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Na^+/Ca^{2+} Exchange and Regulation of Cytoplasmic Concentration of Calcium in Rat Cerebellar Neurons Treated with Glutamate

T. P. Storozhevykh*, E. G. Sorokina, A. V. Vabnitz, Ya. E. Senilova, G. R. Tukhbatova, and V. G. Pinelis

Research Center for Children's Health, Russian Academy of Medical Sciences, Lomonosovskii pr. 2/62, 119991 Moscow, Russia; fax: (495) 134-1308; E-mail: tstor@mail.ru

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Abstract—In the present work, the forward and/or reversed Na^+/Ca^{2+} exchange in cerebellar granular cells was suppressed by substitution of Na^+_o by Li^+ before, during, and after exposure to glutamate for varied time and also using the inhibitor KB-R7943 of the reversed exchange. After glutamate challenge for 1 min, Na^+_o/Li^+ substitution did not influence the recovery of low $[Ca^{2+}]_i$ in a calcium-free medium. A 1-h incubation with 100 μM glutamate induced in the neurons a biphasic and irreversible $[Ca^{2+}]_i$ rise (delayed calcium deregulation (DCD)), enhancement of $[Na^+]_i$, and decrease in the mitochondrial potential. If Na^+_o had been substituted by Li^+ before the application of glutamate, i.e. the exchange reversal was suppressed during the exposure to glutamate, the number of cells with DCD was nearly fourfold lowered. However, addition of the Na^+/K^+-ATPase inhibitor ouabain (0.5 mM) not preventing the exchange reversal also decreased DCD in the presence of glutamate. Both exposures decreased the glutamate-caused loss of intracellular ATP. Glucose deprivation partially abolished protective effects of the Na^+_o/Li^+ substitution and ouabain. KB-R7943 (10 μM) increased 7.4-fold the number of cells with the $[Ca^{2+}]_i$ decreased to the basal level after the exposure to glutamate. Thus, reversal of the Na^+/Ca^{2+} exchange reinforced the glutamate-caused perturbations of calcium homeostasis in the neurons and slowed the recovery of the decreased $[Ca^{2+}]_i$ in the post-glutamate period. However, for development of DCD, in addition to the exchange reversal, other factors are required, in particular a decrease in the intracellular concentration of ATP.

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The Na^+/Ca^{2+} exchanger of plasma membrane (PM) is fundamentally important for regulation of intracellular concentrations of Na^+ ($[Na^+]_i$) and Ca^{2+} ($[Ca^{2+}]_i$) in the central nervous system under both normal (release of neurotransmitters, cell migration and differentiation) and pathologic conditions (neurodegeneration, glutamate neurotoxicity) [1, 2]. In brain neurons three isoforms of K^+-independent exchangers (NCX1-3) and three isoforms of K^+-dependent exchangers (NCKX2-4) have been found, the expression of which in culture depends on $[Ca^{2+}]_i$ and activity of protein phosphatases, in particular, calcineurin [3, 4]. The expression of these isoforms is the most pronounced in nerve cell loci where $[Ca^{2+}]_i$ is markedly changing, e.g., near pre- and postsynaptic membranes [5]. Stimulation of glutamate receptors induces in neurons a transitory calcium signal, which is mainly caused by the Ca^{2+} entry into the cells via the N-methyl-D-aspartate (NMDA) subtype of glutamate channels. However, an excess exposure to glutamate, e.g., on disorders of its reversed uptake by nerve cells under conditions of brain ischemia/hypoxia, resulted in irreversible elevation of cytosolic $[Ca^{2+}]_i$ (the so-called delayed calcium deregulation (DCD)) and death of the neurons [6-9]. The literature data on the role of Na^+/Ca^{2+} exchange in maintenance of the basal level of $[Ca^{2+}]_i$ and its recovery after the exposure to glutamate

Abbreviations: $[Ca^{2+}]_i$, $[Na^+]_i$, $[Ca^{2+}]_o$, $[Na^+]_o$) intracellular and outer concentrations of Ca^{2+} and Na^+; DCD) delayed calcium deregulation; DNP) dinitrophenol; KB-R7943) 2-2-[4-(4-nitrobenzyloxyphenyl]ethylisothiourate methanesulfonate; NMDA) N-methyl-D-aspartate; PM) plasma membrane; $\Delta\Psi_m$) mitochondrial membrane potential.

* To whom correspondence should be addressed.

are rather contradictory. Inhibition of Na^+_o/Ca^{2+}_i exchange by substitution of extracellular Na^+_o by organic cations or Li^+, which are unable to bind with the transport site of the exchanger and, thus, be replaced by Ca^{2+}_i or Ca^{2+}_o [10], caused an increase in the basal $[Ca^{2+}]_i$ and/or deceleration of its recovery after activation of hippocampal neurons, *nucleus basalis*, Purkinje cells, and granular cells of the cerebellum in works [11-18], while no effect was recorded in works [19-22]. Inhibition of the exchanger by synthetic compounds, such as bepridil [18, 23], KB-R7943, SEA0400, amiloride derivatives, etc., also did not clarify this question.

Thus, in the culture of hippocampal and cortical neurons or hippocampal slices the inhibitor KB-R7943 of reversed Na^+/Ca^{2+} exchange decreased the calcium deregulation of neurons caused by glutamate or hypoxia/hypoglycemia and their death [24-26]. In contrast, in [27, 28] inhibition of the exchanger by amiloride or SEA0400, its proteolysis, or suppression of expression aggravated the damage of the brain and neurons in both *in vitro* and *in vivo* models of ischemia. These contradictions might be explained by the ability of the exchanger to transfer Ca^{2+} in a bidirectional way: the so-called *forward* (replacement of one Ca^{2+}_i by three Na^+_o and decrease in $[Ca^{2+}]_i$) and *reversed* (replacement of three Na^+_i by one Ca^{2+}_o and increase in $[Ca^{2+}]_i$) [1, 10]. The electrogenic movement of Na^+ and Ca^{2+} (outward or inward to the cell) mainly depends on the transmembrane gradient of Na^+ concentrations and membrane potential, and to activate the exchange it is necessary to increase $[Ca^{2+}]_i$ and bind Ca^{2+}_i with the intracellular regulatory site of the exchanger [1, 10]. Therefore, the effect of inhibition of Na^+/Ca^{2+} exchange depends on the direction of Ca^{2+} transfer: into the cell or out of it. During glutamate challenge, a rapid reversal of the exchange is possible because not only Ca^{2+} but also Na^+ enters the neurons via glutamate receptor channels and causes depolarization of the PM [18, 29].

