Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 460-46

ERYTHROCYTE MEMBRANE HETEROGENEITY STUDIED USING 1,6 - DIPHENYL - 1,3,5- HEXATRIENE FLUORESCENCE LIFETIME DISTRIBUTION

R.M. Fiorini[1], M. Valentino*, E. Gratton**, E. Bertoli, and G. Curatola

Institute of Biochemistry, Faculty of Medicine,
University of Ancona, Italy

*Occupational Medicine Institute, Faculty of Medicine,
University of Ancona, Italy

**Department of Physics, University of Illinois, URBANA, IL 61801

Received July 23, 1987

SUMMARY. The fluorescence decay of 1,6 - diphenyl - 1,3,5 - hexatriene has been used to characterize the structural organization of erythrocyte membranes. At 37°C a large fraction of the decay (0.96) is associated with a lifetime value of 11.31 ns, while a minor fraction has a short lifetime of 2.63 ns. The distribution analysis approach has shown that the 11 ns component can be described using a Lorentzian distribution function having a full width at half maximum of 0.27 ns. The width of this component is associated with the membrane structural organization since liposomes from erythrocyte total lipid extract exhibit a narrower width. Moreover the distribution width is sensitive to different treatments of erythrocyte membrane.

In contrast with Singer and Nicolson's fluid mosaic model, theoretical considerations and experimental evidence suggest a non-random distribution of membrane components both lipids and proteins. In pure lipid membranes structural heterogeneity has been associated with texture defects and phase separation (see 1,2 for reviews). In biological membranes the presence of proteins and other components such as cholesterol or glicolipids might have a primary role in the

[1] To whom correspondence should be sent.

ABBREVIATIONS: DPH: 1,6-diphenyl-1,3,5-hexatriene; DPPC: L- -dipalmitoyl-phosphatidyl- choline; DMPC: L- -dimyristoyl-phosphatidylcholine; FWHM: full width at half maximum.

0006-291X/87 $1.50

stabilization of structural defects, in the segregation of membrane plane domains as well as in preventing the formation of well defined macroscopic phases (3). In intact cells, membrane heterogeneity can be regulated by cytoskeleton linkages to membrane components (4); in particular heterogeneity in erythrocyte membrane is suggested by the interaction of spectrin with phospholipids in the membrane inner face (5). At present, evidence of biological membrane structural heterogeneity is only indirect(6), and there is no technical approach sensitive enough to detect heterogeneity in a quantitative and/or qualitative way. In our previous studies we have shown that multifrequency phase fluorometry is a technique sensitive for the study of microheterogeneity in model and natural membranes (7,8). Therefore we thought of interest to investigate the use of multifrequency phase fluorometry to study erythrocyte membrane organization. The heterogeneity of DPH decay has been analysed using both a sum of exponentials or continuous lifetime distribution.

MATERIALS AND METHODS

DPH was from Molecular Probes Inc., Eugene (Or) USA.

Hemoglobin free erythrocyte membranes were prepared from fresh human blood by hypotonic haemolysis according to Bramley et al (9).
The extraction of lipids from erythrocyte membranes was carried on according to Zwaall et al (10). The total lipid extract was shown to have the same cholesterol/phospholipid molar ratio of the erythrocyte membrane by two-dimensional thin layer chromatography (11). The phosphate content was determined according to Kates (12) and the cholesterol content by the cholesterol-oxidase assay (13).Multilamellar liposomes were formed resuspending the erythrocyte lipid extract in physiological solution and shaking the suspension vigorously. Erythrocyte membranes were also obtained from erythrocytes incubated at 37°C or at 3°C for one night. The membranes were prepared by washing normal human blood three times with Hank's balanced salt solution and by resuspending the erythrocytes at a hematocrit of 10% in Hank's containing penicillin (100 U/ml). Red cell suspension was mixed with equal volumes of 0.155 M NaCl buffer with albumin (2.0% W/V). After incubation, the samples were washed three times and used to prepare hemoglobin free erythrocyte membranes. Protein concentration was determined by Lowry method (14).

DPH previously dissolved in tetrahydrofuran was added to the membranes to give a 10^{-6}M final concentration, so that phospholipid molar ratio was 1:1000. The suspension was incubated for 1 hour in the dark.

Lifetime measurements were performed, at 37°C, using a GREG 200 phase and modulation fluorometer by using a large range of modulation frequencies from 2 to 70 MHz. The wavelenght of excitation was set at 325 nm (U.V. line of an HeCd laser Liconix Model 4240 NB). The experimental conditions of fluorescence measurements and the method for the derivation of a continuous distribution of lifetime values have been reported (7). 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 least-squares routine for a multiexponential decay has been discussed elsewhere (15).

