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Fluidity of intact erythrocyte membranes. Correction for fluorescence energy transfer from diphenylhexatriene to hemoglobin

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Membranes of intact erythrocytes were labeled by the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) using an improved labeling procedure described previously (Plášek, J. and Jarolím, P. (1987) Gen. Physiol. Biophys. 6, 425–437). The relationship between the steady-state DPH fluorescence anisotropy r and the mean corpuscular hemoglobin concentration (MCHC) was studied. Fluorescence anisotropy increased with increasing MCHC. A linear dependence of $r = 0.0026$ (MCHC) $+ 0.113$ was obtained which enabled us to measure the fluidity of intact red cell membranes. Without this correction for fluorescence quenching by hemoglobin, incorrect conclusions about membrane fluidity could be made. This fact is demonstrated in a group of pyruvate kinase deficient patients compared with a group of healthy blood donors.

Introduction

Most measurements of erythrocyte membrane fluidity have been performed on erythrocyte ghosts. The main reason is the significant quenching of fluorescence of standard fluidity probes, like 1,6-diphenyl-1,3,5-hexatriene and anthroyloxy derivatives of fatty acids, by the nonradiative energy transfer from membrane-bound fluorophores to the cell hemoglobin. On the other hand, lysis of red cells changes intracellular ion and ATP levels and releases some cytoskeletal components [2,3] thus influencing the protein–protein and protein–lipid interactions [4,5]. This may further lead to changes of the cell shape [6,7], the cell membrane fluidity [8] as well as the lateral mobility of membrane components [9].

Comparative studies on ghosts and intact erythrocyte membranes have not given a conclusive answer whether the properties of ghosts closely parallel those of the intact erythrocyte membranes. While some authors claim that the fluidity is the same in both systems [10,11], others have found changes in properties of ghosts compared to intact cells [12–14]. Consequently, it would be desirable to measure membrane fluidity in the intact cells.

One complication of this measurement in a suspension of erythrocytes is the fluorescence depolarization caused by light scattering on the cells [13,15]. However, this can easily be overcome by extrapolation of the measured r to the zero erythrocyte concentration [11].

The second complication is the non-radiative resonance excitation-energy transfer from DPH to hemoglobin [16]. In shortening the fluorescence

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; MCHC, mean corpuscular hemoglobin concentration.

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lifetime, it both lowers the fluorescence intensity and increases the DPH fluorescence anisotropy (for theory see for example, Refs. 17–19). In the first case we can use a more sensitive fluorometer or, in this case, a labeling method with a higher yield which we described previously [1]. The second problem, i.e. changing the anisotropy values, is more serious, since in cells with higher MCHC a higher fluorescence anisotropy value may be expected, indicating thus incorrectly lower membrane fluidity in those cells.

We have measured DPH fluorescence anisotropy in cells with different MCHC looking for a correction which would enable us to compare membrane fluidities in those cells. Furthermore, we found an example of how neglecting this correction could lead to an overestimation of differences in fluorescence anisotropy.

Methods

Preparation of erythrocytes and ghosts

Venous blood was drawn from healthy donors and from patients with pyruvate kinase deficiency into heparinized tubes and washed three times with an isotonic phosphate-buffered saline. Estimation of the MCHC was part of routine hematologic assay. No abnormalities were detectable in donors' erythrocytes used for calculation of the correction (see further Fig. 1). Erythrocyte ghosts were prepared according to Dodge et al. [20] by hypotonic lysis with 20 mM sodium phosphate (pH 7.6), followed by five subsequent washes with the same buffer. Concentration of erythrocytes in samples did not exceed $5 \cdot 10^6$ cells per ml, while ghost protein concentration was less than 10 μg/ml.

