs at 950 bar. As is clearly visible, the differences in τ_c values between the two probes are smaller compared to those in the system DPPC/cholesterol. At 1 bar an increase in τ_c of TMA-DPH from 5.3 to 8.2 ns with the addition of 50 mol% cholesterol is observed. The slope of $\tau_c(x_{chol})$ is less pronounced at 1000 bar—it increases from 5.8 to 7.2 ns.

The τ_c values for the probe TMAP-DPH in POPC vesicles do not vary much as a function of cholesterol concentration.

Hindered rotation analysis of the time-resolved anisotropy data of TMA-DPH and TMAP-DPH in DPPC and POPC lipid vesicles without and with 30 mol% cholesterol as a function of pressure

In Figs. 8-11 the rotational correlation time ϕ of TMA-DPH, including its fractional amplitude f in DPPC and POPC vesicles without and with 30 mol% cholesterol, is shown as a function of pressure (from 1 to 1000 bar). Because of the constraints of motional freedom of fluorophores embedded in a lipid bilayer, we have analyzed the time-resolved anisotropy data with a hindered rotation fit routine, which is based on the "wobble-in-cone" model. The fluorescence anisotropy has been analyzed in terms of the parameters r_o, ϕ, f, r_∞, and Θ, where r_o is the anisotropy at time 0, ϕ is the rotational correlation time of the fluorophore, f is its fractional amplitude, r_∞ is the residual anisotropy, and Θ is the average wobble-in-cone angle. The mean second rank-order parameter $S = \langle P_2 \rangle$ of the fluorescent

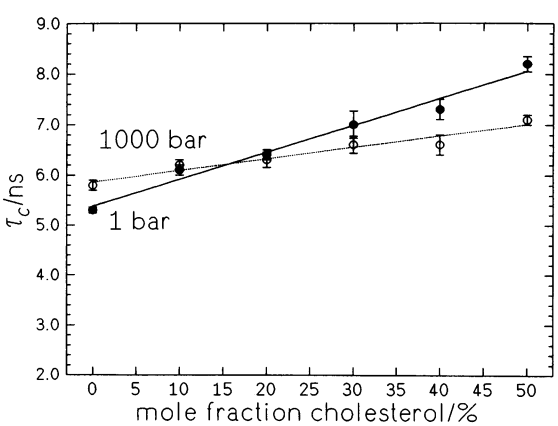

FIGURE 6 Fluorescence lifetime center τ_c of TMA-DPH in POPC vesicles as a function of cholesterol concentration at 1 bar and 1000 bar (T = 15°C).

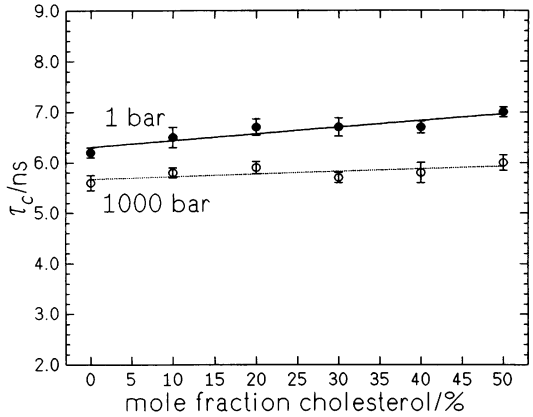

FIGURE 7 Fluorescence lifetime center τ_c of TMAP-DPH in POPC vesicles as a function of cholesterol concentration at 1 bar and 1000 bar (T = 15°C).

probe in the bilayer has been calculated from the residual and initial anisotropy values ($S = (r_\infty/r_o)^{1/2}$).

The residual anisotropy values r_∞ of TMA-DPH and TMAP-DPH in DPPC at 58°C are similar: approximately 0.1 (S = 0.5, Θ = 36°) in the liquid-crystalline state at 1 bar; they increase to about 0.13 (S = 0.6, Θ = 32 °) upon pressurization to 700 bar, and reach a value in the pressure-induced gel state of about 0.25 (S = 0.8, Θ = 21°).

