Organization of membrane lipids and proteins in human En(a-) erythrocytes that lack the major sialoglycoprotein, glycophorin A

A spin-label study

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Membrane fluidity was studied by electron-spin-resonance techniques in human En(a-) erythrocytes that lack the major membrane sialoglycoprotein, glycophorin A. By using stearic acid spin labels with a doxyl group in the C-12 or C-15 positions, we demonstrated that the hydrophobic core in these cells was more fluid than in normal cells. Surface-located regions in isolated En(a-) membranes, when probed with stearic acid labelled in the C-5 position, appeared more stable than in normal membranes. In isolated En(a-) membranes, protein motion was decreased when probed with a nitroxide derivative of maleimide. After incubation with anti-(glycophorin A) antibodies protein motion and membrane fluidity were increased in normal membranes. This effect was observed also after spectrin depletion, which by itself increased protein motion but decreased membrane fluidity in the hydrophobic core of the membrane. The results show that membrane proteins influence the fluidity of membrane lipids.

The major integral proteins of the human erythrocyte membrane are band 3 (for nomenclature, see Fairbanks *et al.*, 1971) and the sialoglycoproteins PAS1–PAS3 (Marchesi *et al.*, 1972). PAS1 or the dimer of glycophorin A is one of the most extensively studied membrane proteins, and the vast amount of information available makes it an attractive model for the study of structural–functional relationship in membranes.

Erythrocytes of the rare blood group En(a-) lack glycophorin A (Gahmberg *et al.*, 1976; Dahr *et al.*, 1976; Tanner & Anstee, 1976). We have taken advantage of this fact and, by using e.s.r. techniques, attempted to identify, in the En(a-) erythrocyte membrane, functional defects that could be attributed to the lack of this major membrane protein. Membrane lipid regions were labelled with doxyl derivatives of stearic acid, whereas protein thiol groups were labelled with a nitroxide derivative of maleimide. We here report that: (a) in En(a-) erythrocytes, membrane lipid fluidity is higher and exhibits a temperature-dependence different to that in normal erythrocytes; (b) the motion of labelled thiol groups in membrane proteins is slower in

En(a-) membranes than in normal membranes; and (c) an antiserum against glycophorin A affects both membrane lipid and protein motion in normal membranes.

Material and methods

All blood samples were obtained from the Finnish Red Cross Blood Transfusion Service. Both the En(a-) and normal erythrocytes were of blood group AB Rh+. Blood samples taken in CPD solution were kept on ice and processed within 3 days. Altogether three samples, drawn in February, April and June 1979 respectively, were analysed.

Chemicals and reagents

Spin labels were purchased from the Syva Corporation, Palo Alto, CA, U.S.A. Anti-(glycophorin A) antiserum was prepared as described previously (Gahmberg *et al.*, 1978).

Isolation of erythrocyte membranes

Erythrocyte membranes were obtained after hypo-osmotic disruption of the erythrocytes (Dodge *et al.*, 1963; Fairbanks *et al.*, 1971) and were washed free from haemoglobin in sodium phosphate buffer (7 mmol/litre), pH 7.2.

Abbreviations used: Rh+, rhesus-positive; CPD solution, citrate/phosphate/dextrose solution.

Freeze-storage of erythrocytes

Portions of the blood samples were treated with glycerol and submitted to the freeze-storage procedure of Åkerblom & Högman (1974). After storage for up to 30 days, the samples were thawed and the glycerol was washed away with NaCl (150 mmol/litre) before e.s.r. studies.

Spectrin depletion of erythrocyte membranes

Isolated cell membranes were partially depleted of spectrin by overnight incubation in EDTA (0.2 mmol/litre), pH 8.0 at 4°C. After incubation, the membranes were pelleted by centrifugation and resuspended in sodium phosphate (7 mmol/litre) buffer, pH 7.2.

Treatment of erythrocyte membranes with anti-(glycophorin A) antiserum

Normal erythrocyte membranes (100 µl, 2–4 mg of protein/ml) were incubated with 50 µl of anti-(glycophorin A) antiserum or preimmunization serum for 2 h at 0°C. After centrifugation in sodium phosphate buffer the membranes were processed for e.s.r. studies.

