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Effect of DIDS on osmotic properties of bovine erythrocytes

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The effect of DIDS on osmotic properties of bovine erythrocytes was studied. The isoosmotic volume, the amount of intracellular solutes, and the osmotically non-active volume were not influenced by DIDS. An increase of osmotic fragility of erythrocytes upon DIDS treatment was evident and identical both in NaCl and in NaCl + KCl hypotonic solutions. These results suggest that the critical cell volume decreases. The link between the effect of DIDS on the membrane skeleton extractability and on the osmotic fragility was postulated.

Introduction

The band 3 protein, that spans the red cell plasma membrane, catalyzes the exchange of anions across the membrane (see Refs. 1 and 2 for recent reviews). The cytoplasmic domain of this protein acts as an anchor for the membrane skeleton [3,4]. It also has binding sites for some glycolytic enzymes [5,6] and hemoglobin [7].

DIDS, which binds specifically to band 3, and is a well known inhibitor of anion transport [8,9], also decreases the extraction by 0.1 M NaOH of some membrane skeleton components: spectrin and band 2.1 protein [10]. However, the effect of DIDS on the peripheral proteins extractability depends on the extraction method [10]. DIDS does not alter the water transport [11], but the change of membrane properties may influence osmotic properties of erythrocytes.

In this work the effect of DIDS on the osmotic properties of bovine erythrocytes was investigated. The gross conformation of the cytoplasmic domain of bovine band 3 shows an extensive similarity to that of human band 3, although it has some structurally different regions, too [12]. DIDS binds to the bovine band 3 also in an approximate molar ratio of 1 : 1 [13].

Materials and Methods

Fresh, heparinized, bovine blood was centrifuged at 2700 × *g* for 10 min at 4°C, plasma and buffy coat were removed, and the red cells were washed three times with Hepes buffer: 143 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 5 mM Hepes (pH 7.4). Each erythrocyte suspension was divided into two equal parts, one of which served as control and the other was treated with DIDS.

The chemicals were of analytical grade. DIDS was obtained from Calbiochem, and Hepes from Serva Feinbiochemica, Heidelberg.

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Treatment of erythrocytes with DIDS. The red cells, suspended to a hematocrit of 25%, reacted with 2, 4, 8, 16 and 32 μM DIDS in Hepes buffer for 45 min at 37°C under dim white light, which lead to an irreversible binding of DIDS to the membranes [14]. The cells were then washed twice with the buffer.

Anion exchange measurements. The degree of inhibition of the bicarbonate-chloride exchange across the erythrocyte membrane, caused by DIDS, were measured by a stopped-flow method described in detail elsewhere [15].

Small samples of erythrocytes prepared for the osmotic experiments were, after incubation with DIDS and subsequent washing, resuspended in an isotonic NaCl + KCl solution to a 20% hematocrit. Upon the addition of $NaHCO_3$ and carbonic anhydrase (from Sigma) to final concentrations of 4.4 mM and 800 units/ml, respectively, the suspensions were mixed at room temperature with a buffer solution (5 mM KH_2PO_4, 5 mM Na_2HPO_4, 137.5 mM NaCl (pH 6.7)), in a stopped-flow apparatus. The increase of pH in the medium was recorded on a chart recorder. The standard analysis of the traces [15,16] yielded the percent inhibition of the HCO_3^--Cl^- exchange rate constant.

Osmotic fragility. The osmotic fragility was determined by a static method. Equal volumes of erythrocyte suspensions were added to two kinds of phosphate buffered solutions: one containing only Na^+ ions, and in the second K^+ ions constituted 25% of all cations. After 15 min the suspensions were centrifuged at $2700 \times g$ for 10 min, and the absorbance of the solution of released hemoglobin was measured at 540 nm wavelength. The osmotic fragility was expressed as the mean osmolarity, found numerically from the hemolytic curves [17]. The osmolarities of the solutions were calculated as for ideal solutions, taking into account the dissociation constant of the phosphate buffer.