The residual anisotropy values rise considerably with the addition of 30 mol% cholesterol, and increase only modestly with pressure. r_∞ ranges from 0.18 (S = 0.68, Θ = 27°) at 1 bar to about 0.24 (S = 0.78, Θ = 23°) at 1000 bar.

The residual anisotropy and thus the order parameter of TMA-DPH in the fluid phase of POPC at 15°C is significantly larger: r_∞ = 0.17 (S = 0.66, Θ = 29 °) at 1 bar, increasing to r_∞ = 0.21 (S = 0.74, Θ = 26 °) at 1000 bar. Only slightly smaller values are observed for TMAP-DPH as compared to TMA-DPH. Similar results have been obtained from measurements of r_{ss}, which is proportional to r_∞. The addition of 30 mol% cholesterol leads to an increase of r_∞ and S values from r_∞ = 0.17 to r_∞ = 0.25 (S = 0.66 to S = 0.8), which do not vary significantly with pressure.

The rotational correlation time ϕ and its fractional amplitude f of TMA-DPH and TMAP-DPH in DPPC vesicles are shown in Fig. 8. ϕ(TMA-DPH) values range from 0.6 to 0.8 ns in the liquid-crystalline phase of DPPC and decrease by ~0.4 ns at the pressure-induced fluid-to-gel phase transition around 750 bar, i.e., the rate of rotational wobbling motion R first slightly decreases with pressure and then markedly increases in going to the gel state of the DPPC bilayer.

The corresponding data of ϕ(TMAP-DPH) are found to exhibit a similar behavior. No significance should be given to the slight difference in ϕ values of both probes ($\Delta\phi \approx 0.1$ ns), because experimental errors could account for such small deviations.

The rotational correlation time and its fractional amplitude of both fluorescence probes in POPC vesicles at 15°C are depicted in Fig. 9. The ϕ values are much higher and continuously and drastically decrease upon pressurization in the fluid phase up to 950 bar, where the fluid-to-gel transition sets in. Compared to DPPC vesicles, we find a larger difference in the rotational correlation times between the two probes. ϕ(TMAP-DPH) is about 0.3 ns smaller than ϕ(TMA-DPH) over the whole pressure range covered. The pressure dependences of ϕ(TMAP-DPH) and ϕ(TMA-DPH) are similar, however. ϕ(TMAP-DPH) decreases almost linearly from 1.0 ns at 1 bar to about 0.5 ns at 1000 bar, whereas ϕ(TMA-DPH) decreases from about 1.26 ns to 0.8 ns in the same pressure range. The corresponding fractional amplitudes decrease slightly with increasing pressure, for instance from 55% to 45% for TMA-DPH.

Compared to DPPC in the fluid phase, the ϕ values of the two fluorophores in POPC are drastically greater, especially in the case of TMA-DPH, and decrease with increasing pressure, whereas the slope of $\phi(p)$ is slightly positive in the fluid phase of DPPC. The larger ϕ values in POPC might be due mainly to the much lower temperature.

Results of the hindered rotation analysis of the systems DPPC/30 mol% cholesterol and POPC/30 mol% cholesterol are shown in Figs. 10 and 11, respectively. Incorporation of 30 mol% cholesterol into the DPPC bilayer results in a decrease in the rotational correlation time of TMA-DPH from 0.63 ns to 0.47 ns at ambient pressure, and the fraction of these rapidly diffusing fluorophores is much smaller, decreasing from 75% to ~50%. The effect of increased pressures in this system is a slight and steady decrease in ϕ and f, from 0.47 ns (f = 0.53) at 1 bar to 0.34 ns (f = 0.39) at 1000 bar. The faster rotational motion of the fluorophore in the 30 mol% cholesterol-containing sample might be due to the smaller average angle of wobble ($\Delta\phi$ = 9° at 1 bar), which can be inferred from the increase of S from 0.48 to 0.68 at 1 bar, and from 0.59 to 0.77 at 700 bar by the incorporation of 30 mol% cholesterol.

The data for TMAP-DPH in DPPC/30 mol% cholesterol behave similarly, but are slightly smaller.

