Biochimica et Biophysica Acta 812 (1985) 811–815
Elsevier

BBA 72456

Kinetics of water transport in sickle cells

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(Received September 3rd, 1984)

Key words: Water transport; Sickle cell; Transport kinetics; (Erythrocyte membrane)

This paper reports the results of stopped-flow studies on differences in the kinetics of osmotic water transport of sickle and normal erythrocytes. The kinetics of inward osmotic water permeability are similar in sickle and normal red blood cells. In contrast, the kinetics of outward water flux are significantly (approx. 38%) decreased in sickle cells. Deoxygenation does not modify the water influx kinetics in either type of cells, but accelerates considerably the rate of water efflux in sickle cells. No significant variation of water transport kinetics was observed in density-separated cell fractions of either type. The results suggest that membrane-associated hemoglobin may decrease the outward water permeability and that in deoxygenated sickle cells the fraction of hemoglobin S near the lipid bilayer is lower than in oxygenated conditions.

Introduction

In contrast to the well documented structural and functional features of hemoglobin S [1], the membrane abnormalities of the sickle cells are less well understood. It is difficult to determine whether the observed cellular impairements in cation transport [2], deformability [3], and osmotic fragility [4] are due to intrinsic membrane lesions or to direct effects of hemoglobin on the membrane.

Cell dehydration is an important factor determining the intracellular polymerization of hemoglobin S and the clinical manifestations of sickle cell disease [5]. The decreased water content observed in irreversibly sickled cells or in deoxygenated reversible sickle cells [6] results from a perturbated cation transmembrane equilibrium regulated by slow transport processes. Factors such as energy (ATP) levels and regulatory agents (i.e. calmodulin) may also be implicated. Water transport across red cell membranes is a passive process, sensitive to the physico-chemical state and composition of the membrane, as well as to the action of some hydrophobic or covalent reagents [7–12].

In order to detect and characterize more fully possible perturbations in membrane transport of sickle cells, we studied the rapid osmotic water transport through sickle cell membranes as compared to normal erythrocyte membranes and determined the effects of deoxygenation and cell density on this process.

Materials and Methods

The blood samples of patients homozygous for sickle cell disease diagnosed by standard laboratory procedures were obtained after their informed consent according to the code of ethics of the World Medical Association. The studied patients

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Abbreviations: AA cells, hemoglobin A-containing red blood cells; 2,3-DPG, 2,3-diphosphoglyceric acid; PCMB, *p*-chloromercuribenzoate; SS cells, hemoglobin S-containing red blood cells.

were at distance of crisis and transfusion. Normal blood was from volunteers of our laboratories. The blood was collected by venipuncture in a EDTA-containing, isoosmolal medium; red blood cells were immediately separated by centrifugation (10 min, $3000 \times g$) and subsequently washed three times in phosphate-buffered saline at 4°C. Separation of red blood cells was carried out by centrifugation in a stractan density gradient [13]. All experiments were performed at 23°C. The experiments in the absence of oxygen were conducted by adding the cells to phosphate-buffered saline which had been previously deoxygenated by equilibration with pure nitrogen. A small amount of dithionite (final concentration 0.5 mM) was added, just before each experiment.

The osmotic water permeability was estimated by monitoring scattered light (640 mm) of a cellular suspension (0.5% hematocrit) suddenly mixed with an equal volume of an anisosmolal phosphate-buffer saline (obtained by changing the NaCl concentration). The monitoring was carried out at 90°C for inward kinetics or 180°C for outward kinetics. For the study of the outward kinetics the monitoring of 90° scattered light gave similar results as for 180°, but with a lower signal-to-noise ratio. The kinetic measurements were made with a Durrum-Gibson stopped-flow apparatus interfaced to a Data General Nova 2 microcomputer. In order to increase the signal-to-noise ratio, five to ten runs were usually averaged [14].

