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Membranes modification of differentiating proerythroblasts. Variation of 1,6-diphenyl-1,3,5-hexatriene lifetime distributions by multifrequency phase and modulation fluorimetry

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The fluorescence emission of 1,6-diphenyl-1,3,5-hexatriene (DPH) in K562 cell membranes has been studied using multifrequency phase and modulation fluorimetry. The DPH decay data collected at various modulation frequencies were analysed by assuming either a model of discrete exponential components or a model of continuous lifetime distribution. The fits showed smaller values of the reduced chi square using the model of continuous lifetime distribution. The K562 cell membranes dynamics were investigated during the cell differentiation along the erythroid pathway. By using the continuous lifetime distribution method for the analysis of the DPH decay, marked variations were observed during the four initial days of the erythroid differentiation. Namely, the width of the DPH lifetime distribution increased by a factor of about two, while the center value of the distribution remained constant. By using the discrete exponential components model for the analysis of the DPH decay no variations were observed during the K562 differentiation.

Introduction

The cell differentiation process is generally described as the acquisition of structural and functional properties peculiar to the mature, specialized cell. The process is controlled by the gene expression but the related molecular structural and dynamical events are largely unknown. The cell membranes can be intimately involved in the control of the differentiation process and in the expression of specific structural components, such as surface constituents [1,2]. Moreover, the modulation of the activity of some membrane enzymes during physiological processes could be achieved by structural and dynamical modification of the membrane matrix [3]. Since different lipid phases coexist in the membrane matrix, their possible rearrangement could produce modifications of the membrane architecture and the modulation of enzymatic activity.

Many efforts have been devoted to the determination and quantitation of different lipid

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; PNA, parinaric acid; *cis*-PNA, *cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid; *trans*-PNA, all-*trans*-9,11,13,15-octadecatetraenoic acid.

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phases in biological membranes [4–6]. Generally, model systems have been studied to characterize the physico-chemical state of membranes. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) shows marked lifetime differences in phospholipid vesicles of different phases and the determination and the quantitation of the two phases was achieved [6]. Two fluorescence lifetime components were determined, corresponding to the gel and to the liquid-crystalline phase of the bilayer. The quantitation of the two phases and their variation vs. temperature was then obtained by the preexponential factors, values, associated with the two DPH lifetimes. Nevertheless, this approach gave dubious results when applied to natural membranes [7] possibly because of the number of different environments. In the case of natural samples, a separation of the lipid constituents between the gel and the liquid-crystalline phases could represent an oversimplification. Indeed, a variety of different components are present in natural cell membranes and many physically different environments can be experienced by the probe, giving rise to multiple fluorescence decay rates. Then a decay model assuming a sum of discrete exponentials could be inadequate to describe DPH decay when inserted in natural membranes. An alternative model has been recently developed [7], assuming a continuous lifetime distribution and justified by the variety of environments the fluorescence molecules can experience.

In the present study we report results obtained by labeling the K562 cell membranes with DPH. In recent years, our efforts for the determination of the spectroscopic characteristics of membrane probes have been also devoted to the study of the decay behaviour of the parinaric acid (PNA) isomers in isotropic solvents and in phospholipids [8]. The very complex decay observed for both *cis*- and *trans*-PNA isomers in solvents and in model phospholipids vesicles discouraged their further use for the study of natural membranes [8]. Moreover, also using the distributional approach for the analysis of the PNAs decay, a complex behaviour was observed (work in preparation) and further studies are needed before using these fluorophores as natural membrane probes. Instead, the DPH decay was satisfactorily described by a monoexponential decay in isotropic solvents [6] and either by two-exponential components [6] or by continuous lifetime distribution [7] in model phospholipid vesicles.

We used K562 cells, a human proerythroblastic cell line which can be induced to differentiate along the erythroid pathway by various substances, butyric acid and hemin for instance [9,10]. The induced cells have been shown to produce hemoglobin and glycophorin, the major sialoglycoprotein of the erythroid cell surface [11]. The induced cells also show altered agglutinability by concanavalin A [10]. DPH decay was followed during the differentiation of the K562 line and modifications of the membrane dynamics have been observed.

For the determination of DPH lifetimes we used sinusoidally modulated exciting light at frequencies variable from 2 to 160 MHz [12]. The phase and modulation data were analysed using both a model of discrete exponential components [13] and of continuous lifetime distribution [7].

