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CHANGES IN FLUIDITY OF ERYTHROCYTE MEMBRANES AFTER STORAGE OF ERYTHROCYTES AND REGENERATION OF CELLULAR ATP LEVEL

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The membrane fluidity of freshly collected human erythrocytes, of erythrocytes stored for 3–4 weeks and of stored erythrocytes rejuvenated with glucose and inosine was investigated by measuring polarization of fluorescence emission of 1,6-diphenyl-1,3,5-hexatriene and *N*-phenyl-1-naphthylamine. The fluidity of membranes prepared from stored erythrocytes was higher than that of fresh erythrocytes. After rejuvenation of erythrocytes with glucose and with or without inosine the membrane fluidity decreased. These changes were probably due to variations of ATP levels in the erythrocytes.

Introduction

The morphological properties of fresh and stored erythrocytes are different, and consequently the structure and physical properties of their membranes are very probably altered, too. One of the important parameters relating to the structure and functional state of the membrane is the so-called membrane fluidity (for review see Ref. 1) measured by means of emission polarization of fluorescent probes [2]. Fluidity assessed by this method relates to the rotational mobility of the probe and to the arrangement and mobility of membrane components as well [3].

The purpose of this work was to discover whether (a) the membrane fluidity measured by means of emission polarization of fluorescent probes was altered after storage of human erythrocytes; (b) the relevant changes were at least partly reversible; and (c) the changes were related to the altered energy level.

The energy source for different processes in erythrocyte membranes is ATP. The ATP level in freshly collected red blood cells is relatively high, 99 μmol ATP per 100 ml cells, and it decreases during their storage [4]; in stored erythrocytes the increase of ATP level can be achieved by rejuvenation of these erythrocytes; for example, by incubation with inosine [5,6]. We have performed tests to evaluate whether the membrane fluidity of stored erythrocytes after their rejuvenation was altered.

All experiments were done with freshly collected human blood or with blood stored for 3–4 weeks at 4°C in solution A (4 parts of blood per one part of solution A)*. The composition of solution A is: 1.4 g sodium citrate, 0.5 g citric acid, 2.5 g glucose, adjusted with water to 100 ml (pH 4.8–5.2). A part of the stored erythrocytes was rejuvenated by 4-h incubation at 37°C in solution A plasma or solution A plasma enriched with 12

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; NPN, *N*-phenyl-1-naphthylamine; *P*, degree of fluorescence polarization; *I*, fluorescence intensity.

* The term ACD solution is sometimes employed.

mM inosine. Hemolysis of erythrocytes was done according to Bodemann and Passow [7] in this modification: To 5 parts of centrifuged erythrocytes (three-times washed with saline) four parts of saline and 50 parts of water (0°C) were added. The hemolysate was saturated with CO_2, the erythrocyte membranes were isolated by centrifugation and suspended in saline adjusted with phosphate buffer to pH 7.5.

For membrane labeling DPH (Koch Light), (or NPN (ICN Pharmaceuticals)) was used as 2 mM (6 mM) stock solution in tetrahydrofuran (methanol) (respectively). Before measurement DPH was diluted 1:200 (NPN, 1:100) in phosphate-buffered saline; 0.1 ml $1 \cdot 10^{-5}$ M DPH ($6 \cdot 10^{-5}$ M NPN) was then added to 0.9 ml membrane suspension with approx. 90 μg proteins per ml, so that a membrane preparation with approx. 80 μg protein per ml $1 \cdot 10^{-6}$ M DPH (per ml $6 \cdot 10^{-6}$ M NPN) in phosphate-buffered saline was obtained. After 60 min incubation at 27°C the samples were twice washed and resuspended in phosphate-buffered saline. The whole procedure was performed in the dark.

Measurements of fluorescence polarization were performed on a home-made fluorescence spectrometer consisting of 200 W high pressure mercury lamp HBO 200 (Osram), two monochromators SPM 2 (Carl Zeiss, Jena), a thermostatically controlled sample holder, photomultiplier 9789 QB (EMI), high-voltage supply 240 A (Keithley), electrometer 610 C (Keithley) and a recorder. As excitation polarizer a Glan-Thompson prism, and as emission analyzer a dichroic filter (Carl Zeiss, Jena) were used. The whole device was equipped with quartz optics. The samples were continuously illuminated maximally for 20 s using excitation and emission wavelengths of 365 and 425 nm, respectively. All measurements were performed at 27°C. Degree of fluorescence polarization, P, was calculated from the equation

$$P = (I_{VV} - G \cdot I_{VH})/(I_{VV} + G \cdot I_{VH})$$

where I is the fluorescence intensity and the first character of the indices denotes the vertical (V) or horizontal (H) position of the polarizer, and the second one the position of the analyzer. $G = I_{HV}/I_{HH}$ is the correction factor for instrument polarization as described by Azumi and McGlynn [8]. Besides, the total fluorescence intensity $I = I_{VV} + 2G \cdot I_{VH}$ was calculated. The calculation of 'microviscosity' according to Perrin's equation commonly used in current papers dealing with membrane fluidity was not performed, since it was valid for macroscopic systems and was irrelevant for the molecular system 'membrane-probe'. While evaluating the results, we can consider that the fluidity reported in literature is approximately inversely proportional to P. However, we should bear in mind that the fluidity determined from the steady-state fluorescence polarization measurements is not a hydrodynamic parameter but a certain, not precisely defined, quantity which is in relationship both with the membrane structure and dynamics as well. Maximum error of P is ± 0.006.