The purpose of the present work was to clarify by what mode, forward or reversed, the Na^+/Ca^{2+} exchanger operates in cerebellar neurons before, during, and after exposure to glutamate for varied times, or, in other words, whether this exchange protects the cells or aggravates their damage by glutamate. To answer the question, the Na^+/Ca^{2+} exchange was inhibited differently: i) replacing Na^+_o by Li^+ before, during, and after the exposure to glutamate; ii) pharmacologically, using the inhibitor KB-R7943 of the reversed Na^+/Ca^{2+} exchange. The Na^+/Ca^{2+} exchanger was shown to be not involved in the maintenance of the basal $[Ca^{2+}]_i$ level and releasing Ca^{2+} from the cells after both short- and long-term toxic action of glutamate. When functioning by the reversed mode, the exchanger promotes an increase in $[Ca^{2+}]_i$ during the exposure to glutamate and slows the recovery of $[Ca^{2+}]_i$ homeostasis in the post-glutamate period. However, for emergence of DCD, in addition to the exchange reversal and high $[Ca^{2+}]_i$, other factors are also required, including a decrease in the intracellular concentration of ATP.

MATERIALS AND METHODS

In the experiments we used a seven-nine-day-old primary culture of granular cells from the cerebellum isolated from one-week-old Wistar rats (the culture preparation is described in detail in [30]). $[Ca^{2+}]_i$ and $[Na^+]_i$ and mitochondrial membrane potential ($\Delta\Psi_m$) were measured using a system of image analysis consisting of an Axiovert-200 inverted fluorescence microscope (Zeiss, Germany), wheel with light filters, CCD chamber (SnapCool-fx, USA), and Metafluor software (Universal Imaging Corp., USA). The neurons were incubated in a cultural medium with corresponding fluorescence probes: high affinity Fura-2 (K_d = 225 nM) or low affinity Fura-2FF (K_d = 5 μM) to measure $[Ca^{2+}]_i$ (5 μM, 40 min), SBFI to measure $[Na^+]_i$ (8 μM, 1 h), and Rh123 to record $\Delta\Psi_m$ (3 μM, 15 min). Then the culture medium was replaced by a salt buffer (pH 7.4) of the following composition (in mM): NaCl (130), KCl (5.6), $CaCl_2$ (1.8), $MgCl_2$ (1.0), HEPES (20), glucose (5); the glass with the cells was placed into a 0.2-ml perfusion chamber mounted on the microscope table. The solutions in the perfusion chamber were changed several times for each exposure of the cells. The experiments were performed at 23-25°C. In solutions without Na^+, NaCl was fully substituted by 130 mM LiCl (pH of the solution was adjusted to 7.4 with LiOH). For excitation of Fura-2, Fura-2FF, and SBFI, light filters were used with the wavelength of 340 and 380 nm, and for Rh123 a filter with 490 nm was used (illumination for 20-100 msec, the dark phase for 10-20 sec). The emission filter passed the spectrum of 505 ± 15 nm for Fura-2, Fura-2FF, and SBFI and 535 ± 10 nm for Rh123. The fluorescence ratio F_{340}/F_{380} characterized changes in $[Ca^{2+}]_i$ or $[Na^+]_i$ in individual cells. To determine absolute values of $[Na^+]_i$, on termination of the experiments the cultures were successively incubated in solutions (pH 7.2) with the Na^+ concentration from 0 to 120 mM (with equimolar substitution with potassium gluconate) (in mM): gluconate (120), Cl^- (20), Ca^{2+} (1.8), Mg^{2+} (1.0), HEPES (20). These solutions also contained ouabain (200 μM) to suppress the Na^+/K^+-ATPase activity of PM and the Na^+/H^+ ionophore monensin (10 μM) [18, 29]. Changes in $\Delta\Psi_m$ were expressed in percent relative to the baseline fluorescence of Rh123 taken as 100%.

To determine the ATP level, the cells were planted into 24-well plates. Seven-eight days later, the culture was washed twice with salt buffer at room temperature and incubated for 1 h with glutamate under conditions prescribed by the protocol. ATP was extracted with 2% trichloroacetic acid cooled to 0°C in the presence of 2 mM EDTA, neutralized with solution of 3 M KOH/1.5 M Tris, and centrifuged at 3000 rpm. In

aliquots of the supernatant, the level of ATP was determined by chemiluminescence using luciferin–luciferase (40 μl into each sample, Labsystems analytical kit) with a Lucy-1 luminometer (Antos, Austria). No less than three determinations were performed for each specimen in five-six independent experiments. The content of ATP was expressed in % relative to the control taken as 100%.

The resulting images were processed using the MetaFluor Analyst Program (USA) and the Prizma Program, with Student's *t*-test. The probes and reagents were from Sigma, Labsystems, Invitrogen, and Fluka (USA).

RESULTS

Effect of inhibition of Na_o^+/Ca_i^{2+} exchange on basal level of $[Ca^{2+}]_i$ and its recovery after short-term exposure to glutamate. The substitution of Na_o^+ with Li^+ in 92% of the neurons (n = 218 cells, nine independent experiments) did not affect the basal level of $[Ca^{2+}]_i$, and in the other cells there was a weak and transitory elevation of $[Ca^{2+}]_i$ (not shown). These data are consistent with current concepts and can be explained by a low affinity of the exchanger for intracellular Ca^{2+}: K_m varies from 0.2 to 2 μM Ca^{2+} [1, 31].

