J. Membrane Biol. 174, 237–243 (2000)

The Journal of
Membrane
Biology

Lateral Microheterogeneity of Diphenylhexatriene-Labeled Choline Phospholipids in the Erythrocyte Ghost Membrane as Determined by Time-Resolved Fluorescence Spectroscopy

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Received: 22 July 1999/Revised: 6 January 2000

Abstract. Choline phospholipids are the major constituents of the outer layer of the erythrocyte membrane. To investigate their lateral membrane organization we determined the fluorescence lifetime properties of diphenylhexatriene analogues of phosphatidylcholine, choline plasmalogen, (the respective enolether derivative), and sphingomyelin inserted into the outer layer of hemoglobin-free ghosts. Fluorescence lifetimes were recorded by time-resolved phase and modulation fluorometry and analyzed in terms of Continuous Lorentzian distributions. To assess the influence of membrane proteins on the fluorescence lifetime of the labeled lipids in the biomembrane, lipid vesicles were used as controls. In general, the lifetime distributions in the ghost membranes are broad compared to vesicles. Phosphatidylcholine and sphingomyelin exhibit very similar lifetime distributions in contrast to an increased plasmalogen lifetime heterogeneity in both systems. Orientational effects of side chain mobilities on the observed lifetimes can be excluded. Fluorescence anisotropies revealed identical values for all three labeled phospholipids in the biomembrane.

Key words: Plasmalogen — Membrane domains — Fluorescent phospholipids — Fluorescence lifetime distribution

Introduction

The asymmetric distribution of phospholipids between the outer and inner leaflet of biomembranes, in particular in the erythrocyte plasma membrane, has been extensively investigated and is well established. The choline phospholipids are localized mainly in the outer leaflet of the erythrocyte membrane, while the amino phospholipids are confined almost exclusively to the inner leaflet of the bilayer [7, 30]. Vesicles reconstituted from erythrocyte lipids do not spontaneously adopt this asymmetric arrangement [23], indicating that establishing and maintaining transbilayer asymmetry requires an energy-consuming mechanism supplied by the cell. The question whether the cytoskeleton contributes to lipid asymmetry is still a matter of debate [6, 40].

According to a current hypothesis, the distribution of phospholipids may exhibit lateral asymmetry within a single leaflet as well. This concept of lipid domains has been discussed in terms of the biological function for different phospholipid classes [4].

To address this question we chose the erythrocyte membrane as an easily accessible and well-defined system. We investigated the heterogeneity of the lateral distribution of fluorescent analogues of the choline phospholipid classes and subclasses in the bilayer, namely sphingomyelin, phosphatidylcholine and choline plasmalogen.

1,6-Diphenylhexa-1,3,5-triene propionic acid was chosen as a fluorescent ligand, because it is an excellent reporter for lipid mobility and organization in aggregated lipid systems [26]. It was our working hypothesis, that the lifetime heterogeneity (multiplicity of excited states) of the labeled lipids would reflect the heterogeneity of their environment in the biomembrane. In addition, fluorescence anisotropies were determined as a measure of phospholipid acyl chain mobility in the bilayer. Unilamellar vesicles consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), plasmalogen and sphingomyelin (SM), respectively, were used as a protein-free reference membrane system in order to es-

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timate the effect of membrane proteins on lipid organization in the ghost membrane. We found that lifetime heterogeneity of diacyl-PC and SM were very similar in the RBC membranes, whereas choline plasmalogen showed the largest distributional widths. In contrast, fluorescence anisotropies were similar for the three fluorescent phospholipids in the erythrocyte membrane. Thus, we may conclude that the origin of the increased plasmalogen lifetime heterogeneity is not due to effects of the flexibility (orientation) of the phospholipid acyl chains but rather due to differences in its microenvironment within the red blood cell membrane.