Results

First, kinetics of probe incorporation into the membrane of fresh erythrocytes and the dependence of the degree of fluorescence polarization on incubation time with the probe and on washing of the sample was examined. Fig. 1 shows that fluorescence intensity after the initial rapid rise reaches a constant value in about 40 min. The constant value after washing is decreased in DPH- and NPN-labeled membranes by 10% and 40% on average, respectively. The degree of fluorescence

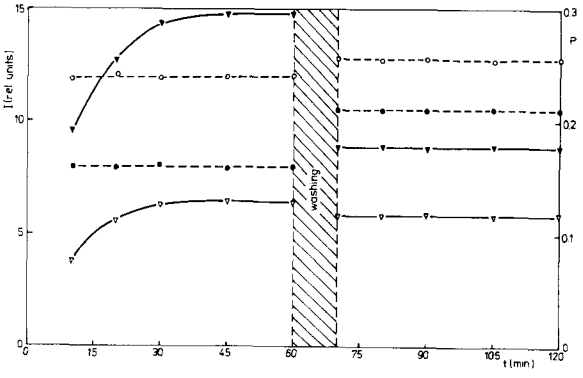

Fig. 1. Dependence of the degree of fluorescence polarization, P (○, ●), and intensity of fluorescence, I (▽, ▼), on incubation time of membranes from fresh erythrocytes labeled with fluorescent probes DPH (○, ▽) and NPN (●, ▼) and on washing of the membranes with phosphate-buffered saline.

polarization remains constant during the whole incubation period and increases after washing for DPH-labeled membranes by about 10% and for NPN-labeled membranes by about 30%. The influence of washing is the same for membranes from fresh and stored erythrocytes.

The considerable increase of the degree of fluorescence polarization in the case of NPN after washing may account for that the part of NPN, due to its amphipatic nature, is bound loosely during incubation to the membrane/water interface where it has a higher rotational mobility. In this way the increased rotational depolarization reduces the total polarization of the emitted light.

Further, the degree of fluorescence polarization was measured in fresh, stored and rejuvenated erythrocytes. The fluorescence polarization was measured twice, at the 55th and 75th min after addition of the probe to the membrane suspension, i.e., 5 min before and 5 min after washing according to the time schedule given in Fig. 1. The results of experiments with erythrocytes from 17 blood donors are given in Table I.

Table I shows that erythrocyte membrane fluidity after 3–4 weeks storage is increased (P is decreased). The relative mean values for fresh, stored and rejuvenated erythrocytes are similar before and after washing of the membranes. In DPH-labeled membranes from stored erythrocytes P is reduced to 91%, in NPN-labeled membranes to 87%, as compared to the fresh membranes. After rejuvenation of the stored erythrocytes with glucose, i.e., after incubation of stored erythrocytes in solution A plasma, P rises slightly (fluidity decreases) in DPH-labeled membranes to 93%, in NPN-labeled membranes to 89% of the normal

TABLE I

INFLUENCE OF STORAGE AND REJUVENATION OF ERYTHROCYTES ON THE DEGREE OF FLUORESCENCE POLARIZATION OF DPH AND NPN INCORPORATED IN MEMBRANES

F, fresh erythrocytes; S, stored erythrocytes (3–4 weeks); RG, stored erythrocytes rejuvenated by 4 h incubation at 37°C with plasma enriched with glucose; RGI, stored erythrocytes rejuvenated by 4 h incubation at 37°C with plasma enriched with glucose and inosine. Erythrocytes from same blood donors are denoted with same indices.

