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The action of stress hormones on the structure and function of erythrocyte membrane

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Abstract. The action of a mixture of hormones (cortisol and adrenaline) on erythrocyte membrane during their binding was investigated. Changes in the membrane structure were elucidated by atomic force microscopy; microviscosity of the lipid bilayer and changes in the activity of Na^+,K^+-ATPase at different concentrations of the hormones in erythrocyte suspension were estimated by the fluorescence method. Cortisol and adrenaline were shown to compete for the binding sites. A hormone that managed to bind nonspecifically to the membrane hindered the binding of another hormone. In a mixture of these hormones, cortisol won a competition for the binding sites; therewith, microviscosity of the membranes increased by 25%, which corresponds to a change in microviscosity produced by the action of cortisol alone. The competitive relationships affected also the Na^+,K^+-ATPase activity, which was indicated by appearance of the second maximum of enzyme activity. It is assumed that an increase in microviscosity of erythrocyte membrane first raises the Na^+,K^+-ATPase activity due to a growth of the maximum energy of membrane phonons, and then decreases the activity due to hindering of conformational transitions in the enzyme molecule.

Key words: Erythrocyte membranes — Stress hormones — Atomic force microscopy — Structural transitions — Na^+,K^+-ATPase activity — Membrane phonons

Introduction

The development of civilization and accelerated pace of living produce the high life stress in modern society. At present, mankind encounters civilization diseases and stress-related pathologies. This can be exemplified by coronary syndrome X that recently became a wide-spread disease (Hurst et al. 2006; Lampendola et al. 2011). It is characterized by exertional angina, ischemic depression of ST segment in ECG, absence of changes in the coronary arteries at angiography, and normal function of the left ventricle of heart. The nature of this phenomenon is being studied intensively (Eshtehardi et al. 2011; Vesely and Dilsizian 2011); however, its mechanism is still not clearly understood. Cardiac syndrome X has been shown to occur in up to 20–30% of anginal patients undergoing coronary arteriography. We suppose that sudden death in sportsmen during high-level competitions can also be related to cardiac syndrome X caused by excessive production of stress hormones (cortisol and adrenaline) (Panin et al. 2012).

In the peripheral blood, cortisol is bound to transcortin and transported by it (Sergeev et al. 1996). Adrenaline is in a free state. A part of the hormones are specifically bound to their receptors on the cell membranes, a part of both hormones in blood can bind nonspecifically to the blood cells, primarily to erythrocytes. Specific and non-specific binding of hormones with membranes changes the membranes microviscosity and the rheological properties of blood. It was demonstrated that CO, OH and NH groups entering the structure of stress hormones can form hydrogen bonds with similar groups of proteins and phospholipids in erythrocyte membranes (Panin et al. 2010). Hydrophobic rings of the hormones can interact hydrophobically with the fatty acid residues of phospholipids. This leads to the formation of complex domains in the structure of membranes and increases their microviscosity (Panin et al. 2010, 2011), which hinders their passage through capillaries and facilitates the

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development of diffusion hypoxia. If such erythrocytes come to coronary vessels and capillaries, this may result in the development of cardiac syndrome X (Panin et al. 2012).

Changes in the structure of erythrocyte membranes can also affect their functions, for example, the activity of membrane-bound Na^+,K^+-ATPase. Earlier it was supposed that the regulatory action of various ligands can be based on certain conformational transformations of Na^+,K^+-ATPase (Jorgensen et al. (1998). The mechanism of stress hormones action on the Na^+,K^+-ATPase activity remains poorly understood.

The present work is the first study devoted to the joint action of cortisol and adrenaline on the structure and function of erythrocyte membrane: changes in the membrane surface relief, relative changes in microviscosity of erythrocyte membranes, and changes in the activity of Na^+,K^+-ATPase at different concentrations of the hormones in erythrocyte suspensions. The mechanism of hormones action on the activity of Na^+,K^+-ATPase is discussed.

Materials and Methods

Atomic force microscopy

The action of two hormones, cortisol and adrenaline (Amersham), was analyzed in the work. Erythrocytes were isolated from fresh blood of Wistar male rats decapitated under light nembutal narcosis. Weight of the three-month-old rats was 180–200 g. The blood was diluted two-fold with a cooled isotonic phosphate buffer containing 0.043 M KH_2PO_4 and 0.136 M Na_2HPO_4, pH 7.35. After sedimentation of the cells by centrifugation at 330 × *g* for 10 min, supernatant was decanted, and the washing procedure was repeated twice more.

All the procedures were performed at 4°C. 20 μl of the resulting erythrocyte suspension was applied on a microscope slide, a thin smear was made, dried in air, and examined in a Solver Bio (NT-MDT, Russia) atomic force microscope at a temperature of 24°C. Silicon cantilevers NSG11 (NT-MDT, Russia) with a resonant frequency between 120 and 180 kHz and spring constant ~ 6 N/m were used (all of these probe parameters were offered by manufacturer). In each experiment, we first tested a control specimen without hormones, and then the experimental one. Images of the surface relief of erythrocyte membrane after absorption of hormones were obtained with the scan size 1 × 1 μm^2 and 1.5 × 1.5 μm^2.

Fluorescence analysis of erythrocyte ghosts

Erythrocyte ghosts were obtained after hemolysis of the cells in a hypotonic phosphate buffer (pH 7.35) containing 2.75 mM KH_2PO_4 and 8.5 mM Na_2HPO_4. The ghosts were washed for 30 min by centrifugation at 5500 × *g*; supernatant was removed. The procedure was repeated six times. All the operations were performed at 4°C.

Fluorescence measurements were carried out on a RF-5301(PC)SCE Shimadzu spectrofluorimeter. A quartz cuvette of size (1 × 1 × 4) cm^3 was filled with 4 ml of a hypotonic phosphate buffer used for hemolysis, erythrocyte ghosts, and pyrene. The concentration of protein ghosts was determined by the Warburg-Christian method from changes in the optical density of the suspension (Dawson et al. 1986). On the average, it varied within 0.100–0.250 mg/ml, the concentration of pyrene in a cuvette was 7.76 × 10^{-6} M. Pyrene was dissolved in dimethyl sulfoxide with ethanol, its initial concentration was equal to 1.5 × 10^{-3} M. The cuvette was shaken vigorously for 1 min and placed in a spectrofluorimeter thermostat for 10 min; then the fluorescence measurements were carried out at 36°C. To measure fluorescence of the ghosts loaded with a different amount of hormones, each time a new specimen was prepared by the same procedure.

