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Interaction of a Phosphatidylcholine Derivative of 1,6-Diphenyl-1,3,5-hexatriene (DPH) with Intact Living Cells: Steady-State Fluorescence Polarization and Phase Fluorometry Studies

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The potential interest of DPH-PC was checked with a macrophagic cell line (P388D1). The uptake of DPH-PC was associated with a rapid increase in both fluorescence intensity and a slow decrease in anisotropy values. A flow cytometry comparative study with DPH revealed in both cases the existence of two cell subpopulations with different labeling levels. The analysis of fluorescence decay of DPH-PC showed two components. The fractional intensity of the main component (9.7 ns) is higher than 92%. The Lorentzian distribution of the main lifetime presents an important homogeneity. The observation that an increase in temperature induced a decrease in steady state anisotropy values but did not affect the lifetime suggests that the anisotropy variations effectively reflect modifications in the cohesion of probe micro-surroundings. A transmembrane diffusional phenomenon of a fraction of fluorescent phospholipids (20%) was suggested by a study with a nonpermeant membrane quencher. The transmembrane diffusion was confirmed by extraction of the phospholipid analog with fatty acid free BSA. The use of inhibitors of endogenous phospholipase A2 showed a progressive hydrolysis of the fluorescent phospholipid. Nevertheless, the hydrolysis can be neglected in the case of short term interactions with cells (< 30 min). Therefore, it can be assumed that DPH-PC can be used as a membrane probe.

KEY WORDS: 1,6-Diphenyl-1,3,5-hexatriene (DPH); living cells; phosphatidylcholine; phase fluorometry; steady-state fluorescence.

INTRODUCTION

DPH has been widely used as a fluorescent probe for assessment of the dynamic properties of biological membranes [10]. Unfortunately, in the case of intact cells, labeling with DPH is associated with a progressive incorporation through the plasma membrane into intracellular membranes [5]. So this probe gives only average information on the whole lipid regions of the cells. In the past years, fluorescent derivatives of phospholipids have been proposed to be able to probe the plasma membrane. In the present work, we were concerned mainly with 1-palmitoyl-2-diphenyl-1,3,5,-hexatrienylpropionyl-*sn*-glycero-3-phosphocholine (DPH-PC), where a DPH moiety replaces a hydrocarbon chain [6,8]. We focused our study on this probe because phospholipids are asymmetrically distributed over the two halves of the membranes of most cell types [3,9,12] and DPH-PC could

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probe one of the two leaflets of the plasma membrane. Using a macrophagic cell line (P388D1), we carried out steady-state and dynamic fluorescence measurements to determine the behavior of DPH-PC molecules with regard to its distribution in a cell population, transverse diffusion rate in plasma membrane [1,7,11], and membrane enzymatic metabolism [2,13].

MATERIALS AND METHODS

Fluorescent Probes and Labeling. DPH-PC stock solution (Lambda, Graz, Austria) in *N,N'*-dimethylformamide (DMF) and DPH (Aldrich, St Quentin Fallavier, France) in tetrahydrofuran (THF) were stored at −18 and 4°C respectively. For labeling, 1 μl of probe solution was added to 1 ml of cell suspension (3×10^6 cells/ml) in a phosphate buffer (PBS; pH 7.2) and cells were incubated for 30 min with DPH-PC and 20 min with DPH. In quenching experiments, bixin-glucosamine (BGA) synthesized in the laboratory was dissolved at a concentration of 3 m*M* in THF and at a final concentration of 15 μ*M* and added to the labeled cell suspensions.

Study of Phospholipid Translocation. Prior to labeling, the cells were incubated with diisopropyl fluorophosphate (DFP) (5 m*M* final concentration; 20 min) or with *N*-ethylmaleimide (NEM) (5 m*M* final concentration; 15 min). In another series of experiments, translocation of the probe to the inner layer was quantified by the back-exchange technique using fatty acid-free serum albumin (BSA) [1].

Fluorescence Instrumentation and Analysis. Steady-state fluorescence anisotropy and fluorescence intensity were measured with a continuous-excitation instrument (Fluofluidimètre, Affibio, Villers les Nancy, France). Excitation was performed at 365 nm when emitted light was determined at 425 nm.

