Fluorescence lifetime distributions of diphenylhexatriene-labeled phosphatidylcholine as a tool for the study of phospholipid-cholesterol interactions

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ABSTRACT Fluorescence lifetimes of 1-palmitoyl-2-diphenylhexatrienylpropionyl-phosphatidylcholine in vesicles of palmitoyloleoyl phosphatidylcholine (POPC) (1:300, mol/mol) in the liquid crystalline state were determined by multifrequency phase fluorometry. On the basis of statistic criteria (χ^2_{red}) the measured phase angles and demodulation factors were equally well fitted to unimodal Lorentzian, Gaussian, or uniform lifetime distributions. No improvement in χ^2_{red} could be observed if the experimental data were fitted to bimodal Lorentzian distributions or a double exponential decay. The unimodal Lorentzian lifetime distribution was characterized by a lifetime center of 6.87 ns and a full width at half maximum of 0.57 ns. Increasing amounts of cholesterol in the phospholipid vesicles (0–50 mol% relative to POPC) led to a slight increase of the lifetime center (7.58 ns at 50 mol% sterol) and reduced significantly the distributional width (0.14 ns at 50 mol% sterol). Lifetime distributions of POPC-cholesterol mixtures containing >20 mol% sterol were within the resolution limit and could not be distinguished from monoexponential decays on the basis of χ^2_{red}. Cholesterol stabilizes and rigidifies phospholipid bilayers in the fluid state. Considering its effect on lifetime distributions of fluorescent phsopholipids it may also act as a membrane homogenizer.

INTRODUCTION

During the past years an increasing number of articles have been published that deal with the analysis of ground-state fluorophor lifetime heterogeneity. Complex fluorescence decays may either be the result of the emission of a mixture of fluorophores, e.g., in solution, or of a single fluorophore encountering different environments during its excited state lifetime (1). The latter situation is highly relevant for studies of biological systems, e.g., tryptophans in proteins (2, 3) or lipid labels such as parinaric acid (4–6) and parinaroyl phospholipids (7) or diphenylhexatriehylpropionyl (DPH) (8, 9) and DPH-phospholipids (10) in membranes. DPH has gained much attention as it might be considered a suitable fluorophor for lifetime studies on membranes with regard to its photophysics. In homogeneous solution, its fluorescence decay is monoexponential. Furthermore, its lifetime depends on the dielectric constant of the medium (11) and it does not undergo solvent-dependent excited state reactions. Therefore, its lifetime heterogeneity may serve as an indicator for the heterogeneity of a more complex system, e.g., membranes, whatever effects might contribute, apart from dielectric properties, to the observed fluorescence decay (see Discussion). Recent studies on fluorescence lifetimes of DPH in artificial and biological membranes showed that the presence of cholesterol leads to a narrowing of lifetime distributions of DPH (12, 13) or DPH-labeled choline phospholipids (14). On the other hand, it was shown that the DPH lifetime distributions became more heterogeneous if the phospholipid and/or protein content of a membrane are more complex, e.g., going from a simple one-component phospholipid vesicle to a biomembrane (15). However, it has to be stressed in this context that DPH as a membrane probe has some essential drawbacks. Its orientation as well as its localization in a phospholipid bilayer above T_c, the gel to liquid phase transition temperature, is not well defined (16, 17). Thus, this uncertainty may give rise to some undesired contributions to lifetime heterogeneity, making interpretation of results very difficult. A much more appropriate probe in this respect is DPH-labeled phosphatidylcholine (DPH-PC) (18). The lifetime properties of this label in vesicles of saturated phospholipids was characterized by Cranney et al. (19) and Parente and Lentz (10) in terms of mono- or biexponential decays depending on the fluidity state of the bilayer. The orientation and localization of DPH-PC in a bilayer is well-defined insofar as it is a phospholipid and, therefore, it should monitor membrane biophysical properties much better as compared with the totally apolar DPH.

Here we present a study on the fluorescence lifetime heterogeneity of DPH-PC in fluid bilayers of palmitoyloleoylphosphatidylcholine (POPC), an unsaturated phospholipid which, compared with the disaturated analogues, resembles much more natural phospholipids. The lifetime measurements were carried out with a commercial multifrequency phase fluorimeter (1–200 MHz modulation

frequency range) (20). The experimental data were analyzed in terms of lifetime exponentials and, according to a more recent and a phenomenologically more convincing concept, continuous lifetime distributions (12, 21). By means of unimodal fluorescence lifetime distributions of DPH-PC in POPC bilayers, we studied the effect of cholesterol on the "homogeneity" of the artificial membranes. We found that increasing amounts of the sterol show only little effect on the lifetime distribution centers but induce significant narrowing of the respective full widths at half maximum. Thus, we conclude that cholesterol, which is known as a membrane stabilizer, may also be considered a membrane homogenizer within the fluorescence time domain.