The addition of 30 mol% cholesterol to POPC at 15°C leads to an even greater reduction of ϕ(TMA-DPH) values, from 1.26 ns to 0.53 ns at ambient pressure, and from 0.8 ns to 0.26 ns at 1000 bar. Compared to the pure phospholipid, the slope of $\phi(p)$ is much smaller, however.

f(POPC) values are about 0.1 greater than the corresponding f values of POPC/30 mol% cholesterol, and the fractional amplitudes of both fluorophores remain almost constant over the whole pressure range.

DISCUSSION

We have used the DPH derivatives TMA-DPH and TMAP-DPH to yield information about the hydrodynamic and dielectric properties of DPPC and POPC at different depths in the bilayer as a function of cholesterol concentration and pressure. The behavior of the fluorescence lifetime and the rotational rate of these probes under pressure allow us to

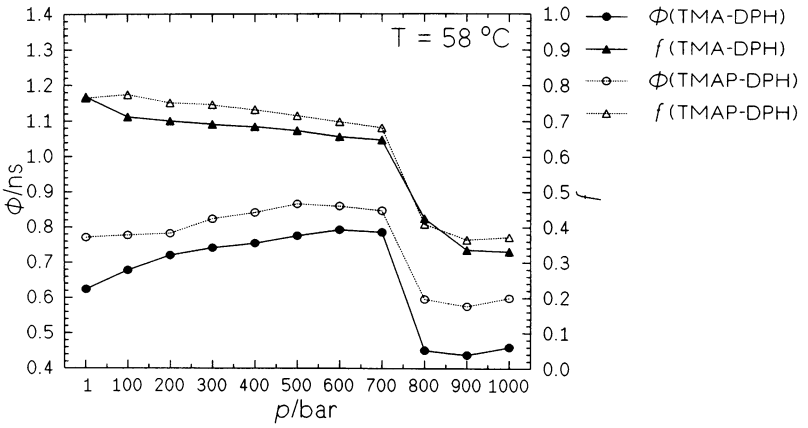

FIGURE 8 Rotational correlation time ϕ and its fractional amplitude f of TMA-DPH and TMAP-DPH in DPPC vesicles as a function of pressure (T = 58°C).

determine the changes in water penetration and the rotational dynamics combined with the free volume and packing that occur in the membrane bilayer as a function of bilayer depth.

The fluorescence lifetime distribution of TMA-DPH, which partitions in the water-lipid interface, is very sensitive to the main phase transition of the saturated phosphatidylcholine DPPC. As the DPPC bilayer transforms from the liquid-crystalline to the gel state upon pressurization, the level of water penetration at the water/lipid interface undergoes dramatic changes (Fig. 2). The fluorescence lifetime in the liquid-crystalline state of DPPC is significantly shorter, suggesting that greater static and/or dynamic molecular disorder is present in the liquid-crystalline phase of the bilayer, which increases the probability of water penetration into the bilayer system. This sharp increase in the TMA-DPH fluorescence lifetime center is accompanied by a broadening of the respective lifetime distribution, which is due to an environmental heterogeneity of the fluorophore excited-state environment in the pressure-induced gel state, and might be explained by packing defects.

Contrary to TMA-DPH, the probe TMAP-DPH, which is localized deeper in the hydrophobic core of the lipid bilayer, is much less sensitive to the liquid-crystalline/gel phase transition of DPPC (Fig. 2). TMA-DPH is more likely to encounter water molecules than is TMAP-DPH. In terms of environmental heterogeneity, expressed by the width of the fluorescence lifetime distribution, TMAP-DPH seems to have a more homogeneous microenvironment in the pressure-induced gel state of DPPC, resulting in a narrower lifetime distribution as compared to that of TMA-DPH. The width of the TMAP-DPH lifetime distribution is constant over the whole pressure range measured, i.e., the environmental heterogeneity of the fluorophore in the upper hydrophobic part of the bilayer does not change upon pressurization.