In the experiments presented in Fig. 1a the cells were several times supplemented with 20 μM glutamate + 2 μM glycine (without Mg^{2+}) for 100 sec. After every challenge, the cells were washed in a calcium-free solution containing calcium buffer EGTA (100 μM) to prevent a possible secondary entry of Ca^{2+} into the neurons by reversed Na_i^+/Ca_o^{2+} exchange or via NMDA channels. All solutions contained the inhibitor cyclopiazonic acid (10 μM) of the endoplasmic reticulum Ca^{2+}-ATPase. To quantitatively characterize the recovery of the basal $[Ca^{2+}]_i$, we determined the time (t) of the ratio F_{340}/F_{380} lowering to 50% of the maximal level achieved on exposure to glutamate (Fig. 1b). In the control (the first and third glutamate challenge, Fig. 1a) t was, on average, 14.5 ± 3.6 sec (Fig. 1b, n = 96 cells, four independent experiments). The recovery of $[Ca^{2+}]_i$ upon substitution of Na_o^+ with Li^+ in the post-glutamate period was virtually the same as the recovery in the control (t = 12.8 ± 2.8 sec). Because the effect of the exchanger inhibition could be compensated by enhanced uptake of Ca^{2+} by mitochondria, the protonophore dinitrophenol (DNP, 300 μM) was added into the solution before the next applications of glutamate. DNP induced depolarization of the mitochondrial membrane and prevented the Ca^{2+} uptake by the mitochondria. DNP caused a slight increase in $[Ca^{2+}]_i$ as a result of Ca^{2+} release from the mitochondria and/or entry of external Ca_o^{2+}. The response to glutamate in the presence of DNP was, on average, 12% higher than in the control ($p < 0.05$). After the exposure to glutamate, $[Ca^{2+}]_i$ decreased nearly

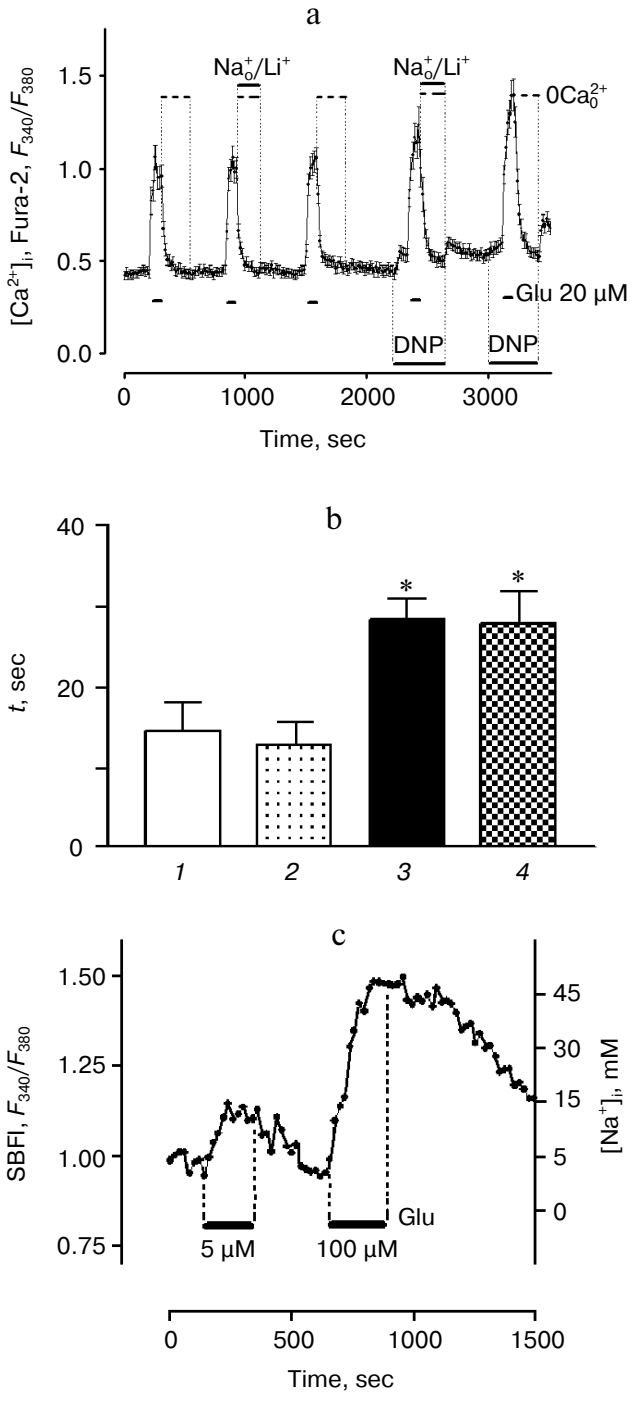

Fig. 1. a) Recovery of the basal $[Ca^{2+}]_i$ level in the cerebellar cells after short-term glutamate challenges (Glu, 20 μM). The figure presents the result of one experiment (n = 12 neurons, M ± m), and similar data were obtained in the other three independent experiments. b) The average time of decrease in F_{340}/F_{380} to 50% of the normal response to glutamate under the following conditions: *1*) control; *2*) Na_o^+/Li^+ substitution; *3*) control + DNP; *4*) Na_o^+/Li^+ substitution + DNP (n = 96 cells, M ± m, four independent experiments). * $p < 0.05$ with respect to the control. c) Increase in $[Na^+]_i$ in the cerebellar neurons in response to the 3-min application of glutamate (Glu) in the concentration of 5 and 100 μM. In the figure the results of one experiment are presented (n = 18, M ± m), similar data were obtained in two other independent experiments.

twofold more slowly than in the control (Figs. 1a and 1b), but this effect was similar in both the Na_o^+-containing medium and upon the Na_o^+/Li^+ substitution (t = 28.3 ± 2.6 and 27.7 ± 3.9 sec, respectively).

