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Membrane heterogeneity in isolated rat hepatocytes and liver plasma membrane subfractions: a comparative study using DPH and its cationic derivative TMA-DPH

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The fluorescence decay of 1,6-diphenyl-1,3,5-hexatriene (DPH) and of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) has been studied in hepatocytes isolated from rat liver and in isolated plasma membrane subfractions (cLPM, canalicular membranes and bLPM, basolateral membranes) using frequency domain fluorometry. The decay has been analyzed either by using a model of discrete exponential components or a model that assumes a continous distribution of lifetime values in order to study different aspects of membrane heterogeneity. The results obtained by the two analyses are practically superimposable but the distributional approach allows an evaluation of membrane heterogeneity through the width of the distribution that has shown particularly significant differences when freshly hepatocytes are compared with in vitro aged hepatocytes. Moreover, the comparison of the distributional analysis of the two probes has shown in cLPM a tendency to higher values of the main lifetime component and a narrower distribution width with respect to bLPM. These results indicate changes of membrane domain organization that have been discussed in relation with the specific lipid composition that characterizes the two membrane subfractions. Our results indicate that frequency domain fluorometry may be used to study membrane heterogeneity in intact cells and isolated membranes.

Introduction

Hepatocytes, like many epithelial and secretory cells, are highly-polarized elements characterized by morphologically, biochemically and functionally distinct surface domains [1,2]. To the functional polarity, which depends on the intactness of tight junctions [3], corresponds a specific distribution, between the plasma membrane domains, of the membrane proteins, including enzymes, transport proteins and receptors.

Different techniques have been developed for the separation and the purification of different plasma membrane regions from hepatocytes and other polarized cells such as renal and intestinal epithelial cells [1,4–6]. In the membrane vesicle preparations from renal and intestinal epithelial cells, the functional polarity and the presence of plasma membrane macrodomains, characterized by different lipid composition and fluidity [6–9] have been correlated. In hepatocytes the fluidity of canalicular membranes (cLPM) is lower with respect to the basolateral counterpart (bLPM) [10,11], in agreement with their respective lipid composition [12], in particular the higher cholesterol/phospholipid ratio in the cLPM domain with respect to the bLPM domain.

Superimposed to the macrodomain organization, there is evidence that membrane components are not randomly distributed in the bilayer [13–17], in fact a non homogeneous lateral distribution either for proteins or for membrane lipids has been shown in various membranes. This specific organizational heterogeneity may have functional as well as structural significance therefore the study of membrane heterogeneity is important and may improve our knowledge on membrane structure and its physiological implications. However, the characterization of different microdomains presents many methodologically and technical difficulties, especially in the plasma membrane of intact cells.

The fluorescent probe DPH has been widely used to study the physico-chemical properties of model and natural membranes (for a review, see Ref. 18) and to detect membrane heterogeneity through its fluores-

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cence lifetime decay. In previous works we have proposed the distributional analysis of DPH fluorescence decay as a phenomenological approach to the study of membrane microheterogeneity [19]. In fact, the decay of DPH in model and natural membranes can be described by a continuous distribution of lifetime values whose width can be related to the heterogeneity and to physico-chemical properties of the microenvironment where probe molecules are located [19–23]. In isolated membranes DPH is localized in the membrane hydrophobic core [24], on the contrary the cationic derivative TMA-DPH is located on the polar headgroup region [25,26]. Therefore, in isolated and model membranes, the parallel use of DPH and TMA-DPH on the same system allows to compare the membrane structure and heterogeneity at different depths of the bilayer. In intact cells, DPH penetrates rapidly into the cell apparatus [27] while TMA-DPH, after a rapid incorporation, remains specifically localized in the plasma membrane for a sufficient time to allow plasma membrane fluidity and heterogeneity measurements in the whole cells [28,29].

In the present paper we have applied the study of DPH and TMA fluorescence decay, measured by frequency domain fluorometry to characterize the microheterogeneity of rat liver plasma membrane subfractions. In addition, we applied the same approach to study TMA-DPH fluorescence decay in freshly isolated rat hepatocytes to analyze membrane heterogeneity in the intact cell.

Materials and Methods

DPH and TMA-DPH were purchased from Molecular Probes (Eugene, OR). All other reagents, including Collagenase type IV, were from Sigma (St. Louis, MO).

Two groups of Sprague–Dawley rats CD strain (Charles River Italia) have been considered: group I included young male rats (5 to 6 weeks old; 155 ± 10 g body weight); group II included adult rats (10 to 12 weeks old; 300 ± 15 g body weight).