As compared to DPPC, in the mono-*cis*-unsaturated POPC an increase in the fluorescence lifetime of both probes in the fluid-like state of the lipid bilayer is observed, which leads to a small change in passing the main transition. The much greater τ_c value in the fluid-like phase of POPC might be due to distinctly less water penetration into the POPC membrane, which was unexpected. Our suggestion is that in POPC the acyl chains take on compact and relatively inflexible configurations. Indeed, it has been pointed out by Seelig (1977) that when DPPC and POPC are compared at 19°C above their respective main phase transition temperature (T_m(DPPC) = 41.5°C and T_m (POPC) = −5°C), the ^{2}H-NMR-derived order parameter profiles for various labeled carbon atoms along the chain were accordingly found to be parallel, except for the region between carbon atoms 4 and 10 (near the *cis* double bond of POPC), and under this

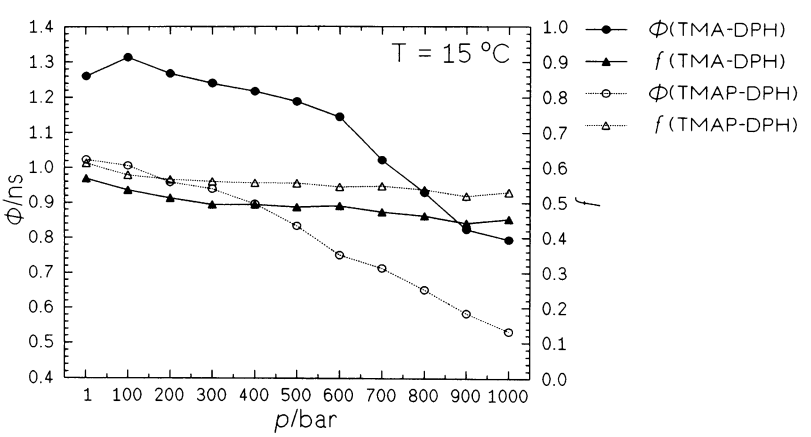

FIGURE 9 Rotational correlation time ϕ and its fractional amplitude f of TMA-DPH and TMAP-DPH in POPC vesicles as a function of pressure (T = 15°C).

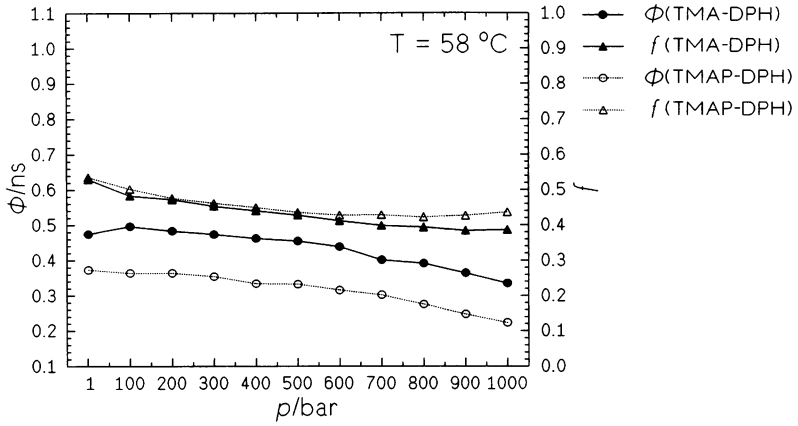

FIGURE 10 Rotational correlation time ϕ and its fractional amplitude f of TMA-DPH and TMAP-DPH in DPPC/30 mol% cholesterol vesicles as a function of pressure (T = 58°C).

condition of comparison the unsaturated system was the more ordered. This suggests a local rigidity caused by the *cis* double bond, which reduces the motional freedom of the CH_2 segments of neighboring chains. The results are also in agreement with recent measurements of the hydrophobicity profiles across phospholipid-cholesterol bilayer membranes using a series of stearic acid spin labels (Subczynski et al., 1994), showing that the introduction of a double bond at C9-C10 of the acyl chain decreases the level of water penetration at all locations in the membrane. Fluorescence lifetime distribution analysis of TMAP-DPH embedded in POPC vesicles has shown that TMAP-DPH is almost indistinguishable from TMA-DPH with regard to the lifetime center and the distributional width (Fig. 2 *b*). This shows that in POPC there is no remarkable change in water penetration and in environmental heterogeneity at different membrane depths. The lifetime data in POPC are also almost independent of pressure. This demonstrates that the dielectric permittivity in the interfacial and hydrophobic acyl chain region is almost constant up to pressures of 1500 bar.

