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Editor
Komarytskyy M.L.
Ph.D. in Economics, Associate Professor

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SHAPE CHANGES OF ERYTHROCYTES IN MEDIUM WITH CHLORIDE ANIONS UNDER IRREVERSIBLE BINDING OF DIDS IN ANION CHANNEL

Ramazanov Viktor Vladimirovich,
Ph.D., senior researcher
Rudenko Sergey Vitalievich,
Ph.D., senior researcher
Institute for Problems of Cryobiology and Cryomedicine NAS of Ukraine,
Kharkov, Ukraine

Abstract. The work investigated the shape change of erythrocytes in media with sodium chloride and sodium sulfate during mechanical mixing of the cell suspension in a in a spectrophotometric cuvette (SP-cuvette). It was shown that in a medium containing sodium chloride, intact erythrocytes are more discoid than cryopreserved cells. In a medium containing sodium sulfate, erythrocytes are spherical. Then, the erythrocytes were treated with an anion channel inhibitor DIDS, in which the specified reagent irreversibly binds to the channel. The resulting DIDS-modified cells in a medium with chloride anions are able to reversibly change their shape from spherical to discoid within ~5 min. This effect is not detected for cryopreserved cells, as well as in a medium with sulfate anions. The results indicate that chloride anions and their binding to specific sites in the membranes are necessary in the medium to maintain the normal discoid shape of erythrocytes. The ability of DIDS-modified erythrocytes to reversibly change their shape indicates that the effect of fluid flow of a stirred cell suspension and the resulting change in chloride binding to specific sites contributes to the modification of the electrostatic (ES) structure of membranes.

Key words: erythrocytes (red blood cell, RBC), chloride, sulfate, DIDS anion channel inhibitor, cell shape.

The anion channel and transporter of erythrocyte membranes is formed by the main integral membrane protein – band 3 protein (accounts for 25% of the total protein mass) [1, p. 1507]. One of the representatives of stilbene disulfonates DIDS is a channel inhibitor. The molecule of this reagent carries two negative charges and can produce an electrostatic (ES) effect when incorporated into the channel inlet cavity, which includes positive amino acid residues [2, p. 1]. It was previously established that the introduction of the anion channel inhibitor DIDS (2 μmol/L) into an erythrocyte suspension in a medium containing sodium sulfate produces a reversible change in cell shape from spherical to discoid within ~5 min. In addition, at a DIDS concentration of 20 μmol/L, reversibility to spherical cell shapes is blocked. The latter is noted for cryopreserved erythrocytes at a DIDS concentration of 2 μmol/L. It has been suggested that binding of negatively charged DIDS molecules causes reversible changes in the geometry of membrane regions adjacent to the anion channel [3, p. 50]. During pH titration of DIDS-labeled echinocytic erythrocytes in a medium with sodium chloride in a cycle of pH 7,4 ↔ 5,6, at the 3rd stage of the titration cycle to pH 5,6, the cells mainly become discocytes [4, p. 247]. These data are probably related to the fact that the negatively charged DIDS reagent can be a modifier of the ES structure of membrane regions adjacent to the anion channel. The presented data allow us to assume the role of modification of the ES structure of membranes in changing the shape of erythrocytes and the possibility that, when exposed to a fluid flow during mixing of cells in a medium with sodium chloride, DIDS-labeled erythrocytes will be able to change their shape.

Objective. To study the shape change of intact and cryopreserved erythrocytes after irreversible modification with the anion channel inhibitor DIDS in media with sodium chloride and sodium sulfate during mechanical mixing of the cell suspension.

Methodology.

In the experiments, we used (mmol/l) media containing 150 NaCl and 110 Na_2SO_4, with the inclusion of 10 mmol/l Tris-buffer (pH 7,4). Erythrocytes from donor blood were precipitated by centrifugation at 1200 g for 3 min at room temperature, followed by removal of plasma and white cells. The erythrocytes

sediment was washed three times by centrifugation in a similar mode with saline solution (0,9% NaCl), the hematocrit of the final sediment was 70-74%. Erythrocytes were cryopreserved in 1 ml tubes with a hematocrit of 35-37% in 3 media containing glycerin (30%, v/v), sorbitol (2,9%) and NaCl (0,63%). The samples were immersed in liquid nitrogen (-196°C), kept for 30 minutes, followed by thawing in a water bath for 3 minutes (40°C). Washing of thawed erythrocytes was carried out using a hypertonic NaCl solution (6%) and an isotonic NaCl solution (0,9%) with the addition of 0,2% glucose, as described in [5, p. 157]. Erythrocyte shape was studied by recording changes in the level and intensity of fluctuations in the optical density (OD) of a cell suspension while stirring in a magnetic stirrer cell at a speed of 420 rpm. Testing was carried out on a spectrophotometer (SP) coupled with a recorder at a wavelength of 720 nm. The OD erythrocytes suspension in the experiments was 0,3-0,35 units, which corresponded to a hematocrit of 0,02% (~$3{,}0\times10^{6}$ cells/ml). Discoid erythrocytes scatter light anisotropically. When they move in suspension, the anisotropy continuously changes, which causes OD fluctuation. Cells represented by spherocytes give isotropic light scattering regardless of their orientation in the light beam, while OD fluctuation is not detected. Cells represented by stomatocytes also do not produce OD fluctuations in solution [6, c. 1767]. No fluctuation is observed in the absence of mixing, which confirms that the OD fluctuation is caused by a change in the orientation of the cells in the light beam. Thus, using this method, it is possible to evaluate the change in erythrocyte shape under the influence of various media and reagents. For irreversible binding of the anion channel inhibitor DIDS, erythrocytes with a hematocrit of 20% were suspended in a medium containing 0,15 mol/l NaCl, 10 mmol/l Tris (pH 7,4). DIDS at a final concentration of 50 μmol/l was added to the erythrocyte suspension, which was incubated at 37°C for 60 min. Then, the erythrocytes were washed 3 times with an incubation medium containing 0,5% albumin, followed by two washes with the same medium without albumin. Such treatment of DIDS leads to its covalent binding to the anion channel [7, p. 31].