Viscosity of the lipid bilayer near proteins (the region of protein-lipid interaction) was measured at the excitation wavelength λ = 281 nm, the spectral slit width was 1.5/5. Microviscosity of the lipid bilayer at a distance from proteins (the region of lipid-lipid interaction) was measured at the excitation wavelength λ = 337 nm, the spectral slit width was 1,5/3. Maxima of the radiation intensity corresponded to λ = 333 nm (the maximum of tryptophan fluorescence), λ = 374 and λ = 393 nm (the vibronic peaks of excited pyrene monomers), and λ = 468 nm (the maximum of excited pyrene dimer fluorescence).

The membrane microviscosity for translational diffusion of a pyrene probe was calculated as a ratio of the fluorescence intensity of pyrene dimer to the fluorescence intensity of pyrene monomer. To calculate specific concentration of hormones, the concentration of hormones was divided by the concentration of protein ghosts in the suspension. For the region of lipid-lipid interaction, the relative microviscosity was calculated by the formula

$$\frac{\eta(A)}{\eta(0)} = \frac{F_{468}(0)}{F_{468}(A)} \cdot \frac{F_{393}(A)}{F_{393}(0)}$$

where η (A), η (0) is the microviscosity of ghosts at the hormone specific concentration A in a suspension and in the absence of hormone in a suspension, respectively; $F_{468}(A)$ is the fluorescence intensity of pyrene at wavelength λ = 468 nm in a specimen at the hormone concentration A in the suspension; $F_{468}(0)$ *is* the fluorescence intensity of pyrene at wavelength λ = 468 nm in a specimen in the absence of hormone in the suspension; $F_{393}(A)$ and $F_{393}(A)$ are the fluorescence intensities of pyrene at wavelength λ = 393 nm at the hormone specific concentration A in the suspension and

in the absence of hormone in the suspension, respectively. The excitation wavelength was equal to 337 nm.

For the region of protein-lipid interaction, relative microviscosity was calculated by the formula

$$\frac{\eta(A)}{\eta(0)} = \frac{F_{468}(0) - I_{468}}{F_{468}(A) - I_{468}} \cdot \frac{F_{393}(A) - I_{393}}{F_{393}(0) - I_{393}}$$

where I_{393} and I_{468} are the fluorescence intensities of tryptophan at wavelength $\lambda = 393$ nm and $\lambda = 468$ nm, respectively. The excitation wavelength was $\lambda = 281$ nm.

Measurement of the Na^+,K^+-ATPase activity

Erythrocytes were isolated from fresh blood of Wistar male rats decapitated under light nembutal narcosis. The blood was diluted 5-fold with a 100 mM isotonic Tris-HCl buffer (pH 7.35). After sedimentation of the cells by centrifugation at 330 × *g* for 10 min, supernatant was decanted, and the washing procedure was repeated twice more.

Erythrocyte ghosts were obtained after hemolysis of erythrocytes in a hypotonic 10 mM Tris-HCl buffer (pH 7.35). The ghosts were washed for 30 min by centrifugation at 5500 × *g*; supernatant was removed. The procedure was repeated six times. All the operations were performed at 4°C.

The Na^+,K^+-ATPase activity in erythrocyte membranes was determined by the method reported in (Kazennov et al. 1991) from accumulation of inorganic phosphorus (P_i) in the ATP-containing medium due to its hydrolysis under the action of ATPase. 20 µl of erythrocyte membrane suspension with a concentration 20 mg protein/ml was supplemented with the hormones and 40 µl of the incubation medium having the following composition (mM): NaCl 125, KCl 25, $MgCl_2$ 3, EDTA 5, ATP 2, and Tris-HCl 50 (pH 7.35). The concentration of ghosts was approximately equal to their blood content. As a control, in another vial the same mixture was supplemented with 10 µl of ouabain (MP Biomedicals, LLC) diluted in a hypotonic Tris-HCl buffer to a concentration of 10^{-2} M. Incubation was performed at 37°C for 1 h. The reaction was stopped by introducing 40 µl of a 20% solution of trichloracetic acid. Protein was sedimented by centrifugation at 330 × *g* for 15 min. To determine the amount of produced inorganic phosphorus (P_i), 100 µl of molybdenum reagent (2.5 g of $(NH_4)_6Mo_7O_2$, 13 ml of concentrated H_2SO_4, and 200 ml of distilled water) and 40 µl of a 7% aqueous solution of ascorbic acid were added in each plate well with 40 µl of supernatant. In 20 minutes, the specimens were scanned photometrically at 630 nm in a STAT FAX-2100 (Awareness Technology Inc.) microplate photometer. The concentration of inorganic phosphorus in each specimen was calculated from the calibration curves. The activity of Na^+,K^+-ATPase was found as the difference between activity of the enzyme measured in the absence and in the presence of ouabain. The activity was expressed in µmol/h *per* mg of protein. Ouabain is a specific inhibitor of Na^+,K^+-ATPase activity, it does not suppress the activity of other biomembrane ATPases; so, control reveals the total activity of all other ATPases.

Measurement errors appeared due to imperfect batching of the ghost suspension specimens and their titration against hormones. Relative measurement errors for absorption and fluorescence intensities were equal to 3%, those for relative microviscosity and activity of Na^+,K^+-ATPase attained 6%.

Results

To calculate specific concentration of hormones, the concentration of hormones was divided by the concentration of protein ghosts in the suspension. The ranges of concentrations of free cortisol and adrenaline in the human blood plasma are 10^{-10}–10^{-9} mol/mg protein and 10^{-12}–10^{-10} mol/mg protein, respectively. The range of changes in hormones concentrations was the same as in previous articles (Panin et al. 2010, 2011). The range of hormones concentrations was wider physiological range for the best study of the influence mechanism of hormones on the membrane.

Erythrocytes of healthy animals scanned by an atomic force microscope looked as large biconcave disks ~6 nm in diameter. At a higher magnification, a minor inhomogeneity appeared on their surface, which was caused most likely by the presence of membrane-bound proteins (Panin et al. 2010, 2011). The addition of a hormone solvent (dimethyl sulfoxide with ethanol, 0.1% of the mixture volume) to erythrocyte suspension increased the surface inhomogeneity, probably due to denaturing effect of the solvent on the surface structural proteins (Fig. 1A,B). Domains of size 0.2×0.2 µm^2 and height 2 nm appeared on the membrane surface.

Adding of cortisol with the specific concentration of 10^{-12}–10^{-10} mol/mg protein to erythrocyte suspension led to pronounced changes in the pattern. Domains of size 500×500 nm^2 and height 2 nm appeared on the membrane surface. Numerous mesostrips of the cell membrane structure loosening appeared on a smooth surface (Fig. 2A,B). These mesostripes are ~7 nm hollows in erythrocyte membrane. These were the zones of hydrostatic tension with emergence of local structural transformations and formation of micropores. They divided erythrocyte membrane into large flat domains.