With increasing cholesterol content, phase separation into cholesterol-rich and cholesterol-poor domains might occur, and thus phase boundaries might be crossed as a function of cholesterol concentration for the temperatures and pressures studied. However, the phase equilibria and the molecular organization of cholesterol in DPPC and other phospholipids are still a matter of debate (see, e.g., Mabrey et al., 1978; Ipsen et al., 1987, 1990; Shin and Freed, 1989; Sankaram and Thompson, 1990; Vist and Davis, 1990; Huang et al., 1993; Finegold, 1993; McMullen et al., 1994, 1995; Virtanen et al., 1995; Tang and Chong, 1992; Tang et al., 1995; Parasassi et al., 1995; Mukherjee and Chattopadhyay, 1996). Several reports have suggested the formation of specific phospholipid-cholesterol complexes. Other studies revealed no complex formation, and still others proposed regular hexagonal superlattice structures at certain sterol concentrations (e.g., at 28, 33, and 50 mol%). Fluorescence lifetime and depolarization data do not seem to be very sensitive to the detection of phase boundaries, so that we will not focus on this point here.

The incorporation of cholesterol into the fluid DPPC membrane at 58°C drastically reduces the dynamic quenching process of TMA-DPH by reducing the probability of water penetration into the bilayer, leading to ~2.5-fold longer fluorescence lifetimes, which are even longer than those found for TMA-DPH in the gel state of the pure DPPC bilayer (Fig. 3 *a*). Indeed, cholesterol has long been recognized to decrease water penetration through phospholipid bilayers (see, e.g., Casal, 1989; Simon et al., 1982; Straume and Litman, 1987a,b). In the pressure-induced gel state of DPPC, a narrowing of the width by the addition of choles-

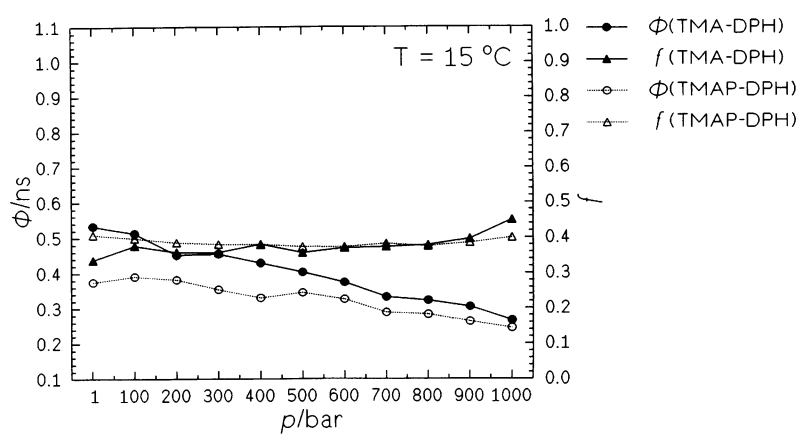

FIGURE 11 Rotational correlation time ϕ and its fractional amplitude f of TMA-DPH and TMAP-DPH in POPC/30 mol% vesicles as a function of pressure (T = 15°C).

terol is observed. Similar results have been found for the temperature-induced gel state of DPPC (Bernsdorff et al., 1996). A homogenizing effect of cholesterol on the lipid bilayer has been reported before (Kalb et al., 1989; Stubbs et al., 1995).

A markedly less pronounced effect of cholesterol on τ_c and thus the hydrophobicity of the membrane is observed for the POPC/cholesterol mixture. The τ_c values are similar for the two probes in the POPC/cholesterol mixture. The width of TMA-DPH in POPC/30 mol% cholesterol is twofold broader than in DPPC/30 mol% cholesterol, indicating a higher environmental heterogeneity in the interfacial region of the *cis*-monounsaturated phospholipid mixture. In contrast, w(TMAP-DPH) in POPC/30 mol% cholesterol is smaller than and indistinguishable from the corresponding values in DPPC/30 mol% cholesterol.