Materials and Methods

DPH was obtained from Molecular Probes Inc. (Oregon) and used without further purification. A stock solution was prepared in tetrahydrofuran (spectroscopic grade) and stored in the dark at −20°C. The K562 subclone S [9], was maintained at 37°C in RPMI 1640 medium, supplemented with 10% fetal calf serum and routinely subcultured every 4 days. For the induction of erythroid differentiation, cells were subcultured to a density of $1 \cdot 10^5$ cells/ml before the addition of butyric acid (the final concentration was 2 mM). Increased hemoglobin synthesis was detected after 3 days following butyric acid treatment [10] by the reported method [10,14]. $1.5 \cdot 10^6$ cells were washed twice with phosphate-buffered saline and resuspended in 3 ml of the same medium with 0.5 mM Ca^{2+} and 0.1% glucose. An aliquot of the DPH tetrahydrofuran solution was previously evaporated in the buffer by vigorous N_2 bubbling, in the dark. The final probe concentration was 0.5 μM. The resuspended cells were incubated in the presence of the DPH suspension for 30–40 min, in the dark and with mild magnetic stirring. DPH fluorescence lifetimes were determined using a multifrequency phase and modulation fluorimeter. The

instrument is a modification of a SLM 4800S apparatus and is similar in design to that described by Gratton and Limkeman [12]. The light source is the 325 nm line of an He-Cd laser (Liconix, Sunnyvale, CA). The modulation of the exciting light was achieved by a Pockels cell (Lasermetrics Inc., Englewood, NJ) installed on a ISS modulation module (ISS, La Spezia, Italy). Phase and modulation data were obtained using an ethanol solution of dimethyl-POPOP (measured lifetime value = 1.4 ns) as the reference. The fluorescence was observed after a Janos Tech. GG cutoff filter cutting below 375 nm. Under our experimental conditions the fluorescence background of unlabeled resuspended cells was less than 0.2% of the total fluorescence. During the measurements the cells were maintained in suspension by mild magnetic stirring. The fluorometer cell holder was kept at 37°C by a water-circulation thermostat. Phase and modulation data were collected by an ISS interface (ISS Inc., Urbana, IL) for an Apple II computer, for 9 to 12 modulation frequencies in the range from 2 to 160 MHz. Data were analysed by a computer program from the ISS Inc. designed for an IBM computer. The least-squares routine for a multiexponential decay has been discussed elsewhere [13]. The description of the method for the derivation of a continuous distribution of lifetime values has been reported [7]. The distribution is characterized by a Lorentzian shape centered at a decay time C and having a width W.

Results

The kinetics of the differentiation process induced by butyric acid on the K562 cell line was determined following the hemoglobin synthesis as described [10]. The presence of hemoglobin was detected after only 3 days from the induction. At that time the hemoglobin concentration varied between 0.02 and 0.06 $\mu g/10^6$ cells. This hemoglobin concentration was still too low to produce quenching of the DPH fluorescence.

Phase and modulation data of the DPH decay in K562 cell membranes were analysed using a discrete and a continuous lifetime distribution model. The goodness of the fit was judged by comparing the value of the reduced chi square [13]. Three lifetime components were necessary to obtain acceptable fits by using the discrete exponentials analysis method.

Using both analysis methods a very short component with the lifetime (or the center) value of around 1 ns and with the fractional intensity of around 0.05 (Figs. 1 and 2) was found, as reported [6,7]. This component has been tentatively attributed to photodecomposition phenomena [6], probably enhanced by the presence of double bonds in the lipids acyl chains and by the presence of oxygen in the medium. The lifetime (or the center) and the fractional contribution of this short component did not show any variation during K562 differentiation.

Two other components resulting from the discrete exponential analysis method had fixed lifetime values of 10.5 and 7.5 nsec and the associated fractional intensities of around 0.2 and 0.8, respectively. These two components have been observed to correspond to the gel and to the liquid-crystalline phase of the bilayer [6] and their associated preexponential factors correspond to the molecular fraction of each phase. Following the differentiation process these components did not show any significant variation of their preexponential factors. Fits obtained by the discrete exponentials analysis always showed larger chi square values

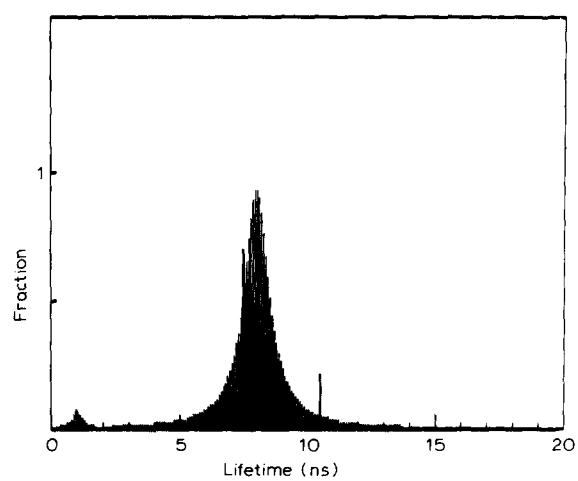

Fig. 1. DPH lifetime distribution in non differentiated K562 cells. The distribution has a FWHM value of 0.7 ns and is centered at 8.0 ns. Superimposed are the lifetime values determined by the discrete exponentials analysis.