METHODS

Preparations

1-Palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5,-hexatrienyl)-phenyl]ethyl]carbonyl]-*sn*-glycero-3-phosphocholine (DPH-PC)

Lysolecithin was prepared by phospholipase-A2 cleavage of dipalmitoylphosphatidylcholine (22). Lysolecithin (20 mg) was acylated for 48 h with 25 mg DPH-propionic acid in the presence of 40 mg dicyclohexylcarbodiimide and 40 mg dimethylaminopyridine in 500 μl chloroform (dried over $CaCl_2$ and Al_2O_3). Chloroform/methanol (2:1, vol/vol) (15 ml) was added to the reaction mixture. The obtained solution was washed three times with 0.1 N HCl/methanol (1:1, vol/vol) and three times with methanol/water (1:1, vol/vol). The product was purified by thin-layer chromatography on silica gel (solvent chloroform/methanol/25% aqueous ammonia = 65:35:5 vol/vol/vol). The pure compound showed only one fluorescent peak and only one peak at the same position after spraying with molybdenum reagent. The preparation was carried out in the dark or under red light and under argon to protect the DPH against decomposition by light and oxygen.

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)

Isomerically pure POPC was prepared according to Hermetter et al. (23)

Vesicle preparation

Unilamellar vesicles were prepared by ethanol injection (24). A solution of the lipid mixture (40 mM) in 25 μl ethanol was injected into 2 ml of a Tris/HCl (pH = 7.4) buffer at 37°C under stirring. Before injection the buffer was deoxygenated with argon. After preparation, the vesicle suspensions were stored under Argon overnight at 4°C. The OD at 360 nm was always lower than 0.2.

The samples prepared as described above contained 1.25 vol% ethanol. It is known that even low amounts of ethanol in a vesicle suspension exert some influence on the thermotropic transitions of lipids and may induce phase separations (25, 26). These effects become significant at temperatures below and at the phase transition. Although the fluorescence measurements were carried out well above the phase transition of pure POPC, we determined the influence of ethanol on the properties of the samples. After dialysis of a sample against ethanol-free buffer, we observed small lifetime differences. However, we cannot exclude some reaction of DPH with oxygen during this procedure. Therefore, we measured the lifetimes at various ethanol concentrations (0.5, 1.25, and 2.5%). In these cases we could not detect any significant differences between the data of the various samples. The label to lipid ratio was 1:300 for all samples. Earlier determinations of DPH-PC lifetimes (10) showed that lifetimes are independent of the label to lipid ratio if it does not exceed 1:150. We have repeated these experiments with a multifrequency instrument and we obtained similar results. Experiments with sonicated vesicles were irreproducible, probably because of label decomposition during ultrasonic irradiation.

Lifetime measurements

Lifetime measurements were performed with a variable frequency phase and modulation fluorimeter (20) from I.S.S. (La Spezia, Italy) ranging from 1 to 200 MHz. A solution of *P*-bis-[2-(5-phenyloxozolyl)]benzene in ethanol served as a lifetime reference (27). A He-Cd laser (model 4207 NB; Liconix, Sunnyvale, CA) was used as an excitation source. The excitation wavelength was 325 nm. The phase and modulation values were determined at 10 different modulation frequencies (5, 10, 15, 20, 35, 50, 70, 100, 140, and 200 MHz). The data were accumulated until standard deviations of phase (Δp) and modulation values (Δm) for frequencies lower than 70 MHz were below $\Delta p = 0.1°$, $\Delta m = 0.004$ and for frequencies above 70 MHz were below $\Delta p = 0.2°$ and $\Delta m = 0.004$, respectively. The sample temperature of 25°C was controlled using an external thermostating bath. The fluorescence was observed through a cutoff filter (model KV370; Schott, Mainz, FRG). A least-squares program from I.S.S. was used for lifetime analysis, minimizing χ^2_{red} defined by

$$\chi^2 = \sum_{\omega} \frac{1}{\sigma^2_{\phi\omega}} (\phi_\omega - \phi_{c\omega}) + \sum_{\omega} \frac{1}{\sigma^2_{m\omega}} (m_\omega - m_{cw}) \qquad (1)$$

$$\chi^2_{red} = \frac{\chi^2}{2N - \gamma - 1}. \qquad (2)$$

ω is the modulation frequency; $\sigma_{\phi\omega}$ and $\sigma_{m\omega}$ are the estimated standard deviations of measured phase angles and demodulations; ϕ_ω and $\phi_{c\omega}$ are the experimental and the calculated phase shifts; m_ω and $m_{c\omega}$ are the experimental and the calculated demodulation factors, respectively; N is the number of measurements per sample; and γ is the number of free parameters (1).