Materials and Methods

Phospholipid Syntheses

The following fluorescent analogues of phospholipids were prepared: 1-palmitoyl-2-[[2-[4-(6-phenyl-trans-1,3,5-hexatrienyl)-phenyl]-ethyl]carbonyl]-*sn*-glycero-3-phosphocholine (DPH-phosphatidylcholine) was prepared as described [18]. N-[[2-[4-(6-phenyl-trans-1,3,5-hexa-trienyl)-phenyl]-ethyl]carbonyl]-trans-4-sphingenine-1-phosphocholine (DPH-sphingomyelin) was synthesized by acylation of sphingosyl-phosphocholine with 1,6-diphenyl-1,3,5-hexatrienyl-propionic acid (Lambda Fluoreszenz Technologie, Graz, Austria) as described for the preparation of fluorescent phosphatidylcholine [18]. Sphingosyl-phosphocholine was prepared from bovine brain sphingomyelin (Sigma, Deisenhofen, Germany) by acidic hydrolysis using a modified version (the reaction time was three times longer) of the procedure described by Kaller [19] and characterized according to Cohen et al. [3]. (1-O-1′-Z-Hexadecenyl)-2-[[2-[4-(6-phenyl-trans-1,3,5-hexatrienyl)-phenyl]-ethyl]carbonyl]-*sn*-glycero-3-phosphocholine (DPH-PL) and 1-palmitoyl-2-N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-aminododecanoyl-*sn*-glycero-3-phosphocholine (NBD-PC) were prepared as described by Loidl et al. [27]. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was synthesized as described [16]; chicken egg yolk sphingomyelin containing primarily palmitic acid was purchased from Sigma (Deisenhofen, Germany).

Erythrocyte Ghost Preparation

Red blood cells were isolated from healthy donors. Hemoglobin was removed from human erythrocytes according to Dodge et al. [5] until the resulting ghost preparation was colorless.

Lipid Analysis

Phospholipids were quantitated according to Broekhuyse [2]. Cholesterol was determined with a commercially available test kit from Boehringer (Mannheim, Germany).

Quasi-elastic Light Scattering

Mean size and size distributions of red blood cell ghosts were determined by quasi-elastic light scattering (QELS) as described previously [37]. Shortly, as particles move in solution due to their Brownian motion the light scattered by them fluctuates. Thus, the time dependence of the scattering intensity, represented by its correlation function, provides information on scatterer motion. If all particles are of the same size, the correlation function is a single exponential function. Its decay is governed by the diffusion coefficient which is obtained either by a single exponential fit or after linearization by a series expansion (cumulant fit) [21]. The diffusion coefficient D is related through the Stokes-Einstein equation to the hydrodynamic radius R_H, the size of an equivalent compact sphere. For a polydisperse sample, this quantity is a mean value putting weight on large particles. Size distributions can be obtained through a Laplace inversion of the correlation function [11, 34]. The intensity distribution D(i) reflects the intensity with which particles of a certain size contribute to the signal.

Preparation of Unilamellar Phospholipid Vesicles

Unilamellar vesicles containing fluorescent phospholipids were prepared by the ethanol injection method [22]. A solution of the lipid (60 nmol) in 25 mL ethanol was injected into 3 mL of Tris/HCl buffer (pH = 7.4) at 37°C under stirring. The preparations were stored over night at 4°C in the dark before use.

Labeling of Erythrocyte Ghosts with Fluorescent Phospholipids

The uptake of DPH-labeled lipid from vesicles into erythrocyte ghosts at 37°C was determined from the increase of fluorescence intensity at 430 nm (excitation 360 nm), using a Shimadzu RF-540 spectrofluorometer (Shimadzu, Kyoto, Japan) equipped with an external thermostatic bath (Haake, Karlsruhe, Germany). The donor membranes (60 nmol phospholipid before incubation) were separated from the ghosts (400 nmol phospholipid) by centrifugation using a Hettich microcentrifuge (Hettich, Tuttlingen, Germany) for 5 min at 15,000 rpm, followed by repeated washings of resuspended membranes with 3 mL portions Tris/HCl buffer, pH 7.4. The extent of labeling was determined after addition of detergent to the biological or artificial membranes. The label to lipid ratio in the ghosts was 1:150 (mol/mol).

