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Activation of Glutamate Receptors Inhibits Na/K-ATPase of Cerebellum Granule Cells

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Abstract—Na/K-ATPase prepared from cerebellum granule cells of 10-12-day-old mice is inhibited by glutamate and its agonists, NMDA (ligand for ionotropic receptors) and ACPD (ligand for metabotropic receptors). The inhibition is specific and prevented by subsequent antagonists (MK-801 for ionotropic NMDA-receptors and MCPG for metabotropic receptors). The inhibiting effect of NMDA is significantly reversed by cysteine and that of ACPD by chelerythrine or indolyl maleimide. It is concluded that ionotropic receptors inhibit Na/K-ATPase because of intracellular production of reactive oxygen species, and metabotropic receptors mediate their effect via protein kinase C.

Key words: Na/K-ATPase, cerebellum granule cells, glutamate receptors, reactive oxygen species, oxidative stress, protein kinase C

Na/K-ATPase, a membrane-bound enzyme supporting asymmetric distribution of Na and K ions across the outer cell membrane, is known to be a target of oxidative stress, the most vulnerable isoforms of the enzyme being those belonging to excitable tissues [1-4]. Excitotoxic effects of glutamate in brain are accompanied by inhibition of Na/K-ATPase, which aggravates the damage of neuronal cells subjected to oxidative stress. Suppression of Na/K-ATPase by glutamate was demonstrated on isolated synaptosomes [5]. On the culture of neuroglial cells glutamate induced neuronal death was described, which was explained by the authors as a result of Na/K-ATPase inhibition [6]. This data, however, was hardly possible to use for analysis of biochemical mechanisms of the interaction of glutamate receptors with Na/K-ATPase because the results were obtained using disordered cell membranes, morphologically heterogeneous brain slices, or cultured neuroglial cells.

In this study, viable cerebellum granule cells were used to elucidate the effect of different glutamate receptors on Na/K-ATPase under exposure of the neurons by specific ligands; Na/K-ATPase activity was measured after such treatment and subsequent disintegration of cellular membranes by fivefold cycles of freezing–thawing. Preliminary publication of the data was recently presented [7].

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MATERIALS AND METHODS

In the experiments, 10-12-day-old mice (AKR/J) of both sexes were used. In each series the animals were combined in groups of 4-5 mice. Cerebellum slices were exposed (40 min at 33°C) to 0.34% dispase II prepared in Tyrode's solution (148 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.3 at 23°C) to disorder intercellular contacts. After washing of slices with fresh Tyrode's solution, the gently rolling and individual cells were separated by filtering the suspension through the nylon mesh of 53 μm size. The cells were restituted 60 min in Tyrode's solution (33°C) and used for measurements for not longer than 4 h.

The suspension was homogenous and represented mainly by granular cells with the portion of viable neurons between 85-95%. The number of cells in the samples was measured in the Bright-Line hematocytometer (USA). Normally, the initial suspension contained ~10^6 cells per ml and each sample contained 50,000 cells. The viability of the cells was determined in the presence of propidium iodide (PI) using a fluorescent microscope or flow cytometry technique [8], during all experimental period the cells keeping their viability [9, 10]. To diminish cell adhesiveness, plastic ware was used in all experiments.

Neurons were exposed to ligands at 37°C for 30 min, this period being necessary for the total saturation of neu-

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ronal receptors. Cell membrane was disintegrated after such procedure using fivefold freezing–thawing in liquid nitrogen. Na/K-ATPase measured after such treatment was similar to that obtained in the presence of the channel former alamethicin. Thus, we concluded that the freezing–thawing procedure de-masks all latent ATPase molecules and does not modify the enzyme.

Measurement of Na/K-ATPase activity was made by inorganic phosphate liberation during ATP hydrolysis by disintegrated cells in the following medium: 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, and 30 mM HEPES (pH 7.4 at 37°C) [2] calculating Na/K-ATPase activity as the difference between total and ouabain independent ATPase. The later was determined in the presence of 1 mM ouabain or in the presence of sodium ions using instead of potassium; the results were similar in the two cases. Specific activity of Na/K-ATPase slightly varied from experiment to experiment in the range of 100-125 nmol P_i/min per mg protein.

To consider direct (nonspecific) effect of agonists and antagonists of glutamate receptors on Na/K-ATPase, the ligands were added to control samples containing disintegrated cells (non-exposed to ligands) and then the enzyme activity was immediatelly measured. The specific effect of ligands was calculated as a difference between their total and nonspecific action. Thus, the data presented are characteristic of specific receptor-mediated effect on Na/K-ATPase of the ligands studied.