Excited-state lifetimes (τ) were determined by the modulation technique with a multifrequency phase fluorometer, SLM AMINCO 48000S (SLM Instruments, Urbana, IL). A large range of frequencies, between 15 and 180 MHz, was used for the lifetime measurements. The data were analyzed with both exponential and continuous components models. The continuous model is a distribution of the lifetime components values which are characterized by a "Lorentzian" form centered on a lifetime with a full width at half-maximum parameter (FWMH). The mean-square test and the chi-square test were used to estimate the goodness of the determination.

The distribution of DPH-PC at the single-cell level was analyzed with a flow cytometer ATC3000 (Bruker Spectrospin, Wissembourg, France) equipped with an argon laser 2025 (Spectra-Physics, Mountain View, CA). For these experiments, the cells were labeled with variable amounts of probe solution (0.5 to 8 μ*M* for DPH-PC, 0.5 to 2 μ*M* for DPH) at 25°C. The analysis was done on 30,000 cells with excitation laser UV light (357 nm, 100 mW).

RESULTS AND DISCUSSION

Incorporation of DPH-PC (4 μ*M*) at 4, 25, and 37°C in P388D1 Cells

The kinetics of incorporation of DPH-PC in P388D1 cells is fast, whatever the temperature. After 2 min of labeling, the fluorescence intensity level corresponds to more than 50% of the final intensity and reaches a plateau after an incubation of about 20 min. As regards the evolution of fluorescence anisotropy $\langle r \rangle$ during the cell labeling, the values are constant after about 30 min of incorporation, and the following experiments were carried out under these conditions.

Distribution of the DPH-PC in Cell Populations

The fluorescence distribution of a cell population labeled with DPH-PC and DPH was analyzed by flow cytometry (Fig. 1). When the DPH-PC concentration increases, a modification of the histogram shape as well as a shift toward high fluorescence levels was noted. For a low concentration of DPH-PC (0.5 μ*M*), there was only one homogeneous, slightly labeled cell population. On the other hand, at higher concentrations (2 and 8 μ*M*) (Fig. 1A), a second highly labeled cell population appeared, which increased with DPH-PC concentrations. A comparative study was performed with DPH. The evolution of the fluorescence distribution was quite similar for equivalent concentrations of fluorophore (Fig. 1B). Thus, the binding of the DPH moiety on the phospholipid does not induce a different macroscopic distribution of the probe in a cell population.

Steady-State and Dynamic Fluorescence

Table I shows the variations in steady-state fluorescence anisotropy and lifetime determinations with temperature. It should be noted that $\langle r \rangle$ values decreased when the temperature increased.

Concerning lifetime measurements, the best fits were obtained with a biexponential analysis. DPH-PC decay is characterized by a main component of about 9.7 ns with a fractional intensity of 92% and a short component

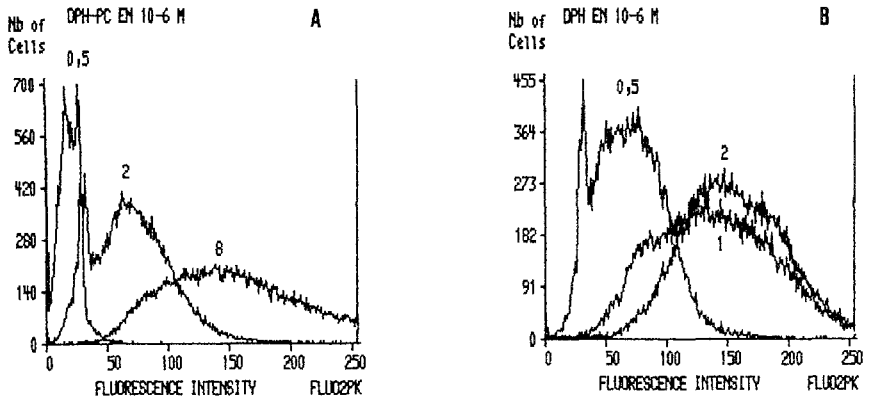

Fig. 1. Fluorescence distribution of a cell population labeled with various amounts of DPH-PC (A) and DPH (B).

Table I. Fluorescence Anisotropy, Discrete Values, and Lorentzian Distribution of the Fluorescence Lifetime of DPH-PC Incorporated in Cells

Temp. (°C)	<r>	Lifetime exponential analysis (ns)			Lifetime Lorentzian distribution (ns)				
								FWMH	
		τ_1	FI. $(\tau_1)^a$	τ_2	τ_1	FI. $(\tau_1)^a$	τ_2	τ_1	τ_2
4°C	0.300	9.63	92.1	0.90	9.67	92.0	0.95	0.002	1.01
25°C	0.250	9.72	92.5	1.03	9.81	92.4	0.92	0.001	1.34
37°C	0.235	9.68	94.6	1.10	9.60	92.5	1.30	0.01	2.20

[a] FI = Fractional Intensity (%)

close to 1 ns. The long lifetime component corresponds to DPH-PC incorporated in the plasma membrane of the cells and the short lifetime, rather, corresponds to DPH-PC molecules present in a very polar environment within the membrane surface or at the interface between two domains [4].