Labeling of erythrocytes and ghosts with DPH

Intact cells and erythrocyte ghosts were labeled according to Ref. 1. A $2 \cdot 10^{-4}$ M solution of DPH in acetone was diluted 1 : 100 (v/v) in a buffer heated to 60 ° C and vigorously stirred for 1 h. This solution was then mixed 1 : 1 with the ghosts or cells and incubated for 1 h at 37 ° C in a shaking water bath. After the incubation, the samples were washed once with the same buffer and r was measured within one hour. All procedures were performed under diminished light and completed on the day of blood collection. A new labeling solution was prepared for each measurement.

Measurement of fluorescence

Fluorescence measurements were made with an SLM-Aminco 4800 spectrofluorometer. Excitation and emission wavelengths were 365 and 450 nm, spectral slit widths 8 and 4 cm, respectively. Temperature was maintained at 37 ° C. An L-type photomultiplier configuration was used and after ten cycles of polarizer positioning the average fluorescence anisotropy was calculated according to Refs. 21 and 22. The maximum error of r was 0.002. The low concentrations of the cells and ghosts enable us to neglect the effect of light scattering.

Results and Discussion

Fluorescence anisotropy values of DPH in intact erythrocyte membranes from 39 donors are shown in Fig. 1 as a function of their MCHC. The theory of energy transfer and fluorescence anisotropy does not predict a completely linear dependence of r on MCHC [18,19,23]. However, it can be shown that for the MCHC range available the deviation from a linear dependence need not be considered. Hence, the data were evaluated by a linear regression analysis [24]. This gives

$$r = 0.0026\ (\mathrm{MCHC}) + 0.113 \qquad (1)$$

with a correlation coefficient 0.63. The relatively great scatter of measured anisotropies is mainly due to the individual variability of membrane

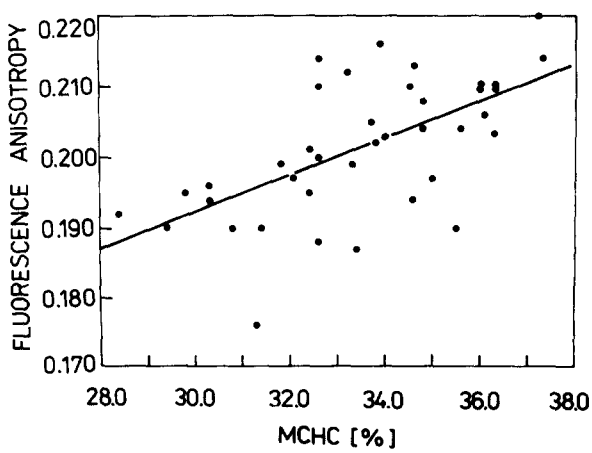

Fig. 1. Fluorescence anisotropy of DPH-labeled intact erythrocytes as a function of mean cell hemoglobin concentration measured at 37 ° C.

TABLE I

FLUORESCENCE ANISOTROPY OF DPH AT 37°C IN INTACT ERYTHROCYTES AND GHOSTS FROM PATIENTS WITH PYRUVATE KINASE DEFICIENCY AND FROM HEALTHY BLOOD DONORS

No.	PK deficiency				Healthy donors			
	MCHC (%)	Erythrocytes		Ghosts	MCHC (%)	Erythrocytes		Ghosts
		r	r_{norm}	r		r	r_{norm}	r
1	32.5	0.193	0.199	0.207	37.3	0.214	0.208	0.211
2	33.4	0.200	0.204	0.220	34.5	0.205	0.206	0.200
3	32.6	0.195	0.201	0.211	33.6	0.203	0.207	0.197
4	35.0	0.208	0.208	0.198	34.9	0.205	0.205	0.199
5	30.3	0.188	0.200	0.207	34.4	0.206	0.208	0.201
6	34.4	0.204	0.206	0.196	36.4	0.205	0.201	0.198
7	31.8	0.198	0.206	0.211	35.5	0.204	0.203	0.202
8	33.6	0.200	0.204	0.209	34.1	0.200	0.202	0.203
Mean	32.95	0.1983	0.2035	0.2074	35.09	0.2053	0.2050	0.2014
S.D.	1.49	0.0063	0.0032	0.0076	1.24	0.0040	0.0027	0.004

fluidities which has been repeatedly observed [25,26] and which may be caused by differences in lipid, protein and ion composition, ATP content etc.