The experimental data were analyzed assuming a sum of lifetime exponentials or a continuous distribution of lifetime values (1, 12, 21, 28). Lifetime (τ) distributions (12) are characterized by uniform (Eq. 3), Gaussian (Eq. 4), or Lorentzian (Eq. 5) shape:

$$f(\tau) = A \text{ from } C - W/2 \text{ to } C + W/2 \qquad (3)$$

$$f(\tau) = A \cdot e\,[2.75(\tau - C)^2/W^2] \qquad (4)$$

$$f(\tau) = A/\{1 + [(t - C)/(W/2)]^2\}. \qquad (5)$$

The essential fitting parameters are the center position (C) and the distribution width at half height (W). The constant A is obtained from the normalization condition.

In order to judge the validity of the different decay models by statistic criteria, an F-test (29) was performed. A significant change in χ^2_{red} was accepted at a probability level $P \leq 0.01$.

TABLE 1 Lifetime parameters determined by fitting various decay functions to the experimental and modulation data obtained with DPH-PC in various POPC/cholesterol mixtures

Discrete monoexponential decay	τ (*ns*)	χ^2_{red}
POPC	6.74 ± 0.08	16.3
POPC/CHOL = 9:1	7.15 ± 0.07	14.2
POPC/CHOL = 8:2	7.21 ± 0.06	13.1
POPC/CHOL = 7:3	7.31 ± 0.05	6.5
POPC/CHOL = 6:4	7.34 ± 0.06	7.0
POPC/CHOL = 5:5	7.54 ± 0.05	7.4

Unimodal Lorentzian distribution	τ_c (*ns*)	FWHM (*ns*)	χ^2_{red}
POPC	6.87	0.57	6.7
POPC/CHOL = 9:1	7.26	0.45	4.8
POPC/CHOL = 8:2	7.27	0.23	5.4
POPC/CHOL = 7:3	7.36	0.18	4.0
POPC/CHOL = 6:4	7.39	0.15	5.6
POPC/CHOL = 5:5	7.58	0.14	4.6

Unimodal Gaussian distribution	τ_c (*ns*)	FWHM (*ns*)	χ^2_{red}
POPC	7.12	3.81	4.5
POPC/CHOL = 9:1	7.46	3.58	4.1
POPC/CHOL = 8:2	7.42	2.91	4.5
POPC/CHOL = 7:3	7.48	2.65	3.3
POPC/CHOL = 6:4	7.51	2.61	4.8
POPC/CHOL = 5:5	7.68	2.53	4.9

Discrete biexponential decay	τ_1 (*ns*)	f_1	τ_2 (*ns*)	f_2	χ^2_{red}
POPC	7.63 ± 0.39	0.85 ± 0.10	3.77 ± 0.87	0.14	4.3
POPC/CHOL = 9:1	7.92 ± 0.42	0.86 ± 0.11	4.13 ± 1.1	0.13	3.9
POPC/CHOL = 8:2	7.91 ± 0.88	0.83 ± 0.35	4.88 ± 2.2	0.16	5.1
POPC/CHOL = 7:3	8.08 ± 1.5	0.76 ± 0.65	5.50 ± 2.3	0.23	4.6
POPC/CHOL = 6:4	8.05 ± 1.7	0.79 ± 0.75	5.43 ± 3.6	0.20	6.0
POPC/CHOL = 5:5	8.39 ± 2.6	0.70 ± 1.22	6.02 ± 3.9	0.29	5.2

Bimodal Lorentzian distribution	τ_{c_1} (*ns*)	$FWHM_1$	f_1	τ_{c_2} (*ns*)	$FWHM_2$	f_2	χ^2_{red}
POPC	7.50	0.05	0.87	3.74	0.05	0.13	4.4
POPC/CHOL = 9:1	7.81	0.05	0.84	4.32	0.25	0.12	3.9
POPC/CHOL = 8:2	7.65	0.05	0.90	4.78	0.30	0.10	5.0
POPC/CHOL = 7:3	7.73	0.05	0.88	5.19	0.20	0.12	4.5
POPC/CHOL = 6:4	7.39	0.15	1.00	5.91	0.05	0.00	7.5
POPC/CHOL = 5:5	7.68	0.05	0.99	3.05	0.13	0.01	6.4

