The Effect of Arsenate and Vanadate Ions on the Critical Cell Volume of Bovine Erythrocytes

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Abstract. Arsenate, used as an inhibitor of glycolysis, decreases the critical cell volume of erythrocytes, whereas orthovanadate, used as an accelerator of dephosphorylation of phosphatidyl-inositol-4,5-biphosphate, exerts an opposite effect. The ATP-dependent changes of the critical cell volume do not depend on the phosphorylation state of phosphatidylinositol. An interaction of the membrane skeleton with the lipid-protein matrix has been proposed as a regulation mechanism of the critical cell volume of erythrocytes.

Key words: Bovine erythrocytes — Critical cell volume — Membrane skeleton — Glycolysis

Introduction

Metabolic starvation of red blood cells leads to shape transformations (Backman 1986; Nakao et al. 1960) and alters the lipid-protein interactions within the erythrocyte membrane (Haest and Deuticke 1975). A decrease of ATP levels facilitates vesicle release induced by dimyristoylphosphatidyl-choline (Bütikofer and Ott 1985). Recently, the critical cell volume of erythrocytes has been found to depend on the rate of glycolysis (Mosior and Gomułkiewicz 1985). A strong dependence of the above alterations on intracellular ATP levels suggests that the phosphorylation of certain elements of the erythrocyte membrane is involved in the effect. If metabolic starvation decreased the surface area of the inner leaflet of the bilayer, the ATP-dependent shape transformations in erythrocytes would be explained by the bilayer couple theory. Ferrel and Huestis (1984) suggest that the ATP depletion-induced conversion of phosphatidylinositol-4,5-biphosphate to

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phosphatidylinositol entails a decrease of the inner leaflet surface area followed by a creation of the erythrocyte. Recently, Backman (1986) has reported a correlation to exist between the rate of echinocytosis and that of dephosphorylation of phosphatidylinositol-4,5-biphosphate. Svetina and Zekš (1983) suggested that changes of the surface area of one bilayer leaflet also alter the critical cell volume of an erythrocyte. According to this hypothesis, a decrease of the surface area of the inner bilayer leaflet increases the critical cell volume of an erythrocyte. However, in a previous paper we reported that an increase of the rate of glycolysis led to an increase of the critical cell volume. Higher glycolysis rates result in higher intracellular ATP levels (Magnani et al. 1983; Rose et al. 1964). Higher phosphorylated phosphatidylinositol will increase the surface area of the inner bilayer surface (Ferrel and Huestis 1984), and, consequently, it will decrease the critical volume of an erythrocyte (Svetina and Zekš 1983). These contradictions between experimental results (Mosior and Gomułkiewicz 1985) and theory (Svetina and Zekš 1983) prompted us to study the effect of vanadate, which accelerates dephosphorylation of phosphatidylinositol-4,5-biphosphate (Backman 1986), on the critical cell volume of bovine erythrocytes. Phosphatidylinositol makes up 3.7% of phospholipids of the bovine red blood cell membrane, a value three times that measured for human erythrocytes (Nelson 1967). It has been of interest therefore what is the effect of the phosphorylation state of phosphatidylinositol on the critical cell volume. The influence of vanadate ions on the critical cell volume was compared with the effects of phosphate (a glycolysis activator (Rose et al. 1964)) and arsenate (a glycolysis inhibitor (Metzler 1977)).

Materials and Methods

Na_2HAsO_4 was purchased from Merck, orthovanadate from Sigma, and Tris from Serva-Feinbiochemica, Heidelberg. Other chemicals were analytical grade reagents from POCH Gliwice.

Fresh heparinized bovine blood was centrifuged at 2700 × *g* for 10 min at 4 °C, the plasma and the buffy coat were removed, and the red cells were subsequently washed three time (2700 × *g*, 5 min, 4 °C) in one of the following solutions, containing (in mmol/l): *A* – 133.5 NaCl; 5 KCl; 1.5 $MgCl_2$; 10 Tris; 10 glucose; pH 7.4; *B* – 112 NaCl; 5 KCl; 1.5 $MgCl_2$; 20 Na_2HPO_4/NaH_2PO_4; 10 glucose; pH 7.4; *C* – 135.5 NaCl; 5 KCl; 1.5 $MgCl_2$ 1 Na_3VO_4; 10 Tris; 10 glucose; pH 7.4; *D* – 125 NaCl; 5 KCl; 10 Na_2HAsO_4; 10 Tris; pH 7.4. All RBC suspensions were then incubated at 37 °C for 2 hours (*A* and *B*) or 3 hours (*C* and *D*). After the incubation, the erythrocytes were washed twice in solution *A* and centrifuged at 2700 × *g* for 5 min at 4 °C. The studied parameters were then measured in all samples and compared with those of a non-incubated control sample suspended in solution *A*.