RESULTS AND DISCUSSION

The DPH decay in erythrocyte membranes and in total lipid extract liposomes were analysed using the exponential and the continuous lifetime distribution approaches. The goodness of the fit was judged by comparing the value of the reduced chi-square (15). In erythrocyte membranes and in liposomes prepared from total lipid extract a double exponential analysis was necessary to obtain an acceptable fit (Table I). Both membranes showed a short lifetime component of about 2 ns with a low fractional intensity and a main component of 11.31 ns in membranes and of 10.63 ns in liposomes. The origin of the short component is still debated and can be tentatively referred to photochemical DPH derivatives (16) or alternatively it can represent a little DPH fraction localised in very polar environments (17). The long lifetime value in erythrocyte membranes was in good agreement with that obtained by Karnovsky et al. (17) by using only three modulation frequencies and a single exponential analysis. The DPH lifetime value of lipid extract was similar to that obtained with saturated phosphatidylcholine (DPPC, DMPC) below the transition temperature (7).

TABLE I - Exponential analysis of DPH fluorescence decay in erythrocyte in membranes (A) and in total lipid extract (B)

(A)	τ_1	f_1	α_1	τ_2	f_2	α_2	X^2
	11.31	0.96	0.85	2.63	0.04	0.15	1.76
(B)	τ_1	f_1	α_1	τ_2	f_2	α_2	X^2
	10.63	0.97	0.81	1.45	0.03	0.19	2.07

(τ, lifetime in nanoseconds; f, fractional intensity; α, pre-exponential factors; X^2, reduced chi-square)

TABLE II - A. Distribution analysis of DPH fluorescence decay in freshly prepared erythrocyte membranes. B. Distribution analysis of DPH fluorescence decay in erythrocyte membranes obtained from erythrocytes incubated overnight at 3°C. C. Distribution analysis of DPH fluorescence decay in erythrocyte membranes obtained from erythrocytes incubated overnight at 37°C.

	C_1	W_1	f_1	C_2	W_2	f_2	X^2
A	11.19	0.27	0.97	2.62	1.55	0.03	1.65
B	11.13	0.25	0.93	1.20	4.55	0.06	1.44
C	10.26	0.05	0.96	1.67	0.43	0.03	2.70

(C, Center of the distribution in nanoseconds; f, fractional intensity; W, Width of the distribution in nanoseconds; X^2, reduced chi-square)

It has been hypothesized that, at the phospholipid phase transition, water permeability increases markedly (18). Since the DPH lifetime value has been shown to be sensitive to the dieletric constant (19) the value of the DPH lifetime in liposomes of erythrocyte total lipid extract suggests a water distribution in the bilayer phase similar to that of saturated crystalline lecithins. The lifetime value was further increased in erythrocyte membranes where the presence of proteins influences membrane structural organization. Membrane heterogeneity can be better analysed by using a distributional analysis which assumes that in principle the fluorophor heterogeneous decay reflectes the multiplicity of microenvironments surrounding the emitting molecule. DPH lifetime distributions in erythrocyte membranes and in multilamellar liposomes from total lipid extract are reported in fig.1A,B and in table II A. In both samples the center of the distribution of the main component had almost the same value which had been obtained using the double exponential analysis. Moreover in erythrocyte membranes the FWHM value (0.27 ns) was larger than that obtained in liposomes (0.05 ns). A second component with a short lifetime value and a very low fractional intensity was necessary to obtain a good fit in both samples(Fig.1A,B). On the basis of the distribution width we can conclude that the erythrocyte membrane is more heterogeneous than the total lipids extracted therefrom. To further characterize the origin of the width of the lifetime

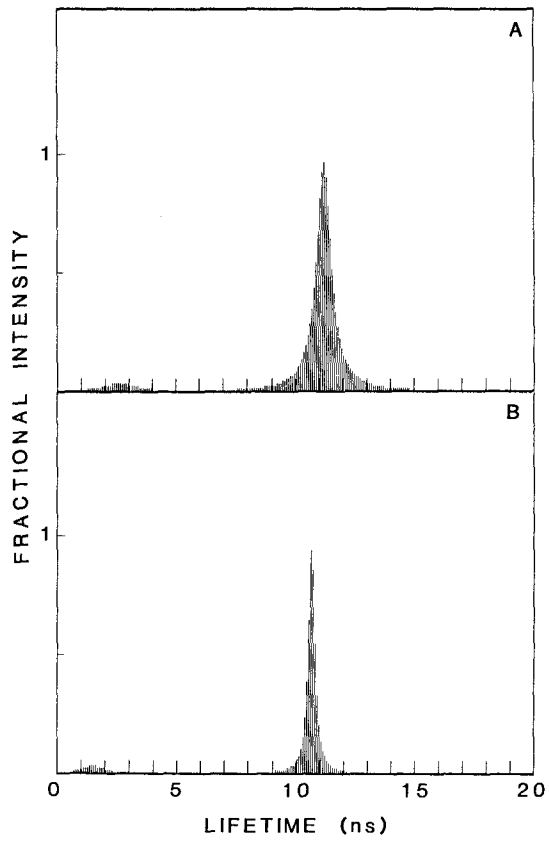

Fig. 1. A - DPH lifetime distribution in erythrocyte membranes.
Main component: distribution central value = 11.19 ns;
FWHM = 0.27 ns; fractional intensity = 0.97
Second component: distribution central value = 2.62 ns;
FWHM = 1.55 ns; fractional intensity = 0.03.
X^2 = 1.65