Upon addition of adrenaline into erythrocyte suspension (Fig. 3A,B), domains of size 250×250 nm^2 and height 25 nm appeared on the membrane surface; this was followed by the formation of smaller domains (fractalization of the surface: size 50×50 nm^2 and height 5 nm). Adrenaline has

a smaller size as compared with cortisol. So, it penetrated deeper into erythrocyte membrane than cortisol and bound to proteins of the retractive spectrin-ankyrin-actin network. Conformational transformation of these proteins became more pronounced, leading to a more strong retraction of the network (Panin et al. 2010, 2011). A stronger retraction of the network and formation of additional hydrogen and Van-der-Waals bonds between proteins and phospholipids produced high mechanical stresses in the membrane as compared to the case of their interaction with cortisol. Deformation of the membrane also increased. Potential energy of elastic stresses in the membrane rose.

Under stress conditions, cortisol and adrenaline act simultaneously in the organism. In this connection, in the next series of experiments we added both hormones to erythrocyte suspension using three variants: 1) first cortisol followed adrenaline; 2) first adrenaline followed cortisol, and 3) a mixture of two hormones. The final concentration of hormones was 3×10^{-12} mol/mg protein for cortisol and 5×10^{-12} mol/mg protein for adrenaline. When cortisol was added before adrenaline, effect of the first hormone dominated in the AFM image. Large domains of size 500×500 nm^2 and height 5 nm appeared on the membrane surface; formed in the membrane structure, pores between them penetrating the entire depth of the membrane (Fig. 4A,B).

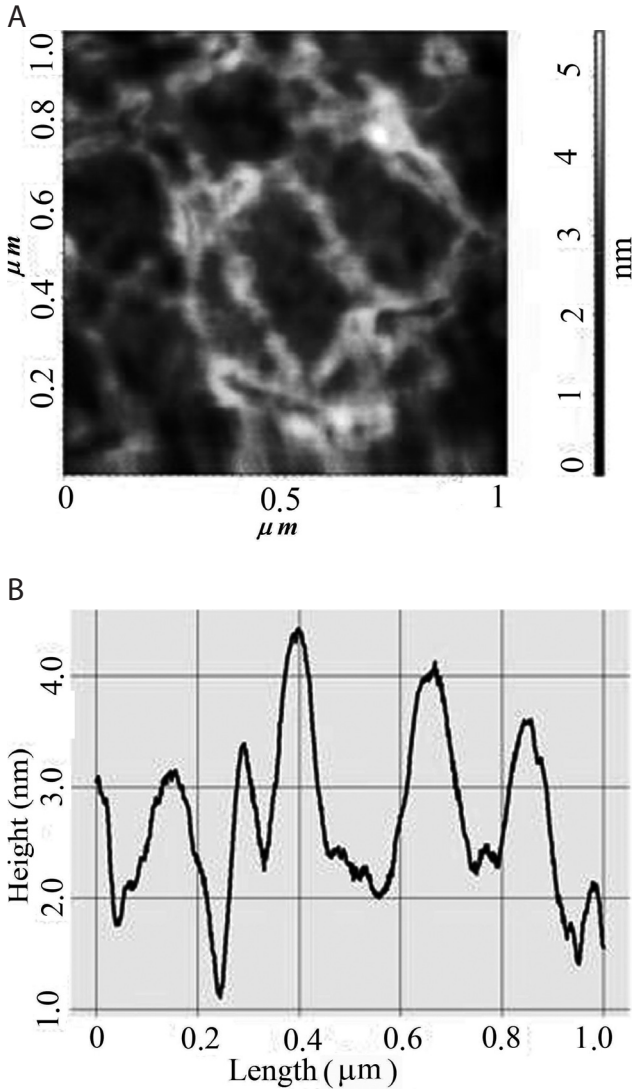

Figure 1. Rat erythrocyte surface after the interaction with a hormone solvent. Atomic force microscopy, scan size 1×1 μm^2 (**A**). Cross-section profile (**B**).

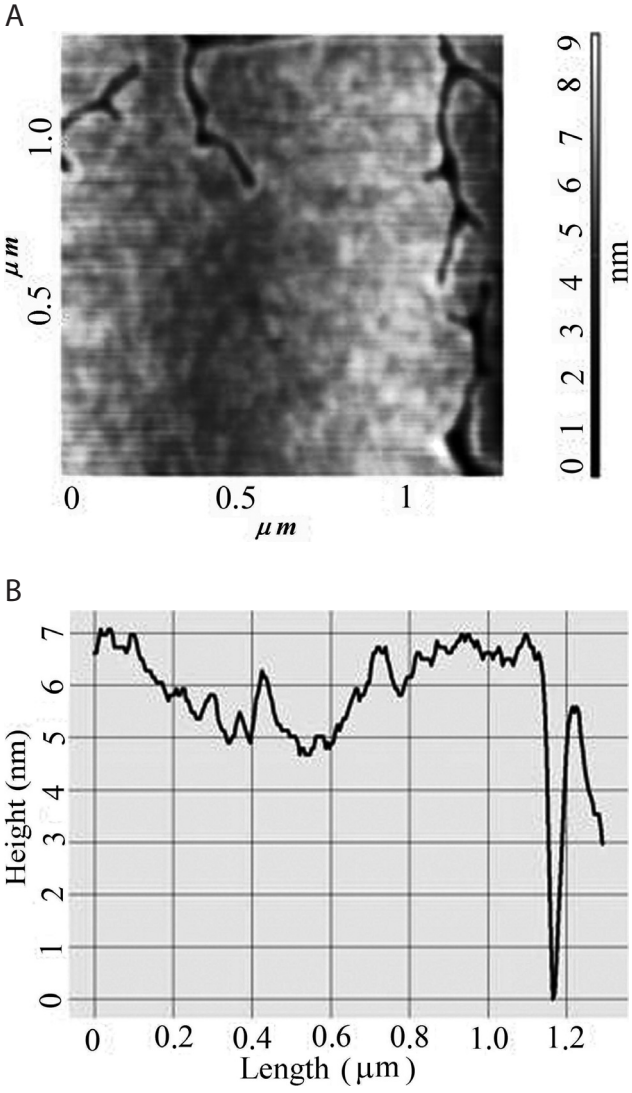

Figure 2. Rat erythrocyte surface after cortisol absorption. Concentration of the hormone 1×10^{-10} mol/mg of protein. Scan size 1×1 μm^2 (**A**). Cross-section profile (**B**).

When adrenaline was introduced before cortisol, domains of size 250 × 250 nm^2 and height 25 nm appeared on the membrane surface; this led to the formation of small domains in the membrane structure, with the penetrating open-ended pores between them (a 'quasi-chessboard structure') (Fig. 5A,B).