Results and discussion. In an isotonic NaCl medium (150 mmol/l) with Tris

buffer, under mechanical mixing of erythrocytes in an SP-cuvette, a certain level of fluctuation in the OD of the cell suspension is observed, which is weaker for cryopreserved cells (Fig. a, b, tracks 1). For a sulfate medium (110 mmol/l Na_2SO_4), such fluctuation is not observed (Fig., tracks 2). With covalent labeling of erythrocytes with DIDS, a reversible change in the shape of the cells from spherical to discoidal is revealed in a medium, containing chloride anions, but not sulfate (Fig. a, tracks 3, 4). For cryopreserved erythrocytes, this change is not detected either in a medium with chloride or in a medium with sulfate (Fig. b, tracks 3, 4).

Positively charged amino acid residues that bind Cl^- anions are localized in the inlet cavity of the anion channel [2, p. 1]. Probably, the association-dissociation of anions on charges produces dynamic screening of these charges. Screening of charges probably contributes to the establishment of the ES structure of local areas adjacent to the channel, which can be included in the formation of the ES structure of the membrane surface. Upon covalent binding of the DIDS inhibitor in the channel, the positive charge of the Lys542 residue is neutralized [8, p. 11844]. In addition, DIDS brings two of its SO_3^- groups into the inlet cavity of the channel, one of which neutralizes Arg730 [1, p. 1507]. All this causes a violation of the above-mentioned screening. The change in the local ES structure of membrane regions adjacent to the anion channel, upon covalent binding of DIDS, may be the cause of a reversible change in the shape of erythrocytes in a medium with chloride anions (Fig. a, track 3).

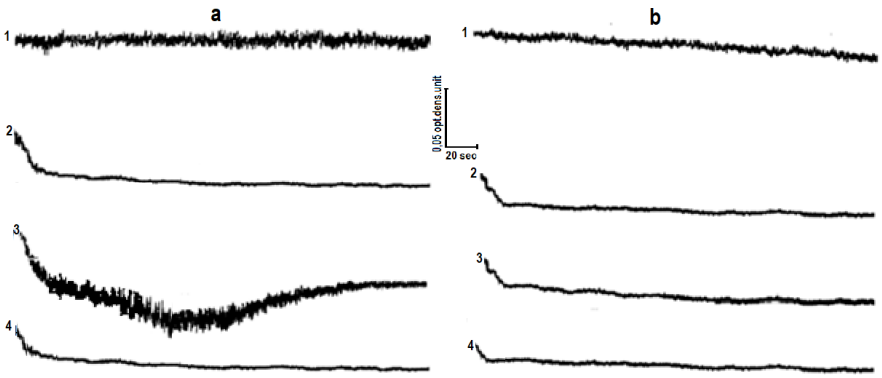

Fig. Change in the intensity of fluctuations OD of a suspension intact (a) and cryopreserved (b) erythrocytes during mixing in a SP-cuvette: 1,3 – chloride medium; 2,4 – sulfate medium. 3,4 – cells treated with DIDS (50 µmol/l, see methodology).

During cryopreservation of erythrocytes in a medium with glycerol or 1,2-propanediol, a decrease in the surface electric potential is observed due to a violation of the ES balance of cell membranes [9, p. 221]. Probably, a violation of the ES structure of membranes during cryopreservation of erythrocytes is the cause of inhibition of shape change, whereas a change in the local ES of membrane areas adjacent to the anion channel may be the basis for targeted modification of the cell shape. During pH titration of DIDS-labeled erythrocytes in a medium with sodium chloride in a cycle of pH 7,4 ↔ 5,6, at the 3rd stage of the titration cycle to pH 5,6, the cells are transformed into discocytes [1]. Transformation into discocytes at an acidic pH is not characteristic of intact erythrocytes, where they become stomatocytes [10, p. 1220]. Therefore, the discogenic effect is associated with the charged DIDS molecule built into the channel, which can be a modifier of the ES structure of membrane areas adjacent to the anion channel, which determines the discogenic effect. Obviously, DIDS is also capable of producing this effect with the assistance of the flow of a stirred cell suspension (Fig. 1a, track 3).

Thus, irreversible binding of the DIDS inhibitor in the erythrocyte anion channel cannot block the reversible change in the cell shape. This change occurs from spherical to discoid shapes and back when simulating flow conditions due to mixing of the cell suspension in a medium with chloride anions. The results indicate that chloride anions and their binding to specific sites in the membranes are necessary for maintaining the normal discoid shape of erythrocytes. The ability of DIDS-modified erythrocytes to reversibly change their shape indicates that the effect of the fluid flow of the stirred cell suspension and the change in chloride binding to specific sites contributes to the modification of the electrostatic structure of the membranes. Probably, the dynamic flow under physiological conditions in the bloodstream causes a change in the degree of association of chloride anions with the residues of charged amino acids of the anion channel. This change can lead to a modification of local electrostatics and the structure of membrane areas adjacent to the channel, which can be the basis for targeted modification of the cell shape.

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