Fluorescence Microscopy

A microscope (Model IM Axiovert 35, Zeiss, Austria) equipped with epifluorescence optics (Filter combination 01 (BP365, FT395, LP397), Zeiss, Austria) and a Plan-Neofluar 100×/1,30 NA oil lens and Ektachrome 400 color slide films (Kodak Limited, England) were used for documentation of fluorescence images. Pictures were taken with a Contax 167MT camera (Kyocera, Japan) equipped with a D-7 data back. Aperture was set 1.4, exposure time was between 5 and 10 sec.

Fluorescence Lifetime Measurements

Fluorescence lifetime measurements were performed using a variable frequency phase and modulation fluorometer [12] from I.S.S. (Champaign, IL) with a frequency range of 1–200 MHz. A solution of p-bis-[2-(5-phenyloxazolyl)]-benzene in ethanol served as a lifetime reference [24]. A He-Cd laser (4207 NB; Liconix, Sunnyvale, CA) was used as an excitation source. The excitation wavelength was 325 nm. Phase shifts and demodulation values were determined at 11 different modulation frequencies (5, 10, 15, 20, 25, 40, 50, 70, 100, 140 and 200 MHz). The data were accumulated until the standard deviations of phase (Dp) and modulation values (Dm) were below Dp = 0.2° and Dm = 0.004. The sample temperature was controlled using an external thermostatic bath. Fluorescence emission was observed through a cutoff filter (KV 370; Schott, Mainz, Germany). The lifetime software

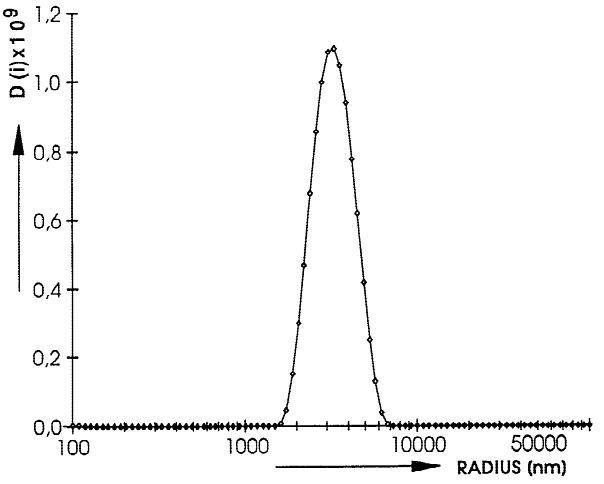

Fig. 1. Intensity distribution of erythrocyte ghosts determined by quasi-elastic light scattering.

package from I.S.S. was used for data analysis in terms of discrete lifetime exponentials and continuous Lorentzian lifetime distributions. Reduced chi-square, χ^2_R, was determined as a measure for the goodness of fit and was minimized as described [17]. Errors in lifetime distribution widths were determined according to [25].

Results

Size heterogeneities of the ghost preparations were characterized by quasi-elastic light scattering. The mean hydrodynamic radius as determined by a cumulant fit was 2950 nm. Furthermore, it gave a polydispersity of 12–15%. Figure 1 shows the intensity distribution of the RBC ghosts with a maximum at about 3100 nm. The difference between these two values may arise from the presence of trace amounts of small particles, being more pronounced in the cumulant fit.

A rather homogeneous size distribution was shown using fluorescence microscopy techniques. NBD-PC, a suitable marker for fluorescence microscopy [10], was incorporated into the ghost membranes. Figure 2 shows the rather homogeneous population of ghosts after the preparation and labeling procedure.

