Biochemistry (Moscow), Vol. 69, No. 4, 2004, pp. 429-434. Translated from Biokhimiya, Vol. 69, No. 4, 2004, pp. 530-536.

Originally published in Biochemistry (Moscow) On-Line, Papers in Press, as Manuscript BM03-157, February 29, 2003.

Functional Relationship between Na/K-ATPase and NMDA-Receptors in Rat Cerebellum Granule Cells

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Received June 19, 2003
Revision received September 17, 2003

Abstract—Activation of rat cerebellum granule cells by N-methyl-D-aspartate (NMDA, 10^{-4}-10^{-3} M) results in progressive increase in reactive oxygen species (ROS) and suppression of the ouabain-sensitive part of Na/K-ATPase activity. When Na/K-ATPase was inhibited by high ouabain concentrations (10^{-5}-$5 \cdot 10^{-4}$ M), an increase in stationary ROS level in neuronal cells was noted, this effect being attenuated by NMDA antagonists, MK-801 and D-AP5. It is concluded that in cerebellum neurons, ouabain-resistant Na/K-ATPase is responsible for suppression of intracellular level of ROS, which, in turn, inhibit ouabain-sensitive Na/K-ATPase.

Key words: reactive oxygen species (ROS), Na/K-ATPase, glutamate receptors, NMDA

Reactive oxygen species (ROS) play in brain neurons the role of second messengers transferring information from the cellular membrane to intracellular metabolic processes [1, 2]. When neuronal cells were studied using flow cytometry the incubation of cerebellum granular cells with glutamate agonist N-methyl-D-aspartic acid (NMDA) was found to result in intracellular accumulation of ROS [3-5]. Na/K-ATPase of neuronal membranes is one of the targets for ROS accumulated in neurons under conditions of oxidative stress [6]. In this work, we have attempted to estimate the interaction between NMDA-receptors and Na/K-ATPase and to characterize the effect of this enzyme on the ROS level in glutamatergic neurons.

MATERIALS AND METHODS

In this study, the interaction between NMDA-receptors and Na/K-ATPase in suspension of neurons prepared from cerebellum of 10-12-day-old rats was studied. Na/K-ATPase activity and level of ROS produced as a result of activation of the cells with NMDA were simultaneously measured. The protocol for preparation of neurons and their characterization have been described elsewhere [5, 6]. Tyrode solution containing 148 mM NaCl, 5 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, and 10 mM glucose (pH 7.3) was used as a medium for preparation and storage of the neuronal cells. Neurons were loaded with fluorescent dyes for 30 min immediately after their preparation [6, 7]. All experiments were done at 37°C. For measuring ROS, dihydrorhodamine 123 (DHR) or 2′,7′-dichlorodihydrofluorescein diacetate (DCF) were used and the ROS levels were measured by spectrofluorimetry (Perkin Elmer LS50B, USA) or flow cytometry (FACStar, Becton Dickinson, USA) [7]. Dead (necrotic) cells in the neuronal suspension were determined with propidium iodide (PI); in different experiments their amount was between 12-18% and did not changed significantly during the experimental procedure. In the tables and figures, specific (receptor mediated) effects of the ligands studied were presented, which were measured after 30 min preincubation of neuronal suspension with the relevant ligand. Nonspecific effect of NMDA on Na/K-ATPase was calculated from experiments where the ligand was added to suspension of neurons disrupted by 5-fold freezing-thawing [6]. Na/K-ATPase activity was measured by inorganic phosphate liberation after incubation of cells disrupted by the abovementioned procedure in the presence of 3 mM MgATP, 130 mM NaCl, and 20 mM KCl (so-called total ATPase) or in the presence of 150 mM KCl alone (Mg-dependent ATPase). Na/K-ATPase was calculated as a difference between these values. Its activity was about 100-125 nmol P_i/min per mg protein.

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The results were statistically analyzed using the computer program "Biostatistika", and the curves of ouabain inhibition of Na/K-ATPase were analyzed using computer program "Origin".