When both hormones were added simultaneously, the effect of cortisol dominated in the pattern: domains of size 500 × 500 nm^2 and height 15 nm appeared on the membrane surface (Fig. 6A,B). Results of the fluorescence analysis allowed us to reveal the mechanism of the domain structure formation in biological membranes.

The addition of cortisol or adrenaline to erythrocyte ghosts increased microviscosity of erythrocyte membranes (Fig. 7A,B). The effect was more pronounced in the presence of adrenaline (a 40% increase) and less pronounced in the presence of cortisol (a 25% increase). In the case of adrenaline, the membrane microviscosity started to grow at a much lower concentration of hormone in the incubation medium. With adrenaline, a plateau was attained at the hormone specific concentration of 1.7×10^{-10} mol/mg protein, whereas with cortisol this occurred at 7×10^{-10} mol/mg protein. For both hormones, microviscosity in the region of lipid-protein interactions increased at lower concentrations and was more pronounced than that in the region of lipid-lipid interac-

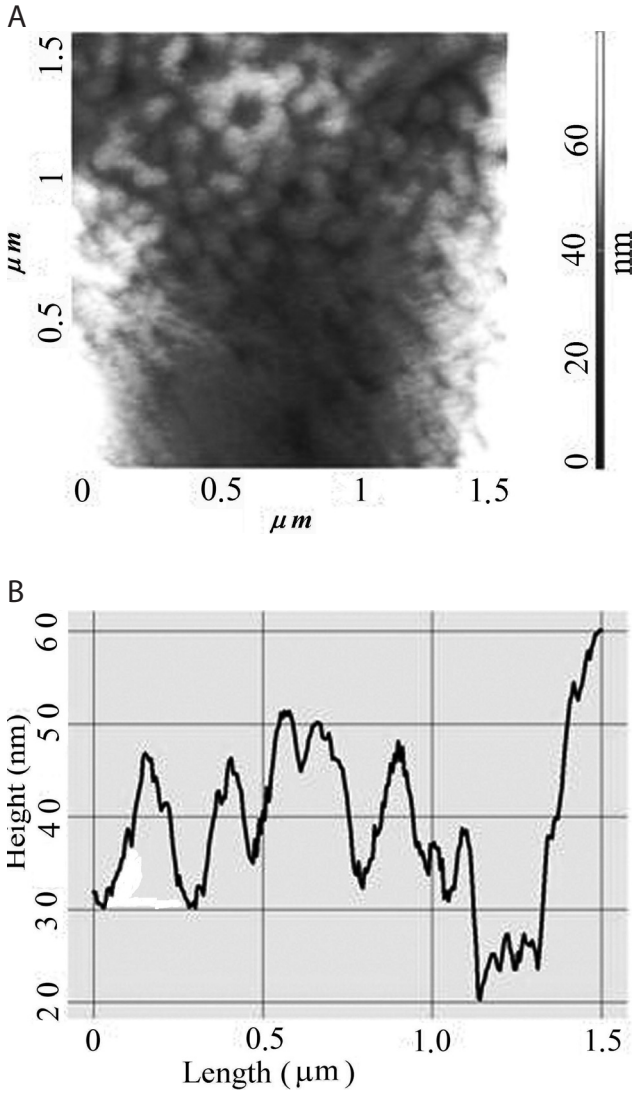

Figure 3. Rat erythrocyte surface after adrenaline absorption. Concentration of the hormone 1×10^{-10} mol/mg of protein. Scan size 1.5 × 1.5 μm^2 (**A**). Cross-section profile (**B**).

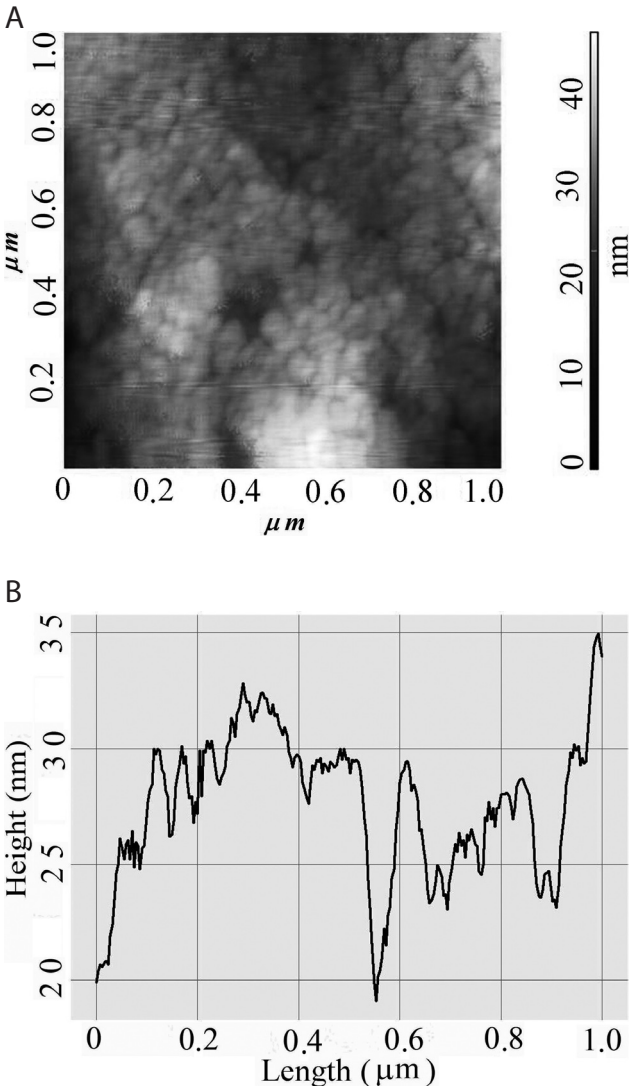

Figure 4. Rat erythrocyte surface after cortisol and adrenaline absorption. Atomic force microscopy, scan size 1 × 1 μm^2. Cortisol was added before adrenaline. Concentration of cortisol 3×10^{-12} mol/mg of protein, concentration of adrenaline 5×10^{-12} mol/mg of protein (**A**). Cross-section profile (**B**).

tions (Fig. 7C). This implies that structural transitions in erythrocyte membranes under the action of stress hormones are initiated to a greater extent in proteins and to a less extent in lipids. Simultaneous introduction of cortisol and adrenaline led to a competition for binding sites between the hormones. Upon binding to the membrane, a hormone initiates its structural rearrangement and prevents another hormone from active binding to the membrane. In the case of free competition, when membranes are incubated with a mixture of hormones, cortisol won the contest for the binding sites. It is surprising, because the constant of cortisol binding to erythrocyte membrane is much lower than that for adrenaline (Panin et al. 2010). A possible explanation is that cortisol, being a more hydrophilic compound, could change the membrane conformation more rapidly upon binding to the polar phospholipid heads, thus blocking the penetration of a more hydrophobic adrenaline into hydrophobic lipid bilayer of the membrane. Measurement of the membrane microviscosity in this case (Fig. 7C) confirms the hypothesis. Cortisol and adrenaline had equal concentrations in a mixture (plotted on the abscissa). At the specific concentration of the mix ranging from 0 to 1×10^{-10} mol/mg of protein, cortisol was not able to block the entire surface of the membrane. In this case, adrenaline penetrated