E.s.r. studies

Surface, intermediate and deep layers of the membranes were probed with *N*-oxyl-4',4'-dimethylxazolidine derivatives of stearic acid with the doxyl groups in the C-5, C-12 and C-15 positions respectively. Membrane thiol groups were probed with a nitroxide derivative of maleimide [3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy]. The spin labels were dissolved in ethanol and pipetted into test tubes, the ethanol was evaporated under a stream of N₂ and the labels were incorporated into the cell membranes by incubation at 37°C for 20 min. The label concentration was 5–20 µg of stearic acid spin label and 2 µg of maleimide spin label per mg of membrane protein (measured as described by Lowry *et al.*, 1951) or per 100 µl of erythrocytes (70% haematocrit). Isolated membranes were suspended in sodium phosphate (7 mmol/litre) buffer, pH 7.2, whereas erythrocytes were suspended in a medium containing NaCl (136 mmol/litre), KCl (5.6 mmol/litre) and sodium phosphate (1.3 mmol/litre), adjusted to pH 7.4 with Tris/HCl buffer (20 mmol/litre). Labelled erythrocytes and membranes were washed by centrifugation, and 20 µl portions were transferred to glass capillary tubes (Mettler Me-18582), which were then sealed by melting. The e.s.r. spectra were recorded at 34, 37 and 41°C on a Varian E-4 spectrometer operating at 9.1 GHz. The polarity-corrected order parameter *S* was calculated as described by Hubbell & McConnell (1971) and the motion parameter τ_0 , corresponding to the rotational correlation time, τ_c , as explained by Henry & Keith (1971).

As spectral parameters in e.s.r. studies are greatly influenced by instrument settings, the En(a-) sample and its respective control were treated as matched pairs, and the significance of the differences between means was tested by the matched-pairs *t* test.

Results

E.s.r. studies on erythrocytes

Alterations in the mobility of the spin label are reflected by changes in the linewidths and peak distances in the e.s.r. spectra. As the mobility of the spin label becomes increasingly restricted by the surrounding membrane, the spectral characteristics change, which is seen as an increase in the spectral order parameter *S* (for its calculation, see Hubbell & McConnell, 1971). Conversely, a decrease in the order parameter *S* indicates an increase in the mobility of the label. The mobility of the label reflects the fluidity of the cell membrane, and in the present study, *S* decreased, i.e. the fluidity of the cell membrane increased, as the label on the fatty acid chain probed deeper regions of the membrane. This is a general feature of biological membranes and has been called the 'fluidity gradient' (Hubbell & McConnell, 1971). In normal erythrocytes at 37°C, the mean value of *S* was 0.639 (three expts.) with the doxyl group situated in the C-5 position (*S*₅) and 0.475 with the label in the C-12 position (*S*₁₂, Table 1). In the deepest fluid regions of the membrane probed with the doxyl group in the C-15 position, the *S* parameter was impossible to measure, and, instead, τ_0 corresponding to the rotational correlation time was measured from the linewidth and amplitudes of the spectra; in normal membranes its value was 1.54 ns at 37°C.

In En(a-) cells, the magnitude of the order parameter was very similar to control values when the C-5 probe was used, indicating that near the membrane surface the bulk organization of membrane lipids did not differ much from that in normal cells. In the hydrophobic core of the membrane, however, a membrane fluidization was revealed by a decrease in the order parameter *S*₁₂ from a mean control value of 0.475 to 0.463 and τ_0 from 1.54 ns to 1.34 ns at 37°C (Table 1). Although the values for *S*₁₂ and τ_0 were differently affected in different experiments, a clear fluidization of the lipid core of the membrane was observed in all experiments (Fig. 1). The ambient temperature greatly affected the fluidity of the cell membrane. In normal cells, *S*₁₂ decreased from 0.475 to 0.441 as the temperature was increased from 37 to 41°C. Correspondingly, as the temperature was decreased to 34°C, *S*₁₂ increased to 0.499. In this temperature range, 34–41°C, the temperature-dependence of the En(a-) cell membrane was similar to that of normal membranes and a fluidization was observed at all

Table 1. *Membrane fluidity in erythrocytes*

Membrane fluidity was probed by stearic acid spin labels with the doxyl group in the C-5, C-12 and C-15 positions respectively. S_5 , S_{12} and τ_0 refer to order and motion parameters respectively. Results are means $\pm$ s.e.m. of three experiments. * $P < 0.05$.

