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Influence of Anticholinesterase Drugs on Activity and Properties of Na^+,K^+-ATPase in Rat Erythrocytes under Stress Caused by Intense Physical Exercise

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We studied the effect of intramuscular injection of physostigmine and neostigmine on Na^+,K^+-ATPase activity in erythrocytes of rats subjected to intense physical exercise. Both anticholinesterase drugs had a significant effect on the development of the stress response, which was expressed in a decrease in the manifestation of its individual components such as the concentration of ascorbic acid in the adrenal glands, stress-related erythrocyte polycythemia, and LPO indicators. Anticholinesterase drugs reverse the stress-induced decrease in Na^+,K^+-ATPase activity, as well as changes in its magnesium-dependent properties. There were no changes in the activity of the studied enzyme in the erythrocyte ghosts. We associate the observed differences with the correction of the functions of the cholinergic components of the hypothalamic–pituitary–adrenal axis leading to the development of a hypoergic type stress reaction.

Key Words: *Na^+,K^+-ATPase; neostigmine; physostigmine; physical exercise*

The Na^+,K^+-ATPase is present in the plasma membranes of all cells and generates the ionic transmembrane gradients and is implemented in fundamental cell functions [1,2]. The ideas about the participation of cholinergic structures in the regulation of Na^+,K^+-ATPase activity were developed over the past decades [3,4]. From the components of choline-reactive systems, mammalian erythrocytes contain only acetylcholinesterase [5], and the direct effect of cholinergic drugs on these cells seems unlikely. However, the blood system is under neuroendocrine control of the hypothalamic–pituitary–adrenal (HPA) axis [6]. Modern data confirm a significant role of the components of cholinergic structures in the functions of the HPA axis: the presence of both muscarinic and nicotinic cholinergic receptors in HPA was shown [7,8] and, as a result, its activity can be modulated by cholinergic blockers [7,9] and anticholinesterase drugs (AD) [10]. AD increase the concentration of extracellular acetylcholine [11], which leads to stimulation of cholinergic receptors and changes in the levels of other neurotransmitters [12]. It was noted that acetylcholine and cholinergic blockers have a significant effect on the production of catecholamines [13] and glucocorticoids [7,9] by rat adrenal glands. And finally, the cholinergic structures of the adrenal glands can be involved in the regulation of secretion of endogenous digoxin-like Na^+,K^+-ATPase inhibitors into the blood plasma [14]. Thus, components of cholinergic structures can mediate their participation through the HPA axis in the regulation of Na^+,K^+-ATPase activity in erythrocytes.

Here we studied the effect of AD (physostigmine and neostigmine) on the activity and properties of Na^+,K^+-ATPase in rat erythrocytes under stress conditions caused by intense physical exercise.

MATERIALS AND METHODS

The experiments were approved by the Local Ethical Committee of the Tyumen State University (Protocol

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No. 10 of December 25, 2023) and were performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes (Strasbourg, 1986).

The studies were carried out on male Wistar rats weighing 200-240 g. The animals were divided into 4 groups (8 rats per group): control rats (group 1) received intramuscular injection of saline (0.2 ml); group 2 rats received 0.2 ml saline intramuscularly and were subjected to physical exercise (PE), group 3 rats received intramuscular injection of neostigmine+PE, group 4 rats received intramuscular injection of physostigmine+PE.

PE was simulated by 40-min swimming in water (28°C) with a load on the tail equal to 4.45% of body weight. Neostigmine and physostigmine (aqueous solutions at a concentration of 0.1 mg/ml) were administered intramuscularly in doses of 0.045 and 0.16 mg/kg body weight, respectively. immediately before PE. The criterion for dose selection was the ability of experimental animals to withstand exercise under the influence of drugs; at rest the doses did not cause clinical manifestations of intoxication.

The animals were decapitated under light ether anesthesia, the blood was collected in porcelain cups, and heparin (150 U/ml) was used as an anticoagulant. Erythrocytes were counted in a Goryaev's chamber; the diameter was determined by averaging the diameter of 100 cells measured using an eyepiece micrometer (Biolam microscope, 100× objective, division value 0.6 μm) in smears stained after Romanowsky. Packed erythrocytes were obtained by centrifugation followed by triple washing with an isotonic solution (0.145 M NaCl in 10 mM Tris-HCl-buffer (pH 7.6) at 20°C). Erythrocyte ghosts were prepared by hypoosmotic hemolysis using the Dodge method. The concentration of ascorbic acid and its fractions was determined by the method of Roe and Kuethler. The concentrations of malondialdehyde and conjugated dienes in erythrocytes and erythrocyte ghosts were measured by the Stalnaya method. The activity of cholinesterases was determined by the Ellman method; acetylthiocholine iodide was used as a substrate. Activity of transport ATPases was measured by the increase in inorganic phosphate (Pi). Na^+,K^+-ATPase activity was calculated as the difference between the total ATPase activity and the Mg^{2+}-ATPase activity determined in the presence of ouabain. Pi was measured by the reaction with ammonium molybdate and tin chloride in the presence of ascorbic acid. Protein concentration in enzyme preparations was measured using the Lowry method.