Bimodal Gaussian distribution	τ_{c_1} (*ns*)	$FWHM_1$	f_1	τ_{c_2} (*ns*)	$FWHM_2$	f_2	χ^2_{red}
POPC	7.55	0.05	0.87	3.61	0.09	0.13	4.1
POPC/CHOL = 9:1	7.83	0.06	0.89	3.97	0.05	0.11	3.5
POPC/CHOL = 8:2	7.79	0.05	0.88	4.59	0.37	0.12	4.4
POPC/CHOL = 7:3	7.92	0.08	0.84	5.16	0.05	0.16	4.1
POPC/CHOL = 6:4	7.77	0.05	0.91	4.52	0.05	0.09	6.1
POPC/CHOL = 5:5	8.10	0.05	0.84	5.41	0.30	0.16	5.8

The data are calculated assuming phase and modulation errors of $\sigma_p = 0.2°$ and $\sigma_m = 0.004$, respectively. The χ^2_{red} values are mean values out of two independent measurements. CHOL, cholesterol.

RESULTS

Phase angles and demodulations were measured at different modulation frequencies between 5 and 200 MHz for DPH-PC in POPC vesicles containing different amounts of cholesterol. The results obtained by fitting various functions to collected data sets are summarized in Table 1. The χ^2_{red}-values are medians of two separate measurements, respectively. In pure POPC and in mixtures with 10 and 20 mol% cholesterol discrete monoexponential fits exhibit significantly higher χ^2_{red}-values ($P \leq 0.01$ obtained from an F-test) as compared with all other functions applied (double-exponential; Gaussian, Lorentzian, and uniform distributions). The difference between monoexponential decays and the more complex models (see above) in the χ^2_{red} values decreases with increasing cholesterol content of the vesicles (Fig. 1). When the cholesterol content exceeds 20 mol%, there are no longer significant differences between the different models ($P \leq 0.1$ obtained from an F-test).

A striking result is that the χ^2_{red} values of the discrete biexponential fits are very similar to those obtained with uniform, Gaussian, and Lorentzian unimodal distributions. On going to bimodal lifetime distributions, there is again no better fit.

Most χ^2_{red}-values are close to 4 or even higher. This shows that deviations in the calculated data of the fitted functions from the experimental phase and modulation values are higher than the estimated standard deviations of the experimental data. Therefore, we examined the deviations to figure out if there are differences in the magnitude and randomization of these deviations depending on the fitted function. Fig. 2 shows the phase and modulation deviations of the discrete mono- and biexponential fit and the Lorentzian, Gaussian, and uniform unimodal distribution functions. All five fitting functions show similar systematic deviations. Deviations of phase angles as a function of frequency exhibit a very similar pattern for biexponential decays and the distributional models. Their magnitude does not change with changes in the cholesterol content of the membranes. The modulation deviations are similar for all decay models tested including the monoexponential model. However, their magnitude increases upon increasing cholesterol levels in the vesicles. There are small but significant differences in the phase deviations, especially between the Lorentzian distribution and the other fitting models. Vesicles of POPC or POPC with 10 mol% cholesterol (the "broader distributions") show deviations for the Lorentzian distribution that are a little bit larger, but vesicles containing between 20 and 50 mol% cholesterol exhibit very small deviations compared with all other fitting functions.

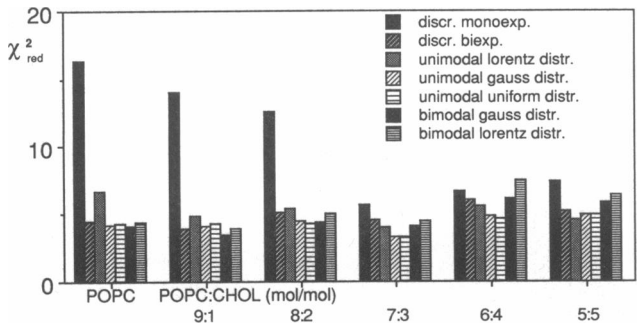

FIGURE 1 χ^2_{red} of various fitting functions applied to the experimental phase shift and demodulation factors of DPH-PC in vesicles consisting of POPC and POPC/cholesterol mixtures at 25°C.

Phase deviations observed for the discrete biexponential function, the uniform and Gaussian distributions, exhibit no big differences at all compared with each other. The modulation deviations determined for the biexponential and the distributional models are similar too.