Temperature ($^{\circ}\text{C}$) ...	S_5		S_{12}			$\tau_0(\text{C-15})$ (ns)		
	34	37	34	37	41	34	37	41
Erythrocyte membrane								
Control	0.652	0.639	0.499	0.475	0.441	1.59	1.54	1.39
	± 0.003	± 0.003	± 0.004	± 0.011	± 0.012	± 0.31	± 0.28	± 0.28
En(a-)	0.650	0.638	0.493	0.463	0.438	1.41	1.34	1.25
	± 0.002	$\pm 0.004^*$	$\pm 0.004^*$	$\pm 0.013^*$	± 0.012	$\pm 0.19^*$	± 0.21	± 0.17

Fig. 1. *Membrane fluidity in En(a-) erythrocytes as a percentage of the control value*

Results are of individual experiments that were performed at 37°C . Stearic acid labels, with the doxyl group in C-5, C-12 and C-15 positions respectively, were used. S_5 , S_{12} and τ_0 (C-15) are order and motion parameters respectively.

Table 2. *Membrane fluidity in isolated erythrocyte membranes*

Membrane fluidity was probed by stearic acid spin labels with the doxyl group in the C-5, C-12 and C-15 positions respectively, as well as with a nitroxide derivative of maleimide. S_5 , S_{12} and τ_0 refer to order and motion parameters respectively. Results are means of two experiments or means $\pm$ s.e.m. of three experiments; * $P < 0.05$.

Temperature ($^{\circ}\text{C}$) ...	S_5		S_{12}		$\tau_0(\text{C-15})$ (ns)		$\tau_0(\text{maleimide})$ (ns)	
	34	37	34	37	34	37	34	37
Erythrocyte membrane								
Control	0.641	0.627	0.490	0.461	2.32	2.23	0.48	0.42
	± 0.005	± 0.005	± 0.005	± 0.004				
En(a-)	0.644	0.629	0.478	0.454	2.29	2.09	0.70	0.58
	$\pm 0.005^*$	$\pm 0.006^*$	$\pm 0.004^*$	$\pm 0.003^*$				

temperatures. The mean values of S_{12} differed from mean control values at $P < 0.05$ at 34 and 37°C , but at 41°C the difference was not significant.

E.s.r. studies on isolated erythrocyte membranes

As judged from the smaller numerical values of S , isolated membranes were less organized than erythrocytes (see Tables 1 and 2). As with intact

cells, the difference in surface layers between En(a-) and normal isolated membranes was small, although a tendency towards stabilization in En(a-) membranes was seen. On the other hand, in intermediate membrane regions probed with 12-doxylstearic acid, En(a-) membranes were more fluid than the control membranes, the respective mean values for S_{12} being 0.454 and 0.461 at 37°C (Table 2 and Fig. 2). This

Fig. 2. Membrane fluidity in isolated En(a-) erythrocyte membranes as a percentage of the control value. Results are of individual experiments performed at 37°C. Stearic acid labels, with the doxyl group in C-5 and C-12 positions respectively, as well as a nitroxide derivative of maleimide, were used. S_5 , S_{12} and τ_0 (maleimide) are order and motion parameters respectively.

Table 3. Effect of spectrin depletion on membrane fluidity in isolated erythrocyte membranes

Erythrocyte membranes were depleted for spectrin as explained in the Material and methods section. Membrane fluidity was probed by stearic acid spin labels with the doxyl group in the C-5 and C-15 positions respectively, as well as by a nitroxide derivative of maleimide. S_5 and τ_0 refer to order and motion parameters respectively, at 37°C. Results are means $\pm$ S.E.M. of three to four experiments. The values in parentheses refer to S and τ_0 in spectrin-depleted membranes as percentages of the control value. * $P < 0.05$; ** $P < 0.01$.