For fluorescence lifetime and anisotropy studies the biomembranes were labeled by incubation with small unilamellar vesicles consisting of DPH propionyl-labeled phospholipid analogues for 3 hr. Vesicles of a distinct size range were prepared by the ethanol injection method [22]. We used choline(glycero)phospholipids, which are the dominant species of the outer layer. Figure 3 shows the diacyl analogue, the ether analogue (1-O-(Z)-1′-alkenyl)-2-acyl-GPC (plasmalogen) and sphingomyelin.

Sphingomyelin and the plasmalogens incorporated into the ghost membrane at a much higher rate compared to DPH-PC (*data not shown*).

Phase and demodulation data as determined for the labeled ghosts at different modulation frequencies were fitted to different exponential [25] and distribution functions [1]. Lifetimes and fractional intensities (t_i, F_i) obtained by exponential fitting were fixed as starting parameters (lifetime centers C_i, fractional intensities F_i) for the distributional fit. Details for the analysis procedure are given elsewhere [32].

The best fits according to χ^2_{red} were obtained using bimodal Lorentzian lifetime distributions. χ^2_{red} values are, in general, higher than unity. We have to stress in this context that the Lorentzian function is most probably not the parent function of the lifetime distributions of the membrane-bound labels. However, it seems to be a good approximation, since it coincides with data obtained by the exponential series method. In two former studies [32, 33], we compared the lifetimes of DPH-PC and -PE in ghosts as determined by the pulse and phase method. The pulse data could be fitted to lifetime distributions with a χ^2_{red} of close to 1.0. A different algorithm was used for the fitting of the phase data, corresponding to symmetric Lorentz distribution functions (as used in this paper!) giving much higher χ^2_{red} values. This is not very surprising taking into account that the latter fitting function is likely not to be identical to the parent function. Despite differences in the measurement and fitting procedures, we obtained the same relative differences in lifetime widths of the labeled lipids in ghost membranes though the absolute values were not the same. Thus despite different qualities of data fitting (the χ^2_{red} of the pulse data were close to 1.0), we arrived at the same conclusions.

In artificial membranes, DPH-PC and plasmalogen exhibit very similar centers of the main lifetime component, whereas SM exhibits shorter values. DPH-PC and DPH-SM show comparable distribution widths in a POPC matrix, whereas a somewhat broader distribution was found for DPH-PL [15]. Thus, the lifetime distribution widths depend on the type of DPH-lipid only to a little extent but are affected very significantly by the composition of the unlabeled membrane matrix. DPH-PC and DPH-PL show similar distribution widths if embedded in the same bilayer (POPC or plasmalogen). However, lifetime heterogeneity is quite different, if for one type of label the matrix is different. Plasmalogen bilayers show, in general, broader lifetime distributions, supporting the assumption that the matrix lipid influences the DPH-lifetime much more as compared to the lipid to which the label is covalently bound. A markedly increased distribution width was found for the PC-marker in the SM matrix.

The centers of the long lifetime component of all fluorescent phospholipids in the biomembrane are shifted significantly (0.5 ns for PC, 0.8 ns for the plasmalogen and 1.0 ns for SM) to higher values as com-