From these deviations it is obvious that the lifetime values resulting from the modulation data are not the same as the lifetime values obtained from the phase data. Therefore, the modulation data and the phase data were fitted separately to a biexponetial decay. The modulation data gave χ^2_{red} values of ~1.6 and the phase data gave χ^2_{red}-values reaching from 1.2 to 4.0. From the modulation data a shorter minor lifetime component and a smaller fraction of this second component was obtained as compared with the phase data. In principle, the same tendency was observed after fitting both data sets (see Discussion).

Alcala et al. (21) described the resolution limits for various distribution functions as compared with discrete exponential fits. They determined the minimum lifetime center/width ratio, allowing discrimination between a unimodal distribution and a monoexponential decay depending on the experimental error. Assuming a random error of 0.1% this ratio for a uniform distribution is 0.32; for a Gaussian distribution, 0.2; and for a Lorentzian distribution, 0.01. For bimodal distributions the lowest resolvable ratios are even higher. Fitting our experimental values to bimodal Lorentzian distribution results in most cases in ratios even below 0.01. Therefore, it is not possible to resolve the bimodal from the discrete biexponential model. With increasing error the resolvable center/width ratio increases dramatically. Because it is very difficult to extrapolate the results of Alcala et al. to our problem, we did some simulations to make comparisons with our data. We chose simulated functions to be very close to our experimental data. Four different sets of random errors have been added to our simulated phase (Δp) and modulation (Δm) data (resulting in $\sigma_{p_1} = 0.1$,