Isolation of hepatocytes

Preparation of isolated hepatocytes was made according to the technique of Berry and Friend as modified by Hardison et al. [30,31]. Microscopic examination of the final suspension showed cell debris and isolated hepatocytes, of which 85–90% excluded Trypan blue. All experiments were carried out within 2–3 h after cell isolation.

Isolation and characterization of canalicular and basolateral liver plasma membranes (LPM) fractions

The procedure for isolation of liver plasma membrane fractions (LPM) was made according to Boyer et al. [32]. Small aliquots of the LPM fraction were also examined for purity after processing for electron microscopy following fixation with 1% osmium tetroxide and embedding in Epon Araldite. No significant contamination by intracellular organelles was present. The procedure for separation of the canalicular (cLPM) from the basolateral LPM (bLPM) subfractions was made according to the technique of Meier et al. [12]. A mixed LPM subfraction was isolated by differential centrifugation. The final pellet was homogenized and the vesiculated LPM elements were separated on a three-step sucrose gradient (31%, 34% and 38% wt/wt). The membranes collected at each interphase (cLPM at 31/34 and bLPM at 34/38) were then centrifuged at $10500 \times g$ for 60 min and resuspended in 0.25 mol/l sucrose, 0.2 mmol/l $CaCl_2$, 5 mmol/l $MgSO_4$, 10 mmol/l Hepes-Tris (pH 7.5). The degree of purification of cLPM and bLPM was analyzed by measuring Na^+/K^+-ATPase enzyme activity as bLPM marker enzyme [12].

Incorporation of fluorescent probe molecules in hepatocytes and plasma membrane

Incorporation of DPH and TMA-DPH in isolated plasma membranes and intact hepatocytes was made as described previously [11]. Plasma membranes subfractions (90–100 μg/ml) resuspended in 1 mmol/l $NaHCO_3$, 0.5 mmol/l $CaCl_2$ buffer (pH 7.5) or hepatocytes (10/ml of Schwartz buffer) were incubated with a stable dispersion of DPH or TMA-DPH (final probe concentration 10^{-6} mol/l). Incorporation of TMA-DPH in isolated hepatocytes was performed as described previously [11]. TMA-DPH from a $2 \cdot 10^{-3}$ mol/l stock solution in ethanol was added to hepatocytes (10^6 cells/ml of Schwartz buffer) and a final TMA-DPH concentration of 10^{-6} M was used.

Fluorescence measurements

Steady-state fluorescence polarization measurements were performed with a Perkin-Elmer MPF66 fluorescence spectrofluorometer. Lifetime measurements were performed by a multifrequency phase fluorometer (ISS GREG200) interfaced with a M24 Olivetti computer, for data collection and analysis [34]. Excitation wavelength was 325 nm (UV line of an HeCd Liconix model 4240 NB laser). A large range of modulation frequencies was used, between 2 and 130 MHz. POPOP in absolute ethanol was used as reference lifetime (1.35 ns) [23]. The fluorescence emission was collected using a Corion LG 370 S filter in emission. Data were accumulated until the standard devation of phase and modulation values at a given frequency were below 0.2° and 0.004, respectively.

The experimental data were analysed using a nonlinear least-square routine for the multiexponential fit [23] and a routine based on the simplex method for lifetime distribution analysis A program provided by

TABLE IA

Exponential analysis of TMA-DPH fluorescence emission decay in hepatocytes

τ_1, τ_2, lifetime in nanoseconds; f_1, f_2, fractional intensity; χ^2, reduced chi-square.

τ_1	f_1	τ_2	f_2	χ^2
4.54	0.79	1.03	0.21	11.3

ISS (La Spezia, Italy) was used for these analyses. The distribution used in this work is characterized by a Lorentzian shape centered at a decay time C and having a Full Width at Half-Maximum (FWHM). The reduced chi-square value was used to judge the goodness of the fit [22]. Given our uncertainty in the evaluation of the standard deviations of the individual points, a value of less than 3 is considered acceptable, while a value of the order of 10 indicated a large deviation between experimental and calculated values [23]. As discussed by Fiorini et al. [19], when using different functions for the fit the important parameter is the change in χ^2 rather than its absolute value. For both the exponential and distribution analysis, the programs minimize the reduced chi-square defined by an equation reported elsewhere [19]. The applicability of distribution analysis in comparison with exponential approach to study lifetime heterogeneity has been already discussed in detail by Alcala et al. [34].

