Effect of Lipid Matrix and Cytoskeleton Proteins on Ca^{2+}-Activated K^+ Channels in Erythrocytes of Alcoholic and II Type Diabetes Mellitus Patients

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We studied the effect of changes in erythrocyte volume and irreversible thermal denaturation of cytoskeleton proteins and lipid matrix on activity of Ca^{2+}-activated K^+ channels in erythrocytes of alcoholic and patients with II type diabetes mellitus. Changes in Ca^{2+}-dependent potassium permeability of erythrocyte membrane in alcoholic patients and patients with II type diabetes mellitus are related to modification of cytoskeleton, rather than to changes in lipid matrix.

Key Words: *erythrocyte; Ca^{2+}-activated K^+ channels; cytoskeleton proteins; lipid matrix*

Deformability of erythrocytes plays an important role in O_2 and CO_2 transport. Various pathologies including diabetes mellitus [10] and alcoholism [8] are associated with changes in erythrocyte shape and decrease in their deformability, which aggravate the severity of these diseases. Calcium-activated potassium channels ($K^+(Ca^{2+})$-channels) in erythrocyte membrane contribute to changes of deformability. It was shown that Ca^{2+}-induced decrease in erythrocyte deformability can be corrected by reducing K^+ gradient [7]. In some hereditary anemias (*e.g.* sickle-cell disease), enhanced Ca^{2+}-dependent potassium permeability of erythrocyte membrane results in their dehydration, rigidity, and hemolysis [2,4,5]. Changes in erythrocyte volume are accompanied by deformation of the cytoskeleton [6]. It is widely known that cytoskeleton proteins control some ion-transporting systems in erythrocyte membrane, such as Na^+/H^+ ion exchanger or $Na^+,K^+,2Cl^-$-cotransporter [11]. There is an indirect evidence that these proteins regulate $K^+(Ca^{2+})$ channels as well [1]. Activity of some erythrocyte transport systems such as Ca^{2+}-ATPase and Na^+, K^+-ATPase depends also on the state of lipid environment and, in particular, microviscosity of the lipid bilayer [3,9,14]. However, the role of lipid matrix in the regulation of $K^+(Ca^{2+})$-channels was not studied.

Our aim was to study the role of lipid matrix and cytoskeleton proteins in the regulation of Ca^{2+}-dependent potassium permeability of erythrocyte membrane in patients with alcoholism and type II diabetes mellitus.

MATERIALS AND METHODS

The study was carried out on patients with II type diabetes mellitus (n=13), alcoholism (n=13), and on virtually healthy donors (n=12). The state of $K^+(Ca^{2+})$ channels was assessed indirectly by pH shifts in erythrocyte suspension induced by addition of a protonophore. Under these conditions, the change of pH in the incubation medium is directly related to membrane potential [1].

Blood was taken from the ulnar vein after overnight fast using heparin (25 U/ml blood) as an anticoagulant. Erythrocytes were isolated in a medium with 150 mM NaCl and 5 mM phosphate buffer at pH

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7.4 (both agents from BDH). Preparation of erythrocyte suspension was described previously [1].

Packed erythrocytes (0.25 ml) were added to 4.75 ml incubation medium (150 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 50 μM $CaCl_2$, all agents from BDH) and incubated for 5 min at 37єC under continuous stirring. Then protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP, Calbiochem) was added in final concentration of 20 μM, and Ca^{2+}-ionophore A23187 (Sigma) was added 2 min later in a concentration of 0.5 μM. Solutions of CCCP and A23187 were prepared on ethyl alcohol, whose final concentration was no more than 0.5% and produced no effect on $K^+(Ca^{2+})$ channels. Other solutions were prepared on deionized water.

An increase of intracellular concentration of Ca^{2+} results in opening of $K^+(Ca^{2+})$ channels and exit of K^+ from erythrocytes, and hyperpolarization of the erythrocyte membrane. This leads to alkalization of the incubation medium. Restoration of membrane potential results from a decrease of Ca^{2+} concentration in cytosol induced by activation of calcium pump in erythrocyte membrane [1]. The state of $K^+(Ca^{2+})$ channels was assessed by hyperpolarization shift (ΔE).

Measurements of pH were carried out with a HI 1332-combined pH-sensitive electrode (HANNA Instruments) and a TYP N517 pH-meter.

The volume of erythrocytes was measured by varying osmolarity of incubation medium from 220 mosmole (by decreasing NaCl concentration to 100 mM) to 520 mosmole (by addition of sucrose to isoosmotic incubation medium).

Irreversible thermal denaturation of spectrin was performed by a 10-mim-long incubation of erythrocytes in isoosmotic medium (150 mM NaCl and 5.0 mM Na-phosphate buffer at pH 7.4) at 50єC [12].