Statistical processing of the results was carried out using the Microsoft Excel 2016 and Statistica 6.0 software (StatSoft, Inc.). The data are presented as the arithmetic mean and standard error of the mean (*M±SEM*). The significance of differences was assessed using the Mann–Whitney *U* test at a significance level of $p<0.05$.

RESULTS

Anticholinesterase effect seems to be the main manifestation of biological activity of both neostigmine and physostigmine [15]. In this regard, the activity of cholinesterases was primarily assessed (Table 1). Both drugs, after a 40-min interval during which PE was performed, did not cause significant changes in cholinesterase activity in packed erythrocytes, whole blood, and the adrenal glands of rats in the experimental groups compared to the control. At the same time, neostigmine and physostigmine had a significant effect on indicators that serve as markers in assessing the effect of stress in mammals. In the PE group, a

TABLE 1. Cholinesterase Activity and Stress Markers in Rats Subjected to PE after AD Administration (*M±SEM*)

Parameter		Control	PE	Neostigmine +PE	Physostigmine +PE
Ascorbic acid, mg%		385.97±25.93	286.87±16.61***	332.07±18.35	323.38±21.11
Erythrocytes, 10^{12}/liter		7.02±0.09	8.14±0.27**	7.39±0.23$^+$	7.55±0.29
Average erythrocyte diameter, μm		6.17±0.04	5.93±0.04**	6.03±0.05	6.02±0.06
MDA	erythrocytes, nmol/ml	9.31±0.82	10.85±0.94	10.15±0.82	10.04±0.81
	erythrocyte ghosts, nmol/mg protein	0.98±0.10	1.10±0.09	1.06±0.11	0.93±0.09
CD	erythrocytes, nmol/ml	12.56±1.02	18.49±1.61*	14.12±1.23	14.68±1.01
	erythrocyte ghosts, nmol/mg protein	3.08±0.27	4.05±0.37^o	3.44±0.33	3.33±0.30
AChE	in erythrocytes, μmol ATCh/h/ml	58.51±5.51	56.32±5.36	53.10±3.90	55.64±5.14
	in the whole blood, μmol ATCh/h/ml	67.78±4.87	72.59±6.35	61.94±3.78	69.43±5.55
	in the adrenal glands, μmol ATCh/h/mg protein	0.59±0.06	0.65±0.06	0.54±0.04	0.56±0.05

Note. AChE: acetylcholinesterase; ATCh: acetylthiocholine; CD: conjugated dienes; MDA: malondialdehyde. $^o p=0.052$, *$p<0.05$, **$p<0.01$ in comparison with the control; $^+p<0.05$ in comparison with PE.

TABLE 2. Activity of Transport ATPases in Erythrocytes of Rats Subjected to PE after AD Administration (μmol Pi/ml/h; *M±SEM*)

Groups	Erythrocytes		Erythrocyte ghosts	
	Na^+,K^+-ATPase	Mg^{2+}-ATPase	Na^+,K^+-ATPase	Mg^{2+}-ATPase
Control	18.65±1.65	20.12±1.87	1.43±0.11	1.34±0.09
PE	12.85±1.29*	23.61±2.41	1.57±0.15	1.32±0.13
Neostigmine+PE	19.74±2.01	22.02±2.23	1.46±0.16	1.30±0.13
Physostigmine+PE	18.47±1.92	20.60±2.41	1.35±0.11	1.23±0.10

Note. *p<0.05 in comparison with the control.

significant decrease in ascorbic acid concentration in the adrenal glands was observed, which indicates the development of stress [16]. Moreover, this indicator does not change significantly in animals treated with AD, which suggests the development of a stress reaction according to a hypoergic scenario with insufficient synthesis of catecholamines. The effect of anticholinergic drugs on the production of catecholamines by the adrenal glands was reported previously [13].