B - DPH lifetime distribution in multilamellar liposomes obtained from erythrocyte total lipid extract.
Main component: distribution central value = 10.65 ns;
FWHM = 0.05 ns; fractional intensity = 0.97
Second component: distribution central value = 1.52 ns;
FWHM = 0.62 ns; fractional intensity = 0.03.
X^2 = 2.35

distribution, we investigated the effect of temperature treatments on the distribution width. The DPH lifetime value in membranes prepared from erythrocytes stored one night at 3°C and 37°C have been analysed with the distribution approach. The average lifetime values of the two samples were different, and also the widths of the distributions showed a different pattern (Table II). In the membranes obtained from erythrocytes stored one night at 3°C the lifetime was centered at 11.13

ns and the FWHM value was 0.25 ns similarly to the values obtained from freshly prepared membranes, while in the membranes prepared from erythrocytes stored one night at 37 °C the lifetime was centered at 10.26 ns and the FWHM value is 0.05 ns . In all membrane samples the short component showed changes of the distribution center and width but they cannot be adequately discussed due to the low fractional intensity of this component. The release of membrane skeleton proteins at 37°C (20) and the consequent release of cytoskeleton constraints could explain the decrease of distribution center value, which was similar to that of liposomes obtained from erythrocyte lipid extract.

Our experimental results suggest that the decay of DPH fluorescence can be used as a sensitive tool to study membrane heterogeneity. In particular the lifetime distribution analysis is a better approach than the exponential one. In erythrocyte membrane the center and the width of DPH lifetime distribution are related to the membrane organization and sensitive to treatments commonly used in erythrocyte manipulations.

ACKNOWLEDGEMENTS

This work was supported by Regione Marche - Ricerca Sanitaria Finalizzata N. 7005 - 22/12/1986

REFERENCES

1. Jain, M.K. (1983) Membrane Fluidity in Biology, pp.1-37, Academic Press, New York
2. Curatola, G., and Bertoli E. (1987) Biomembrane and Receptor Mechanism, pp. 143-161, Springer-Verlag, New York
3. Klausner, R.D., and Kleinfeld, A.M. (1984) Cell Surface Dynamics, pp. 23-40, Marcel Dekker, New York
4. Loor, F. (1981) Cytoskeletal Elements and Plasma Membrane Organization, pp. 253-335, Elsevier, North Holland
5. Sackman, E., Sui Sen-fang, Wirthensohn, K., and Urumow, T. (1987) Biomembrane and Receptor Mechanism, pp. 97-111, Springer-Verlag, New York
6. Sklar, L.A. (1984) Membrane Fluidity, vol. 12, pp. 99-126, Plenum Press, New York
7. Fiorini, R.M., Valentino, M., Wang, S., Glaser M., and Gratton, E. (1987) Biochemistry in press
8. Parasassi, T., Conti, F., Gratton, E., and Sapora, O. (1987) Biochim. Biophys. Acta 898, 196-201
9. Bramley, T.A., Coleman, R., and Finean, J.B. (1971) Biochim. Biophys. Acta 241, 752-769
10. Zwaall, R.F., Roelofsen, B. (1976) Biochemical Analysis of Membrane, pp. 352-377, Wiley and Sons. Inc., New York

11. Kritshevsky, L., Yamamoto, A.,and Rouser, L. (1967) Lipid Chromatographic Analysis, vol.1, pp.99-162,Marcel Dekker, New York
12. Kates, M. (1972) Techniques in Lipidology, vol. 3, pp. 355-360, Elsevier, New York
13. Cooper, R.A., Arner, E.C., Wiley, J.S., and Shattil, S.J. (1975) J. Clin. Invest. 55, 115-126
14. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
15. Lakowicz, J.R., Gratton, E., Cherek, H., and Limkeman, M. (1984) Biophys. J. 46, 463-477
16. Parasassi, T., Conti, F., Glaser, M., and Gratton, E. (1984) J. Biol. Chem. 259, 14011-14017
17. Karnovsky, M.J., Kleinfeld, A.M., Hoover, R.L., and Klausner, R.D. (1982) J. Cell. Biol. 94, 1-6
18. Deamer, D.W., and Bramhall, J. (1986)Chem. Phys. Lipids 40, 167-188
19. Zannoni, C., Arcioni, A., and Cavatorta, P. (1983) Chem. Phys. Lipids 32, 179-250
20. Elgsaeter, A., and Branton, D. (1974) J. Cell. Biol. 63, 1018-1036