Evaluation of the osmotic parameters of red cells. The relative critical volume of red cells, V_c, was calculated from the Van't Hoff's law modified for erythrocytes [18]:

$$V_c = \frac{c}{\pi_h} + b \qquad (1)$$

where c is the sum of products of osmotic coefficients and the amounts of internal solutes, π_h is the cells' osmotic fragility. b is the volume of osmotically non-active part of erythrocyte. The parameters c and b were evaluated by the linear-regression method from the modified for erythrocytes van't Hoff's law. A modified microhematocrit method [17] was used to obtain the relation between the cell volume and inverse osmolarity. Six NaCl solutions with relative osmolarities of 0.60–1.84 (unity corresponds to 310 mosM) were mixed in the ratio 2:3 with an isoosmotic erythrocyte suspension of a 75% hematocrit. Final osmolarities were calculated using the hematocrit of the isoosmotic cell suspension, final hematocrits of cell suspensions with varying osmolarities, and the volume of the solution trapped between erythrocytes. All hematocrits were measured after centrifugation at $15000 \times g$ for 5 min. In order to calculate the final osmolarity it was necessary to know the relative volume of intracellular water. This was determined by an application of the Brouwer theorem on the fixed point of representation [19] to a modified linear regression operator, which also included the evaluation of the osmolarity of the cell suspensions. The volume of the osmotically active water, $V_w = 1 - b$, was found by iteration.

Relative isoosmotic volume. The relative isoosmotic volume of erythrocytes was determined from the compared absorbances of hemoglobin released from control and modified erythrocytes in suspensions with identical hematocrits [17].

Trapped solution volume. 100.0 μl of isoosmotic NaCl solution, with or without 0.2 mM hemoglobin, was added to six tubes, each with 5.00 ml of erythrocyte suspension of 50% hematocrit. The hematocrits were measured upon centrifugation of capillaries at $15000 \times g$ for 5 min. Following the centrifugation of erythrocyte suspensions at $2700 \times g$ for 10 min, the absorbances of supernatant were read at $\lambda = 540$ nm. The absorbance difference between samples with and without hemoglobin added, was compared with a reference curve to determine the volume of the solution in the suspension. The difference between hematocrit measured by a capillary method and that indicated by hemoglobin concentration in supernatant, was the measure of the volume of solution trapped between packed cells.

Results

The dependence of the increase of the osmotic fragility on DIDS concentration (2–32 μM) is shown in Table I. The osmotic fragility of bovine red blood cells increased with an increasing DIDS concentration in an incubation medium in a saturable manner. To test whether the observed alterations of osmotic fragility were associated with interaction of DIDS with band 3 protein, where it binds, we have compared them with the percent inhibition of the bicarbonate-chloride exchange in the same erythrocyte samples (Table I).

In order to check if DIDS alters the KCl efflux when it decreases the osmotic fragility [20,21] we have measured it in two series of hypotonic solutions: one with only Na^+ ions, and the other containing also 25% of K^+ ions (in the intracellular solution of bovine erythrocytes potassium ions constitute no more than 25% of all cations [22]). The hemolysis curves, obtained for both control and DIDS treated erythrocytes, were indistinguishable (Fig. 1).

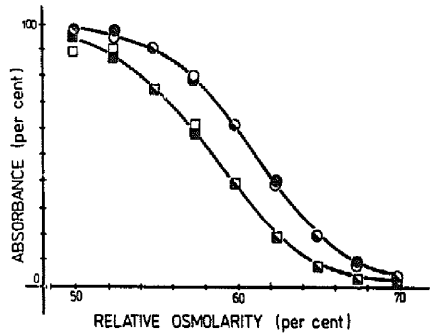

Fig. 1. The effect of DIDS on the hemolysis of erythrocytes in NaCl, and NaCl + KCl, solutions. The identical control curves (squares) were shifted upon 20 μM DIDS treatment of the erythrocytes (circles) to the region of higher fragilities. The shift was similar in both NaCl (filled points), and in NaCl + KCl solutions.

TABLE I

INFLUENCE OF DIDS ON OSMOTIC FRAGILITY AND BICARBONATE-CHLORIDE EXCHANGE IN BOVINE ERYTHROCYTES

The values shown are means ± S.E., $n = 6$. The coefficient of correlation between the rate constant inhibition and osmotic fragility is, up to 16 μM DIDS, $r = 0.88$, which is statistically significant at $P < 0.001$.

DIDS concn. (μM):	2	4	8	16	32
Percent inhibition of HCO_3^-/Cl^- exchange rate	17±7	37±8	77±8	100	100
Increase of osmotic fragility (percent of maximal shift)	19±7	49±9	74±10	95±4	100

TABLE II

THE EFFECT OF 20 μM DIDS ON THE OSMOTIC PROPERTIES OF BOVINE RED BLOOD CELLS

The number of experiments $n = 10$, the confidence limits are given for the confidence level $1 - P = 0.95$. All values are relative with respect to the control values ($b = 0.420 \pm 0.026$ V_i; $V_c = 138.4 \pm 2.0\%$ V_i; $\pi_h = 57.5 \pm 3.3\%$ (100% = 310 mosM); mean ± S.D., $n = 10$).