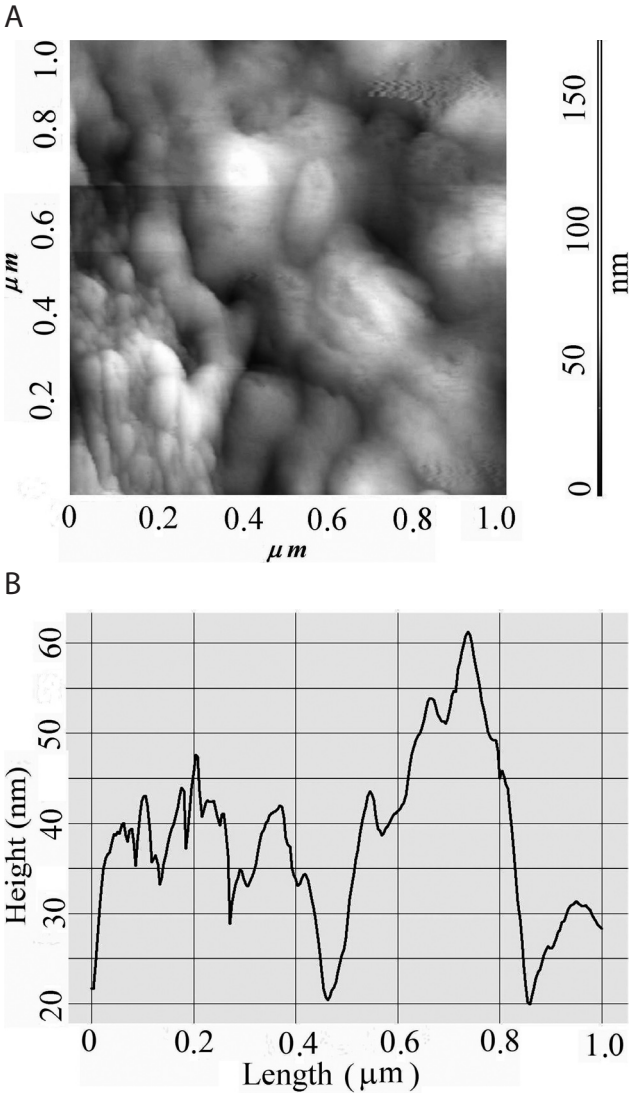

Figure 5. Rat erythrocyte surface after cortisol and adrenaline absorption. Atomic force microscopy, scan size 1×1 μm^2. Adrenaline was added before cortisol. Concentration of cortisol 3×10^{-12} mol/mg of protein, specific concentration of adrenaline 5×10^{-12} mol/mg of protein (**A**). Cross-section profile (**B**).

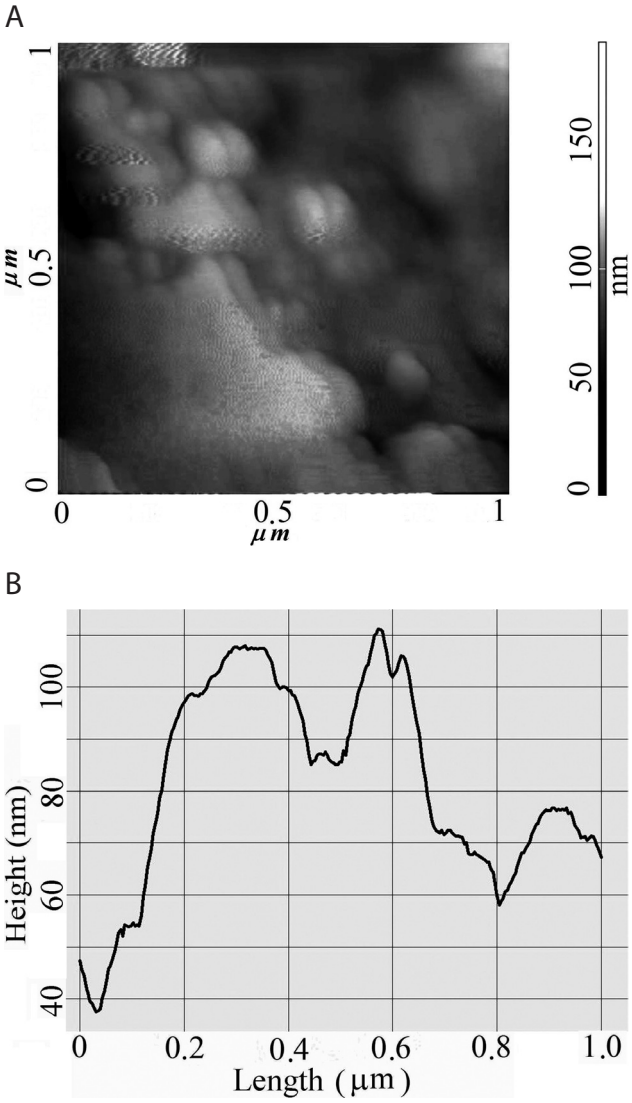

Figure 6. Rat erythrocyte surface after cortisol and adrenaline absorption. Atomic force microscopy, scan size 1×1 μm^2. Both hormones were added simultaneously. Concentration of cortisol 3×10^{-12} mol/mg of protein, specific concentration of adrenaline 5×10^{-12} mol/mg of protein (**A**). Cross-section profile (**B**).

into hydrophobic bilayer of the membrane and increased its microviscosity by 15% in the region of lipid-protein interactions. When specific concentration of hormones mix exceed 1×10^{-10} mol/mg of protein, microviscosity was the first maximum after that cortisol actively blocked the penetration of adrenaline into the membrane. When specific concentration of hormones mix exceeded 2×10^{-10} mol/mg of protein, microviscosity decreased to the case of control. Therewith, its microviscosity increased by ~25%, similar to the case of cortisol alone.