	S_5	τ_0 (C-15) (ns)	τ_0 (maleimide) (ns)
Control membranes	0.650	2.86	0.94
	± 0.004	± 0.50	± 0.33
Spectrin-depleted membranes	0.649 (99.8%)	3.85 (134.7%)	0.67 (71.1%)
	± 0.005	$\pm 0.92^*$	$\pm 0.32^{**}$

difference was significant at $P < 0.05$. Only two experiments were performed with the 15-doxyl-stearic acid, but as judged from the results, the deepest membrane layers did not clearly differ in En(a-) and control membranes.

The spectrum obtained after labelling thiol groups in membrane proteins with the nitroxide derivative of maleimide revealed at least two classes of binding sites for the label: one permitting relatively free motion of the label, as revealed by three sharp central lines separated by 1.6 mT, the other permitting only restricted movement, as indicated by shallow lateral peaks separated by 6.5 mT. The motion parameter (τ_0) for label movement at sites permitting unrestricted movements was, in En(a-) membranes at 37°C, 138% of the control value (Table 2), indicating that, in the En(a-) membrane, protein motion was restricted.

Effect of anti-(glycophorin A) antiserum on normal and spectrin-depleted membranes

A partial extraction of spectrin from normal membranes by incubation in EDTA-containing

buffer did not influence the organization in superficial membrane regions, as revealed by experiments with the 5-doxylstearic acid, but the hydrophobic core of the membrane was stabilized, as judged from an increase in τ_0 value for the C-15 label from 2.86 to 3.85 ns at 37°C (Table 3). Spectrin depletion had no effect on the strongly bound maleimide spectrum, but τ_0 for the freely moving maleimide label was decreased from 0.94 to 0.67 ns at 37°C, indicating an increased mobility of some membrane protein(s).

Anti-(glycophorin A) antiserum had no effect on the organization in superficial regions of either untreated or spectrin-depleted normal membranes, but in the hydrophobic core, a fluidization was observed as a 4–5% decrease in τ_0 for the 15-doxyl label. However, the effect of the antiserum was most obvious on those membrane proteins that allowed free motion of the maleimide label. The mobility of these proteins, as judged from the maleimide spectra, was increased, especially in spectrin-depleted membranes, where τ_0 was decreased by 25% as compared with a 10% decrease in normal membranes (results of two experiments).

In two experiments, the specificity of the effect of the anti-(glycophorin A) antiserum was tested by making use of erythrocyte membranes from a subject whose erythrocytes contained only half the normal amount of glycophorin A [Gahmberg *et al.*, 1976; En(a-) heterozygote]. In these experiments, treatment with the antiserum decreased the motion parameter for the freely moving maleimide label in normal membranes by 8%, whereas the antiserum had practically no effect on En(a-) heterozygote membranes (about 1% decrease in τ_0), thus demonstrating the specificity of the antiserum effect.

Effect of freezing on membrane order and motion parameters in normal and En(a-) erythrocytes

When normal erythrocytes were submitted to a commonly used freeze-storage procedure (Åkerblom & Högman, 1974) and thereafter thawed, e.s.r. studies revealed that whereas the lipid organization in surface membrane regions was unaltered, that in intermediate regions was decreased, as indicated by a lowered (1.7%, two expts.) value for S_{12} . In contrast with this observation, En(a-) cells reacted on the same freeze-storage procedure by rather increasing their membrane organization (increase in S_{12} value by 1.5%; two expts.) in intermediate membrane regions, whereas the surface was largely unchanged. In parallel experiments it was observed that whereas 7.5% of normal cells haemolysed during the freeze-thaw procedure, 22.8% of En(a-) cells were irreversibly damaged by the procedure. The concentrations of ATP and 2,3-diphosphoglycerate in freeze-stored cells (0.14 and 2.8 mmol/litre respectively) were 55–70% of that in fresh erythrocytes, but there were no differences between normal and En(a-) erythrocytes.