Isoosmotic volume, V_i (%)	Amount of intracellular solution, c (%)	Osmotically non-active volume, b (%)	Osmotic fragility, π_h (%)	Critical cell volume, V_c (%)
100.0±0.5	101.2±3.6	98.8±4.8	104.4±2.0	97.6±1.3

The isoosmotic volume, the osmotically non-active volume, and the amount of intracellular solutes did not change significantly upon DIDS treatment. DIDS did influence the osmotic fragility (Tables I and II) and the critical cell volume (Table II).

The volumes of trapped extracellular solution were equal (2.0 ± 0.6)% and (1.9 ± 0.6)% (mean ± S.D., $n = 5$) of the volume of packed control and DIDS treated cells, respectively.

Discussion

The osmotic fragility of erythrocytes depends on the critical cell volume, the amount of intracellular solutes, the osmotically non-active volume [23], and the hemolysis-protecting potassium efflux [21,24]. The critical cell volume, calculated from Eqn. 1 depends on the ratio of membrane surface area to the isoosmotic cell volume [23], and the membrane extensibility [25].

The lack of any difference between the hemolysis curves obtained in NaCl, and NaCl + KCl,

solutions (Fig. 1) points out that the potassium efflux, observed in human erythrocytes, is not measurable in bovine red cells.

The chloride anion exchange does not influence the amount of the intracellular solution and, therefore, the osmotic fragility of erythrocytes. The netto chloride flux is too small [1] to influence significantly the osmotic properties. Gary-Bobo and Solomon [26] concluded that the apparent non-osmotic water was to be attributed to Cl^- shifts across the red cell membrane. However, Solomon et al. [27] in a recent isotopic experiment failed to confirm this. The lack of a significant change of the osmotically non-active volume of erythrocytes upon DIDS treatment (Table II) also contradicts the former conclusion of Gary-Bobo and Solomon. DIDS does not influence the water transport across the red blood cell membrane either [11], and, therefore, the osmotic fragility shift was, in this experiment, not associated with the water transport properties of erythrocyte membrane, altered by DIDS.

Although the calculated critical cell volume depends on the trapped volume, the dependence is rather weak. The maximal absolute experimental error of the trapped volume, equal 0.7% of the volume of packed cells, results in a 0.25% change of the critical cell volume. The increase of the trapped solution volume decreases the value of the osmotically non-active volume but it increases the amount of internal solutes. Since the osmotic fragility was close to 50% of the isotonic osmolarity, it follows from formula (1) that the relative error of the critical cell volume was about three times smaller than the relative error of the osmotically non-active volume, which is in turn about thirty times smaller than the relative error of the trapped volume.

The observed decrease of the critical erythrocyte volume upon DIDS treatment was significant (Table II). The increase of the osmotic fragility, due to interaction of DIDS with erythrocyte membrane, was also evident, but interestingly, it was not noticed by Slobozhanina et al. [28]. They wrote that the osmotic fragility, up to 5 μM DIDS, "practically did not change". Our findings indicated that the change at those DIDS concentrations was approx. 2% (which corresponds to about 3 mosM). It was small but significant statistically (Table I). Slobozhanina et al. did not specify the meaning of their term 'practically', nor the method of measuring the difference. Moreover, their data: a 50% inhibition of sulphate ion transport achieved by 0.07 μM DIDS, and a maximal inhibition at less than 1 μM DIDS (Fig. 1, and text) are far below any known values [10]. So great an inhibition may have been caused by DIDS at such small concentrations due to a low hematocrit during incubation However, no appropriate numerical information could be found in the paper.

The isoosmotic volume of erythrocytes remained constant in the whole range of DIDS concentrations used. The amount of the internal solutes, and the osmotically non-active volume did not change significantly, as is seen in Table II. The increase of the osmotic fragility, observed upon cell incubation in solutions with 2–32 μM DIDS, was, thus, a result of a decrease either of the membrane surface area, or of its extensibility. The band 3 protein occupies no more than 15% of the membrane surface [29], therefore, a 12% decrease of the area occupied by this protein would be necessary to explain the 2.4% decrease of the critical cell volume (Table II). Thus, the change of the membrane extensibility seems to be more probable. A correlation between the increasing osmotic fragility and the degree of anion transport inhibition (Table I) indicates that an important role in this process may be played by the binding of DIDS to the band 3 protein. The band 3 protein binds specifically, and with high affinity, to band 2.1 protein [30,31], which, in turn, strongly interacts with spectrin [32] creating thus a spectrin-band 2.1-band 3 complex, firmly bound to the lipid bilayer.