Transformation of the membrane structure upon interaction with the stress hormones produced changes in its functions. To this end, we studied changes in the activity of membrane-bound Na^+,K^+-ATPase, which determines the formation of transmembrane potential. The activity of erythrocyte membrane Na^+,K^+-ATPase was examined in dependence on the concentration of hormones in the suspension (Figs. 8, 9). Similar to microviscosity, activity of the enzyme first increased with raising the concentration of hormones. After attaining a maximum value, it started to decrease. Peak activity of the enzyme corresponded to the concentration at which microviscosity curve was at a middle of the climb to a plateau. Then the enzyme activity decreased. The more pronounced was an increase in erythrocyte membrane microviscosity caused by the hormone, the higher was the Na^+,K^+-ATPase activity. With cortisol – at a concentration of 3.5×10^{-10} mol/mg protein (Fig. 8A), whereas with adrenaline, the maximum of Na^+,K^+-ATPase activity was observed at 0.5×10^{-10} mol/mg of protein (Fig. 9A). A maximum value of activity under the action of adrenaline was 5 times higher than in the absence of hormone. Cortisol increased the activity by a factor of 2.5. The hormones also exerted a biphasic effect on the activity of other membrane-bound ATPases (Figs. 8B and 9B). Adrenaline increased their activity 1.5-fold, and cortisol by 10%.

At the joint action of the hormones, the behavior of erythrocyte membrane Na^+,K^+-ATPase activity also correlated with the dependence of microviscosity on the concentration of hormones in the suspension (Fig. 10). Two maxima were observed, which were caused most likely by the action of cortisol and adrenaline on the membrane. First peak activity of the enzyme corresponded to the concentration at which microviscosity curve was the first peak. The second peak activity of the enzyme corresponded to the concentration at which microviscosity curve was at a middle of the climb to a plateau (7×10^{-10} mol/mg of protein).

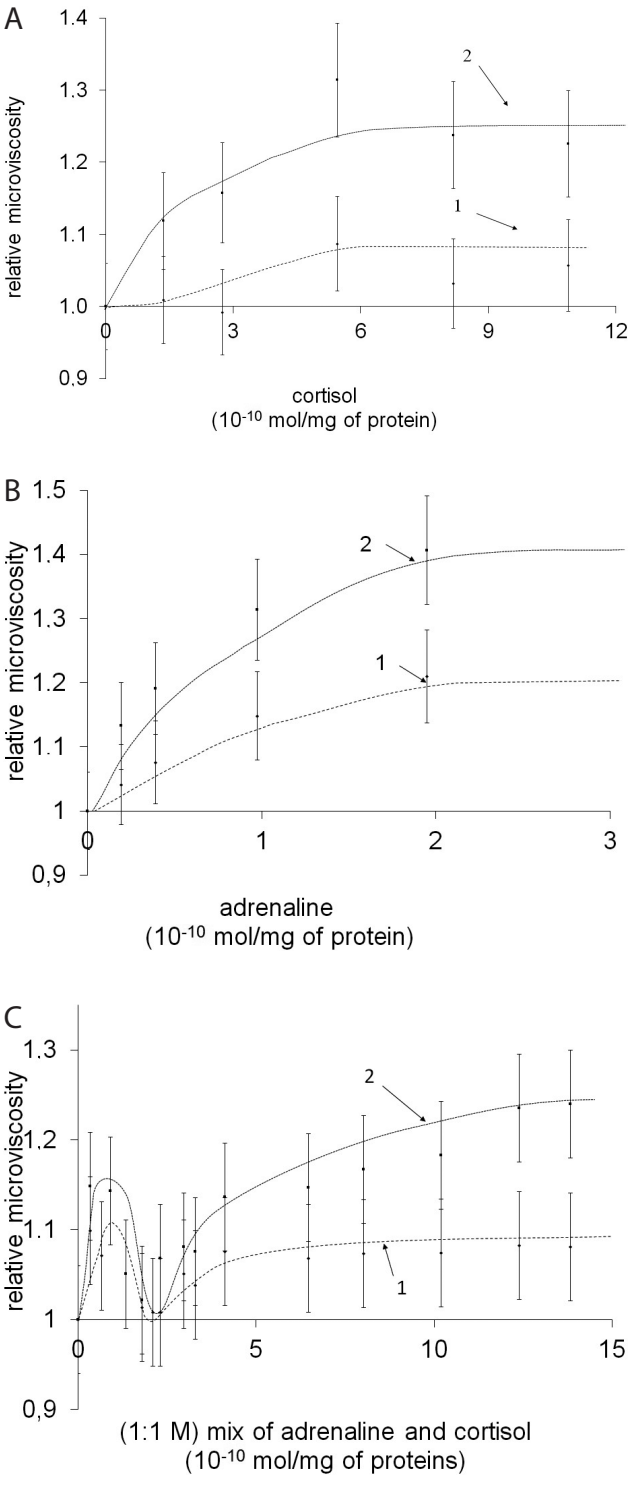

Figure 7. Changes in the relative microviscosity of rat erythrocyte ghost membranes under the action of hormones: cortisol (**A**), adrenaline (**B**), a mixture (1:1 by moles) of cortisol and adrenaline (**C**). Line 1, changes in relative microviscosity in the region of lipid-lipid interaction; line 2, changes in relative microviscosity in the region of protein-lipid interaction. Relative measurement errors for relative microviscosity attained 6%.

Discussion

Earlier, the application of IR spectroscopy allowed us to reveal the mechanism of erythrocyte deformation (Panin et

al. 2010). The zones of local hydrostatic tension were shown to result from the formation of domains in the membrane structure. The domains formed due to interaction of the CO and OH groups of cortisol with the CO, OH and NH groups of proteins and phospholipids. Hydrophobic rings of the hormones enhanced hydrophobic interactions in the domains. The appearance of new and strengthening of the existing bonds produced domains (zones of hydrostatic compression) with an increased membrane microviscosity around the proteins. Molecularly bound water was displaced to the domain boundaries, where it loosened the cell membrane structure. Strips of hydrostatic tension formed between the domains. Full destruction of erythrocyte membrane further occurred along such strips. A 'quasi-chessboard' distribution of tangential stresses emerged in the membrane. This led to the membrane bending, its surface relief became folded. Apexes of the folds formed in the zones of hydrostatic compression.

