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EVIDENCE FOR STRUCTURAL CHANGES IN ERYTHROCYTE MEMBRANES OF SPONTANEOUSLY HYPERTENSIVE RATS. A FLUORESCENCE POLARIZATION STUDY.

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SUMMARY

Diphenylhexatriene (DPH) was used as a fluorescent probe to detect possible structural differences between erythrocyte membranes of spontaneously hypertensive (SHR) and normotensive (WKY) rats which differ by several functional and chemical aspects. Fluorescence polarization was found constantly higher in SHR than in WKY erythrocyte membranes. In adult rats, the activation energy of the fluorescent probe motion appeared lower in SHR than in WKY erythrocyte ghosts. These results represent an additional evidence for an erythrocyte membrane abnormality in genetic hypertension of the rat, especially so as the difference is observed in young animals before the rise in blood pressure.

INTRODUCTION

Spontaneously hypertensive rats (SHR) of the Okamoto strain are considered to be a model of human essential hypertension. Erythrocyte membranes from both SHR and essential hypertensive patients exhibit various functional and structural abnormalities such as altered fluxes of monovalent cations measured under various conditions (1-8), changes in calcium binding and transport (9-12) and modified phosphoinositide content (11). Some of these changes were shown to be of genetic origin since they could be found also in some normotensive offspring of hypertensive parents (8) and in young SHR when hypertension had not yet developped (3, 10). The aim of this study was to examine whether any differences in the physico-chemical properties of the erythrocyte membrane exist between SHR and the normotensive substrain (WKY). This comparison was carried out on young rats in order to avoid possible consequences of elevated blood pressure as well as the erythrocytosis which is reported to develop in

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older hypertensive rats (12). Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) has been used to provide information on membrane structure in erythrocytes of normotensive (WKY) and hypertensive (SHR) animals. Due to the restricted mobility of this fluorophore when it is embedded in the phospholipid bilayer, its fluorescence emission is highly polarized. Therefore, fluorescence polarization permits the study of the movement of DPH in its local environment (13-16). Consistent differences in fluorescence polarization and in activation energy were observed in SHR erythrocyte membranes as compared to WKY controls.

MATERIAL AND METHODS

Male Okamoto spontaneously hypertensive rats (SHR) and normotensive Kyoto rats (WKY) derived from the NIH stock were supplied by Iffa-Credo (France). Systolic arterial pressure was recorded by tail sphygmomanometry. Blood was sampled by cardiac puncture on stunned animals and collected on heparin 10 IU/ml) or citrate as anticoagulant. Red blood cells were washed three times with a pH 8 buffer containing 5 mM phosphate and 140 mM NaCl. The white buffy layer was carefully eliminated and lysis performed as described by Steck and Kant (17). Sealed inside-out vesicles were prepared according to the method of Steck and Kant (17) modified by Blostein and Chiu (18). Sideness and sealing quality were measured by the activity of acetylcholine esterase as previously described (17).
DPH was dissolved in freshly distilled tetrahydrofuran at a concentration of $2x10^{-3}M$. This solution was dispersed in the incubation buffer to reach a concentration of $2x10^{-6}M$ and then mixed with an equal volume of membrane suspension and incubated for 30 minutes at 37°C. The absorbance (due to light scattering) of the membranes suspension was adjusted to 0.15 at 456 nm (wavelength of DPH fluorescence maximum).

Fluorescence emission and excitation spectra were recorded with a FICA 55000 absolute differential spectrofluorimeter using the membrane preparation without DPH in the reference compartment. Emission from DPH dispersed in the buffer was always less than 3 % of that from DPH in the membrane containing samples. Polarized fluorescence emission and excitation spectra were recorded with the same apparatus using Glan-Thomson polarizers. With an excitation wavelength of 352 nm, no variation in fluorescence polarization was detected as a function of the emission wavelength. There was no marked difference between the polarization ratio determined using this equipment and that obtained using an Elscint MV I apparatus. Polarization ratios were then systematically determined with this last apparatus using square 5 mm optical pathlength cells. When the temperature dependence of polarization was investigated, no alteration in the membrane characteristics was observed when the polarization ratio was measured at 35°C after each temperature cycle. The same value was obtained except when the membrane preparation was cooled below 5°C.