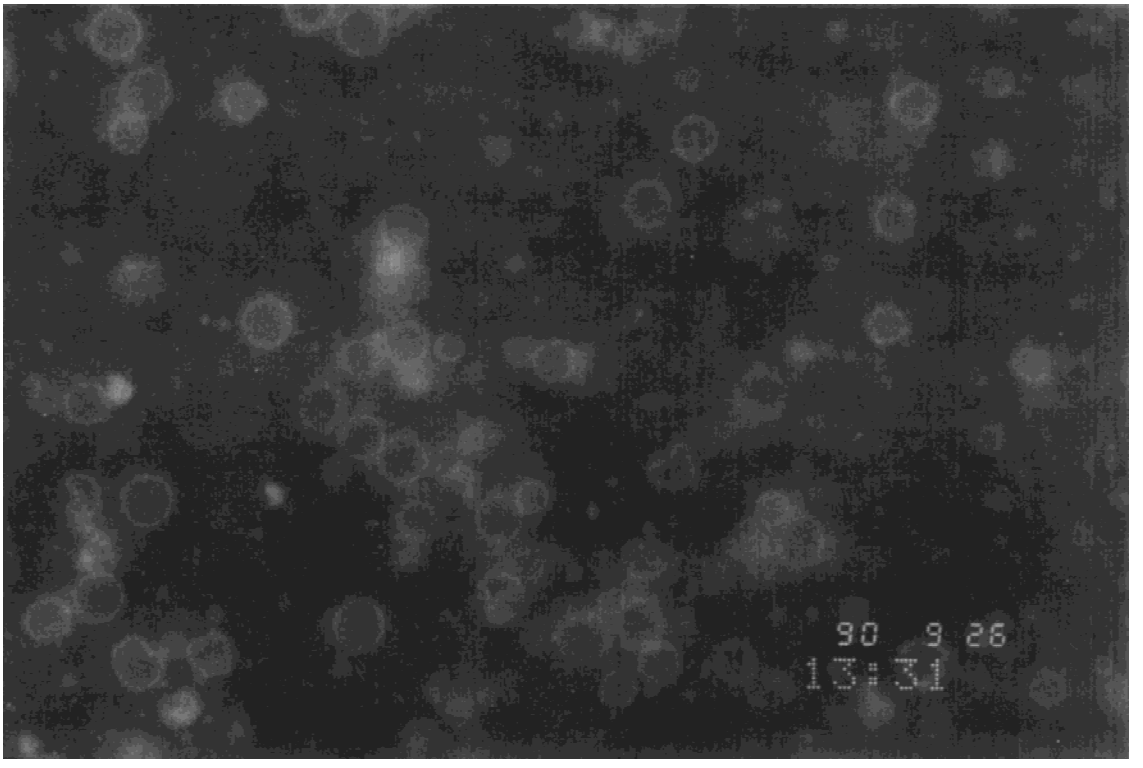

Fig. 2. Fluorescence image of erythrocyte ghosts labeled with NBD-PC (1,000×).

pared to the centers observed with phospholipid vesicles (Table 1). The respective distribution widths are increased for all labels, DPH-PC and DPH-SM revealing similar values, whereas DPH-PL exhibits a much broader lifetime distribution. This difference is significant, taking into account comparative data we have already obtained for DPH-PC and -PE in erythrocyte ghosts using the pulse and the phase method. Under the same experimental conditions we obtained excellent χ^2_{red} values for the pulse data which showed the same differences between both lipids as compared to the phase data irrespective of the higher χ^2_{red} values for the latter results. In contrast to the protein-containing biomembrane, fluorescence lifetimes are more homogeneous in vesicles composed of the main lipids (PC, SM, cholesterol) of the outer erythrocyte membrane layer (Table 1).

To obtain information about the mobility of the DPH-choline lipids we measured their fluorescence anisotropies in artificial and biological membranes. The respective values for the three markers in POPC vesicles are very similar (Table 2). In general, higher anisotropy values were found for the three labeled lipids residing in a SM matrix, consistent with an increased rigidity of SM [13, 20] compared to the POPC matrix. Fluorescently labeled PC, SM and choline plasmalogen show identical anisotropies in the biomembrane (Table 2).

Discussion

It was the aim of our study to investigate the lateral organization of phospholipid classes and subclasses in a biological membrane, which on a molecular level is still a matter of much debate. We chose erythrocyte ghosts as an easily accessible and well-characterized model of a biomembrane. Furthermore, inside-outside asymmetry of membrane phospholipids with choline phospholipids residing mainly in the outer and aminophospholipids in the inner layer, respectively, is still maintained to a significant extent [35] under the preparation conditions used.