RESULTS AND DISCUSSION

Increasing the NMDA concentration in the incubation medium from 0.1 to 1 mM results in a dose-dependent increase in ROS measured in the neuronal suspension by both steady-state level of DHR fluorescence (Table 1) and DCF fluorescence of single neurons using flow cytometry (Fig. 1, black bars). Addition of 10 μM NMDA antagonist MK-801, which prevented operation of ion channels that are dependent on NMDA-receptors, simultaneously suppressed accumulation of ROS; this effect of MK-801 was demonstrated by two methods (Fig. 1 and Table 1).

One can also see from Fig. 1 (gray bars) that activation of neurons by increased concentrations of NMDA resulted in progressive inhibition of Na/K-ATPase (to 50% of the initial enzyme activity at 0.5 mM NMDA). This inhibiting effect was also prevented by addition of MK-801. Ten-minute incubation of neurons activated by NMDA with 1 mM cysteine totally restored the activity of Na/K-ATPase.

Comparison of dose-dependence of both processes demonstrated that inhibition of Na/K-ATPase by NMDA reached its maximum at 0.5 mM NMDA while further increase in ROS signal was registered when NMDA concentration continued to increase (Fig. 1). This means that the rest of Na/K-ATPase molecules operating in the presence of 0.5 mM (or higher concentrations) NMDA are not sensitive to ROS.

It is known that brain tissues contain several isoforms of Na/K-ATPase differing in number and location of SH-groups in the α-subunit. One of the isoforms (α1) is relatively resistant to ouabain and more stable against free radical oxidation; other isoforms (α2 + α3) are more sensitive to ouabain, contain easily oxidized SH-groups, and are modified by ROS more easily [8, 9]. While in brain ontogeny (α2 + α3)-isoforms appear later than α1, all three isoforms of Na/K-ATPase are revealed in the brain of 10-12-day-old rats [10]. Based on these facts one can suggest that the portion of Na/K-ATPase that is oxidized by ROS on activation of NMDA-receptors is represented mainly by (α2 + α3)-isoforms because this activity is suppressed by ROS and restored by cysteine. Accessibility of this portion of Na/K-ATPase to ROS generated when neurons were activated by NMDA suggested a possible physiological significance of free radical regulation of the Na-pump.

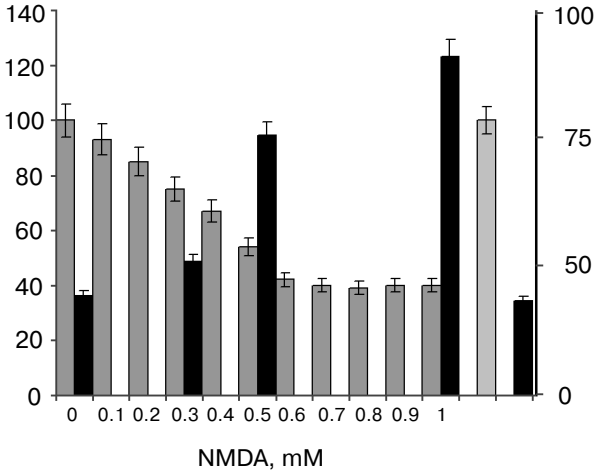

Fig. 1. Effect of NMDA on Na/K-ATPase activity (nmol P_i/min per mg protein) (ordinate, left) and ROS level (mean fluorescence value measured using flow cytometry, arbitrary units) (ordinate, right) in cerebellum granule cells. Freshly prepared neurons were exposed to different NMDA concentrations for 30 min, then the cells were broken and used to measure Na/K-ATPase activity (gray bars); in parallel samples, neurons preloaded with DCF were used to measure ROS by flow cytometry technique (black bars). The last two bars to the right correspond to Na/K-ATPase activity measured after addition of 1 mM cysteine and ROS level measured in the presence of 1 mM NMDA and 10 μM MK-801.