RESULTS AND DISCUSSION

Sealed inside-out membrane vesicles were compared to ghosts obtained under four different conditions. Lysis was carried out in a 5 mM phosphate buffer

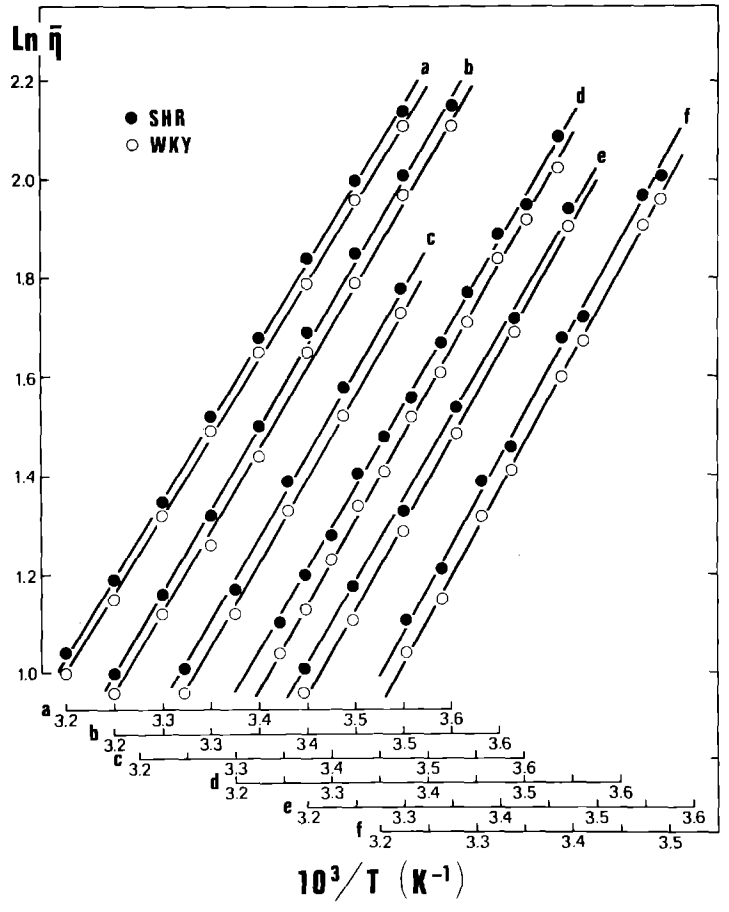

Figure 1

Arrhenius plots of 'equivalent microviscosity' Ln $\bar{\eta}$ vs (1/T), for erythrocyte ghosts in a 5 mM phosphate buffer pH 8.

a, b, c, d, : erythrocyte ghosts from 3, 4-5, 8 and 12 week-old SHR (●) and WKY (○) rats respectively, prepared in the presence of 1 mM Mg SO_4.
e : erythrocyte ghosts from 12 week-old SHR (●) and WKY (○) rats, prepared in the presence of 0.2 mM EDTA.
f : inside-out vesicles prepared from 12 week-old rats in 0.5 mM phosphate buffer pH 8. Values of the correlation coefficients were between 0.999 and 1. These curves represent the result of one out of at least 4 independent experiments with similar results.

Blood pressures were as follows (mm Hg)

age (weeks)	3	4-5	8	12
WKY	105 ± 3	108 ± 5	127 ± 4	130 ± 3
SHR	115 ± 3	123 ± 5	182 ± 4	178 ± 3

(pH 8) in the absence or presence of 1 or 5 mM $MgSO_4$ or 0.2 mM EDTA. In all cases, steady state fluorescence polarization was measured in parallel on membrane preparations from the two rat substrains at different temperatures.

As shown in Figure 1 the polarization ratio of DPH fluorescence was higher in hypertensive SHR than in normotensive WKY rats. The difference was small

TABLE 1 Activation energy calculated from Ln $\bar{\eta}$ versus 1/T plots obtained in erythrocyte membranes. Significant differences in activation energy between SHR and WKY erythrocytes ghosts were only observed in adult animals ($p < 0.01$, paired t-test).