Protein was measured by the Lowry method using a Sigma standard set (USA).

In the experiments, the following reagents were used: HEPES, ouabain, ATP, glutamate, N-methyl-D-aspartate (NMDA), glycine, propidium iodide, chelerythrine, and alamethicin from Sigma (USA); *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), α-amino-2,3-dihydro-5-methyl-3-oxo-4-izoxazolepropanoic acid (AMPA), 6,7-dinitroquinoxaline (DNQX), (+)-α-methyl-4-carboxyphenylglycine (MCPG), and dizocilpine maleate (MK-801) from RBI (USA); other reagents (of analytical grade) were from Reakhim (Russia).

Each value presented is calculated as an average from 3-4 parallel measurements and presented as $m \pm$ SD. Each experimental series was repeated no less then 3 times using cell suspension prepared from different sets of animals. Statistical analysis was made using Student's *t*-test taking for significant differences those characterized by $p < 0.05$.

RESULTS AND DISCUSSION

Glutamate demonstrates distinct dose-dependent inhibition of Na/K-ATPase, which, however did not result in total suppression of the activity. In the presence of 100 μM glutamate in the pre-incubation medium the activity was suppressed by 15-20% and the highest inhibition (in the presence of 0.8-1 mM glutamate) was 35-45%. ACPD, the ligand activating most metabotropic glutamate receptors, was similarly effective. At the same time, N-methyl-D-aspartate affecting the ionotropic receptors of NMDA-subtype taking at maximal concentration (1 mM) provided higher inhibiting action (Fig. 1). When glutamate and NMDA were added to the samples immediately before ATPase measurements (1 mM), inhibition of the activity was neglected, in contrast to ACPD, which itself significantly suppressed the enzyme (by about 80% at 1 mM). Taking into account the nonspecific effect of ACPD, we used this ligand at concentrations lower than 500 μM where its direct effect on the enzyme was significantly lower.

The inhibiting effect of glutamate was dependent on the presence of calcium ions. When calcium was substituted by 1 mM EGTA, the inhibiting effect of glutamate decreased twofold while the viability of the cells was not changed during the experimental period (table).

To estimate specificity of glutamate inhibition, we measured its action on Na/K-ATPase in the presence of several antagonists—MK-801 (for ionotropic NMDA-receptors) and MCPG (for metabotropic receptors). The data presented in Fig. 2 demonstrate that inhibiting effect of glutamate on Na/K-ATPase can be the result of activation by both ionotropic and metabotropic receptors. In both cases the inhibition is a specific because it can be prevented by subsequent antagonist demonstrating the presence of two different modes of inhibition via different subtypes of glutamate receptors.

Activation of glutamate receptors on the neuronal membrane switches on protein kinases, which are under

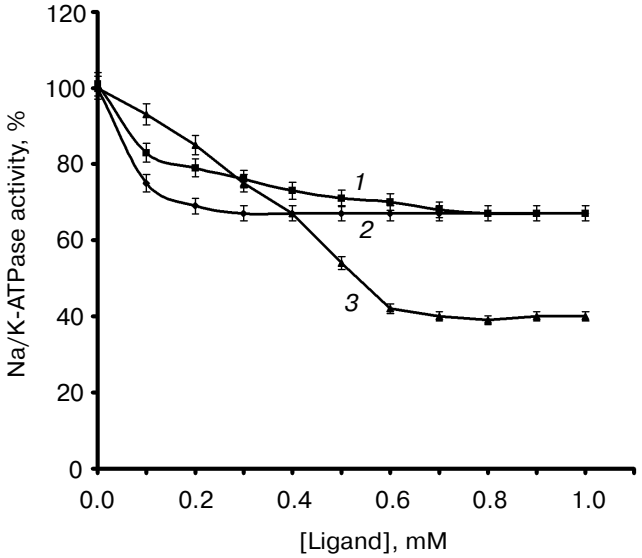

Fig. 1. Specific effect of glutamate (*1*), ACPD (*2*), and NMDA (*3*) on Na/K-ATPase in cerebellum granule cells.