From the rate constant of light scattering variation $k(s^{-1})$, we calculated the osmotic water permeability P (cm/s) at 23°C according to the formula [15]:

$$P(\mathrm{cm/s}) = (5.6 \cdot 10^{-2}) \frac{(1-b)V_0/A}{\pi^2 \cdot C_0} k \qquad (1)$$

where $(1-b)$ is the osmotic active cellular water fraction, V_0/A is the erythrocyte volume to surface ratio at isotonicity (in cm), π is the relative final osmolality and C_0 is the isotonic concentration (osM/cm^3). In order to calculate $(1-b)$, the changes in erythrocyte volume resulting from osmotic variations were estimated by microhematocrit measurements in the osmotic range 300–225 mosM. The V_0 was evaluated with a Coulter Counter S Plus II.

All the chemicals were reagent grade. The osmolality of solutions was measured from freezing-point depression with a Roebling osmometer.

Results

A typical osmotic swelling experiment with normal red blood cells is presented in Fig. 1 (line a); the mean rate constant of six runs is $k = 1.33 \pm 0.05\ s^{-1}$. Using the experimentally determined cellular parameters $(1-b) = 0.65$ and $V_0 = 94\ \mu m^3$ and Eqn. 1, we obtained the inward osmotic permeability, $P = 1.79 \cdot 10^{-2}$ cm/s. This value is in good agreement with the values reported by others [8,10,14–19]. The corresponding experiment for a sickle cell sample is presented in Fig. 1 (line b): ($k = 1.29 \pm 0.09\ s^{-1}$, 6 runs). In this case the permeability is $P = 1.62 \cdot 10^{-2}$ cm/s for $(1-b) = 0.60$, $V_0 = 97\ \mu m^3$. The results obtained on four matched pairs of AA and SS cells indicated no significant difference ($p > 0.05$) between the in-

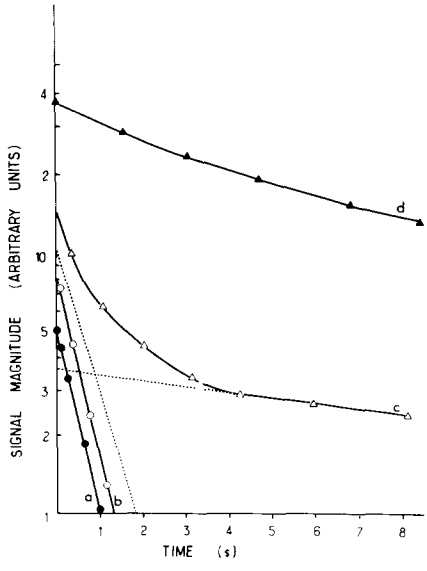

Fig. 1. Light scattering variation at 90° versus time in swelling experiments performed by rapidly mixing equal volumes of an isoosmolal erythrocyte suspension (0.5% hematocrit) and an hypoosmolal phosphate-buffered saline: (a) AA cells, final osmolality 235 mosM; (b) SS cells, final osmolality 235 mosM; (c) AA cells, final osmolality 150 mosM; the complex curve is decomposed in two single exponentials (interrupted lines); (d) AA cells initially equilibrated at 200 mosM, final osmolality 105 mosM.

TABLE I

INWARD AND OUTWARD OSMOTIC WATER PERMEABILITY IN AA AND SS CELLS IN OXY AND DEOXY CONDITIONS

The results represent the mean of four experiments on different blood samples with the standard deviation in parenthesis. The results in presence of PCMB (1 mM) are the mean of two values.

Cell type	P(cm/s) ($\times 10^2$)			
	Inward		Outward	
	oxy and deoxy	PCMB	oxy	deoxy
AA	1.79 (0.06)	0.38	1.49 (0.03)	1.53 (0.05)
SS	1.72 (0.05)	0.36	1.01 (0.07)	1.58 (0.06)