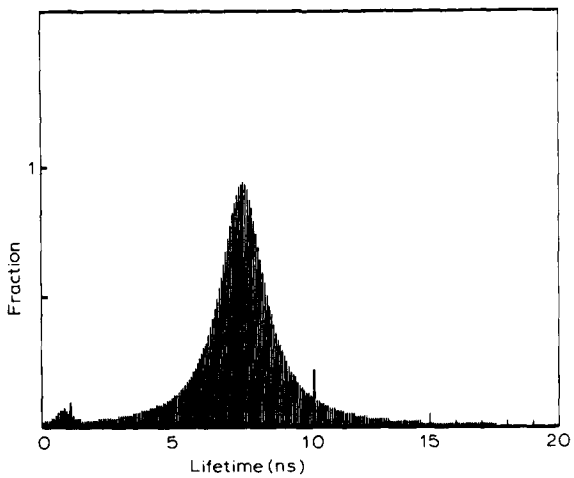

Fig. 2. DPH lifetime distribution in K562 cells 100 h after the induction of differentiation. The distribution has a FWHM value of 2.1 ns and is centered at 7.8 ns. Superimposed are the lifetime values determined by the discrete exponentials analysis.

with respect to those obtained by the continuous lifetime distribution method (average chi square value reduction of around 40%).

In Figs. 1 and 2 the DPH lifetime distributions in non-differentiated and in differentiated K562 cells are reported. Superimposed are the lifetime values obtained by a three-component analysis. In non-differentiated cells the distribution appears relatively broad (full width at half maximum (FWHM) of around 0.8 ns, Fig. 3 and centered around 8 ns. The width of the distribution is of relevance since it can qualitatively explain the necessity of using more than one exponential term in the case of the discrete components analysis.

Following the differentiation process the FWHM values of the DPH lifetime distribution increased by about a factor of two. In Fig. 2 the DPH lifetime distribution in K562 cells during the fourth day after the induction of the differentiation is reported. The value of the center was constant while the FWHM increased to a value of 2.1 ns. Superimposed are the lifetime values obtained by a three exponentials analysis. In Fig. 3 the behaviour of the FWHM value during the four days after the butyric acid induction of differentiation is reported. The FWHM value increased from the second day, reaching a plateau

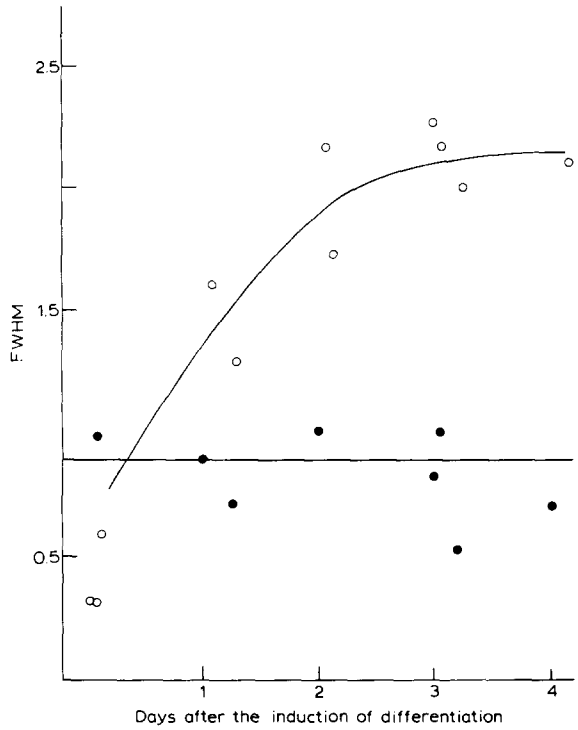

Fig. 3. FWHM value of DPH lifetime distribution during differentation of the K562 cells. Full circles represent the FWHM values obtained in parallel control experiments on non-differentiated cells.

during the third day. In Fig. 4 the behaviour of the associated value of the center is reported. During the four days after the induction of differ-

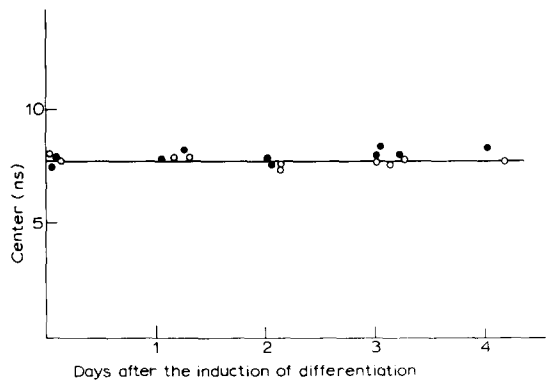

Fig. 4. Center value of DPH lifetime distribution during K562 differentiation. Full circles represent the center values obtained in parallel control experiments.

entiation no significant changes were observed from the average value of 7.8 ns.