In this study a fluorescence approach was applied using labeled analogues of choline phospholipids occurring naturally in the RBC membrane (PC 27%, SM 22%, cholineplasmalogen 2.5% of PC) [29, 38]. These choline lipids can be transferred from phospholipid vesicles to the outer layer of the RBC membrane, where they remain during the time needed for fluorescence measurements. Translocation to the inner layer should be negligible [6, 31]. Therefore, differences in lipid organization as detected by lipid fluorescence are due to differences in the lateral label environment (influence of heterogeneous lipid composition, presence of sterols and proteins, effects of water penetration, and lipid mobility).

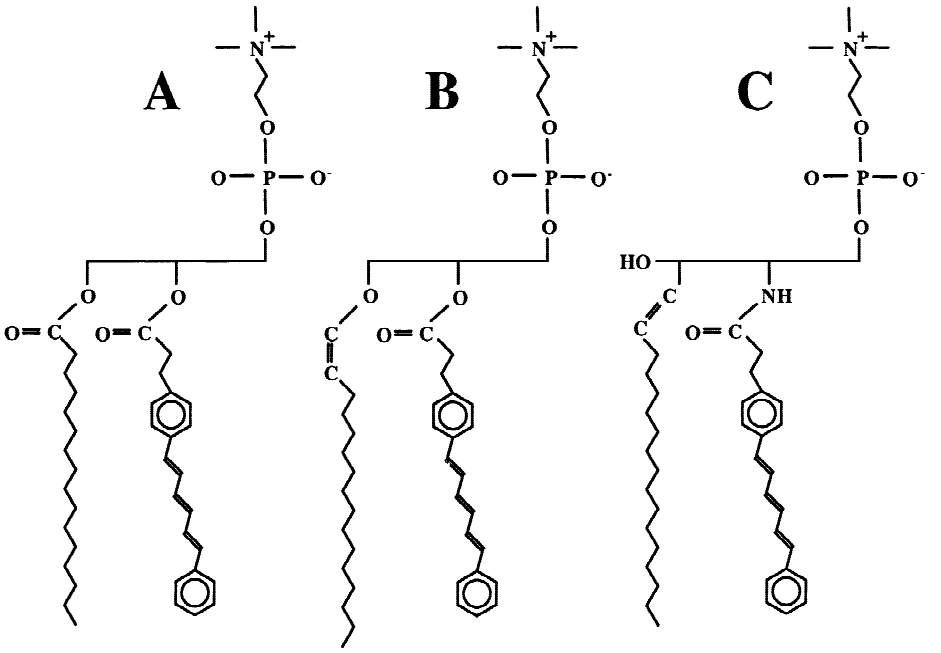

Fig. 3. Chemical structures of fluorescent choline phospholipids. (*A*) 1-Palmitoyl-2-[[2-[4-(6-phenyl-trans-1,3,5-hexatrienyl)-phenyl]-ethyl]-carbonyl]-*sn*-glycero-3-phosphocholine (DPH-phosphatidylcholine). (*B*) 1-O-1′-Z-hexadecenyl-2-[[2-[4-(6-phenyl-trans-1,3,5-hexatrienyl)-phenyl]-ethyl]carbonyl]-*sn*-glycero-3-phosphocholine (DPH-Plasmalogen). (*C*) N-[[2-[4-(6-phenyl-trans-1,3,5-hexatrienyl)-phenyl]-ethyl]carbonyl]-trans-4-sphingenine-1-phosphocholine (DPH-sphingomyelin).