Probe	Expt.	P							
		Before washing				After washing			
		F	S	RG	RGI	F	S	RG	RGI
DPH	1	0.238	0.211	0.229 [a]	0.237 [a]	0.276	0.233	0.265 [a]	0.280 [a]
	2	0.238	0.212	0.225 [b]	0.233 [b]	0.254	0.244	0.231 [b]	0.248 [b]
	3	0.239	0.215	0.218 [c]	0.233 [c]	0.255	–	0.228 [c]	0.247 [c]
	4	0.234	0.210 [d]	0.217 [d]	0.215 [d]	0.266	0.245 [d]	0.252 [d]	0.249 [d]
	5	0.229	0.207 [e]	0.226 [e]	0.244 [e]	0.262	0.239 [e]	0.257 [e]	0.287 [e]
	6	0.233	0.201 [f]	0.205 [f]	0.228 [f]	0.263	0.232 [f]	0.236 [f]	0.258 [f]
	7	0.235	0.214 [g]	0.222 [g]	0.232 [g]	0.268	0.237 [g]	0.244 [g]	0.264 [g]
	Mean	0.235	0.210	0.220	0.232	0.263	0.238	0.245	0.262
	Relatively	1.00	0.89	0.94	0.99	1.00	0.91	0.93	0.99
NPN	1	0.161	0.145	0.144 [a]	0.149 [a]	0.228	0.190	0.217 [a]	0.215 [a]
	2	0.161	0.144	0.126 [b]	0.153 [b]	0.210	0.194	0.152 [b]	0.196 [b]
	3	0.159	0.141	0.126 [c]	0.154 [c]	0.209	0.190	0.152 [c]	0.197 [c]
	4	0.166	0.124 [d]	0.155 [d]	0.156 [d]	0.217	0.182 [d]	0.217 [d]	0.213 [d]
	5	0.151	0.138 [e]	0.149 [e]	0.149 [e]	0.218	0.189 [e]	0.198 [e]	0.200 [e]
	6	0.170	0.130 [f]	0.146 [f]	0.145 [f]	0.222	0.183 [f]	0.199 [f]	0.197 [f]
	7	0.168	0.145 [g]	0.165 [g]	0.164 [g]	0.216	0.193 [g]	0.220 [g]	0.220 [g]
	Mean	0.162	0.138	0.144	0.153	0.217	0.189	0.194	0.205
	Relatively	1.00	0.85	0.89	0.94	1.00	0.87	0.89	0.95

value. After rejuvenation of stored erythrocytes with glucose and inosine, P rises again in DPH-labeled membranes almost to the original value (to 99%), in NPN-labeled membranes to 94%, as compared with the fresh membranes.

Discussion

The results show that there is a certain difference when using DPH and NPN probes. The alterations found are a little more pronounced with polar NPN than with non-polar DPH. While, in the membrane, DPH is probably situated in the middle of the lipid bilayer [9] and on hydrophobic domains of proteins [3], NPN is most probably located on carbonyl groups of phospholipids and proteins [10]. From our results we can therefore judge that during storage and subsequent rejuvenation of erythrocytes the particular changes are both in the hydrophobic and hydrophylic domains of the membrane, but the changes in the hydrophylic domain are more pronounced.

Cooper et al. [11] established that great changes in the proportion cholesterol/phospholipids (molar ratio 0–2) strongly influenced the fluidity of the membranes of human erythrocytes. The changes found by them are much greater than changes reported by us, but the alteration of the membrane composition due to their treatment is much more essential, too. Our results are difficult to explain by formation and release of vesicles and resulting alteration of the cholesterol/phospholipids ratio, because the composition of lipids in the membrane and in the released vesicles is very similar [12], and because the fluorescence intensity is the same in the membranes from fresh and stored erythrocytes before as well as after washing.

By monitoring the electron paramagnetic spectra of fatty acid spin labels incorporated into the membrane decreased membrane fluidity of the in vivo aged human erythrocytes was found by Shiga et al. [13]. Using spin-label studies, Bartosz [14] found a decrease in membrane fluidity after in vivo aging of cow erythrocytes, too. On the other hand, after storing human erythrocytes, we found increased fluidity. The processes during in vivo aging, however, differ substantially from those at play during storage of erythrocytes at 4°C.

The membrane fluidity of stored erythrocytes partially returns to normal after rejuvenation with glucose and more markedly after rejuvenation with glucose and inosine. Since under these conditions the ATP level increases, more after rejuvenation with both glucose and inosine than with only glucose, we are lead to believe that the energy level of the membrane plays an important role in fluidity regulation. In our view, the regeneration of ATP level influences the conformation of certain membrane proteins and can cause a change in their shape or their ability to interact with other membrane components. Due to ATP, also some polar group in protein could be exposed and thus the mobility of polar probe could be affected.

In the case of erythrocyte membrane, this particular protein is very probably spectrin. Spectrin is phosphorylated by ATP [15–17], and is bound to a number of membrane proteins, e.g., F-actin, proteins denoted as band 2.1 (ankyrin, spectrin-binding protein) and band 4.1 (matrix-associated protein) [18]; most probably spectrin is also bound to membrane phospholipids [19]. Our idea is in accordance with the results of Sheetz et al. [20]. They found that mouse spherocytic erythrocytes that contained less spectrin, spectrin-binding protein and matrix-associated protein had about 50-times higher coefficient of lateral diffusion than mouse erythrocytes with normal amounts of these proteins.

According to Singer [21] the membrane fluidity decreases when within the membrane ordered complexes are formed, or when interactions with structures attached to the membranes take place. In our view, the low membrane fluidity of freshly collected erythrocytes is due to the presence of functional complexes in the membrane, where spectrin has an important role, and to the high degree of order of these membranes – all of which requires energy. The membranes of stored erythrocytes, on the other hand, show a sort of 'loosening' of individual components and some decrease in the degree of order.

The membrane fluidity influences a number of cellular functions, and presumably, any significant change of fluidity seems to be undesirable for the optimum cell functions. Our results suggest that the increase of erythrocyte membrane fluidity occurring during blood storage may be favorably influenced by regeneration of the ATP level.

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