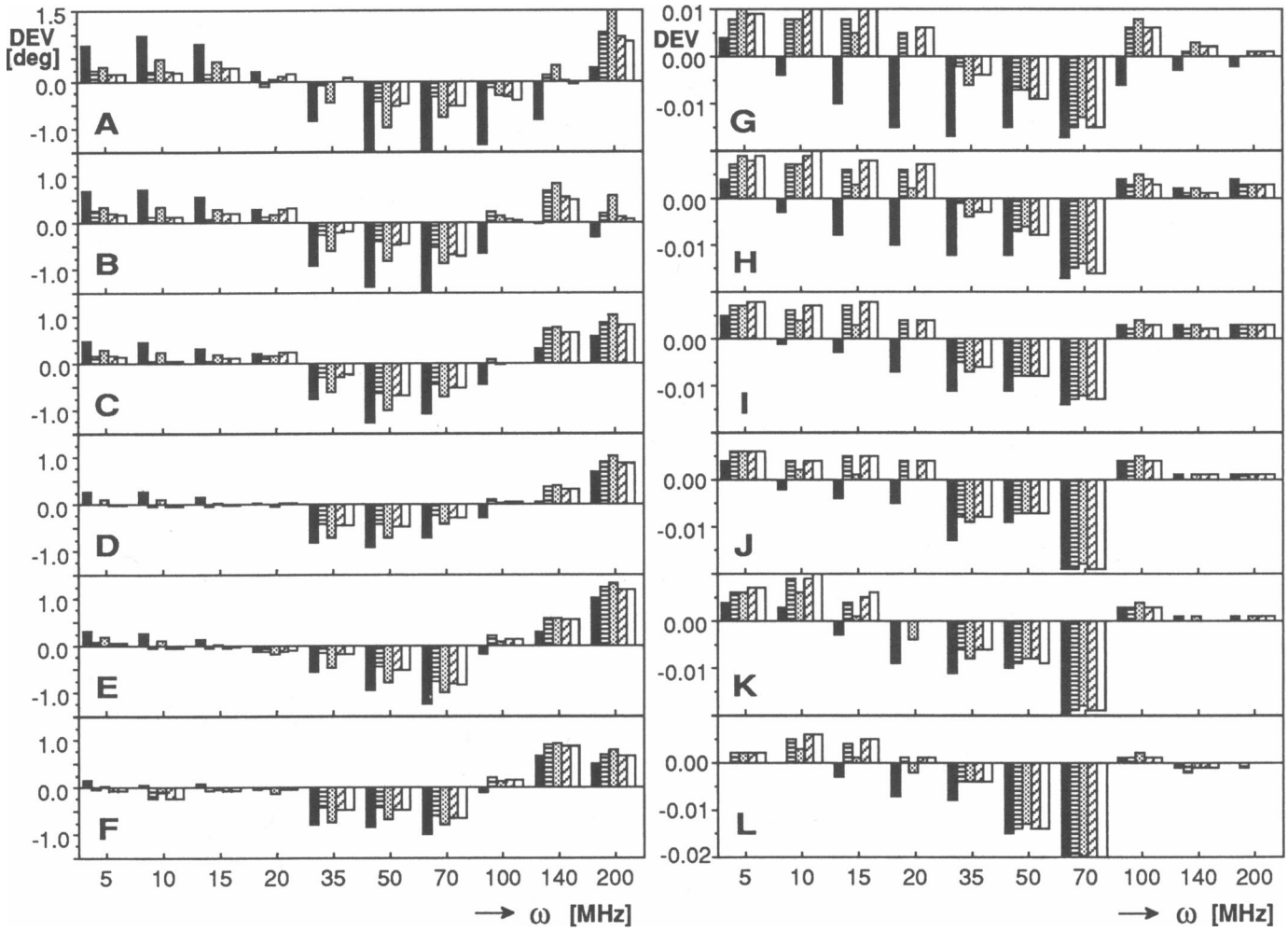

FIGURE 2 Phase (*A–F*) and modulation deviations (*G–L*) of the calculated values for discrete monoexponential (■), biexponential (▤), unimodal Lorentzian distribution (□), Gaussian distribution (▨), and uniform distribution (□) fits from the experimental values obtained for DPH-PC in vesicles of pure POPC (*A*, *G*) and POPC/cholesterol mixtures [9:1 (*B*, *H*); 8:2 (*C*, *I*); 7:3 (*D*, *J*); 6:4 (*E*, *K*); 5:5 (*F*, *L*)]

$\sigma_{m_1} = 0.001$; $\sigma_{p_2} = 0.2$, $\sigma_{m_2} = 0.004$; $\sigma_{p_3} = 0.5$, $\sigma_{m_3} = 0.01$; $\sigma_{p_4} = 1$, $\sigma_{m_4} = 0.01$). The results of a few representative sets of simulations are shown in Table 2. According to the χ^2_{red} values obtained with a simulated double exponential decay or a unimodal Lorentzian distribution (FWHM = 0.5 ns), it is not possible to decide which of the four fitting functions is the parent or better one ($P \leq 0.1$ except for the uniform distribution fitted to the Lorentzian distribution [$P \leq 0.05$]). If one goes to shorter second lifetime components ($\tau 2$) or/and higher fractions of the long lifetime component ($\tau 1$), it is possible to resolve the discrete biexponential system from the unimodal Lorentzian distribution. In general, we have to assume larger fractions of the second component to resolve the discrete biexponential decay from the Gaussian distribution. On the other hand, a Lorentzian distribution can be resolved ($P \leq 0.01$) if its width becomes larger (Table 2, FWHM = 1.3 ns).

In principle, our results are supported by the conclusions of Lakowicz et al. (30), implying that on the basis of χ^2_{red} a biexponential fit is indistinguishable from unimodal Lorentzian or Gaussian distribution (this conclusion refers of course to data obtained with today's available accuracy).

Accordingly, our data (estimated errors: $\sigma_p = 0.2$, $\sigma_m = 0.004$) do not provide satisfactory information on the validity of the fitting functions on the basis of χ^2_{red}. Nevertheless, there may be other criteria that could help select the most adequate model for the fluorescence decay of a DPH-phospholipid in a phospholipid membrane on a more physical and phenomenological basis (12) as discussed below.

TABLE 2 Analysis of simulations of a discrete biexponential fluorescence decay and Lorentzian decay distributions with two different widths (FWHM).

Simulated function: discrete two-exponential decay: $\tau_1 = 7.0$ ns, $f_1 = 0.9$, $\tau_2 = 3.0$ ns, $f_2 = 0.1$					
Fitted function: discrete 2-exp.:	$\tau_1 = 7.07$	$f_1 = 0.879$	$\tau_2 = 3.19$	$f_2 = 0.121$	$\chi^2 = 0.95$
Lorentzian distr.:	$\tau_c = 6.45$	$w = 0.701$			$\chi^2 = 1.77$
Gaussian distr.:	$\tau_c = 6.70$	$w = 3.826$			$\chi^2 = 1.19$
Uniform distr.:	$\tau_c = 6.71$	$w = 6.160$			$\chi^2 = 1.58$
Simulated function: Lorentzian distribution: $\tau_c = 7.000$ ns, $FWHM = 1.300$ ns					
Fitted function: discrete 2-exp.:	$\tau_1 = 7.60$	$f_1 = 0.921$	$\tau_2 = 2.38$	$f_2 = 0.079$	$\chi^2 = 3.54$
Lorentzian distr.:	$\tau_c = 7.02$	$w = 1.321$			$\chi^2 = 0.98$
Gaussian distr.:	$\tau_c = 7.36$	$w = 4.909$			$\chi^2 = 2.86$
Uniform distr.:	$\tau_c = 7.47$	$w = 8.490$			$\chi^2 = 5.55$
Simulated function: Lorentzian distribution: $\tau_c = 7.000$ ns, $FWHM = 0.500$ ns					
Fitted function: discrete 2-exp.:	$\tau_1 = 7.21$	$f_1 = 0.960$	$\tau_2 = 2.29$	$f_2 = 0.040$	$\chi^2 = 1.74$
Lorentzian distr.:	$\tau_c = 7.00$	$w = 0.510$			$\chi^2 = 1.04$
Gaussian distr.:	$\tau_c = 7.18$	$w = 3.383$			$\chi^2 = 1.82$
Uniform distr.:	$\tau_c = 7.17$	$w = 5.336$			$\chi^2 = 2.19$