Table 1. Continuous Lorentzian lifetime distributions of DPH-labeled lipids in unilamellar vesicles and erythrocyte ghost membranes

Lipid	Matrix	C_1[*ns*]	F_1	ω_1[*ns*]	C_2[*ns*]	F_2	ω_2[*ns*]	χ^2R
DPH-PC	PC/SM/CHOL	8.09 ± 0.09	0.98 ± 0.01	0.13 ± 0.05	0.67 ± 0.07	0.02	0.39 ± 0.10	0.62
DPH-PC	POPC	7.37 ± 0.08	0.94 ± 0.01	0.34 ± 0.13	0.73 ± 0.18	0.06	0.56 ± 0.24	1.45
DPH-PL	POPC	7.08 ± 0.05	0.95 ± 0.01	0.42 ± 0.12	1.04 ± 0.07	0.05	1.46 ± 0.33	2.05
DPH-PL	PL	6.50 ± 0.05	0.95 ± 0.01	0.89 ± 0.11	0.80 ± 0.15	0.05	1.20 ± 0.31	5.16
DPH-SM	POPC	6.16 ± 0.11	0.96 ± 0.01	0.28 ± 0.09	0.31 ± 0.16	0.04	0.19 ± 0.07	10.1
DPH-PC	SM	7.97 ± 0.10	0.97 ± 0.01	0.62 ± 0.11	0.18 ± 0.17	0.03	0.26 ± 0.06	4.89
DPH-SM	SM	6.60 ± 0.13	0.97 ± 0.01	0.36 ± 0.10	0.44 ± 0.27	0.03	0.48 ± 0.16	11.4
DPH-PC	GHOSTS	7.94 ± 0.07	0.95 ± 0.01	0.64 ± 0.09	0.68 ± 0.05	0.05	0.68 ± 0.09	2.40
DPH-PL	GHOSTS	7.90 ± 0.10	0.92 ± 0.01	1.02 ± 0.11	0.78 ± 0.05	0.08	0.90 ± 0.09	2.62
DPH-SM	GHOSTS	7.13 ± 0.08	0.93 ± 0.01	0.61 ± 0.09	0.63 ± 0.05	0.07	0.57 ± 0.07	3.91

C_i = Center of the Lorentzian lifetime distribution.
F_i = Fractional intensity.
ω_i = Full width at half maximum of the Lorentzian lifetime distribution.

We chose DPH propionic acid covalently linked to PC, SM or choline plasmalogen as a fluorescent reporter for potentially different lipid environments in the red blood cell membrane. Its anisotropy, mean lifetime and lifetime heterogeneity (in terms of the lifetime distribution width) reflect the mobility, polarity and environmental heterogeneity, respectively, within a lipid bilayer. Our working hypothesis was based on the fact that we looked at different phospholipids, carrying the same fluorescent label embedded in the same biomembrane. Thus, differences in the above fluorescence parameters must be due to a different microenvironment of the labeled lipid in the bilayer. We studied the fluorescence properties of the labeled lipid analogues in ghost membranes and protein-free phospholipid vesicles to determine the properties of the labeled lipid analogues, the influence of the surrounding membrane lipid and membrane proteins of the biomembrane. The centers of the main lifetime component (>90% fractional intensity) of a bimodal Lorentzian lifetime distribution were different

Table 2. Fluorescence anisotropy values of DPH-labeled phospholipids in unilamellar vesicles and erythrocyte ghost membranes

Lipid	Matrix	Anisotropy
DPH-PC	POPC	0.111 ± 0.007
DPH-PL	POPC	0.115 ± 0.008
DPH-SM	POPC	0.104 ± 0.006
DPH-PC	SM	0.123 ± 0.007
DPH-SM	SM	0.133 ± 0.008
DPH-PC	GHOSTS	0.122 ± 0.001
DPH-PL	GHOSTS	0.120 ± 0.001
DPH-SM	GHOSTS	0.120 ± 0.001

Temperature was 37°C; optical density was below 0.2 for all samples.

for all labeled lipid analogues in vesicles of PC and SM, respectively. Therefore, they reflect effects of the polarity of the surrounding lipid matrix and to some extent the polarity of the labeled lipids. The shorter lifetime of DPH-SM can probably be explained by the polar (OH) substitution of the shingosine backbone.