Osmotic properties: Osmotic fragility, isoosmotic volume, osmotically non-active volume, critical cell volume, and the volume of the solution trapped between packed cells were measured using methods described elsewhere (Mosior and Gomułkiewicz 1985; 1986; Mosior et al. 1988). Six NaCl solutions with relative osmolarities between 0.60–1.84 (unity

corresponds to 310 mOsmol/l) were mixed in 2:3 ratio with an isoosmotic erythrocyte suspension of 75% hematocrit. Final osmolarities were calculated from the initial osmolarity, from the hematocrit of the isoosmotic cell suspension, and from the volume of the solution trapped between erythrocytes (Mosior and Gomułkiewicz 1985). The relative volume of intracellular water was determined by iteration of a modified linear regression operator, which also included the evaluation of osmolarities of the cell suspensions. This allowed to avoid measuring the osmotic pressures of the RBC suspensions. The critical cell volume was calculated according to van't Hoff's law modified for erythrocytes. The measure of osmotic fragility was the mean osmolarity of solutes in which hemolysis occurred. The hemolysis was determined in hypotonic NaCl or 75% NaCl + 25% KCl solutions buffered with 5 mmol/l phosphate, pH 7.4. The relative isoosmotic volume of erythrocytes was determined from the absorbance of hemoglobin released from control and modified erythrocytes in suspensions with identical hematocrits.

ATP concentration: To assess the ATP concentration, the erythrocyte suspension was mixed with an equal volume of trichloroacetic acid (12% w/w), left for 15 min and then centrifuged to clarity. The supernatant was assayed for ATP by the phosphoglyceric-phosphokinase/glyceraldehyde-phosphate-dehydrogenase method using a standard kit from Sigma. The determination of the ATP concentration was not affected by vanadate, or arsenate remaining after the wash following the incubation.

Morphological index: Erythrocyte suspensions were diluted to a hematocrit of 2% and fixed in solution *A* containing 0.5% glutaraldehyde. Cell samples were then examined under a phase contrast microscope. Echinocytes were assigned a morphological score from +1 to +5, based on Bessis' nomenclature (Bessis 1973). Discocytes were assigned a score of 0, and stage I stomatocytes were assigned a score of −1. The average score for 100 cells is called the morphological index (MI).

Table 1. Effect of incubation of bovine erythrocytes at 37 °C in media on the critical cell volume (CCV), cell shape, and intracellular ATP concentration. For media composition see the text. CCV and ATP values shown were related to the respective control parameters (mean ± S.D.): $V_c = (144.1 \pm 5.1)\%\ V_i$; ATP concentration 410±35 μmol/l, morphological index (MI) +0.1. Means and confidence intervals at $1 - P = 0.95$, $n = 10$.

	Incubation media			
	A	*B* phosphate	*C* vanadate	*D* arsenate
CCV	100.3 ± 0.8	102.6 ± 1.5	102.5 ± 0.9	98.9 ± 0.7
ATP level	92 ± 12	126 ± 14	74 ± 11	48 ± 10
MI	+0.1	+0.1	+0.9	+0.6

Results

The effect of vanadate ions on the erythrocyte membrane was checked under a light microscope. The morphological index (MI) of cells incubated in solution *C* (with orthovanadate) was +0.9. Cells incubated in solution *A* or *B* did not change

their shape. Solution *D* caused a slight change of cell shape (Table 1). The effects of all modifications tested on the critical cell volume (CCV), and the intracellular concentrations of ATP, are also summarized in Table 1. Values of all parameters used to calculate CCV are shown in Table 2 (relative to the corresponding control parameters). The values of the critical volume and osmotically inactive volume were also multiplied by the relative isoosmotic volume of the modified cells to allow a direct comparison of data.

Table 2. Effect of incubation of bovine erythrocytes at 37 °C in media on the osmotic parameters of the cells. See text for media composition. All values shown were related to the respective control parameters, (mean ± S.D.): $b = (44.5 \pm 2.2)\%$ V_t, $\pi_h = 56.0 \pm 2.5\%$ (100% = 310 mOsmol/l); since osmolarity and cell volume are relative units, the amount of the intracellular solutes is $1 - b$ for control cells. Means and confidence intervals at $1 - P = 0.95$, $n = 10$.

	Incubation media			
	A	*B* phosphate	*C* vanadate	*D* arsenate
Isoosmotic volume, V_t	100.1 ± 0.7	98.6 ± 1.0	101.0 ± 0.9	98.9 ± 0.9
Amount of internal solutes	100.8 ± 1.3	101.3 ± 3.3	103.7 ± 3.0	93.4 ± 2.2
Osmotically inactive volume	99.7 ± 1.7	95.1 ± 3.8	97.3 ± 3.5	108.5 ± 3.0
Osmotic fragility	99.6 ± 0.9	95.8 ± 2.2	98.5 ± 1.1	98.9 + 0.4

Activation of glycolysis by incubating the cells with phosphate (solution *B*) raised the intracellular concentration of ATP and increased CCV while inducing no significant shape changes. Metabolic starvation of cells (incubation in glucose-free solutions) caused a slight crenation of the cell surface and resulted in a considerable decrease of ATP levels and a small decrease of CCV. Strong crenation of cells in the presence of vanade was accompanied by an increase of CCV and a decrease of ATP levels.