In general, the addition of cholesterol at levels up to 50 mol% to DPPC at 58°C has a greater effect on the increase in hydrophobicity in the interfacial region of the bilayer than the application of hydrostatic pressure of several hundred to 1000 bar (Fig. 4). The change is much less significant in the inner chain region, the region detected by the probe TMAP-DPH (Fig. 5). The same trend is observed in the corresponding system POPC/30 mol% cholesterol (Figs. 6 and 7). The τ_c(TMA-DPH) values are more pressure dependent in the low-pressure regime than τ_c(TMAP-DPH), whereas at 1000 bar this difference in behavior of the two probes vanishes.

For the system DPPC/cholesterol, we observe a nonmonotonic behavior of the fluorescence lifetime as a function of cholesterol concentration. However, we do not want to discuss these differences in terms of possible phase separation phenomena or superlattice theories here, as these changes observed in the fluorescence lifetime as a function of cholesterol concentration are rather small (see Fig. 12). They might be significant, however, as shown by the persistence of the lifetime changes as the pressure is changed.

In addition to the fluorescence lifetime studies, we have investigated the rotational dynamics of the two fluorescence probes embedded in DPPC and POPC lipid bilayers and their mixtures with 30 mol% cholesterol to determine their rotational correlation time ϕ (reciprocal to the rotational rate) as well as their motional freedom within the bilayer (expressed by the order parameter S) at different depths as a function of pressure (Figs. 8–11).

Increased hydrostatic pressure has a progressive ordering effect upon the environment of the probe and thus the order parameter of the lipid acyl chains, which is due to the reduction in free volume of the bilayer under pressure and a reduction in the cross-sectional area per lipid molecule. The bulk compressibility of DPPC multilamellar vesicles is 5.6×10^{-5} bar^{-1} at 50°C and ambient pressure. As the bilayer thickness increases with pressure (Winter and Pilgrim, 1989), the lateral compressibility of the bilayer plays the dominant role in pressure-mediated effects. Pressurization in the fluid phase of DPPC at 58°C leads to a 40% increase in the average order parameter of the fluorescence probes per kilobar. The corresponding value of S(POPC) at 15°C is ~10%/kbar. S-values rise considerably with the

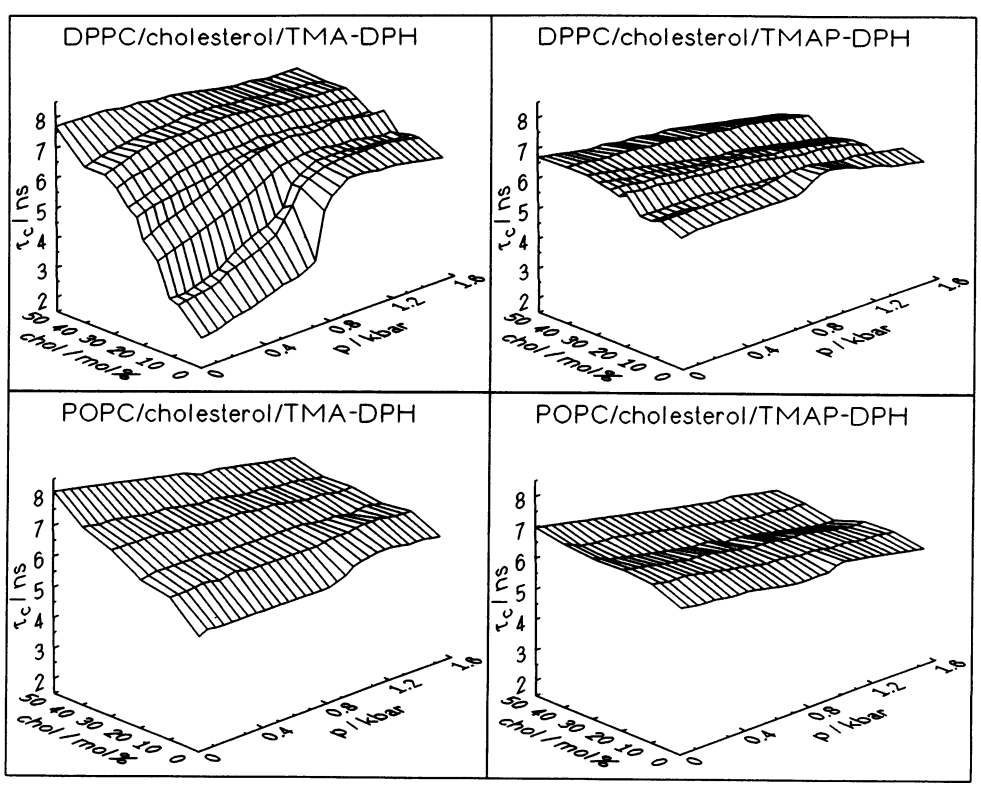

FIGURE 12 Fluorescence lifetime center τ_c of TMA-DPH and TMAP-DPH in DPPC and POPC vesicles as a function of cholesterol concentration and pressure at T = 58°C and T = 15°C, respectively.