Recently, increase in the ROS level in myocyte culture was demonstrated as a result of addition to the medium of 10^{-5}-10^{-4} M ouabain [11]. As seen from Table 2, ouabain also increased the intracellular ROS in suspensions of single neuronal cells; this effect was clearly dose dependent. The effect of ouabain on the intracellular ROS level starts to be visible from concentration higher than 10^{-5} M and underwent further increase at 10^{-4} M. Moreover, it is attenuated by the NMDA antagonist MK-801, which is known to inactivate ion channels associated with NMDA-receptors, or D-AP5 (D-(−)2-amino-5-

Table 1. ROS level (arbitrary units, m ± S.D.) in cerebellum neurons measured by stationary fluorescence spectroscopy after 30-min incubation with different concentrations of NMDA

NMDA, μM	50	100	250	500	1000	
					− MK-801	+ MK-801
DHR fluorescence, arbitrary units	120 ± 1	170 ± 3	168 ± 5	215 ± 3	237 ± 4	128 ± 3

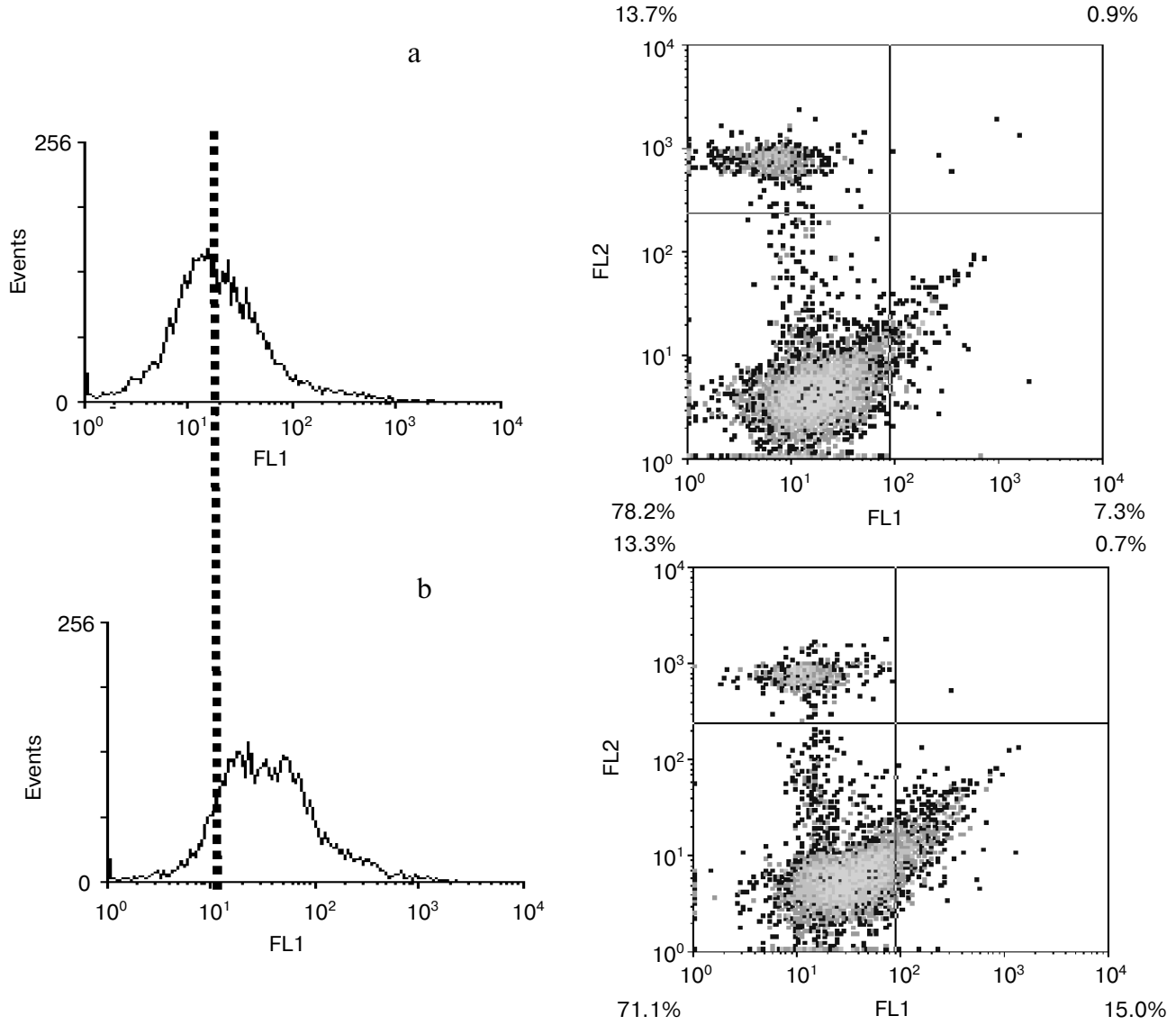

Fig. 2. Ouabain increases ROS production in suspensions of cerebellum granule cells. a) Mean DCF fluorescence (MF) measured by peak position (left, MF = 20.1), and distribution of neuronal population between viable cells labeled with DCF (right, FL1 axis, lower left and right quadrants) and necrotic, PI-labeled cells (FL2 axis, upper left and right quadrants) (control); b) the same after 30-min incubation with 0.1 mM ouabain (left, MF = 35.3). At the corner of each quadrant, % of cells in it is noted. Left side of the figure: the ordinate corresponds to the number of cells measured (events), the abscissa to DCF fluorescence (FL1) in logarithmic scale; the vertical line notes the position of the maximum (MF) and its shift after ouabain treatment. Right side of the figure: ordinate (FL2) corresponds to PI fluorescence, abscissa (FL1) to DCF fluorescence.

phosphonopentanoic acid), which prevents NMDA binding to its receptors.