These data suggested that the decrease in $[Ca^{2+}]_i$ after the exposure to glutamate should be mainly caused by the mitochondrial uptake of Ca^{2+} and its ejection outwards by Ca^{2+}-ATPase of PM, while the exchanger was not involved in the Ca^{2+} *release*. In particular, this could be due to a rapid increase in $[Na^+]_i$ in the presence of glutamate which suppressed the Ca_i^{2+} exchange for Na_o^+. Figure 1c shows the change in $[Na^+]_i$ upon 3-min application of glutamate in two concentrations, 5 and 100 μM. The initial $[Na^+]_i$ was 6.7 ± 2.6 mM (n = 79 cells, three independent experiments). Glutamate at the concentration of 5 μM induced, on average, a 2.5-fold increase in $[Na^+]_i$ and at the concentration of 100 μM caused nearly an eightfold increase, to 49.3 ± 7.2 mM. It should be emphasized that $[Na^+]_i$ remained high for 5-10 min after the removal of glutamate from the solution (Fig. 1c).

Effect of inhibition of the forward and/or reversed Na^+/Ca^{2+} exchange on $[Ca^{2+}]_i$ during and after the toxic action of glutamate. In was earlier shown [7-9, 32] that a prolonged incubation of neurons with 100 μM glutamate induced an irreversible increase in $[Ca^{2+}]_i$ and $[Na^+]_i$ and a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$). Our experiments were designed to find out whether the Na^+/Ca^{2+} exchange in neurons was preferentially realized by the forward or reversed mode during and after the prolonged toxic action of glutamate.

Glutamate caused a biphasic $[Ca^{2+}]_i$ rise: after a slight primary increment of $[Ca^{2+}]_i$ in 71.7 ± 8.0% of the neurons a differently delayed secondary increase in $[Ca^{2+}]_i$ was recorded which was associated with a pronounced decrease in $\Delta\Psi_m$, i.e., the development of DCD (Fig. 2, a and b). Such neurons failed to recover the basal levels of $[Ca^{2+}]_i$ and $\Delta\Psi_m$ after the abolishment of glutamate. To elucidate, whether the secondary increase in $[Ca^{2+}]_i$ was caused by the external Ca_o^{2+} exchange for intracellular Na_i^+, we measured $[Na^+]_i$ in the presence of glutamate

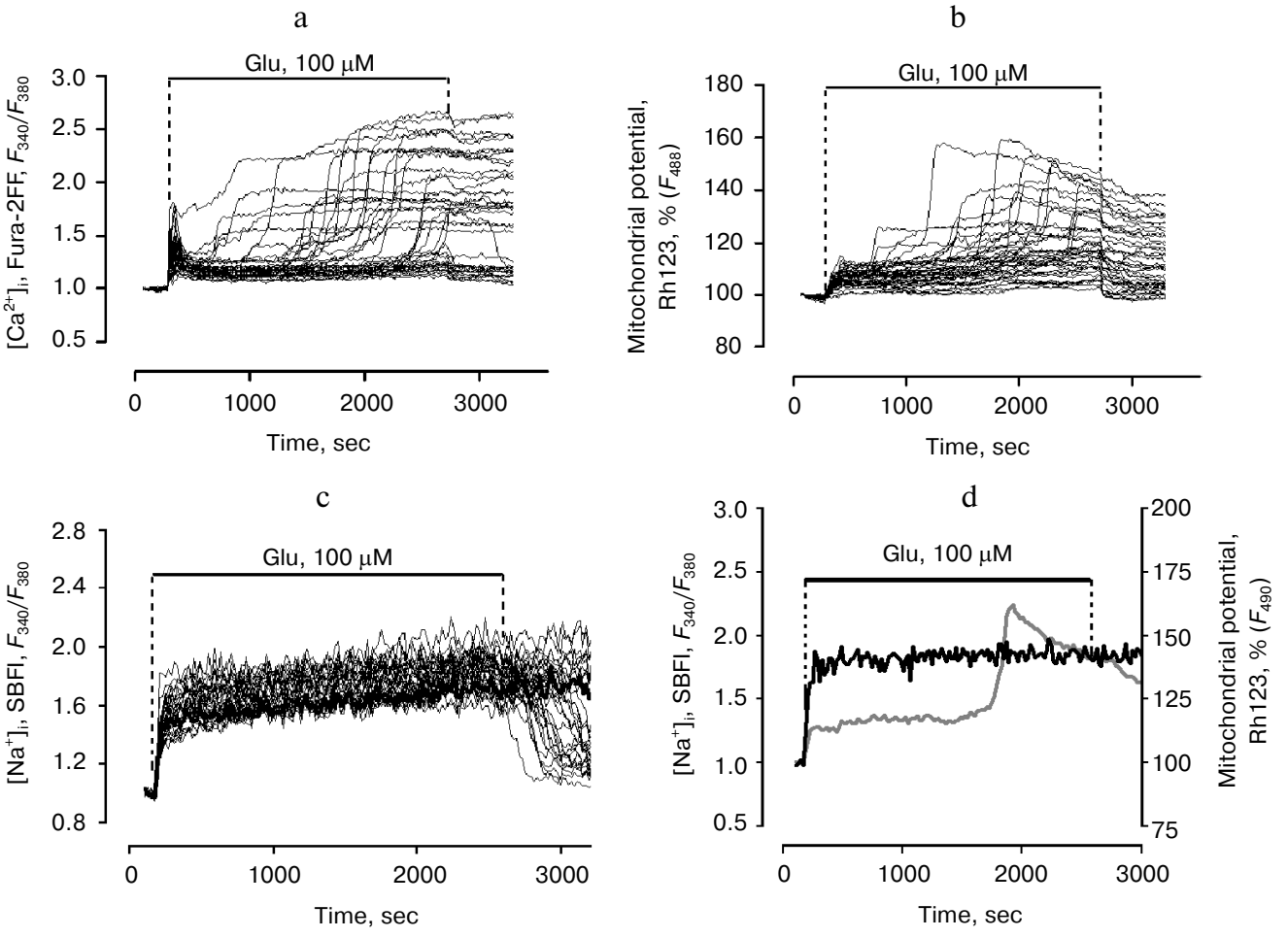

Fig. 2. Changes in $[Ca^{2+}]_i$ (a), mitochondrial potential $\Delta\Psi_m$ (b), and $[Na^+]_i$ (c) in individual neurons during and after the exposure to 100 μM glutamate (Glu). The enhanced fluorescence of Rh123 indicates a decrease in $\Delta\Psi_m$. $[Ca^{2+}]_i$ and $\Delta\Psi_m$ were measured concurrently in the same cells, while $[Na^+]_i$ was determined in the sister culture. $[Ca^{2+}]_i$ was recorded using the low affinity probe Fura-2FF. d) The biphasic change in $\Delta\Psi_m$ (gray line) in the neuron in response to glutamate is accompanied by a monophasic change in $[Na^+]_i$ (black line).

