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Study of Plasma Membrane Heterogeneity Using a Phosphatidylcholine Derivative of 1,6-Diphenyl-1,3,5-Hexatriene [2-(3-(Diphenylhexatriene)propanoyl)-3-palmitoyl-L-α-phosphatidylcholine]

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The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and DPH derivatives have been used to characterize structural and physicochemical properties of specific membrane domains. Steady-state and fluorescence decay measurements of three probes, DPH (1,6-diphenyl-1,3,5-hexatriene), TMA-DPH [1-(4-trimethyl-ammonium-phenyl)-6-phenyl-1,3,5-hexatriene], and a phosphatidylcholine derivative of DPH, DPH-pPC [2-(3-(diphenylhexatriene)propanoyl)-3-pamitoyl-L-α-phosphatidyl choline], have been performed in erythrocyte membranes and in lymphocyte plasma membranes. The steady-state fluorescence polarization of the three probes showed a similar trend in both membranes. In fact either in erythrocyte or in lymphocyte plasma membranes the fluorescence polarization values of DPH-pPC and TMA-DPH were similar, but significantly higher with respect to DPH. A better characterization of erythrocyte and lymphocyte plasma membranes was possible by using fluorescence decay measurements. The data suggest the possible use of different DPH derivatives to characterize specific domains in biological membranes.

KEY WORDS: Fluorescent phospholipid; plasma membrane heterogeneity; fluorescence lifetime decay.

INTRODUCTION

Much experimental evidence has been accumulated suggesting a heterogeneous organization of plasma membrane, with membrane molecules not randomly distributed in the plane of the bilayer but, rather, organized in distinct domains [1,2]. In a biological membrane, microenvironments of different dielectric costants could be formed due to the presence of cholesterol and/or protein molecules and as a consequence of microdomains of specific phospholipid composition. Moreover, the linkages of membrane molecules with cytoskeleton can be involved in the formation of compositional and functional domains.

DPH (1,6-diphenyl-1,3,5-hexatriene) has been widely used as a fluorescent probe to investigate membrane molecular order and it has also been proposed as a probe to analyze membrane heterogeneity. Its decay from the excited state, strongly influenced by the dielectric costant of the solvent [3], can be described by a continuous distribution of lifetime values whose width is related to the heterogeneity of chemical and physicochemical properties in the microenvironments of the probe. However, DPH shows some drawbacks in its application. In fact, it diffuses in the membrane hydrophobic core during the lifetime and therefore averages microenvironments of different dielectric costants; more-

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over, its orientation as well as its localization in the lipid bilayer is rather uncertain [4,5].

DPH derivatives, which include a charged species [1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene; TMA-DPH], and phospholipid derivatives, which localize the chromophore at different positions within the bilayer, have been proposed as more suitable probes to investigate structural and physicochemical properties of specific membrane domains [6,7]. In particular, fluorescent derivatives of phospholipids, as the phosphatidylcholine analogue (DPH-pPC), being identical to natural phospholipids with respect to headgroup structure and polarity, may reflect the behavior of their natural prototypes in biological membranes, as suggested by the results obtained with other phospholipid derivatives [8,9]. Moreover, DPH derivatives remain in the plasma membrane without internalization long enough to allow fluorescence measurements [10,11].

To characterize membrane heterogeneity with different probes, we studied the properties of DPH and DPH derivatives in erythrocyte membranes and lymphocyte plasma membranes using both static and dynamic fluorescence.

EXPERIMENTAL PROCEDURES

Erythrocyte membrane were obtained according to the method of Steck and Kant [12]. Lymphocyte plasma membranes were isolated following Kaever *et al.* [13]. Protein concentration was determined by the method of Lowry *et al.* [14]. Membranes (200–250 μg protein/ml) were labeled with DPH or derivatives following Stubbs *et al.* [15]; a probe/phospholipid ratio of 1:800 mol/mol was used. The incorporation of DPH-pPC in plasma membranes was described by Ferretti *et al.* [16], by studying the decrease in self-quenching. Briefly, suspensions composed exclusively of DPH-pPC, not showing well-defined fluorescence emission spectra, were incubated with membrane samples. The transfer of the probe molecules to membranes causes dequenching and the reexpression of fluorescence, and after an incubation of about 30 min at room temperature, the fluorescence intensity reached stable values. Unincorporated probe was removed by centrifugation and the fluorescence measurements were performed in the membrane pellet resuspended in buffer [16].

Fluorescence spectra and steady-state fluorescence polarization measurements were performed with a Perkin–Elmer MPF66 fluorometer [λ_{ex} = 360 nm and λ_{em} = 430 nm). Steady-state fluorescence polarization (P) was calculated following Shinitzky and Barenholz [17]. Excited-state lifetimes (τ) were determined by the phase modulation technique using a multifrequency phase fluorometer (ISS GREG200).

Measurements were done following Fiorini *et al.* [18], with an excitation wavelength of 360 nm and a 430-nm-long pass emission filter (Corion LG-370). The lifetime in membranes was measured over a large range of modulation frequencies, between 2 and 200 MHz. Data were accumulated and analyzed following Ferretti *et al.* [16]. The analysis software was provided by ISS Inc., according to the models and equations described by Alcalà *et al.* [19]. The distribution is characterized by a Lorentzian shape centered at a decay time C and having a full width at half-maximum w.