This effect was carefully analyzed using flow cytometry and DCF as an intracellular ROS marker. It was demonstrated that 30 min incubation of neurons with 100 μM ouabain resulted in increase of ROS signal by 75% (from 20.1 ± 2.4 to 35.3 ± 2.8 arbitrary units) and in simultaneous appearance of neuronal population with extremely high ROS fluorescence (Fig. 2). At the same time, the portion of dead neurons labeled with PI was unchanged during the whole time of the experiment. The concentration dependence of the ouabain effect was found to be similar to that found earlier with DHR in the measurements of stationary fluorescence (see Table 2).

The effect of 100 μM ouabain on the intracellular ROS level was not additive to that of 500 μM NMDA, which also resulted in appearance of a cell population with very high DCF fluorescence; these data in addition to the dependence of the ouabain effect on NMDA antagonists MK-801 and D-AP5 suggested functional connections between NMDA-receptors and Na/K-ATPase.

Table 2. Concentration dependence of ouabain effect on ROS generation (arbitrary units) in cerebellum neurons under different conditions (measurements were done after 10-min incubation with ouabain of neuronal suspension pre-loaded with DHR)

Ouabain, M	No additions	+ D-AP5, 10 μM
No additions	0	0
$1 \cdot 10^{-6}$	0	0
$1 \cdot 10^{-5}$	26 ± 4	8 ± 3
$1 \cdot 10^{-4}$	66 ± 3	31 ± 4
$2.5 \cdot 10^{-4}$	103 ± 5	82 ± 3
$5 \cdot 10^{-4}$	263 ± 7	94 ± 5

Table 3. $K_{0.5}$ values obtained for ouabain inhibition of Na/K-ATPase under different conditions (the correlation coefficient for the curves used to calculate $K_{0.5}$ was 0.992)

Na/K-ATPase source	$K_{0.5}$, M	
	ouabain-sensitive Na/K-ATPase	ouabain-resistant Na/K-ATPase
Microsomes from adult rat brain	$1.1 \cdot 10^{-7}$	$1.3 \cdot 10^{-4}$
Cerebellum neurons from 12-day-old rats	$2.2 \cdot 10^{-7}$	$1.9 \cdot 10^{-4}$
The same after 30-min incubation with 0.5 mM NMDA	$2.9 \cdot 10^{-6}$	