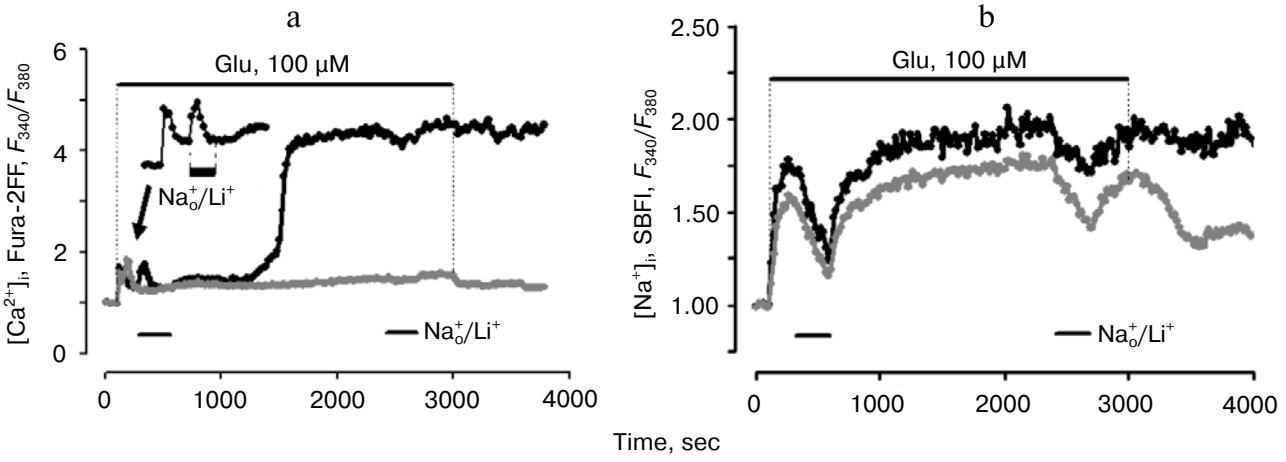

Fig. 3. Effect of 5-min Na_o^+/Li^+ substitution on changes in $[Ca^{2+}]_i$ (a) and $[Na^+]_i$ (b) in individual cerebellar cells during their incubation with glutamate. The black line shows the neuron with DCD, the gray line corresponds to the neuron without DCD.

(Fig. 2c, sister cultures). In all cells changes in $[Na^+]_i$ were monophasic, and the appearance of DCD was not accompanied by a decrease in $[Na^+]_i$, i.e. the release of Na^+ from the cells. In the post-glutamate period no recovery of $[Ca^{2+}]_i$, $[Na^+]_i$, and $\Delta\Psi_m$ took place in the overwhelming majority of the neurons with DCD (Fig. 2, a-c). When $[Na^+]_i$ and $\Delta\Psi_m$ were measured in the same cells (Fig. 2d), the decrease in $\Delta\Psi_m$ simultaneous with the emergence of DCD was not accompanied by pronounced changes in $[Na^+]_i$. These data suggested that the secondary increase in $[Ca^{2+}]_i$ should not be caused by the Ca^{2+} entry into the cells in exchange for intracellular Na^+. But the question of the contribution of the exchanger to disorders in the calcium homeostasis of neurons during the toxic action of glutamate is still open. Does the exchanger remove Ca^{2+} from the cells or promote its accumulation in the cytosol and mitochondria (the reversal of exchange)?

To elucidate whether the exchanger operates by the *forward* mode in the presence of glutamate, Na_o^+ was replaced by lithium for 5 min initially in the first phase of the $[Ca^{2+}]_i$ increase and then in the second phase (Fig. 3). This substitution, which inhibited the *forward* Na_o^+/Ca_i^{2+} exchange, caused no additional increase in $[Ca^{2+}]_i$, either in the first phase of the $[Ca^{2+}]_i$ rise or during the DCD. Figure 3a presents changes in $[Ca^{2+}]_i$ in two typical neurons, with DCD (black line) and without DCD (gray line) (altogether $n = 486$ cells, five independent experiments). In the first phase the inhibition of Na_o^+/Ca_i^{2+} exchange resulted in a slight transitory increase in $[Ca^{2+}]_i$ in 14% of the cells, and in the other cells $[Ca^{2+}]_i$ decreased or did not change. The substitution of Na_o^+ for lithium during the secondary increase in $[Ca^{2+}]_i$ either did not affect $[Ca^{2+}]_i$ or slightly decreased it. During the substitution of Na_o^+ by Li^+ in the solution, $[Na^+]_i$ was considerably decreasing in all cells, with DCD and without it (Fig. 3b, sister culture which was concurrently stained with Rh123 to follow the development of DCD). The Na^+ return into the solution was associated with increase in $[Na^+]_i$. The transitory increase in $[Ca^{2+}]_i$ and the concurrent decrease in $[Na^+]_i$ indicated that this increase was due to a transitory enhancement of the *reversed* Na_i^+/Ca_o^{2+} exchange. It is known that Li^+ not only inhibits the forward component of the exchanger but also strengthens the reversed one, because univalent cations ($Li^+ > K^+ = Rb^+ > NH_4^+$) enhance the affinity of the transport site of the exchanger for external Ca^{2+} [10]. In our experiments the increase in the reversal was transitory, obviously owing to the gradual decrease in the perimembrane $[Na^+]_i$ (Fig. 3b). If the $[Ca^{2+}]_i$ rise were caused by suppression of the Ca^{2+} *release*, the $[Ca^{2+}]_i$ rise would be steady.