RESULTS AND DISCUSSION

The photophysical properties of DPH-pPC have been characterized by Parente and Lentz [20]. These authors demonstrated that this fluorescent phospholipid has photophysical properties similar to those of DPH [21,22]. It was shown that both steady-state and fluorescence decay parameters of DPH-pPC are sensitive to structural changes in the phospholipid bilayer, showing a pattern of fluorescent decay and steady-state polarization data similar to those of the related molecules DPH and TMA-DPH. Moreover, Pap *et al.* [23] showed that the fluorescence decay lifetime of all DPH probes is sensitive to polarity. The DPH moiety is thought to be located in the hydrocarbon core of the lipid bilayer, while the cationic TMA-DPH is at the lipid–water interface [24]. Cheng [21] showed that the emission moment of DPH-pPC fluorophore, in phosphatidylcholine/phosphatidylethanolamine mixtures, is located in the hydrophobic region of lipid layers, spanning approximately from the third carbon position to the end of the chain. For this reason this fluorescent phospholipid derivative appears to be particularly useful because it is likely located in the membrane plane, parallel to the membrane normal, and it would display motional behavior similar to that of neighboring lipids.

In erythrocyte membranes the fluorescence polarization value (P) of DPH-pPC was similar to that obtained with TMA-DPH (0.354 ± 0.002 vs 0.359 ± 0.007 at 25°C), but significantly higher than that for DPH (0.296 ± 0.006; $P<0.02$) (Table I). Slightly higher values of P for DPH-pPC and TMA-DPH (0.337 ± 0.005 and 0.343 ± 0.005, respectively) with respect to DPH (0.301 ± 0.004) were also observed in lymphocyte membranes (Table I). Comparison of the data in the two kind of membranes suggests that TMA-DPH and DPH-pPC

Table I. Fluorescence Polarization Values (P) of DPH and Derivatives in Erythrocyte and Lymphocyte Plasma Membranes[a]

	Membranes	
	Erythrocyte	Lymphocyte
DPH	0.296 ± 0.006	0.301 ± 0.004
DPH-pPC	0.354 ± 0.002	0.337 ± 0.005
TMA-DPH	0.359 ± 0.007	0.343 ± 0.005

[a]Measurements were made at 25°C. Results are expressed as mean ± SD.

Table II. Distributional Analysis of the Fluorescence Emission Decay in (A) Lymphocyte and (B) Erythrocyte Plasma Membranes[a]

	τ_1	w_1	f_1	τ_2	w_2	f_2
			A			
DPH	10.49	0.05	0.93	3.76	0.70	0.07
TMA-DPH	6.24	0.05	0.84	2.69	0.89	0.16
DPH-pPC	6.74	0.93	0.88	1.18	0.43	0.12
			B			
DPH	10.75	1.39	0.99	1.58	0.50	0.040
TMA-DPH	6.37	0.05	0.99	0.28	1.24	0.016
DPH-pPC	8.04	2.99	0.97	0.32	0.90	0.027

[a]Measurements were made at 25°C. Reduced chi-square ranged from 2.62 to 5.89.

microenvironments are less "fluid" in erythrocyte than in lymphocyte membranes.

Following the model of Fiorini *et al.* [25], which proposed analyzing membrane heterogeneity in terms of the distribution width of DPH lifetime decay, we extended this approach to DPH derivatives.

DPH fluorescence decay is characterized by a long component of 10.75 ns in erythrocyte membranes and 10.49 ns in lymphocyte plasma membranes, with a fractional intensity of about 95% in both systems (Table II). A short lifetime component with a low fractional intensity was also observed, whose origin has been discussed extensively [17]. Due to its low contribution to the total fluorescence, the short component is not discussed further.

No changes in longer lifetime values for probes embedded in erythrocyte and lymphocyte plasma membranes were evident for TMA-DPH (6.37 and 6.24 ns, respectively) (Tables IIA and B). However, the lifetime value for DPH-pPC was 8.04 ns in erythrocyte membranes and 6.74 ns in lymphocyte plasma membranes, suggesting different DPH-pPC microenvironments in these two membranes (Tables IIA and B).

DPH and DPH derivative lifetime values are sensitive to the lipid phase, decreasing during the transition from the gel to the liquid-crystalline phases, in agreement with an increased water penetration in the bilayer [23,25,26].

Moreover, since DPH-pPC is likely not randomly distributed in the membrane plane, but preferentially located in the fluid phase [19], these results could indicate that DPH-pPC probes the presence of more fluid domains in lymphocyte plasma membranes.

Beside the values of the main lifetime, comparison of the decay of the three probes has also shown differences in the distribution width. In lymphocyte membranes, both DPH and TMA-DPH showed a narrow distribution width (w_1 = 0.05 ns), compared to the distribution width associated with the DPH-pPC longer lifetime (w_1 = 0.93 ns) (Table IIA). In erythrocyte membranes a large distribution width was associated with longer lifetimes of both DPH and DPH-pPC (w_1 = 1.39 ns and w_1 = 2.99 ns, respectively) (Table IIB), suggesting a higher heterogeneity, for the microenvironments probed by these molecules, in this kind of membrane compared with lymphocytes. Differences in the fluorescence decay of these probes, with different membrane localizations, could be discussed in the light of the linkage among membrane composition, order, and interaction with cytoskeletal elements.

Further studies are necessary to increase the knowledge of membrane microheterogeneity, however, our data suggest that the continuous lifetime distribution model may be usefully applied to this research field. The possible use of various DPH derivatives may be helpful to characterize different environments of the membrane. Moreover, different phospholipid species labeled with DPH may be used to investigate specific membrane domains.

Since it has been suggested that fluorescent phospholipid derivatives could reflect the behavior of the natural prototypes in biological membranes [8,9], their applicability in intact cells could open a new research field in the study of membrane structural and functional organization.

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