As we noted earlier, in vertebrate brain two enzyme populations are present, being characterized by different affinity to cardiac glycosides. Ouabain-resistant ATPase (α1-isoform) interacts with these drugs in the range of 10^{-6}-10^{-3} M, whereas for ouabain-sensitive (α2 + α3)-isoforms this range lies between 10^{-9}-10^{-6} M [10, 12]. These data are in good correlation with the biphasic curve of inhibition of Na/K-ATPase by ouabain (Fig. 3). Comparison of the dependence of Na/K-ATPase activity on increased ouabain concentrations measured in neuronal suspension before and after its 30-min incubation with 0.5 mM NMDA revealed specific change in the shape of the inhibiting curve. In the case of control (not activated neurons), the curve has again a biphasic character corresponding to two populations of the enzyme with high and low affinity to the cardiac glycoside. After 30-min activation of the neurons with NMDA the curve transforms into a monotonous one and the inhibition starts from higher concentrations of ouabain (Fig. 3). One can suggest that pre-incubation of the cells with NMDA results in preferable inhibition of the ouabain-sensitive isoforms of Na/K-ATPase.

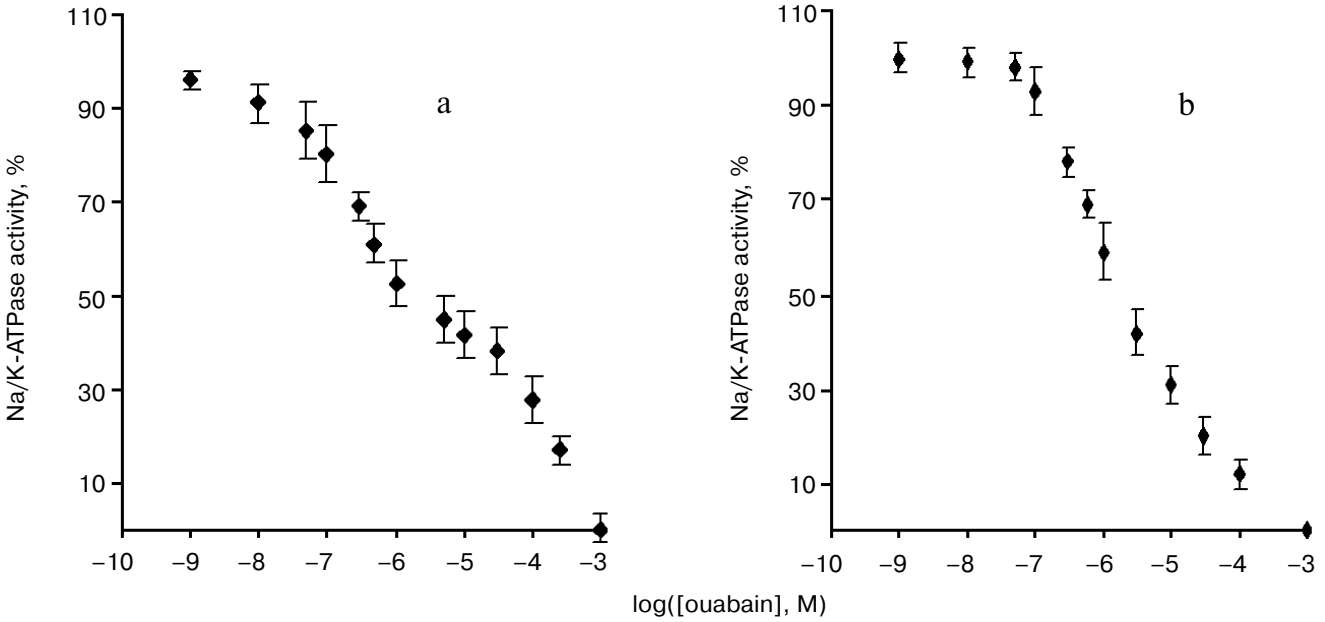

Fig. 3. Dependence of Na/K-ATPase activity of control (a) and NMDA-treated neurons (b) on ouabain concentration (measured after 30-min incubation of neuronal cells with 0.5 mM NMDA).