In the Evans-Skalak model [33] the erythrocyte membrane is a mixture of compressible and non-compressible elements. The membrane skeleton, especially spectrin, may locally regulate the membrane rigidity and, therefore, its extensibility, too. Li Hsu and Morrison [10] have found that the extractability of the spectrin-band 2.1 complex from the erythrocyte membrane decreases upon DIDS treatment. The binding of the membrane skeleton to the lipid bilayer is then stronger. This may diminish both the membrane extensibility and the critical cell volume, altering thus the osmotic fragility of erythrocytes.

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References

1 Passow, H. (1986) Rev. Physiol. Pharmacol. 103, 61–203.
2 Knauf, P.A. (1986) in Physiology of Membrane Disorders (Andreoli, S.G. et al., eds.), pp. 191–220, Plenum Medical Book Company, New York and London.
3 Bennett, V. and Stenbuck, P.J. (1979) Nature 280, 468–473.
4 Luna, E.J., Kidd, G.H. and Branton, D. (1979) J. Biol. Chem. 254, 2526–2532.
5 Murthy, S.N.P., Liu, T., Kaul, R.K., Köhler, H. and Steck, T.L. (1981) J. Biol. Chem. 256, 11203–11208.
6 Yu, J. and Steck, T.L. (1975) J. Biol. Chem. 250, 9170–9175.
7 Murthy, S.N.P., Kaul, R.K. and Köhler, H. (1984) Hoppe-Seyler's Z. Physiol. Chem. 365, 9–17.
8 Cabantchik, Z.I. and Rothstein, A. (1972) J. Membr. Biol. 10, 311–330.
9 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membr. Biol. 15, 207–226.
10 Hsu, L. and Morrison, M. (1983) Arch. Biochem. Biophys. 227, 31–38.
11 Macey, R.I. (1984) Am. J. Physiol. 246, c195–c203.
12 Moriyama, R., Kitahara, T., Sasaki, T. and Makino, S. (1985) Arch. Biochem. Biophys. 243, 228–237.
13 Makino, S., Moriyama, R., Kitahara, T. and Koga, S. (1984) J. Biochem. 95, 1019–1929.
14 Lepke, S., Fasold, H., Pring, M. and Passow, H. (1976) J. Membr. Biol. 29, 147–177.
15 Chow, E.-I., Crandall, E.D. and Forster, R.E. (1976) J Gen. Physiol. 68, 633–652.
16 Lambert, A. and Lowe, A.G. (1980) J. Physiol. 306, 431–433.
17 Mosior, M. and Gomułkiewicz, J. (1985) Stud. Biophys. 107, 169–178.
18 Dick, D.A.T. (1959) Int. Rev. Cytol. 8, 387–448.
19 Musielak, J. (1976) An Introduction to Functional Analysis, p. 204, Państwowe Wydawnictwo Naukowe, Warszawa, in Polish.
20 Seeman, P., Sauks, T., Argent, W. and Kwant, W.O. (1969) Biochim. Biophys. Acta 183, 476–489.
21 Jay, A.W.L. and Rowlands, S. (1975) J. Physiol. 252, 817–832.
22 Bartosz, G., Świerczyński, B. and Gondko, R. (1981) Experientia 37, 723.
23 Ponder, E. (1948) Hemolysis and Related Phenomena, pp. 53–170, Churchill, London.
24 Richieri, G.V. and Mel, H.C. (1985) Biochim. Biophys. Acta 813, 41–50.
25 Evans, E., Waugh, R. and Melnik, L. (1976) Biophys. J. 16, 585–595.
26 Gary-Bobo, G.M. and Solomon, A.K. (1968) J. Gen. Physiol. 52, 825–853.
27 Solomon, A.K., Toon, M.R. and Dix, J.A. (1986) J. Membr. Biol. 91, 259–273.
28 Slobozhanina, E.I., Fedorovich, I.E., Kozlova, N.M., Yamaikina, I.V., Kiranova, S.M. and Chernitsky, E.A. (1986) Biophysica 31, 800–803.
29 Weinstein, R.S., Khodadad, J.K. and Steck, T.L. (1978) J. Supramol. Struct. 8, 325–335.
30 Bennett, V. and Stenbuck, P.J. (1980) J. Biol. Chem. 255, 6424–6432.
31 Hargreaves, W.R., Giedd, K.N., Verkleij, A. and Branton, D. (1980) J. Biol. Chem. 255, 11965–11972.
32 Tyler, J.M., Reinhard, B.N. and Branton, D. (1980) J. Biol. Chem. 255, 7034–7039.
33 Evans, E. and Skalak, R. (1980) Mechanics and Thermodynamics of Biomembranes, pp. 175–233, CRC Press, Inc., Boca Raton, FL.