Thus, the exchanger loses the ability to *release* Ca^{2+} from the cerebellar neurons during long-term exposure to glutamate. This conclusion is consistent with calculations of Czyz et al. [33] that the shift in equilibrium potentials on PM for Ca^{2+} and Na^+ during exposure to high concentrations of glutamate or NMDA inevitably results in the exchange reversal, notwithstanding a very high $[Ca^{2+}]_i$.

In the experiments presented in Fig. 4 we compared the development of DCD in experiments corresponding to three protocols (four-five independent experiments per protocol): in the first protocol the inhibitor KB-R7943 of the reversed exchange was used [34], in the second protocol Na_o^+ was replaced by Li^+ before addition of glutamate, and in the third protocol the glutamate-containing solution was supplemented with the inhibitor ouabain (0.5 mM) of PM Na^+/K^+-ATPase. The preliminary substitution of Na_o^+ by Li^+ prevented the glutamate-induced increase in $[Na^+]_i$ and, consequently, during the exposure to glutamate suppressed not only the *forward* Na_o^+/Ca_i^{2+} exchange but also the *reversed* Na_i^+/Ca_o^{2+} exchange. In contrast, ouabain preserved the high $[Na^+]_i$ during the exposure to glutamate and theoretically had to enhance

or, at least, not affect the reversed exchange. Figure 4 (a-d) shows the changes in $[Ca^{2+}]_i$ recorded under these conditions, and the table presents number of the neurons with DCD in percent of the total number of neurons in the image on the 10th and 40th min from the beginning of the glutamate action. KB-R7943 (10 μM) was added to the cells before introduction of glutamate (Fig. 4a) and also on the 10th min after it (Fig. 4b) to prevent a possible inhibition by this compound of the Ca^{2+} entry through the NMDA channels [35]. KB-R7943 did not affect the number of neurons with DCD but increased 7.4-fold ($p < 0.001$ with respect to glutamate) the number of neurons which succeeded in recovery of the basal $[Ca^{2+}]_i$ in the post-glutamate period (compare Figs. 2a and 4a). The removal of external Na^+ decreased the number of neurons with DCD nearly fourfold, to 15.8 ± 4.9% (Fig. 4c and table). Against the expectation, ouabain also lowered the number of such neurons 1.5-fold, as compared to the number of the cells with DCD exposed only to glutamate (Fig. 4d and table).

Thus, the KB-R7943-induced suppression of the exchange reversal did not decrease the number of neurons with DCD, whereas the Na^+_o/Li^+ substitution before the application of glutamate displayed a protective effect. Ouabain, unable to suppress the reversed Na^+/Ca^{2+} exchange, also had a protective effect. It seems that in addition to high $[Ca^{2+}]_i$, which can be attributed to the Na^+/Ca^{2+} exchange reversal, other factors are important for development of DCD, first of all a decrease in the intracellular concentration of ATP. It was shown [36] that the veratridine-caused increase in $[Na^+]_i$ in isolated nerve terminals enhanced hydrolysis of ATP with the plasma membrane Na^+/K^+-ATPase, and this promoted a rapid depolarization of mitochondria and increase in $[Ca^{2+}]_i$. We supposed that the effects of ouabain and Na^+_o/Li^+ substitution on the development of DCD should be similar because they both decreased the activity of the plasma membrane Na^+/K^+-ATPase, whereas lithium on entrance into the cell activated the Na^+-pump an order of magnitude more weakly than sodium [37]. Na^+/K^+-ATPase is the main consumer of ATP in the nerve cell [38]; therefore, ouabain and Na^+_o/Li^+ substitution which suppress hydrolysis of ATP by Na^+/K^+-ATPase can prevent the development of DCD.

After the exposure to glutamate for 1 h (Fig. 5), the ATP level was, on average, 48.1% decreased, and this is