The continuous Lorentzian distribution analysis of the decay is in agreement with discrete analysis. This analysis gives another parameter (FWMH) that allows the appreciation of the homogeneity of the two lifetime components' distribution. The FWMH of the main component is very low and means a high homogeneity in the lifetime component value, while the FWMH of the second component reflects heterogeneity in the distribution.

Regarding the values of the long lifetime component, they give information on the location of the probe within the membrane. The values of τ_1 are included between the value found for DPH (11.5 ns) and that for its cationic derivative TMA-DPH (8.6 ns) [11]. The data suggest that the fluorophore is located in an intermediate area of the plasma membrane between those characterized by TMA-DPH (polar areas) and DPH (nonpolar areas). The <r> values support this hypothesis (data not shown) and confirm the assumption of Parente *et al.* that the fluorophore part of DPH-PC is located much farther down in the lipid hydrocarbon region of the bilayer than the carbon close to the glycerol backbone [8].

Finally, it is important to note that the values of both lifetime components as well as their fractional intensities are independent of the temperature. Thus, it may be assumed that steady-state anisotropy variations represent a real modification in the cohesion of the probe surroundings.

Partitioning of DPH-PC in Cells

To verify if the whole DPH-PC molecule is located in the plasma membrane, experiments with a nonpermeant membrane quencher (BGA) were carried out. Figure 2 shows that immediately after the addition of the

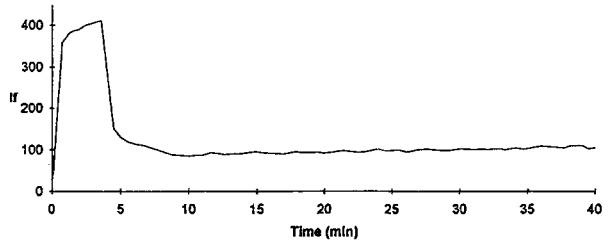

Fig. 2. Effect of BGA on the fluorescence intensity of a DPH-PC-labeled cell suspension.

quencher (15 μM) to the cells previously labeled at 25°C for 2 min with DPH-PC, the fluorescence intensity decreases very rapidly and reaches a plateau after 5 min, indicating that a constant amount of probe molecules (about 20%) was not quenched. The proportions of unquenched probe molecules varied weakly (20 to 27%) when the temperature range was 4–37°C. Therefore, it can be assumed that about 80% of the fluorescent phospholipid was in the plasma membrane.

Kinetics of Outside–Inside Translocation of DPH-PC in P388D1 Cells

The existence of transmembrane diffusion was confirmed by the back-exchange technique using cell labeling for various periods (0–6 h). Figure 3A shows that the percentage of probe molecules that were not extracted by BSA increased with time and with temperature. The pretreatment of cells with NEM, which is known