Age (weeks)	Membrane Preparation	Experimental Conditions	Activation energy (kJ. mole^{-1})		
			WKY	SHR	n
3	Ghosts	5P8* + 1 mM $MgSO_4$	27.4 ± 0.9	27.0 ± 1.0	4
4-5	Ghosts	5P8* + 1 mM $MgSO_4$	27.5 ± 0.2	27.5 ± 0.3	4
8	Ghosts	5P8* + 1 mM $MgSO_4$	28.0 ± 0.4	28.0 ± 0.4	4
12	Ghosts	5P8* + 1 mM $MgSO_4$	28.2 ± 0.4	27.5 ± 0.3	5
12	Ghosts	5P8*	30.2	28.4	3
12	Ghosts	5P8* + 0.2 mM EDTA	29.0	27.8	2
12	Ghosts	5P8* + 5 mM MgH SO_4	28.0	27.0	2
12	Inside-out vesicles	0.5 mM Phosphate buffer pH 8	29.7	29.6	2

* 5P8 : 5mM Phosphate buffer, pH 8.
mean ± S.E. is given only when $n \geq 4$.

but reproducible and significant (p 0.001, $n = 18$, paired Student t-test) and did not depend upon the methods used to prepare the membranes. The difference was unlikely to be related to superficial proteins which could have been released by EDTA treatment as indicated by the lack of effect of EDTA. From the polarization ratio, an "equivalent microviscosity" $\bar{\eta}$ (in poise) can be derived according to the following relationship (15).

$$\bar{\eta} = \frac{2p}{0.46 - p}$$

This equation is based upon several assumptions which are discussed in reference 15. As shown in Figure 1, plots of Ln $\bar{\eta}$ versus I/T gave accurate linear fits from which activation energies were calculated. As shown in Table 1, the difference in activation energies between SHR and WKY ghosts of adult rats (12 weeks and above) existed independently of whether $MgSO_4$ or EDTA was present or not. This difference was not observed in the case of inside-out vesicles.

In order to determine whether the observed difference in fluorescence polarization was preexistent to the rise in blood pressure, experiments were performed on rats of various ages. Results are presented in Figure 1 together with blood pressure in the two substrains. The difference in DPH fluorescence polarization between SHR and WKY membranes was observed already at 3 weeks of age before the rise in blood pressure which is highly suggestive of a primary phenomenon. A difference in activation energies was observed in adult rats but not in young animals. Nevertheless, in the temperature range investigated, 5-45°C, the polarization ratio remained higher for SHR than for WKY rats independently of age.

A difference in the physico-chemical properties of SHR and WKY erythrocyte membranes has previously been reported using a spin-labelled fatty acid as a probe for structural modifications (19). The rotational mobility of the spin label was found to be higher in SHR than in WKY erythrocytes, and the activation energy for the rotation of the probe molecule was higher for SHR than for WKY erythrocytes (19). These results differ from those obtained in the present investigation (higher polarization (implying lower rotational mobility) and lower activation energy for SHR as compared to WKY membranes). Spin-label experiment were carried out on intact erythrocytes whereas fluorescence polarization data concern isolated membranes. However, the difference between spin labels and fluorescence probes most probably reflects a difference in the localization of the two probes in the membranes. It has been recently suggested that DPH can bind to hydrophobic cores of membrane proteins as well as to the aliphatic part of phospholipids (16). Spin-labelled fatty acids could also anchor to proteins and to some phospholipid polar heads through interactions involving their carboxylic groups. For these reasons it is difficult to interpret both spin-label and fluorescence polarization data in terms of general fluidity of biological membranes. Nevertheless, the present data confirm that SHR and WKY erythrocytes exhibit differences in their membrane structure which may have some relationship with the other membrane abnormalities previously described. Moreover, they demonstrate that this alteration already exists in very young animals prior to the rise in blood pressure. Preliminary experiments performed on plasma membranes of various tissues have shown that this difference between SHR and WKY is not restricted to erythrocytes. This finding supports the hypothesis that the membrane defect is a diffuse abnormality, possibly involved in the pathogenesis of the disease (6).

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