Discussion

Membrane fluidity depends on the amount and classes of phospholipids and glycolipids present in the membrane, on membrane proteins, on the degree of fatty acid-chain saturation, on the tilt angle of the fatty acid chains and on the cholesterol content of the membrane. Two possibilities are readily ruled out as causes for the altered fluidity in En(a-) membranes; the total amount of the various phospholipid classes as well as the total cholesterol content in these membranes are equal to those of normal cells (Jokinen & Gahmberg, 1979; M. Jokinen & C. G. Gahmberg, unpublished work). On the other hand, two facts are known about the En(a-) membrane that could result in the observed alterations. Firstly, the lack of the major membrane sialoglycoprotein, glycophorin A, which spans the membrane, could alter membrane fluidity, especially in the hydrophobic core of the membrane, as was observed in the present study. Further, the lack of

this major membrane protein could result in a decreased amount of boundary lipids, i.e. the layer of highly organized lipids surrounding the protein, which could result in a net fluidization of the En(a-) membrane. Secondly, although the total amount of phosphatidylethanolamine is normal in En(a-) membranes, this phospholipid is apparently abnormally organized, as revealed by its increased labelling in intact cells with the amino-group-reactive non-permeant reagent trinitrobenzenesulphonate (Jokinen & Gahmberg, 1979). The cause of this abnormality is unknown; further speculations do not seem justified.

Besides a more fluid hydrophobic membrane core, the mobility of those unidentified membrane proteins that allowed free motion of the maleimide label was decreased in En(a-) cells. In En(a-) membranes, band 3 protein is over-glycosylated (Gahmberg *et al.*, 1976; Tanner *et al.*, 1977), and in this case the decrease could be explained by a more bulky sugar chain restricting protein motion. This possibility is, however, not supported by the observation that the rotational diffusion of band 3 protein of En(a-) membranes does not seem to be changed from normal (Nigg *et al.*, 1980a).

When normal membranes were treated with antiserum against glycophorin A, an increased maleimide label motion was observed. At the same time, the hydrophobic core became more fluid. The antiserum is specific for glycophorin A (Gahmberg *et al.*, 1978; Gahmberg *et al.*, 1979). When tested with membranes containing only half the normal amount of glycophorin A [En(a-) heterozygote], the effect was marginal. The effect on normal membranes can be interpreted in either of three ways. Firstly, the antiserum is known not to induce a capping phenomenon (Gahmberg *et al.*, 1979), but it could alter the molecular conformation of glycophorin A, allowing increased mobility of glycophorin A or of membrane proteins other than glycophorin A. Secondly, the antiserum could quench the electrostatic properties of glycophorin A, thereby hindering it from contributing to the normal random distribution of intramembrane particles (Gahmberg *et al.*, 1978). Thirdly, the antiserum could block binding sites for the maleimide label; the increased label motion could originate from swiftly moving proteins other than glycophorin A. This possibility is unlikely, however, because depletion of the membranes for spectrin (which contains a major proportion of the maleimide label binding sites) did not qualitatively alter the effect of the antiserum. It is noteworthy that the rotational movement of band 3 protein was recently shown to decrease by anti-(glycophorin A) antiserum, indicating a close association between the two proteins (Nigg *et al.*, 1980b).

The results of the experiments with spectrin-

depleted membranes merit a few more comments. As was expected, spectrin depletion increased membrane protein motion by eliminating the supposedly regulatory association between spectrin and other proteins (Bennett & Stenbeck, 1979; Luna *et al.*, 1979). What was not expected, however, was that spectrin depletion stabilized the hydrophobic core, but not the surface, of the membrane. This indicates a regulatory action of peripheral membrane proteins on membrane fluidity.

As has been frequently pointed out, it is amazing that the lack of glycophorin A, perhaps due to compensatory alterations in the membrane, causes no clinical symptoms. A blood transfusion, even with ABO-compatible blood, would, however, be hazardous, and therefore attempts have been made to store En(a-) homozygote blood for possible future autotransfusions. Unfortunately, En(a-) cells do not lend themselves well for this procedure since considerable haemolysis occurs. We do not know the reason for the increased haemolysis, but the present results show that the En(a-) membrane reacts in an abnormal manner to the subzero temperatures used in the freeze-storage procedure. We hypothesize that the abnormal response to low temperatures reflects the lack of glycophorin A. It is known that glycophorin, in common with the 'anti-freeze' glycoproteins of some species of arctic fish, contains large amounts of O-glycosidic oligosaccharides (Feeney & Yeh, 1978), and such oligosaccharides may somehow be involved in temperature-dependent functional changes.

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