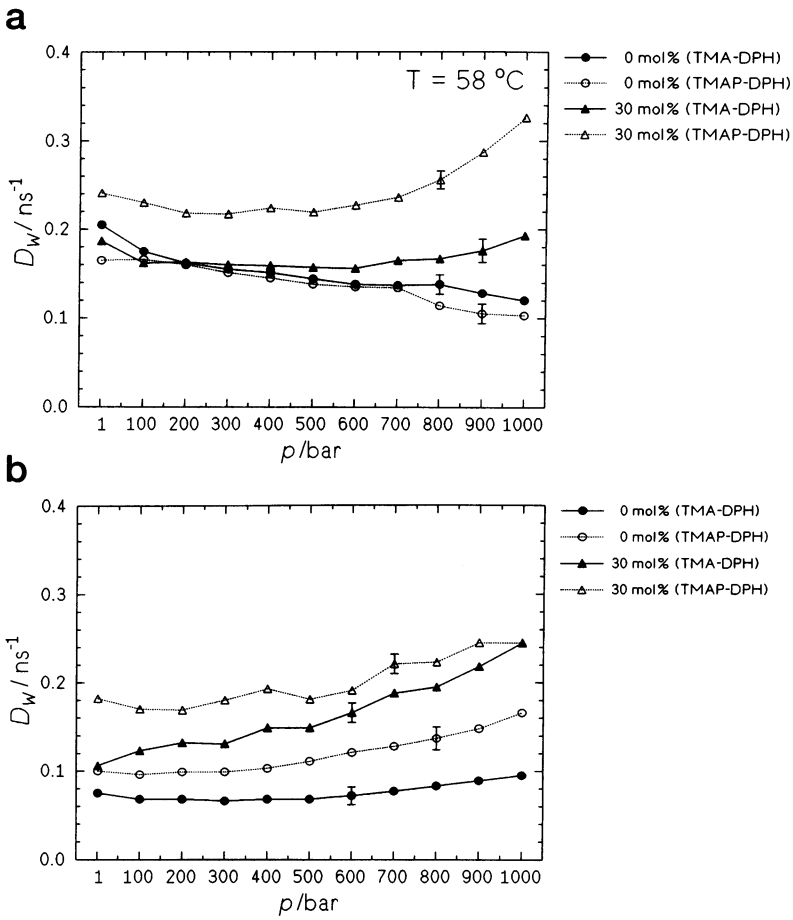

FIGURE 13 Pressure dependence of the wobbling diffusion coefficient D_w of TMA-DPH and TMAP-DPH in (*a*) DPPC and DPPC/30 mol% cholesterol and (*b*) POPC and POPC/30 mol% cholesterol vesicles at 58°C and 15°C, respectively.

addition of 30 mol% cholesterol (30% for DPPC and 19% for POPC), and increase only modestly with pressure.