The improved labeling procedure together with Eqn. 1 enables the assessment of the DPH fluorescence anisotropy in intact erythrocyte membranes, which may better reflect differences among erythrocytes from various groups of patients. On the other hand, Eqn. 1 shows that great care must be taken when comparing results of fluidity measurements in hemoglobin containing cells. As an example, results of fluidity measurements of both intact erythrocytes and ghosts from healthy donors and from patients with pyruvate kinase deficiency are compared. The results for eight individuals from each group are shown in Table I.

For intact erythrocytes, both uncorrected r values as well as the normalized values $r_{norm} = r - 0.0026\,(MCHC - 35)$ are shown, r_{norm} being the measured anisotropy value r corrected for the deviations caused by variation in MCHC. MCHC = 35% was chosen arbitrarily as a reference value.

Patients with pyruvate kinase deficiency are often anemic and have lower MCHC values. If we compare only the mean uncorrected r values, we find that the mean r value of pyruvate kinase deficient patients is 3.5% lower than the mean uncorrected r for normal cells. However, if we take into account the MCHC values (i.e. if we compare mean r_{norm} values), the difference is only 0.7%. Statistically, applying the Student's t-test whilst neglecting the differences in MCHC, causes the difference in r between two groups to be statistically significant at the 5% significance level. However, after correction for MCHC, the difference is no longer statistically significant.

This is further illustrated in Fig. 2, in which anisotropy values for both groups are plotted vs. MCHC. These values clearly split into two almost disjunct groups. If these groups were evaluated only in terms of anisotropy, the mean fluidities

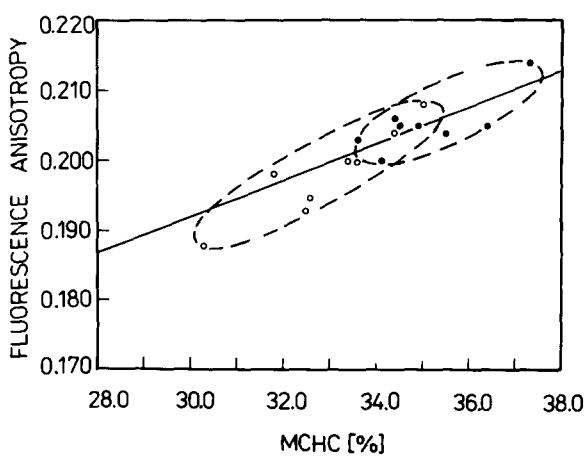

Fig. 2. DPH fluorescence anisotropy in intact erythrocytes from healthy donors (●) and patients with pyruvate kinase deficiency (○).

would be found different. However, position of both groups of points along the correction curve indicates no significant difference between the cell fluidities of normal and pyruvate kinase deficient cells.

With the ghosts the situation was more complicated. While we were able to prepare white ghosts from all normal erythrocytes, some pyruvate kinase deficient ghosts remained pink even after additional washes. This was previously described for some other types of anemias as well [27,28]. Thus, even if we presume that ghosts fluidity parallels the fluidity of intact cells, residual hemoglobin may still be a source of error. For these reasons, it was more precise to use the intact cells for measurements of fluidity.

But even if intact cells are used, great care must be taken whenever hemoglobin is present. This holds for all measurements with erythrocytes and other hemoglobin containing cells. For example, if we measure membrane fluidity during differentiation of an erythroleukemic cell line, we find a decrease in fluidity (Jarolím and Plášek, unpublished observation). This again may be an artifact caused by synthesis of hemoglobin after induction of differentiation.

In conclusion, the use of an appropriate correction for changes in fluorescence anisotropy caused by hemoglobin allows the accurate determination of membrane fluidity in intact cells when combined with a high yield fluorescence labeling procedure. This applies not only for DPH but for all fluorescent probes emitting close to the Soret band of the hemoglobin absorption spectrum.

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