Specific effect of glutamate on Na/K-ATPase activity (nmol P_i/min per mg protein) in cerebellum granule cells

Experimental conditions	With Ca^{2+}	Without Ca^{2+}	*p*
Control	122 ± 2	112 ± 6	
Glutamate (1 mM)	75 ± 2	91 ± 6	
Inhibition, %	38	19	< 0.05

immediate control of metabotropic receptors (via G-proteins of different classes) or mediated by calcium ions (in the case of ionotropic receptors) [11]. For this reason, the inhibiting effect of glutamate on Na/K-ATPase activity can be explained as a result of activation of cellular protein kinases. Phosphorylation-dephosphorylation of cellular proteins is known to be one of the important regulatory mechanisms and such mechanism may take place in the case of Na/K-ATPase. Phosphorylation of the enzyme by protein kinases A and C results in its suppression, whereas dephosphorylation of Na/K-ATPase by protein phosphatases can restore its activity [12]. Moreover, it was demonstrated that toxic effect of glutamate on cultured neurons is decreased by protein kinase C inhibitors [13]. All these data confirm an importance of interactions between glutamate and Na/K-ATPase for regulation of neuronal viability.

To check possible involvement of protein kinases in inhibition of Na/K-ATPase by activation of glutamate receptors of different kinds, we tested the effect on the phenomenon described of chelerythrine and indolyl maleimide, penetrating the cell membranes and inhibiting intracellular Ca-dependent protein kinases, both inhibitors being taken at concentration (10 μM) sufficiently enough for total effect on cellular suspension [14].

The results obtained in such experiment with chelerythrine are presented in Fig. 3 as an example. A protecting effect was found in the case of ACPD (activator of metabotropic receptors) but not of NMDA (activator of ionotropic NMDA-receptors), whereas this inhibition is totally prevented by subsequent antagonists (MCPG for ACPD-receptors or MK-801 for NMDA-receptors). Indolyl maleimide at the same concentration demonstrated similar (but not total) effect—when it was added to the incubation medium together with ACPD its inhibiting action was 2 times less (data not presented).

Inhibition of Na/K-ATPase by NMDA is only partially prevented by chelerythrine. This suggests that the inhibiting action of ionotropic receptors during their activation by NMDA is realized only partially. Thus, such an effect can be explained by both of protein kinase activation [12] and, independently, by direct oxidation of protein molecules by reactive oxygen species [2-4] being generated in the neuron whose ionotropic receptors are activated [9].

Recently it was found by other authors that Na/K-ATPase can be suppressed by oxidation of protein SH-groups by means of oxygen radicals and this effect can

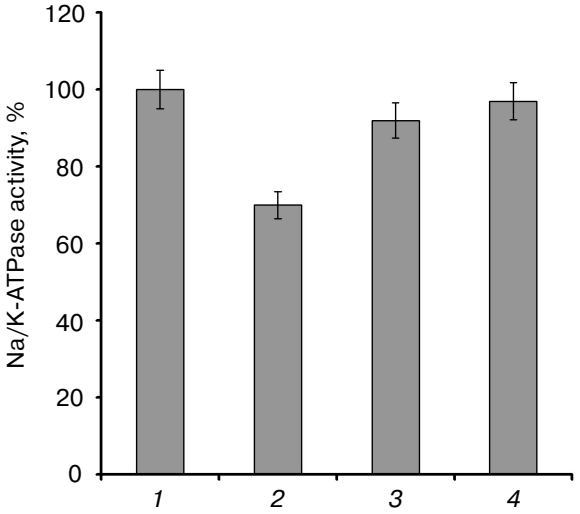

Fig. 2. Activity of Na/K-ATPase in cerebellum granule cells measured after their incubation under different conditions: *1*) control; *2*) 30 min after incubation with 1 mM glutamate; *3*, *4*) the same as *2* with addition of 10 μM MCPG or MK-801 (% of control).