The distribution widths were very similar in all cases except that for labeled PC in SM, which was much broader. A similar effect has already been observed with choline plasmalogen bilayers. It was interpreted in terms of a less steep dielectric gradient across the hydrophobic-hydrophilic membrane interface [14, 15].

For the three labeled lipid analogues lifetime centers are higher in RBC membranes than in pure phospholipid vesicles. This increase is most pronounced with labeled SM. This effect might be due to the presence of proteins [39] and cholesterol [18] in the biomembrane. A similar shift has been reported by Fiorini et al. using DPH as a reporter molecule in erythrocyte membranes and liposomes prepared from extracted erythrocyte lipids [8, 9]. In addition there was a significantly broader distribution in the biomembranes compared to the liposomes [8, 9]. DPH-PC fluorescence lifetimes are more homogeneous in vesicles composed of the main lipids (PC/SM/cholesterol) of the outer erythrocyte membrane layer as compared to the protein-containing biomembranes. Thus, the large lifetime widths observed with erythrocyte ghosts are due to membrane proteins to a significant extent. Cholesterol is unlikely to contribute to lifetime broadening of DPH-lipids in the cell membranes. In agreement with previous data obtained with lipid vesicles [18], the cholesterol-containing bilayers showed smaller lifetime distribution widths as compared to the sterol-free lipid membranes.

The distribution width of the major fluorescence lifetime component depends on the type of label (DPH-PC od -PL) and to a much larger extent on the unlabeled lipid matrix. Plasmalogen vesicles always show larger distribution widths as compared to POPC. For DPH-PL in RBC membranes, we found very broad lifetime distributions, corresponding to those observed for DPH-PL in plasmalogen vesicles and much broader than DPH-PL widths in POPC. On the other hand, DPH-PC in the biomembrane shows much less lifetime heterogeneity similar to the value of DPH-PC in SM vesicles which is larger than that of the same label in POPC. The lifetime width of DPH-PC in the ghost membrane is not surprising, since on the one hand DPH-PC might be in contact with SM (and proteins) and on the other hand it "sees" a considerable number of different PC species differing by their acyl chains. PC is probably not in contact with plasmalogen in the RBC membrane. In plasmalogen vesicles its lifetime width is larger as compared to the RBC membrane. The lifetime properties of DPH-PC and -SM are very similar in RBC membranes. Thus, we may speculate that both phospholipid classes might be intermixed in the biological membrane. In contrast to this, but in agreement with the data discussed above, choline plasmalogen might undergo considerable phase separation.

We can exclude cholesterol effects in this respect, since cholesterol would rather lead to smaller widths (Table 1) [15, 18]. Mobility (orientational) effects, e.g., immobilization of lipids by proteins and/or cholesterol, can be excluded as well, because the fluorescence anisotropies are almost identical for labeled PC, SM and plasmalogen in the ghost membrane.

Therefore, we speculate that biophysical membrane properties of the alkenylacyl glycerophospholipids may contribute to this increase in lifetime heterogeneity of plasmalogen in the biomembrane. The conformation of the 2-acyl side chain is different for enolether lipids as compared to ester derivatives [14, 28] and differences of the hydrophilic-hydrophobic interface (depth of water penetration) between alkenyl and diacyl-GPC have been reported [36] that influence the dielectric gradient and subsequently the width of the fluorescence lifetime distribution. The above effects occur only in a plasmalogen matrix. In artificial membranes much broader lifetime distribution widths are observed with DPH-plasmalogen in a plasmalogen matrix as compared to a diacyl-PC bilayer [15].

The authors thank Mr. Herbert Stütz for expert technical assistance. Financial support by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (Projekt F0107) and by the Österreichische Nationalbank (Projekt 6939) is gratefully acknowledged.

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