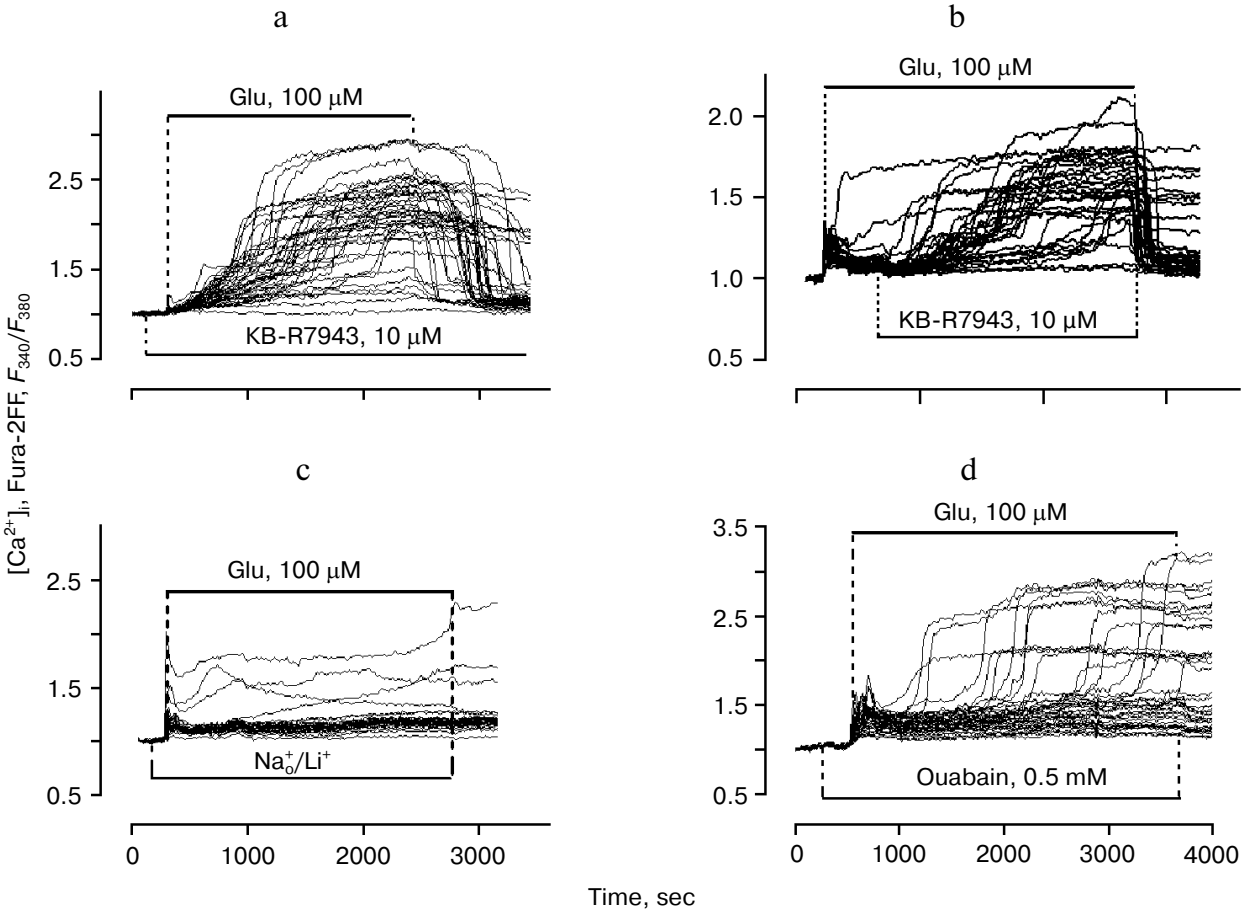

Fig. 4. Changes in $[Ca^{2+}]_i$ in individual neurons in response to glutamate (Glu) in the presence of the inhibitor KB-R7943 of the reversed Na^+/Ca^{2+} exchange (a, b) under conditions of preliminary Na^+_o substitution by Li^+ in the solution (c) or incubation with ouabain (d).

Effects of different exposures on the number of neurons (in %) with the secondary $[Ca^{2+}]_i$ rise (DCD) on the 10th and 40th min from beginning of the exposure to glutamate (n = four-five independent experiments for each exposure)

Exposure	10th min	40th min
Glutamate (100 μM)	9.2 ± 1.6	71.7 ± 8.0
Glutamate + 10 μM KB-R7943	11.8 ± 2.7	86.0 ± 12.2
Glutamate + Na_o^+/Li^+ substitution	1.2 ± 0.7*	15.8 ± 4.9*
Glutamate + ouabain (0.5 mM)	4.7 ± 2.6	40.4 ± 11.3**
Glutamate + deoxyglucose	30.2 ± 6.9	98.7 ± 1.3
Glutamate + Na_o^+/Li^+ + deoxyglucose	1.6 ± 0.2***	72.4 ± 9.1****
Glutamate + ouabain + deoxyglucose	2.6 ± 1.1***	96.8 ± 2.9

* $p < 0.01$ and ** $p < 0.05$ with respect to glutamate.
*** $p < 0.01$ and **** $p < 0.05$ with respect to glutamate + deoxyglucose.

consistent with the available data [9, 39]. Incubation with ouabain and Na_o^+/Li^+ substitution lowered the ATP loss caused by glutamate, on average, by 32.7 and 51.9%, respectively ($p < 0.05$, as compared to the effect of glutamate alone).

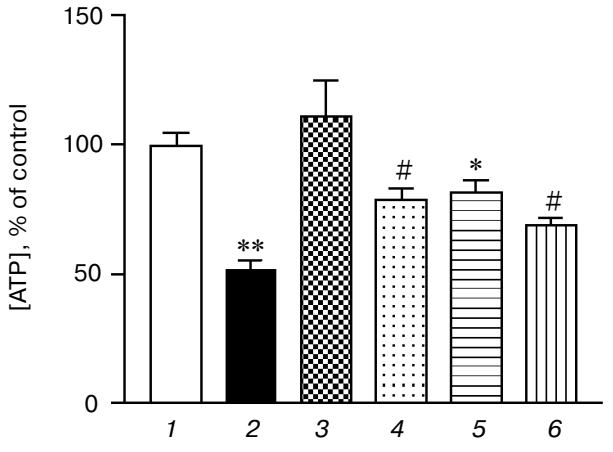

Fig. 5. Changes in the ATP concentration (in % of control) upon 1-h incubation of cultures under the following conditions: *1*) control; *2*) glutamate (100 μM); *3*) Na_o^+/Li^+ substitution; *4*) glutamate + preliminary Na_o^+/Li^+ substitution; *5*) ouabain (0.5 mM); *6*) glutamate + ouabain (0.5 mM); * $p < 0.05$ and ** $p < 0.01$ with respect to control; # $p < 0.05$ with respect to glutamate; n = five-six cultures for each column. [ATP] was determined using luciferin–luciferase chemiluminescence.

Effect of glucose deprivation on development of DCD during exposure to glutamate. Because the Na_o^+/Li^+ substitution and ouabain lowered the glutamate-caused loss of ATP (Fig. 5), it was interesting to determine whether the glucose deprivation markedly decreasing the ATP stores in the nerve cells [39] would abolish the protective effect of ouabain and the Na_o^+/Li^+ substitution on the development of DCD. The table presents the numbers of neurons with DCD on the 10th and 40th min from the beginning of the glutamate action in the solutions where glucose was equimolarly replaced by 2-deoxy-D-glucose. In the glucose-free medium, the number of neurons with DCD was sharply increased and reached nearly 100% by the 40th min of the incubation with glutamate. The number of cells with DCD under conditions of the Na_o^+/Li^+ substitution in the glucose-free medium also was increased, but reached the level observed in the glucose-containing medium only by the 40th min of the exposure to glutamate. A similar effect on the number of neurons with DCD in the presence of ouabain was observed in the glucose-free medium (table). Thus, a decrease in the ATP level in the presence of glutamate is the most significant factor for the DCD development, and this decrease is partially caused by the increase in the Na^+/K^+-ATPase activity and enhancement of ATP hydrolysis.