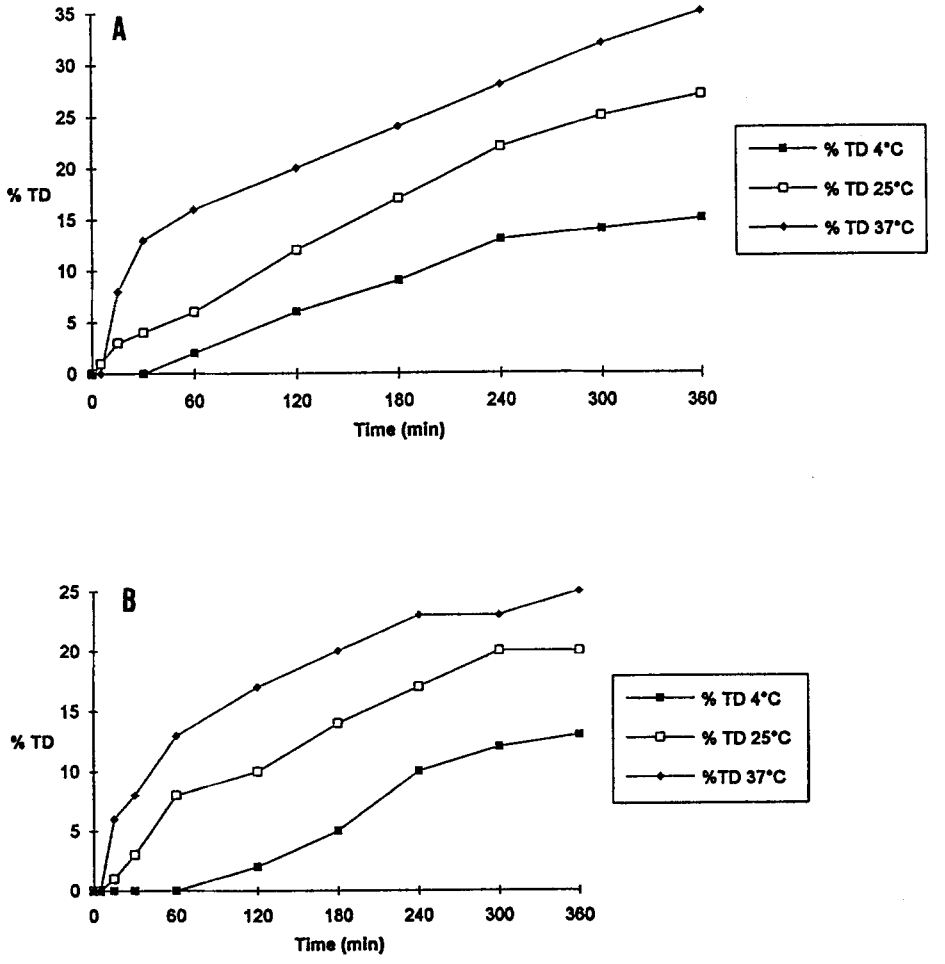

Fig. 3. Evolution of the percentage of transmembrane diffusion of the DPH-PC in P388D1 cells, without (A) or with (B) pretreatment with DFP, as a function of time and temperature.

Table II. <r> Value (Before and After Extraction) Evolutions of Labeled Cells During Translocation Experiments With or Without DFP Pretreatment of the Cells

Labeling time	<r>					
	Before extraction			After extraction		
	4°C	25°C	37°C	4°C	25°C	37°C
60 min						
−DFP	0.300	0.255	0.235	0.300	0.250	0.230
+DFP	0.300	0.260	0.242	0.300	0.255	0.235
180 min						
−DFP	0.295	0.245	0.225	0.290	0.235	0.215
+DFP	0.300	0.255	0.240	0.290	0.250	0.235
360 min						
−DFP	0.290	0.237	0.215	0.285	0.225	0.208
+DFP	0.300	0.250	0.235	0.290	0.245	0.235

to be an inhibitor of phospholipid translocase, did not show any significant effects on the translocation rate, suggesting that the phenomenon is not protein dependent.

In another series of experiments, it was considered that DPH-PC might be the target of membrane enzymatic metabolism as native phospholipids. Thus, a study with another inhibitor, DFP, was carried out to display the enzymatic activity related to phospholipase A2 (PLA2), which could cleave the molecule and induce release of free DPH.

Preincubation of the cells with DFP (Fig. 3B) demonstrated a marked effect on the outside–inside diffusion rate, which was lower than that observed with cells not treated with DFP, suggesting the possible involvement of a membrane enzymatic protein responsible for DPH-PC cleavage, liberating the fluorophore (DPH).

The hypothesis is confirmed in Table II, which summarizes the results of the experiments before BSA extraction and after extraction with or without pretreatment with DFP. Before extraction of the DPH-PC present in the outer leaflet and in the absence of DFP, it must be noted that <r> regularly decreased as a function of time and the decrease is more important at 37°C. On the other hand, following pretreatment with DFP, <r> remained constant at 4°C and decreased slightly at 25 and 37°C, suggesting that DFP treatment inhibited enzymatic activity. It is worth noting that pretreatment of the cells with DFP had no significant effect when cells were taken after a 30-min labeling with the fluorescent probe, which corresponds to the usual incorporation conditions of the probe.

CONCLUSION

From this work, it can be assumed that DPH-PC can be used as a membrane probe. However, the interpretation of modifications of steady-state or dynamic properties in terms of membrane cohesion of living cells must also take into account variations in transmembrane diffusion which may be associated with cell function or pathological situations. In the same way, it is necessary to check the activity of endogenous phospholipases.

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