A frequency-independent error of phase angles $\sigma_p = 0.2°$ and demodulation factors $\sigma_m = 0.004$, respectively, was added to the calculated data at 10 different modulation frequencies (the same set of modulation frequencies was used as in the experimental part). All data were fitted with an assumed error of $\sigma_p = 0.2°$ and $\sigma_m = 0.004$. The values shown are the average values out of three simulations.

DISCUSSION

With respect to χ^2_{red} values, four different functions (biexponential, uniform, Lorentzian, and Gaussian distributions) can be fitted equally well to phase and modulation data of DPH-PC in vesicles of POPC or POPC/cholesterol mixtures containing up to 20 mol% sterol. Keeping in mind that the χ^2_{red} is a measure for the discrepancy between an estimated function and the parent function and, in addition, for the deviation of the experimental data from data associated with the parent function, it is not possible to attribute any of these functions to the parent one. On the basis of statistics, the model with the minimum of free-fitting parameters is the most probable, if χ^2_{red} does not become significantly lower upon introduction of additional fitting parameters. In this respect, we have to give preference to the unimodal lifetime distribution model with two free-fitting parameters compared with a biexponential decay or bimodal distributions with three or five fitting parameters, respectively. It is interesting to note that the fluorescence decay of DPH in saturated phospholipids above T_c is equally well fitted to a biexponential and a bimodal lifetime distribution on the basis of χ^2_{red}, whereas the bimodal distribution gives the better fit below T_c (12). However, there is no information available on the goodness of a unimodal fit for DPH lifetimes in a bilayer. Our lifetimes obtained from the biexponential model can be compared with data for DPH-PC in saturated phosphatidylcholines (10). These authors found a long-living fraction of 6.8 ns for the label in DPPC above T_c. This value is somewhat lower compared with the corresponding lifetime of DPH-PC in POPC reported herein. However, apparent lifetimes in fluid DPPC were measured at higher temperatures (above 40°C) compared with our measurements on fluid POPC carried out at 25°C.

If the cholesterol content of the bilayer matrix exceeds 20 mol%, χ^2_{red} for monoexponential decays of DPH-PC is no longer significantly different from χ^2_{red} for the more complex decay models (Table 1, Fig. 1). Thus, a monoexponential decay should be considered as the statistically most probable model as compared with the other ones in these cases. Nevertheless, if we wish to describe consistently the effect of cholesterol on the biophysical properties of a phospholipid bilayer by means of fluorescence lifetime studies, we have to address the question as to the physical and phenomenological meaning of the selected decay models. DPH (8, 31) and DPH-PC (10) are known to exhibit biexponential fluorescence decays even in "homogeneous" phospholipid bilayers (13). Although the origin of the minor short-living lifetime component is unknown, there is no clear reason why the DPH fluorophors should probe two distinct environments in such systems. Another important aspect has to be considered. The lowest lifetime for DPH was measured in glycerol (~3.9 ns [11]) and, on the other hand, DPH is nearly quantitatively quenched in water. Therefore, the existence of distinct membrane environments with DPH lifetimes of ~2 ns is not very likely! A much more useful description could be provided by a unimodal lifetime distribution corresponding to a distribution of states experienced by a label within its lifetime. The possible

effects responsible for lifetime heterogeneity have already been discussed in the literature by Williams and Stubbs (15) and Fiorini et al. (12): a dielectric gradient along the membrane normal, motional freedom, organizational and compositional heterogeneity. Whereas the first two effects may become effective in membranes containing only a single species, the latter one accounts for heterogeneity in phospholipid composition and the presence of sterols and/or proteins.