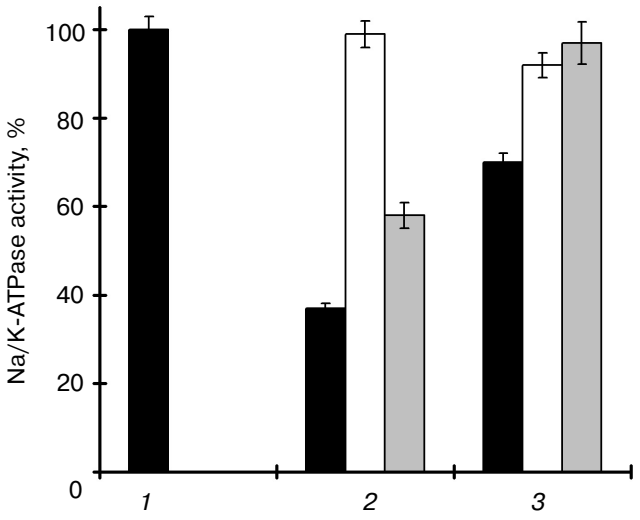

Fig. 3. Activity of Na/K-ATPase in cerebellum granule cells (% of control, *1*) in the presence of 1 mM NMDA (*2*) or 300 μM ACPD (*3*); black bars, initial activity; white bars, in the presence of 10 μM subsequent antagonist (MK-801 (*2*) or MCPG (*3*)); gray bars, in the presence of 10 μM chelerythrine.

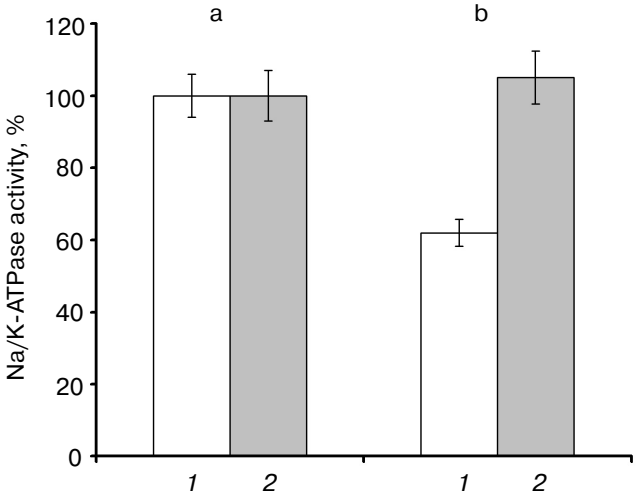

Fig. 4. Na/K-ATPase activity in control (a) and NMDA treated cerebellum cells (exposed to 1 mM NMDA during 30 min) (b) measured before (1) and after (2) addition to the samples of 1 mM cysteine.

be reversed by SH-group reducers like cysteine or dithioerythritol, DTT [15, 16]. We have examined such possibility by measuring the effect of cysteine on Na/K-ATPase after its inhibition by NMDA. The experiments were done in the following manner. Cerebellar granule cells were incubated for 30 min with 1 mM NMDA, after that the suspension was subjected to freezing–thawing and 5 min before Na/K-ATPase measuring 1 mM cysteine was added to the samples. Addition of cysteine was found to reverse the inhibiting effect of NMDA (Fig. 4). Thus, we conclude that inhibition of the ATPase by incubation of the cells with NMDA is directly connected with rise in reactive oxygen species and oxidative modification of protein SH-groups. The total restoration of the activity by cysteine addition can be explained by its possible action on other SH-group containing proteins, for example NMDA-receptors, which are known to be regulated by sulfur-containing excitotoxic amino acids [17].

The results presented demonstrate that glutamate induces in cerebellum granular cells a receptor-dependent suppression of Na/K-ATPase using two different mechanisms of inhibition: one connected with ionotropic receptors and realized predominantly via oxidative modification of ATPase and another via metabotropic receptors, protein kinase C, and regulatory phosphorylation of the enzyme.

These mechanisms may partially overlay each other because activation of ionotropic receptors results in both increase in free radicals production and calcium ion entry into the cell [11, 18], which, in turn, activates protein kinase C. At the same time, a number of protein kinase activated reactions can generate reactive oxygen species and related compounds (superoxide anion, hydrogen peroxide, etc.), which may additionally inhibit Na/K-ATPase.

It is important to stress that in the experiments on cerebellum granule cells isolated from young animals we did not observe activating effect of glutamate (from 100 to 1000 μM) on Na/K-ATPase, in contrast with that described on mature neurons cultured [19] or microsomes prepared from brain of adult animals [7]. In the latter case, activation of Na/K-ATPase was observed at low (100 μM) and inhibition at high (250-1000 μM) concentrations of the neuromediator. This discrepancy may be possibly addressed to the age and morphological features of preparations studied, for example with increased activity of calcineurin possessing activity of Ca-dependent protein phosphatase, which can restore Na/K-ATPase activity after increase in concentration of calcium in neuronal cells activated by glutamate [19].

We must emphasize that both modes of Na/K-ATPase inhibition described in this study (oxidative modification and regulatory phosphorylation) are reversible and can provide native ways of its regulation in the living neurons.

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