We find an increase in the rotational rate of the fluorophores in passing the pressure-induced gel phase of DPPC, contrary to the behavior observed when the temperature is lowered at ambient pressure (Bernsdorff et al., 1995). At first glance this seems to contradict the ordering effect of pressure described above. It is possible, however, that the pressure-induced constraint imposed upon the rotational wobbling amplitude of the fluorophores may lead to an increase in the rate of motion within the restricted cone, if the wobble angle, in which the probe can rotate on the nanosecond time scale, decreases upon pressurization. A decrease in the cone angle has also been proposed for the incorporation of cholesterol into DPPC and DMPC membranes by Pasenkiewcz et al. (1990). Alternative explanations may also account for this effect, however. The probe molecules (in particular TMAP-DPH) may take a different conformation or may come to occupy a slightly different site upon pressurization, one in which rotational motion is less hindered. The latter should be accompanied by a decrease in r_∞, however. The effect could also be explained by changes in the rotational characteristics of the probe. This consideration led us to the concept of a "sticking" to "slipping" change in the rotational mode (Hu and Zwanzig, 1974), which has already been discussed before (Chong and Cossins, 1983; Chong et al., 1985). Under sticking boundary conditions (at low pressures), the rotations of the probe are damped because of strong interactions with neighboring molecules, such as with solvent molecules, or the fluorophore molecules become entangled with neighboring lipid chain molecules. The rotational mode thus depends on the strength of the hydrogen bond network within the interfacial region of the bilayer and on the molecular order parameter of the acyl chains. Under slipping boundary conditions (at higher pressures), the rotations could occur with less frictional force and therefore be faster, as pressure squeezes water out of the interfacial region of the lipid bilayer (Reis et al., 1996), and the fluorescence probe becomes progressively more aligned by the more ordered hydrocarbon chains. A better measure of the degree of the rotational rate might be the average wobbling diffusion constant D_w of these rodlike fluorescence probes, which is approximately given by $D_w = R(r_o - r_\infty)/r_o$ (Wieb van der Meer et al., 1986). The pressure dependence of D_w is depicted in Fig. 13. Contrary to ϕ or R, D_w does not change significantly when passing the pressure-induced liquid-crystalline/gel transition. As expected, D_w of TMA-DPH and TMAP-DPH slightly decreases with increasing pressure.

In contrast to DPPC, the rotational rates of the two probes in POPC vesicles at 15°C show no abrupt change at the pressure-induced liquid-crystalline/gel phase transforma-

tion. R increases (wobble angle decreases) almost continuously upon pressurization, with little change in the fractional amplitude (Fig. 9). This difference in behavior might be due to the *cis* double bond in the sn-2 oleoyl acyl chain of POPC, which causes significant conformational disorder, even in the L_β gel phase of the bilayer (Winter and Pilgrim, 1989), thus leading to a minor change in the rotational mode at the main transition only. Comparing the two fluorescence probes in POPC at each pressure measured, we note that the rotational correlation time ϕ of TMA-DPH is about 0.3 ns higher than that of TMAP-DPH. By contrast, DPPC bilayers do not show as great a difference in the ϕ values of the two fluorophores (Fig. 8). The corresponding D_w values at 1 bar are about 0.07 ns^{-1} for TMA-DPH and 0.1 ns^{-1} for TMAP-DPH, respectively, and D_w increases slightly with increasing pressure, particularly above about 500 bar (Fig. 13 *b*).

The incorporation of 30 mol% cholesterol leads to an adaptation not only of the dielectric, but also of the hydrodynamic properties of both lipid systems. Both the rotational correlation time and the fractional amplitude of TMA-DPH and TMAP-DPH and their pressure dependences are similar in DPPC/30 mol% cholesterol and POPC/30 mol% cholesterol vesicles, as shown in Figs. 10 and 11. These results clearly demonstrate the ability of cholesterol to also adjust the dynamic properties of biomembranes composed of different phospholipids.

By comparison with the pure phospholipid vesicles, the rotational correlation time is found to be smaller in the phospholipid/cholesterol systems, and ϕ decreases only slightly with increasing pressure. A change in the rotational mode in response to the pressure perturbation occurs to a much lesser extent. The wobbling diffusion constant of TMA-DPH in DPPC at 58°C (Fig. 13 *a*) does not change significantly upon the addition of 30 mol% cholesterol in the lower pressure range. D_w increases slightly at higher pressures, however, particularly for TMAP-DPH above about 600 bar. This effect is even more pronounced in the case of POPC/30 mol% cholesterol, where D_w increases continuously with increasing pressure (Fig. 13 *b*). The increase in D_w with increasing pressure can be explained by the effects mentioned above. Pressure-induced changes in the phase state of the lipid mixture might be invoked as well.

These results clearly demonstrate the ability of sterols to efficiently regulate lipid conformation, motional freedom, and hydrophobicity of membranes, so that they can withstand even drastic changes in environmental conditions, such as high external hydrostatic pressure.

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