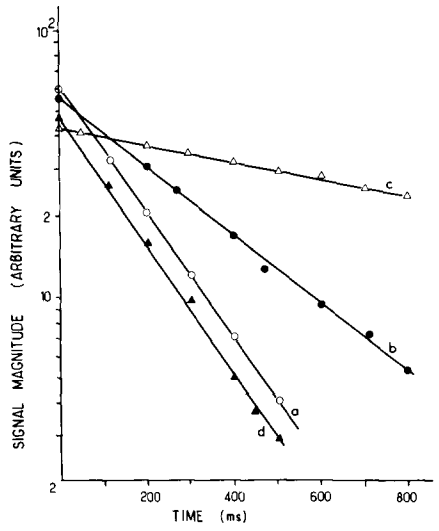

Fig. 2. Light scattering variation at 180° versus time in shrinking experiments, final osmolality 430 mosM. (a) AA cells; (b) SS cells; (c) SS cells after 30 min incubation at room temperature with 1 mM PCMB; (d) deoxygenated SS cells.

ward osmotic water permeability for the two types of cells (Table I).

We next compared the sensitivity of inward water transport to inhibition by a classical sulfhydryl-specific reagent, PCMB, for normal and sickle cells. Incubation for 30 min with 1 mM PCMB of diluted cell suspensions produces the same inhibition (approx. 80%) in AA and SS cells (Table I). The inward osmotic water permeability was found to be also independent of oxygenation in both types of cells (not shown).

For final osmolalities less than 220 mosM, we observed a biphasic light scattering pattern (Fig. 1, curve c) implying two kinetically different processes. The complex curve was analysed interms of two exponentials (the two dotted lines in the Fig. 1). The rapid phase corresponds to the above discussed swelling phenomenon. The slower component represents a lysis event; indeed, when a suspension of erythrocytes, equilibrated at 200 mosM, was mixed in the stopped-flow apparatus with a 10 mosM buffer we obtained a simple exponential curve (line d, Fig. 1) similar to the above, slower phase. The slower rate constant ($k = 0.052\ s^{-1}$) agrees well with the kinetic values of hemolysis, determined by hemoglobin leakage measurements [20]. This last experiment was necessary in order to verify that the permeability measurements were not altered by a slow scattering phase induced by hemolysis.

The study of the outward osmotic water permeability was performed using final hyperosmolal conditions (shrinking experiments). The results obtained in this way are presented in Fig. 2 and Table I. Both oxy AA and SS cells exhibited a lower outward water permeability ($P = 1.49 \cdot 10^{-2}$ cm/s and $1.01 \cdot 10^{-2}$ cm/s, respectively) as compared to inward permeability; this is known as the rectification of the water transport [9]. The rate constant of the shrinking kinetic in SS cells (line b) was significantly lower than in AA cells (line a). For four pairs of AA and SS samples, this decrease has a mean value of 38%.

While deoxygenation has no effect on the shrinking kinetics for normal erythrocytes, the rate constant for sickle cells increases by approx. 60% upon deoxygenation (Fig. 2, line d). Since the permeability computed from Eqn. 1 depends on the isoosmolal volume of cells it was necessary to ascertain that SS cells did not significantly change their volume upon deoxygenation during the brief period of the experiments. An absence of volume change was established by finding no detectable variation in light scattering intensity, when the red cell suspension was deoxygenated in the stopped-flow apparatus with an isoosmolal PBS containing 0.5 mM dithionite. Therefore, the deoxygenation-induced changes in the shrinking kinetics for SS

cells are a result of a true permeability change and not of a variation in the initial cell volume (V_0) (see Eqn. 1).

Swelling and shrinking experiments performed on density separated AA and SS cell fractions revealed that water permeability is independent of cell density (not shown).

Discussion

Our results indicate that the inward osmotic water permeability is not significantly perturbed in oxygenated, nor deoxygenated sickle cells, by comparison with normal erythrocytes. The outward permeability for water osmotic or diffusion transport in normal red blood cells was shown to be lower than inward permeability [11,15,17,21]; the inward/outward permeability ratio is denoted as the rectification ratio (see Ref. 11 for a detailed discussion). For AA cells we found a mean rectification ratio of 1.20 which is comparable with the values reported by others [11,15,17]. In SS cells, the reduction in outward osmotic permeability was further reduced and corresponded to a rectification ratio of 1.70 (mean for four samples).