To test whether the experimental procedures alter the hemolysis-protective potassium efflux (Jay and Rowlands 1975; Seeman et al. 1969), the osmotic fragilities of all samples were measured in hypotonic NaCl and NaCl+KCl solutions. The hemolysis curves obtained for all modifications tested showed no dependence on the cationic composition of the hypotonic solutions. Three examples are given in Fig. 1. The volumes of the trapped extracellular solution were within 2.2–2.8% of the isoosmotic cell volume.

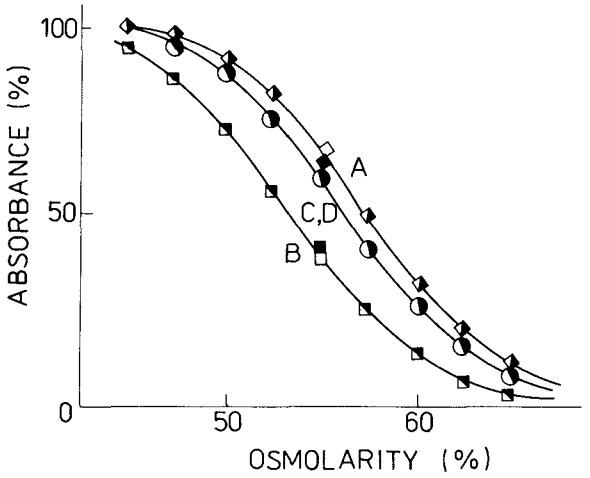

Figure 1. Effect of the cationic composition of the hypotonic solution on osmotic fragility of bovine erythrocytes. Open symbols: hemolysis in NaCl solutions. Closed symbols: hemolysis in 75% NaCl + 25% KCl solutions. Semi-filled symbols represent overlapping points.

Discussion

The calculated critical cell volume of the erythrocyte depends on the amount of the intracellular solutes, the osmotically inactive volume, the osmotic fragility (Ponder 1948; Dick 1959), as well as on the hemolysis-protective potassium efflux (Seeman et al. 1969; Jay and Rowlands 1975). The lack of difference between the hemolysis curves obtained for NaCl and NaCl+KCl; solutions (Fig. 1) in all samples measured indicates that the potassium efflux, observed in human erythrocytes (Seeman et al. 1969; Jay and Rowlands 1975), has no influence on the osmotic properties of bovine erythrocytes. Therefore, the other parameters measured can be used to calculate the erythrocyte CCV. The observed changes of CCV were small; nevertheless they were 2 to 4 times larger than the accuracy of the method of calculation as estimated by computer simulation (Mosior et al. 1990). The osmotic fragility of erythrocytes decreases upon incubation with arsenate, despite the decrease of the critical cell volume, since the osmotically inactive volume increases. This latter effect and a decrease of the osmotically inactive volume upon glycolysis activation suggest that the inactive volume depends on the state of glycolysis. Such a dependence may be explained by an influence of intracellular pH, related to the metabolic state of the cell, on the osmotically inactive volume (Ponder 1948).

According to the bilayer couple hypothesis, as developed by Svetina and Zekš

(1983), a decrease of the inner surface area of the bilayer would cause the erythrocyte membrane to crenate and CCV to increase. According to Svetina and Zekš (1983), desphosphorylation of phosphatidylinositol-4,5-biophosphate due to vanadate treatment followed by a strong crenation of the cells (Ferrel and Huestis 1984; Backman 1986), with no permeability change of the cell membrane under the conditions of our experiments (Heller et al. 1987), would therefore be expected to be accompanied by an increase of CCV. This indeed occurred (Table 1). However, CCV similarly increased after glycolysis activation without any significant change of the cell shape. Moreover, the uncoupling of glycolysis by arsenate, accompanied by a slight crenation of the cells, paralleled the decrease of CCV. Therefore, the ATP-dependent changes of CCV cannot be related to differences between the surface areas of the two leaflets of the bilayer under the influence of phosphatidylinositol phosphorylation state (Ferrel and Huestis 1984; Backman 1986). The question arises whether there may be another mechanism responsible for all changes of CCV observed. Phosphorylation of band 4.1 protein by membrane kinase reduces the affinity of the protein for spectrin (Eder et al. 1986), and so does phosphorylation of ankyrin (Lu et al. 1985). However, dephosphorylation of phosphatidylinositol biphosphate reduces the affinity of band 4.1 protein for glycophorin (Anderson and Marchesi 1985). Presumably, in all cases shown a decreased affinity results in a weaker interaction between the membrane skeleton and the lipid-protein matrix providing for a flexible cytoskeleton, important for the cell function. Incubation of the cells both with phosphate (solution *B*) and vanadate (solution *C*) led to an increase of the erythrocyte CCV (Table 1). Also, thermal denaturation of spectrin, resulting in a loss of the binding ability of the beta subunit of the protein to the erythrocyte membrane (Yoshino and Minari 1987) increased the CCV value (Mosior et al. 1990). The opposite effect of dephosphorylation of phosphatidylinositol biphospate and of skeletal proteins on membrane skeleton-lipid bilayer interactions may be the reason for the smaller CCV change seen upon ATP depletion.

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