A correlation between changes in microviscosity of erythrocyte membrane and activity of Na^+,K^+-ATPase allowed us to propose the next mechanism of effect of stress hormones on the activity of membrane Na^+,K^+-ATPase. According to modern concepts, the sodium pump cycle is accompanied by conformational rearrangement of its α-subunit, which

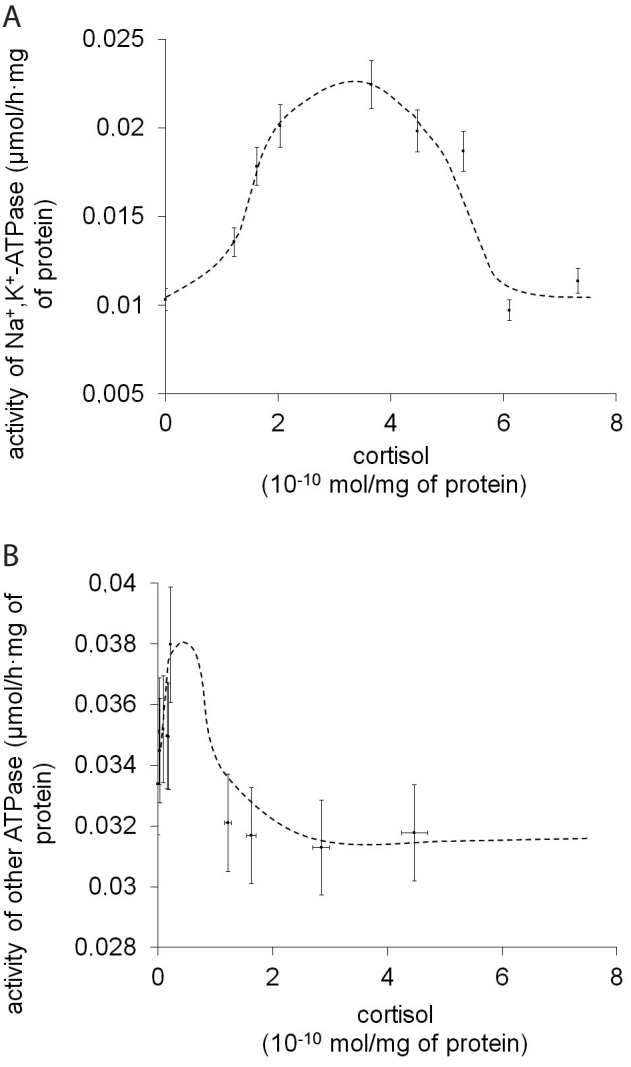

Figure 8. A. Changes in the activity of rat erythrocyte membrane Na^+,K^+-ATPase *versus* the specific concentration of cortisol in a suspension. **B.** Changes in the activity of other erythrocyte membrane ATPase *versus* the specific concentration of cortisol in a suspension. Relative measurement errors for activity of Na^+,K^+-ATPase and other erythrocyte membrane ATPase attained 6%.

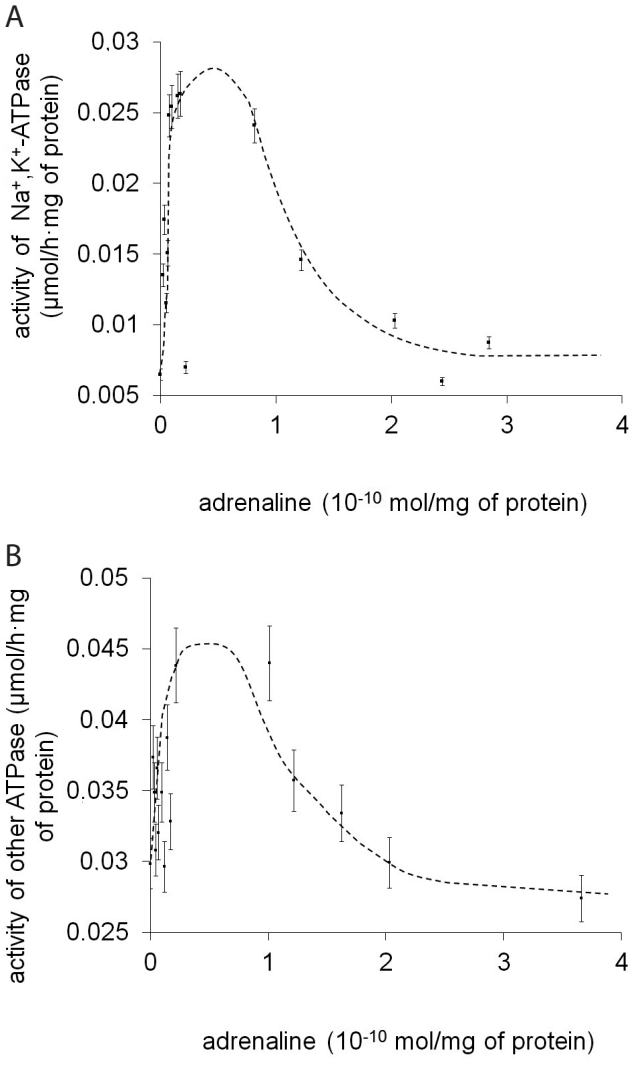

Figure 9. A. Changes in the activity of rat erythrocyte membrane Na^+,K^+-ATPase *versus* the specific concentration of adrenaline in a suspension. **B.** Changes in the activity of other erythrocyte membrane ATPase *versus* the specific concentration of adrenaline in a suspension. Relative measurement errors for activity of Na^+,K^+-ATPase and other erythrocyte membrane ATPase attained 6%.

leads to rearrangement of ionic sites and subsequent migration of the ion binding site loop into or outside the cell. ATP hydrolysis serves as an energy source for the Na^+,K^+-ATPase operation. The migration of binding site is strongly facilitated by mechanical vibrations of the enzyme subunits (Bouvrais et al. 2012). Such mechanical vibrations (phonons) are transferred to subunits from the phospholipid bilayer.

The plasmatic membrane can be considered as a liquid crystal. Properties of biomembranes can be considered using ideas of the theory of solids (Rowlands et al. 1982; Rheinstadter et al. 2009). An increase in the concentration of hormones in the suspension raises density of the membrane, which underlies a growth of the membrane microviscosity. According to the Debye theory (Mason 1965), a maximum energy of phonons in the membrane is directly proportional to density of the body. Thus, loading of the membranes with stress hormones increases the maximum energy of their phonons. The higher is the energy of membrane phonons, the easier is the migration of lipid loops through the membrane. The Na^+,K^+-ATPase activity also increases. This is one of the mechanisms affecting the enzyme activity.

Stability of the domain complex is caused by both the hydrophobic and electrostatic interactions. This model implies that relative movements of subunits in the Na^+,K^+-ATPase protein-lipid complex would result in deformation of the lipid bilayer (Tverdislov and Yakovenko 1980). The process consumes a part of the energy produced by ATP hydrolysis. A considerable growth of the membrane stiffness is expected to increase this part of the energy. Migration frequency of the loops though the membrane and the enzyme activity will decrease. A decrease in the enzyme activity (ATP hydrolysis rate) with the growth of membrane stiffness is caused also by hindering a close contact between ATP and the enzyme active site due to local growth of elastic shear constants of the membrane.

Thus, when the membrane microviscosity increases, the enzyme activity is affected simultaneously by diversely directed mechanisms. Initially, the dominant process is the growth of activity due to increasing the membrane phonon energy. After attaining a critical value of membrane microviscosity, the enzyme activity starts to decrease because now the dominant process is the loss of energy delivered to the enzyme during ATP hydrolysis and hindering of the lipid bilayer deformation.