Results

Intact hepatocytes

In hepatocytes isolated from fasted rats, TMA-DPH fluorescence emission decay as studied by exponential analysis (Table IA), is characterized by a long component of 4.54 ns with a fractional intensity of 0.79 and a short component of 1.02 ns with a fractional intensity of 0.21. The central value of the distribution (Table IB) is lower with respect to the value obtained with exponential analysis (3.87 ns). Although the results obtained for the lifetime values do not show remarkable differences between the two analysis, the distributional analysis has shown a chi-square reduction from 50% to 70% compared to the exponential analysis. No significant differences between fasted/fed rats and old/young rats, as studied by exponential and distributional analysis, have been observed (data not shown). However, sex-related changes which deserve further investigation have been observed (data not shown), therefore for the present study we have chosen 12 weeks old animals in the fasted state.

TABLE IB

Distribution analysis of TMA-DPH fluorescence emission decay in hepatocytes

τ_1, τ_2, lifetime in nanoseconds; f_1, f_2, fractional intensity; C, center of the distribution in nanoseconds; W, width of the distribution in nanoseconds; χ^2, reduced chi-square.

C_1	W_1	f_1	C_2	W_2	χ^2
3.87	1.56	0.89	0.88	0.91	3.3

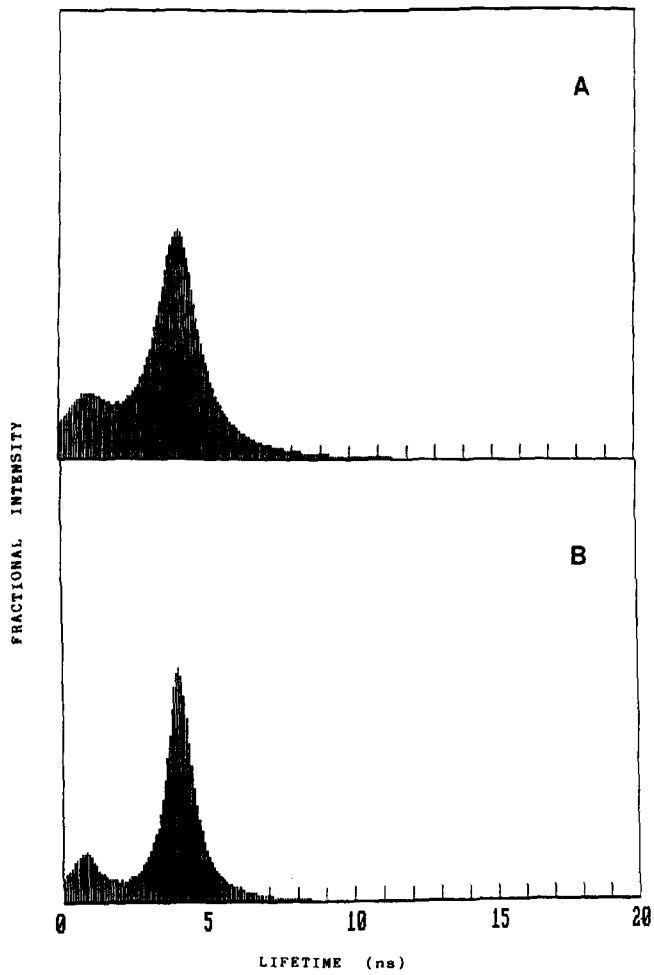

Fig. 1. Distribution analysis of TMA-DPH fluorescence emission decay ii, freshly isolated hepatocytes (A) and in hepatocytes after a incubation of 60 min at 37°C (B). (A) the distribution width of the main lifetime component has a center at 4.04 ns and a FWHM of 1.39 ns. χ^2 is 9.40. (B) the distribution width of the main lifetime component has a value of 0.51 ns and is centered at 3.90 ns and χ^2 is decreased to 2.90.

Freshly isolated hepatocytes exclude Trypan blue, indicating the intactness of the plasma membrane. After 1 h pre-incubation at 37°C in buffer, more than 15–20% of the cells become viable to the dye, indicating a loss of plasma membrane integrity [35]. After incubation at 37°C, using the distributional analysis we observed modifications of the fluorescence decay of TMA-DPH, while the long component did not show significant changes of the central value of distribution, a slight decrease for the short component was observed

TABLE IIA

Exponential analysis of the fluorescence emission decay of DPH and of TMA-DPH in basolateral (bLPM) and canalicular (cLPM) membrane fractions

τ_1, τ_2, lifetime in nanoseconds; f_1, f_2, fractional intensity; W, width of the distribution in nanoseconds; χ^2, reduced chi-square.