Fluorescence of pyrene was measured at 25єC on a Hitachi-850 spectrofluorimeter in standard rectangular cuvette (path 1 cm) in a medium containing 145 mM NaCl and 10 mM Tris at pH 7.0. Pyrene(final concentration 10 μM) was added to the cuvette with erythrocytes ghosts obtained by the method [13]. Protein concentration was 0.35 mg/ml 10 min prior to fluorescent measurements. The wavelength of excitation light was 336 nM, and the slit was 3/3 nm. Pyrene fluorescence spectrum had three peaks at 373, 385, and 392 nm corresponding to fluorescence of pyrene monomeric form (F_M) and a peak at 465 nm caused by fluorescence of pyrene excimer (F_E). The peak values at 465 and 392 nm were used to calculate the degree of pyrene excimerization F_E/F_M, correspondingly [15].

The data were analyzed statistically using Statistics 5.0 software for dependent and independent variables.

RESULTS

In patients with diabetes mellitus, the hyperpolarization step ΔE decreased, while in alcoholic patients it increased in comparison with the control ($p<0.02$, Fig. 1). These data attest to inhibition of activity of erythrocyte $K^+(Ca^{2+})$-channels in II type diabetes mellitus patients and to activation of these channels in erythrocytes of alcoholic patients.

Published data on the regulation of other membrane ion-transporting systems suggest that activity of erythrocyte $K^+(Ca^{2+})$-channels depends on the state of membrane lipid bilayer and cytoskeleton proteins.

The study of microviscosity of erythrocyte membrane with pyrene, a fluorescent probe, showed that pyrene excimerization constant F_E/F_M significantly decreased in alcoholic patients and patients with diabetes mellitus in comparison with healthy controls (0.710± 0.027 and 0.640±0.028 *vs.* 1.100±0.039, $p<0.01$). This constant was significantly higher in alcoholic patients than in diabetic patients ($p<0.05$). These data attest to increased microviscosity of erythrocyte membranes in both groups of patients in comparison with healthy individuals, the effect being more pronounced in diabetic patients.

Thus, we observed similar changes in the state of lipid bilayer of erythrocyte membrane in both pathologies, which manifested in its increased microviscosity due to decreased motility of lipid acid chains and decreased hydrophobic volume.

Permeability of $K^+(Ca^{2+})$-channels in diabetes mellitus and alcoholism varied in opposite directions (Fig. 1). This is an indirect argument in favor to the hypothesis that modification of lipid bilayer in these diseases is not the main cause of changes in permeability of $K^+(Ca^{2+})$-channels. Probably, more important role in

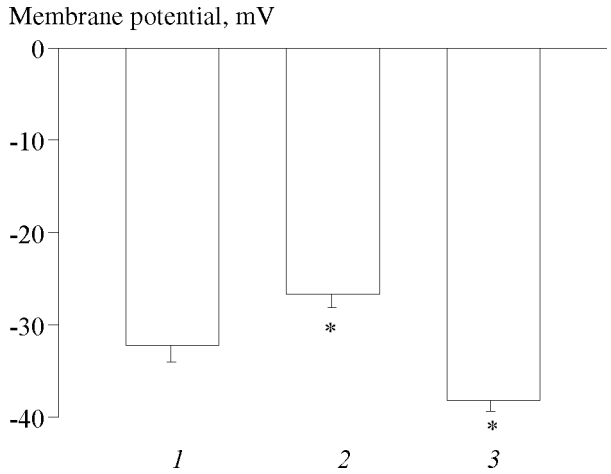

Fig. 1. Hyperpolarization response of erythrocytes from (*1*) healthy donors, (*2*) patients with insulin-independent diabetes mellitus, and (*3*) alcoholic patients. The hyperpolarization responses were recorded in isoosmotic medium (320 mosmole) containing 50 μM $CaCl_2$. *$p<0.02$ compared to healthy donors.