As a result, a domelike dependence of the erythrocyte membrane Na^+,K^+-ATPase activity on the concentration of hormones in the suspension (Figs. 8, 9, 10) is formed. When the membrane is affected by a mixture of low-concentrated cortisol and adrenaline, the number of binding sites is sufficient for the hormone molecules. In this case, most pronounced in the action of adrenaline due to its strong effect on the membrane. This shows up as the first maximum on the curve of Na^+,K^+-ATPase activity (Fig. 10). As the concentration of hormones increases, cortisol goes ahead in the competition for binding sites. However, it is bound to the surface and exerts a weaker effect on the membrane. So, the membrane microviscosity decreases to some extent and activity of the enzyme starts to grow again. A further increase in the cortisol concentration restarts the suppression, which leads to appearance of the second maximum on the curve of Na^+,K^+-ATPase activity.

This hypothesis is supported by the fact that operation of Na^+,K^+-ATPase stops in the absence of lipid bilayer,

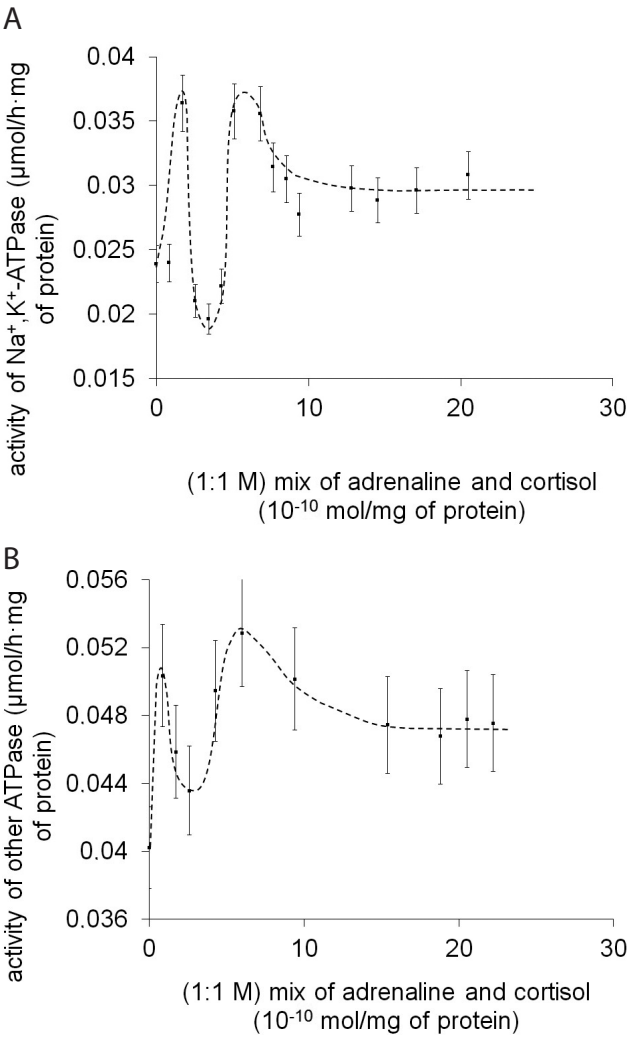

Figure 10. A. Changes in the activity of rat erythrocyte membrane Na^+,K^+-ATPase *versus* the specific concentration of a mixture (1:1 by moles) of cortisol and adrenaline in a suspension. **B.** Changes in the activity of other erythrocyte membrane ATPase *versus* the specific concentration of a mixture (1:1 by moles) of cortisol and adrenaline in a suspension. Relative measurement errors for activity of Na^+,K^+-ATPase and other erythrocyte membrane ATPase attained 6%.

the removal of spectrin-ankyrin-actin network from the membrane leading to a more than twofold decrease in the Na^+,K^+-ATPase activity (Kazennov and Maslova 1991). In the absence of lipid bilayer, elastic vibrations cannot be transferred to Na^+,K^+-ATPase. As was demonstrated in (Panin et al. 2010), microviscosity of the entire membrane is determined by conformational changes of the membrane proteins. If proteins are removed or denatured, the membrane microviscosity sharply decreases; a decrease is observed also in the Young's modulus and in the maximum energy of membrane phonons. Migration of the ions is hindered, and the Na^+,K^+-ATPase activity diminishes.

Changes in the structure and function of erythrocyte membranes during their interaction with an excessive amount of stress hormones (cortisol and adrenaline) in the blood may be disastrous for the organism. It is known that diameter of erythrocyte is comparable with the diameter of small blood capillaries. To pass through the capillary system, erythrocyte should have an increased fluidity. Earlier it was shown that in capillaries a part of cholesterol goes from erythrocyte membrane into the blood plasma (Panin and Panin 2008). This decreases the membrane microviscosity and increases the fluidity of erythrocytes. Such erythrocyte readily moves through the capillary system. An inverse process occurring in the veins restores the amount of cholesterol in erythrocyte membranes. Thus, erythrocyte membrane can be considered as a diffusion-hardened composite material.

Steroid hormones are the derivatives of cholesterol. However, in distinction to cholesterol, they have a much greater number of active CO and OH groups, which readily form hydrogen bonds with CO, OH and NH groups of proteins and phospholipids of erythrocyte membranes. This leads to the formation of complex domains in the membrane structure. The enhancement of hydrophobic interactions in the domains due to hydrophobic rings of the hormones increases the compressive elastic stresses in them and, accordingly, the membrane microviscosity. Such erythrocytes serve as microthrombi – they occlude the capillaries and lead to the development of diffusion hypoxia. Distortions of the membrane structure change the activity of Na^+,K^+-ATPase and suppress the formation of transmembrane potential, which may result in aggregation of erythrocytes. We think that high incidence of cardiac syndrome X and frequent deaths in sportsmen during important competitions are related to structural and functional disturbances of erythrocytes under the action of excess stress hormones.

Conclusion

At a simultaneous binding of adrenaline and cortisol, the hormones compete for the binding sites. Cortisol wins the competition. This is accompanied by a structural transition in the membrane, and adrenaline molecules cannot penetrate into hydrophobic region of biomembrane. Changes in the membrane structure affect the activity of erythrocyte membrane Na^+,K^+-ATPase. A domelike dependence of the erythrocyte membrane Na^+,K^+-ATPase activity on the hormone concentration in a suspension was obtained in the experiments. A hypothesis was proposed according to which raising of the hormones concentration, when the membrane microviscosity and its Young's modulus grow, increases energy of the membrane phonons. The growing energy of phonons facilitates migration of the ion binding site loop into or outside the cell, which raises the activity of Na^+,K^+-ATPase. Then, the increasing microviscosity of the membrane starts to suppress the operation of Na^+,K^+-ATPase active site, which decreases its activity.

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