The dependence on oxygenation of water efflux in SS cells suggests that the differences observed in the water transport properties of AA and SS cells cannot be attributed to irreversible membrane damages alone. The fact that the inward water flux and the PCMB-inhibition effect are similar in both types of cells are consistent with this suggestion. Extramembraneous factors seem necessary to explain the results.

The mechanism of water transport in human erythrocytes is still largely uncharacterized. It is mediated by membrane proteins since natural membranes show water permeabilities 10-times greater than lipid model membranes [9]. From studies performed with radioactively labeled inhibitors of water transport, it was shown that band 3-protein, which forms a transmembrane complex with glycophorin, may be part of the structure of the hypothetical aqueous pores [22]. One of the hypothetical explanations for the rectification of water transport is that water efflux produces a transient accumulation of cytoplasmic proteins, mainly hemoglobin, on the cytoplasmic surface of the membrane, resulting in steric hindrance of the aqueous pore [11].

Evidence that hemoglobin binds to band 3 in erythrocyte membrane in vivo, has been recently obtained by fluorescence quenching experiments [23] and by measuring directly the affinity of the purified, 43 kDa, cytoplasmic fragment of band 3 with hemoglobin at physiological pH and ionic strength (Chetrite, G. and Cassoly, R., unpublished data). It has also been suggested, from independent experiments, that membrane bound hemoglobin could mediate the state of aggregation of band 3 protein [24], i.e. the formation of higher order structures of band 3 in the membrane which could represent pores for water transport. Finally, while having a rotational motion 400-times slower than the free tetramer [25], membrane-bound hemoglobin is considerable more mobile than the integral part of band 3 protein [26]. As a result hemoglobin may flow onto the membrane during the outward water flux and this process could result in an inhibition of aqueous pathways. It has previously been reported that the rate of water influx in resealed ghosts and intact human red cells was the same [19], but the efflux was 50% larger in ghosts when compared to intact erythrocytes [14]. This suggests that depletion of erythrocyte cytosolic proteins significantly decreases the rectification of water transport. The difference we have measured in water outward permeability according to the presence of hemoglobin S or hemoglobin A in the cytoplasm (Fig. 2 lines a, b) is consistent with the reported increase in hemoglobin S membrane interaction as compared with hemoglobin A [27].

We have shown that the rectification ratio measured for normal and sickle cells was not significantly different for density-separated cells whose hemoglobin concentration was between 4 mM and 7 mM (per tetramer). This result is consistent with the fact that in this range of concentration the fractional saturation of band 3 with hemoglobin in the cells only changes by 10% (Chetrite and Cassoly, unpublished data).

The deoxygenation of SS cells significantly increases the water efflux permeability practically eliminating the transport asymmetry (Table I). In the deoxygenated state approx. 70% of the intracellular hemoglobin S is in a gel phase [28], which may result a significant decrease in the quantity of hemoglobin tetramers bound to the

membrane. As a consequence, the water efflux should increase (line d in Fig. 2).

By measurements of resonance energy transfer from membrane-incorporated fluorescent probes to hemoglobin heme in the cytoplasm it was recently found [29] that there are more hemoglobin molecules at the internal membrane interface in oxygenated SS cells than AA cells and, furthermore, that deoxygenated hemoglobin S withdraws from the membrane surface. Our results, obtained by a quite different method, are in accord with these facts.

In conclusion, our results suggest that the decrease in the rate of the osmotic water outward transport in oxygenated sickle cells and the effects observed on deoxygenation do not result form perturbations of the structure of the membrane. Rather, they suggest strongly that the properties of intracellular hemoglobin and its interaction with membrane play a key role in modifying the outward water flux.

Acknowledgements

We thank Mrs. M. Segear and A.M. Dulac for preparation of the manuscript. We are indebted to Professor S. Edelstein and Dr. B. Lubin for reading the manuscript and for their comments.

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