Probe	Sample	τ_1	f_1	W_1	τ_2	χ^2
DPH	bLPM	7.84	0.74	0.31	1.24	20.00
	cLPM	8.89	0.81	0.36	1.17	22.50
TMA-DPH	bLPM	5.13	0.74	0.36	1.06	12.70
	cLPM	5.58	0.85	0.52	1.09	15.30

(Fig. 1). For the longer lifetime, the distribution width was significantly modified, from 1.70 ns to 0.49 ns while the shorter lifetime was not modified (from 0.91 ns to 0.78 ns).

Fluorescence lifetime in bLPM and cLPM

DPH and TMA-DPH fluorescence lifetimes in cLPM and bLPM are shown in Table IIA and IIB. In both the membranes the majority of DPH fluorescence was associated with a long lifetime component. With exponential analysis in bLPM and cLPM the values observed were, respectively, 7.84 ns and 8.89 ns for DPH. The cationic derivative TMA-DPH showed, with respect to DPH, lower values of the main lifetime component in both subfractions (5.13 ns in bLPM and 5.58 ns in cLPM). A short lifetime component of about 1 ns was observed either in cLPM and bLPM with the two probes.

The same data were also analyzed using a continuous distribution of lifetime values. In agreement with the exponential analysis, the bLPM have shown a lower value of the long component with respect to the cLPM (6.05 ns vs. 7.68 ns for DPH and 3.98 ns vs. 4.80 ns for TMA-DPH). The study of the distributional widths with both probes has also shown a narrower distribution for cLPM compared with bLPM (Table IIB). The use of Global Analysis and the linkage of the w_1 parameter between cLPM and bLPM subfractions has shown an increase of χ^2 associated to bLPM and represents an evidence of a difference between the widths in the two subfractions.

Discussion

Most of the studies concerning membrane microheterogeneity have been performed in model and isolated natural membrane fractions [14,15,19–22]. Although the study of membrane properties in isolated membranes has given an important contribution to the elucidation of the structure and function of biological membranes, an estimation of membrane activities in intact cells remains a necessary step for elucidating the relationship between cell physiology, plasma membrane structure and physico-chemical properties. In fact during isolation of biological membranes, regulatory properties are likely to be partially lost.

Hepatocytes are in vivo highly polarized cells containing morphologically and functionally distinct surface domains [1]. Following tissue dissociation, some underlying mechanisms, responsible for polarity, remain operational, even if less effective [1], moreover, in whole hepatocytes, the relationships between the cell membrane and cytoplasmic cytoskeletal elements are maintained therefore these cells can represent an useful model to study membrane heterogeneity in living cells.

TMA-DPH molecules show an homogeneous distribution in the plasma membrane of whole cells in suspension [28] and the probe has been widely used to study plasma membrane fluidity in intact cells [28,29,35–37]. Using discrete exponential and distribution analysis, we have shown that TMA-DPH decay in hepatocytes can be characterized by two components. The value of the long component of about 4.54 ns, obtained by exponential analysis, is comparable with values observed in pulmonary artery or aortic endothelial cells (4.40 ns and 4.90 ns, respectively) [37], but it appears shorter with respect to the values in other cells in suspension (6.29 ns in MDCK cells and 6.50 ns in PMNs) [36,29]. These results could be related to differences of plasma membrane lipid composition in various cells as suggested for DPH [15]. A TMA-DPH short component (1.03 ns with a fractional intensity value of 0.2) was also observed. The meaning of the second lifetime component is still debated: for DPH, this lifetime has been related to photochemical derivatives

TABLE IIB

Distribution analysis of the fluorescence emission decay of DPH and of TMA-DPH in basolateral (bLPM) and canalicular (cLPM) membrane fractions

C, center of the distribution in nanoseconds; W, width of the distribution in nanoseconds; f, fractional intensity; χ^2, reduced chi-square.

Probe	Sample	C_1	W_1	f_1	C_2	W_2	χ^2
DPH	bLPM	6.05	4.17	0.89	0.58	0.10	2.50
	cLPM	7.68	3.98	0.93	0.47	0.07	2.40
TMA-DPH	bLPM	3.98	2.35	0.90	0.58	0.10	0.99
	cLPM	4.80	1.99	0.96	0.41	0.10	1.14

of the probe [38], or to a fraction of probe molecules localized in a very polar environment [13]. In our recent work we have hypothesized that, in the case of TMA-DPH, this short lifetime might be related to different sets of microenvironments for the probe and to different pattern of heterogeneity in the bilayer [22]. Significant changes of the distribution width of both lifetimes and the decrease of chi-square have been observed in hepatocytes labelled with TMA-DPH after an incubation of the cells for about 45–60 min at 37°C. These findings suggest a decrease of plasma membrane heterogeneity and could be related to modifications of plasma membrane organization that have been described during incubation of isolated hepatocytes [39].