If we look at the fluorescence properties of DPH-PC in POPC bilayers above T_c in terms of unimodal lifetime distributions, we observe a profound effect of cholesterol on the bilayer properties. Whereas lifetime centers only slightly increase upon increasing cholesterol concentration, the widths of the distributions decrease significantly (Table 1, Fig. 3). Such effects have been observed for DPH itself in bilayers above T_c. However, it is very difficult to make interpretations for DPH lifetimes, as the localization of the label in bilayers above T_c is ill defined (16, 17), thus giving rise to lifetime heterogeneity. The situation is much better defined in the case of DPH-PC being oriented in a defined way like any other membrane phospholipid in a bilayer. Therefore, the effect of the sterol on DPH-PC lifetimes might also be interpreted in a somewhat simpler way and should be connected with the phenomena already mentioned above: the sterol expels water from the bilayer interface (32), thus affecting the dielectric gradient in this region; it reduces motional freedom of the phospholipids (33), and might finally lead to more homogeneous lateral lipid entities in a bilayer. The latter assumption is not necessarily at variance with data that shows that lateral distribution of cholesterol itself (for a review, see reference 33) or a fluorescent cholesterol analogue (34) can be heterogeneous in a membrane. Our data obtained with DPH-phosphatidylcholine instead provide a picture of the membrane as "seen" by a phospholipid molecule. We looked at our data also from another point of view. Assuming that the long lifetime component and its fraction in the biexponential analysis are a measure for the homogeneity of the sample, we can see a bridge between the biexponential function and the unimodal distributions. With increasing cholesterol content, the short lifetime and the calculated associated standard deviations are increasing and/or the fraction of the short component is decreasing. In the unimodal distribution analysis, a little change in the lifetime center is associated with a large decrease in the width of the distribution with increasing cholesterol content. This behavior is not only observed with DPH-PC in POPC-cholesterol mixtures, but also with plasmalogen-cholesterol bilayers and other markers (Kalb, E., F. Paltauf, and A. Hermetter, manuscript in preparation). In addition, if we fit only phase or modulation data measured at various frequencies the same tendency is visible. Even if we reduce the number of frequencies and fit the different functions to these reduced data sets, we get the same tendencies. It is also important to note that if we analyze data from different individual samples, containing the same amount of cholesterol, in terms of a discrete biexponential decay, especially the values of the short lifetime components and the associated fractions are sometimes increasing or decreasing. In terms of heterogeneity, this means that if the difference between the two lifetime components is smaller, the fraction of the short lifetime is larger, and if the lifetime difference is larger the fraction of the shorter lifetime is smaller. If we analyze this with unimodal distributions, in most cases we get similar widths for the corresponding individual samples.

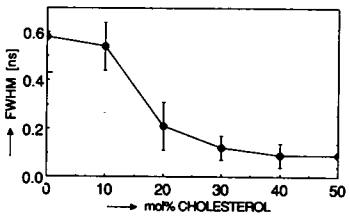

FIGURE 3 Dependence of the FWHM of unimodal Lorentzian distributions of DPH-PC lifetimes on the cholesterol content in POPC vesicles made by ethanol injection. The data shown represent the average values out of two measurements. The bars indicate the estimated uncertainty of the values.

Thus, cholesterol exerts a homogenizing effect on phospholipid bilayers as detected by DPH-PC (this work) and DPH (12) as well as in biomembranes (13) as determined by DPH fluorescence. The same sterol effect could also be observed in bilayers of alkenyletherphospholipids (plasmalogens), although the latter did not reach the same low degree of lifetime heterogeneity at the maximum of 50 mol% sterol compared with the diacyl phospholipid POPC (14).

With regard to the possible biological significance of our data, we conclude that unimodal distributions are superior in describing lifetime data obtained with DPH-PC in membranes, and that the widths of the distributions may be interpreted as a measure for the heterogeneity of the sample. This final conclusion is in good agreement with the results of Williams and Stubbs (15). On the other hand, we have to stress that none of the used distribution functions will be the parent function. Intuitively we think a Lorentzian or Gaussian distribution is more probable than a uniform distribution and that an asymmetric function would be the best. In a first approximation one can take a particular distribution, e.g., the Lorentzian distribution, and compare the center and width values in terms of lifetime-heterogeneity.

Biological implications

By means of DPH lifetime distributions Fiorini et al. (13) already observed increased "homogeneity" of erythrocyte membranes with the natural cholesterol content as compared with the same membranes whose cholesterol content was artificially reduced. Fluorescence anisotropy and resonance energy transfer studies by Molotkovsky et al. (35) provided evidence for cholesterol-induced randomization of separate choline phospholipid pools in high density lipoproteins, differing in fluidity and interaction with apolipoprotein A. Therefore, the effect of cholesterol as a "membrane homogenizer" is not only restricted to artificial bilayer systems, but might also play an essential role in biological membrane systems.

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