Discussion

The DPH lifetime value has been shown to be sensitive to the physical state and to the dielectric constant of the molecule environment [6,7]. Although the reasons for the changes in lifetime are unclear, DPH has been used as an indicator of the membrane state. In natural membranes many different lipids, phospholipids are fatty acids exist, apart from the various proteins. The association of these components can give rise to a multiplicity of dynamical interactions, with the temporary creation of different physical states and properties. Since DPH is assumed to partition equally between the different environments [15], its lifetime values should reflect such a complexity. Indeed, the DPH decay in non diffferentiated K562 cell membranes was better described using a continuous lifetime distribution (Fig. 1) rather than in terms of discrete exponentials. The value of the reduced chi square serve as the criterion to judge the goodness of the fits [13]. For the data presented in Fig. 1 a chi square value of 3.2 was obtained using the distributional method while a chi square value of 4.9 was obtained using three exponentials.

During K562 differentiation, the FWHM value of the DPH lifetime distribution increased (Fig. 3) indicating a heterogeneity increase in the probe environment. The increase of the FWHM value started from the first day after the induction or erythroid differentiation and reached the plateau during the second day. The presence of hemoglobin was detected only during the third day. Thus, the observation of the membrane heterogeneity modification by the FWHM value variation constitutes a relevant early indication of the differentiation process. On the contrary, the center value of the distribution remained constant (Fig. 4) during the four days after the induction of differentiation, probably reflecting the constance of the averaged physico-chemical properties of the membranes around the same central value.

In the discrete exponentials analysis method two lifetime components of 10.5 and 7.5 ns were fixed, corresponding to the DPH lifetime values in pure gel and in pure liquid-crystalline phase, respectively [6]. The associated preexponential factors did not show any significant variation during the K562 differentiation process. Labeling with DPH vesicles composed of pure phospholipids showing lateral phase segregation (acyl chains differing of 4 carbon atoms), the fluorophore decay was succesfully resolved using this exponential method. Fixing the 10.5 and 7.5 ns lifetime values corresponding to the two phospholipid phases, the variation of the phospholipid molar fraction in each phase was followed by the associated variation in preexponential factor value as a function of temperature [6]. The cited results implied the basic assumption of two discrete phospholipid phases, coexisting and topographically separated, quantitatively detectable by the DPH molecules. Indeed, recent results obtained by labeling with DPH single phospholipid vesicles [7] showed a continuous fluorescence lifetime distribution with considerably high FWHM value in the gel phase. The FWHM value decreased with increasing temperature, reaching lowest value after the phospholipid phase transition. A possible interpretation is that the membrane heterogeneity detected by the the probe reflects a continuous variation of the interaction dynamics between the probe and the neighbouring phospholipid molecules. In this respect, a slower kinetic of the DPH–phospholipid interaction, at temperatures below the gel to liquid-crystalline phase transition, will originate larger FWHM values. The possibility of a continuous variation of the probe–phospholipid interaction kinetics should be reasonably attributed to the variety of the allowed conformations of both molecular species, increasing with the membrane heterogeneity increase. Moreover, the results obtained with K562 cells suggest that the distributional analysis can better fit the decay data.

The increase of the FWHM value during K562 cells differentiation suggests a heterogeneity increase around the probe molecules. By comparison with the data obtained in single phospholipid vesicles [7] the FWHM value increase suggests a decrease of the DPH-membrane lipids interaction kinetics, that could be attributed to a restricted lipid mobility during differentiation. These findings are in agreement with previous results ob-

tained labeling K562 cell membranes by the *cis* and *trans* isomers of the parinaric acid [10]. A rearrangement was also observed during differentiation but the further resolution of the observed variations in terms of the membrane physical state was hindered by the observation of the complex decay of both parinaric acid isomers [8].

A further explanation of the biological meaning of the increase in heterogeneity observed in K562 cell membranes during differentiation requires more investigations. The basic questions future studies are expected to answere should be: the type of cell membrane from which the observed heterogeneity increase originates, the molecular mechanism of action of the differentiation inductors, the influence of new membrane structures and properties on membrane architecture, and the activity variation of the enzymes involved in the differentiation process.

At present, the observed effects must be referred to the bulk of cellular membranes. Under the experimental conditions used in this work the K562 cells were maintained in a viable state, subsequently endocytotic phenomena are present. The DPH labeling of the membranes will occur not only by the probe dissolving and passively diffusing but also by active endocytosis. Consequently, the observed DPH fluorescence originates from plasma and subcellular membranes.

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