In the present study, using a discrete exponential analysis, we have observed that the decay of DPH and of its cationic derivative TMA-DPH requires, in cLPM and bLPM, at least two exponentionals for adequate description. In both fractions, TMA-DPH lifetime values were lower with respect to DPH. These results, in agreement with the results obtained in other membranes [41,22], could be related to the membrane localization of the two probes. TMA-DPH because the bulky charged trimethilammonium group is localized in a more superficial region of the bilayer, and therefore, is much more exposed to the quenching effect of water molecules with respect to DPH. Moreover the comparison of the distributional analysis of the two probes has shown in cLPM a tendency to higher values of the main lifetime component with respect to bLPM.

The study of the properties influenzing the DPH fluorophore lifetime distribution using model membranes and microsomes and the bimodal Lorentzian fluorescence lifetime distribution, has shown that increasing complexity of lipid composition is associated with a broader width of the main lifetime component [42] that indicates a greater environmental heterogeneity. It has also been proposed that the width of the distribution can be taken as an indication of the degree of membrane heterogeneity related to structural defects which induce microenvironments of different dielectric constant [20]. The cholesterol content of biological membranes also exerts a relevant effect on membrane microheterogeneity, as revealed by a broader width in cholesterol depleted erythrocytes with respect to control membranes [20].

The characterization of the two LPM subfractions (cLPM and bLPM) has demonstrated considerable differences in membrane lipid composition and enzyme activities [1,2]. cLPM that account for only 13% of the total hepatocyte surface membrane, is characterized by a higher content in sphingomyelin than in bLPM (23.9 vs. 14.3%) with a concomitant increase of the sphyngomyelin/phosphatidylcholine ratio that is 2-fold higher in cLPM with respect to bLPM. cLPM also show an higher cholesterol/phospholipid molar ratio (0.54 vs. 0.42) and a significant increase in the content of sialic acids (86 vs. 42 nmol/mg of protein) that indicates significant differences in the content of acidic glycolipids [12]. Using the fluorescence polarization of DPH and TMA-DPH we have shown that rat cLPM are more rigid with respect to the bLPM [11] suggesting the presence of a lateral fluidity gradient in the plasma membrane of rat liver cells. The contemporary use of the two fluorescent probes has also allowed to observe a vertical gradient of fluidity. In fact, the inner core portion of the bilayer, probed by DPH, was more fluid with respect to the surface probed by TMA-DPH [11] in agreement with theoretic calculations regarding the movements of acylchains and with experimental observations [41]. Therefore the differences of the fluorescence decay of DPH and TMA-DPH in bLPM and in cLPM could be discussed in the light of the relation between membrane composition, order and permeability. In agreement with the membrane lipid composition, cLPM show an increase of order with respect to bLPM [10,11] that is associated with a narrower width suggesting that a decrease of solvent penetration could be the major contributing factor. A decrease of water penetration inside the bilayer is also supported by the higher lifetime values of DPH and TMA-DPH in cLPM when compared with bLPM. Differences in the fluorescence decay of DPH or other fluorescent probes, associated with changes in lipid composition, have been observed in membrane subfractions isolated from other polarized cells such as renal membranes and bovine thyroid plasma membrane [43,44] (Table III). Altogether our data in comparison with those obtained from literature have shown that fluorescence decay analyzed by distributional approach can be considered a useful tool to analyze membrane heterogeneity along with the characterization of membrane structure and functions.

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TABLE III

DPH fluorescence lifetime (τ_1) in plasma membrane subfractions isolated from different tissues

τ_1 is expressed in nanoseconds.

Cell/Tissue	Membrane subfraction	τ_1	Ref.
Hepatocyte	canalicular (cLPM)	8.9±0.2	
	basolateral (bLPM)	7.8±0.2	
Thyroid	luminal	9.3±0.3	43
	basolateral	9.2±0.2	
Kidney (rat)	cortical brush border	10.8±0.2	39
	basolateral brush border	9.3±0.1	
Kidney (rabbit)	basolateral (ALM)	8.9±0.3	44
	brush border (BBM)	9.3±0.4	

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