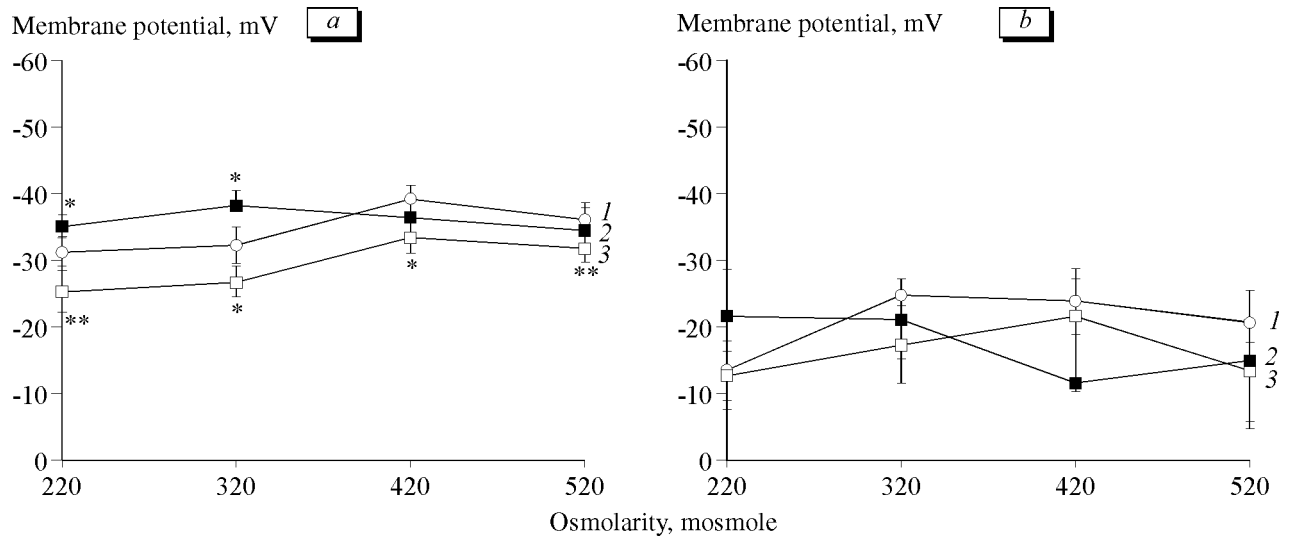

Fig. 2. Effect of osmolarity of incubation medium on hyperpolarization response of intact erythrocytes (*a*) and erythrocytes with heat-denaturated spectrin (*b*). *1*) healthy donors; *2*) patients with insulin-independent diabetes mellitus; *3*) alcoholic patients. *$p<0.02$, **$p<0.05$ compared to healthy donors.

the regulation of $K^+(Ca^{2+})$-channels can be played by non-lipid factors such as the state of erythrocyte cytoskeleton proteins. It cannot be excluded that the differences in permeability of erythrocyte $K^+(Ca^{2+})$-channels in diabetic and alcoholic patients can result from the differences in the state of these proteins.

The changes in erythrocyte volume in response to variations in osmolarity *in vitro* and during various pathologies lead to structural rearrangement of cytoskeleton proteins [6]. The study of volume-dependent control of activity of $K^+(Ca^{2+})$-channels showed that dependence of ΔE on osmolarity of the incubation medium in patients with diabetes mellitus and healthy donors was similar and differed from that in alcoholic patients (Fig. 2, *a*). In hypoosmotic medium (220 mosmole) inducing swelling of erythrocytes, ΔE of healthy donors and diabetic patients did not differ from ΔE measured in isoosmotic medium, although it was significantly lower in diabetic patients than in healthy controls. By contrast, in alcoholic patients ΔE at 220 mosmole was significantly lower than ΔE measured in isoosmotic medium. At the same time, ΔE of alcoholic patients was significantly higher than that of healthy donors measured in the same media (Fig. 2, *a*).

In hyperosmotic medium the maximum increase in ΔE was observed at 420 mosmole both in diabetic patients and in healthy donors. Further increase in osmolarity to 520 mosmole decreased ΔE. In diabetic patients ΔE was lower than that in healthy donors at all examined values of osmolarity.

In alcoholic patients, ΔE decreased with increasing the osmolarity of the incubation medium (Fig. 2, *a*). At 420 and 520 mosmole, ΔE of alcoholic patients did not differ from the corresponding values of healthy donors.

These data attest to different sensitivity of $K^+(Ca^{2+})$-channels to erythrocyte volume in alcoholic and diabetic patients. Presumably, preexisting differences in the organization of erythrocyte cytoskeleton in alcoholic and type II diabetic patients determine different structural changes in cytoskeleton during cell swelling and shrinkage. This hypothesis was tested using an approach proposed by V. L. Shnyrov [12]. Participation of spectrin, a basic cytoskeleton peptide, in the volume-dependent reactions of the cell was excluded because of its thermal denaturation. Heating significantly decreased ΔE in healthy donors and in alcoholic and diabetic patients. Moreover, the dependence ΔE on osmolarity disappeared in all examined groups (Fig. 2, *b*).

It was concluded that changes in Ca^{2+}-dependent potassium permeability of erythrocyte membrane in patients with type II diabetes mellitus and alcoholism are primarily related to modification of cytoskeleton proteins, rather then changes in membrane lipids.

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