Activity of Na^+,K^+-ATPase in erythrocytes of rats subjected to PE significantly decreased (-31%) in comparison with the control (Table 2). In addition, we also observed a loss of enzyme sensitivity to high magnesium concentrations in the solution for measuring enzyme activity (Fig. 1, *a*). At the same time, these changes were not revealed in erythrocytes of animals subjected to PE after AD treatment. Reduced enzyme activity in erythrocytes under conditions of acute stress was described elsewhere [17] and was associated with the action of a number of factors: changes in the composition of the circulating erythrocyte population and changes in the erythrocyte membrane composition [18], as well as the action of endogenous digoxin-like factors that specifically reduce activity of Na^+,K^+-ATPases [2]. The main question is which of the existing factors is canceled under the influence of AD. In our experiment, practically no changes in the circulating erythrocyte population typical of stress reaction (PE group) [19] as well as in indicators reflecting oxidative modification of erythrocyte membranes were found in the groups treated with AD.

In addition, no differences in the enzyme activity were found in all groups, and its sensitivity to high concentrations of magnesium ions in membrane preparations (ghosts) of erythrocytes was preserved (Fig. 1, *b*). Hence, stress-related polycythemia and oxidative cell membrane modification play a secondary role in the decrease of Na^+,K^+-ATPase activity. In addition, there are reports on similar effects on the enzyme of high magnesium ion concentrations [20] as well as cardiotonic steroids [2,3] stabilizing it in the E2P conformation. This suggests that an endogenous factor that mediates the decrease in enzyme activity in erythrocytes under stress conditions is removed from their membranes during isolation of these cells. Based on previous report on the cholinergic regulation of the production of endogenous ouabain-like factors in the adrenal glands [14], we can assume that their

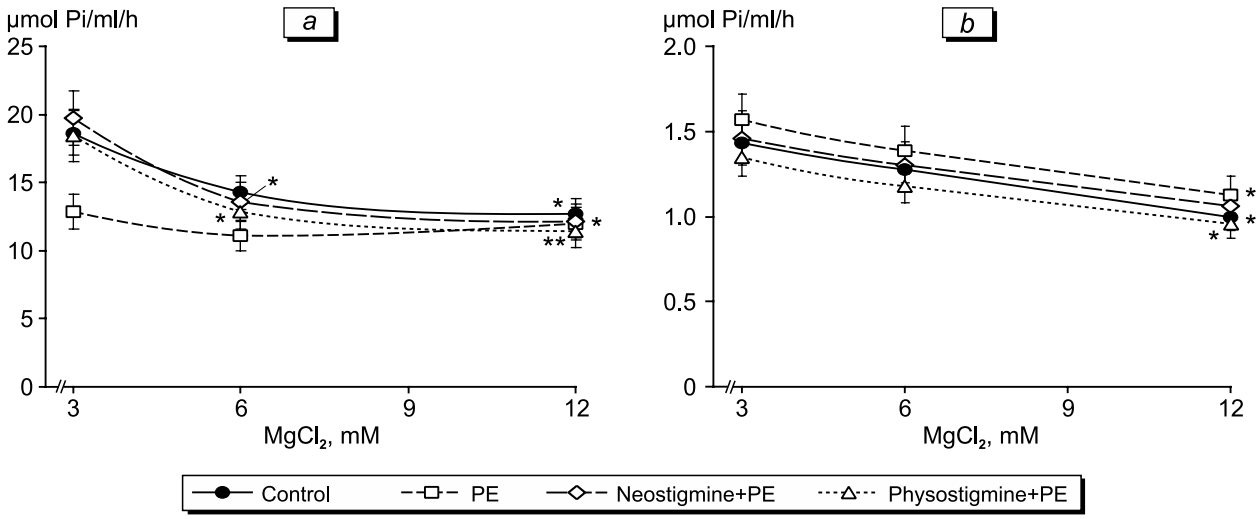

Fig. 1. Activity of Na^+,K^+-ATPase in erythrocytes (*a*) and erythrocyte ghosts (*b*) of rats depending on Mg^{2+} concentration in the incubation medium.*p<0.05, **p<0.01 in comparison with the control (3 mM Mg^{2+}).

secretion is less pronounced in groups of animals subjected to PE after administration of AD.

The results show a significant difference in the activity of erythrocyte Na^+,K^+-ATPase, as well as in the manifestation of classical markers of stress in rats exposed to PE in groups treated and not treated with AD. The observed differences are primarily attributed to the correction of functions of the HPA axis by influencing its cholinergic components, which prevents the development of an adequate response to stress in animals.

Conflict of interest. The authors have no conflicts of interest to declare.

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