The interaction of ouabain with both populations of Na/K-ATPase in the neurons prepared from the brain of 12-day-old animals as well as from that of adults was described well by a biphasic curve with an intermediary plateau (correlation coefficient 0.993). The same dependence for the 12-day-old brain neurons after their activation with 0.5 mM NMDA was described by a monotonous curve with correlation coefficient 0.992. Ouabain concentrations inducing half-maximal effect ($K_{0.5}$) on each Na/K-ATPase population are presented in Table 3.

As seen from Table 3, these values for the adult and 12-day-old animals are similar and in both cases, two Na/K-ATPase populations are found differing in their sensitivity to ouabain. After activation of the neurons with NMDA and subsequent transformation of the biphasic curve into the monophasic one, the $K_{0.5}$ corresponds to an intermediate value ($2.9 \cdot 10^{-6}$ M), thus demonstrating that sensitivity of the rest of the ATPase to ouabain is modified by NMDA. The indirect data suggest that this resting activity is represented by an ouabain-resistant α1-isoform. This isoform is more stable against oxidative modification [9], and after such modification enzyme activity can be restored by 1 mM cysteine. Moreover, activation of NMDA-receptors is known to result in calcium ion entry into the intraneuronal space and in activation of protein kinase C (PKC) [2]. Na/K-ATPase is one of the primary targets for this kinase, the ouabain-resistant α1-isoform being more sensitive to PKC. It is known that ouabain sensitivity of Na/K-ATPase is increased after its regulatory phosphorylation [13], which was if fact demonstrated in our experiments (see Table 3).

The mode of control of ROS formation by Na/K-ATPase is not yet clear. Attenuation of the ouabain effect by NMDA antagonists MK-801 and D-AP5 suggests participation of NMDA-receptors in this process. The relatively high concentration of ouabain inducing ROS generation (10^{-5} M or more) pointed out participation of the α1-isoform in this process. Thus, NMDA-receptors and Na/K-ATPase interact functionally, the latter being involved in signaling mechanisms of interneuronal transduction [14, 15].

It was demonstrated recently that endogenous ouabain-like cardiac glycosides existing in animal tissues can regulate Na/K-ATPase activity [16]. This is in good correlation with the physiological significance of the described effect directed to regulation of intraneuronal ROS level.

It is known that oxidative damage to glutamatergic brain regions results in long-term activation of glutamate receptors by the mediator released from damaged cells and in development of oxidative stress, namely because of hyperactivation of NMDA-receptors [17]. We have demonstrated that 30-min activation of NMDA-receptors and subsequent rise in intracellular ROS results in inhibition of ouabain-sensitive Na/K-ATPase. At the same time, an ouabain-resistant population of the enzyme limits the ROS generation, presumably via NMDA-receptors. The functional relation between Na/K-ATPase and NMDA-receptors is also supported by the recent demonstration that endogenous ouabain-like glycoside prevents interaction of MK-801 with NMDA-receptors [18]. This relation is under careful analysis in many laboratories [6, 7, 19]. Protein kinases of the PKC family may possibly be involved in these interactions. As recently demonstrated the PKC inhibitors indolyl maleimide and chelerithrine prevented inhibition of cerebellum granule cell Na/K-ATPase induced by activation of the glutamate receptors of these neurons [6].

The data obtained show that Na/K-ATPase in neuronal cell is important not only to support asymmetric distribution of the ions across the neuronal membrane, but also to regulate the intracellular ROS level and to protect neurons against oxidative stress. One indirect evidence on the importance of the Na-pump to support oxidative stability of neurons is a well-known phenomenon of toxic influence of ouabain on brain. As demonstrated recently, such action of ouabain does not result from membrane depolarization or dissipation of ionic gradient but does reflect participation of Na/K-ATPase in antioxidant defense of the neurons [20]. In this article, the native mechanism is described, which may explain participation of this enzyme in protection of neurons against oxidative damage.

This work was supported by the German Scientific Foundation DAAD (grant No. S-325) and Russian Governmental Science Programs (grant Nos. 03-04-48767, 03-04-47947, and 1760.2003.4).

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