DISCUSSION

Our studies have shown that the Na^+/Ca^{2+} exchanger is not involved in maintenance of $[Ca^{2+}]_i$ in resting granular cells of the cerebellum and loses the ability to *release* Ca^{2+} outwards after both short- and long-term toxic challenge with glutamate. During the exposure, the exchanger is working in the reversed mode and enhances DCD. This is proved by the following findings: i) inhibition of the forward exchange failed to affect the $[Ca^{2+}]_i$ at rest and the rate of the $[Ca^{2+}]_i$ lowering in the calcium-free medium after the removal of glutamate (Fig. 1); ii) KB-R7943-induced inhibition of the reversed exchange improved 7.4-fold the recovery of $[Ca^{2+}]_i$ in the post-glutamate period (Fig. 4). Thus, the reversal of the Na^+/Ca^{2+} exchange is concluded to slow down the recovery of the basal level of $[Ca^{2+}]_i$. It seems that the reversal is caused by glutamate which, in addition to the $[Ca^{2+}]_i$ rise, induces an increase in $[Na^+]_i$ which is maintained for rather a long time after the removal of glutamate (Fig. 1c) and also depolarization of PM and intracellular acidosis [9, 18, 40]. In the presence of glutamate, $[Na^+]_i$ increases through Na^+ entry via ionotropic glutamate channels [40] and also as a result of the Na^+ transport together with glutamate into the cell by protein carriers of glutamate and glycine [41]. The decrease in the transmembrane Na^+ gradient, depolarization of PM, and acidosis inhibit the forward mode of the Na^+/Ca^{2+} exchange and concurrently enhance the reversed mode [1, 10]. Similar data on the

effect of KB-R7943 on the rate of the $[Ca^{2+}]_i$ decrease after the mechanical stimulation of astrocytes were obtained in work [42]. KB-R7943 can partially inhibit Ca^{2+} entry through the NMDA and depot-regulated channels of PM [35, 43], but in our experiments the addition of the antagonist of NMDA receptors into the washing solution did not affect the decrease in $[Ca^{2+}]_i$ after the long-term exposure to glutamate [30].

During the exposure to glutamate the Na^+/Ca^{2+} exchange also seems to be reversed, or at least the majority of the exchanger molecules, possibly certain isoforms NCX1 or NCKX, are working in this mode [4, 25, 44]. This was indicated by the findings that the inhibition of $[Ca^{2+}]_i$ *release* by the Na_o^+/Li^+ substitution during the cell incubation with glutamate did not affect $[Ca^{2+}]_i$ or induced a slight transitory increase in $[Ca^{2+}]_i$ with subsequent decrease (Fig. 3a). These changes in $[Ca^{2+}]_i$ can be associated with two opposite effects of the Na_o^+ removal on the neurons. On one hand, the forward exchange is suppressed and the reversed one is enhanced [10], and on the other hand, Li^+ lowers the current through the NMDA channels more strongly than Na^+ [45, 46], possibly owing to inhibition of phosphorylation of subunit NR2B of the NMDA receptor [47]. The short-term increase in $[Ca^{2+}]_i$ upon the Na_o^+/Li^+ substitution during the incubation with glutamate (Fig. 3a) seemed to be caused by enhancement of the reversed Na_i^+/Ca_o^{2+} exchange. This increase was short-term because of a gradual decrease in $[Na^+]_i$ (Fig. 3b) which could be due to: i) exchange of intracellular Na^+ for extracellular Ca^{2+} and H^+; ii) termination of the Na^+ entry into the cells during the continuous pumping outwards by Na^+/K^+-ATPase; iii) entry of Na_i^+ into the mitochondria through mediation of the Na^+/H^+ antiporter or mitochondrial Na^+/Ca^{2+} exchange [48].

The other significant conclusion of the present work is that reversal of the Na^+/Ca^{2+} exchange is an important but insufficient condition for appearance of the second phase of the $[Ca^{2+}]_i$ increase, i.e. DCD. In the works of Kiedrowski et al. [4, 33] it was supposed that reversal of the Na^+/Ca^{2+} exchange could provide for not only the maintenance of the high level of $[Ca^{2+}]_i$ plateau during the exposure to glutamate (on the gradual inactivation of the NMDA channels) but also Ca^{2+} accumulation in mitochondria with their subsequent depolarization resulting in the secondary increase in $[Ca^{2+}]_i$ and death of the neurons. However, our findings suggest that, in addition to the high $[Ca^{2+}]_i$, for development of DCD a decrease in the ATP level is necessary. These finding are as follows: i) no dependence was revealed by concurrent determinations of $[Na^+]_i$ and $\Delta\Psi_m$ between the decrease in $\Delta\Psi_m$ simultaneous with the second phase of the increase in $[Ca^{2+}]_i$ and change in $[Na^+]_i$ (Fig. 2d); ii) the inhibitor KB-R7943 of the reversed exchange did not affect the number of cells with DCD (table); iii) glucose deprivation significantly enhanced the number of cells with DCD in the absence of the reversed Na_i^+/Ca_o^{2+} exchange (Na_o^+/Li^+ substitution performed before the application of glutamate, Fig. 4c and table). The pronounced suppression of development of DCD upon the Na_o^+/Li^+ substitution (table) does not contradict the above-presented data. As it has been said earlier, Li^+ can decrease the probability of the glutamate channel location in the open state, and this, in addition to the inhibition of the exchange reversal, can explain the positive effect of the Na_o^+/Li^+ substitution on the development of DCD. It should be emphasized that ouabain, unable to affect the reversal, displayed a similar protective effect (Fig. 4d and table). Ouabain (0.5-1 mM) slightly increased the current entering through the NMDA channels [49] and had almost no influence on the $^{45}Ca^{2+}$ entry into the cerebellar neurons during the action of NMDA, whereas in the presence of Li^+ the $^{45}Ca^{2+}$ entry was decreased nearly twofold [33].

The decrease in the number of cells with DCD upon the incubation with ouabain or in sodium-free solution could be caused by a decrease in ATP hydrolysis as a result of inhibition of Na^+/K^+-ATPase (Fig. 5). Ouabain (0.5 mM) seems to inhibit all known isoforms of the sodium pump in the brain. The activity of Na^+/K^+-ATPase (mainly λ_2/λ_3 isoforms) increases during the first minutes of exposure to glutamate and/or in brain ischemia [50, 51], and this is likely to be one of main reasons for the glutamate-induced decrease in the ATP level. We have recorded the total changes of ATP level in cultures, but in some perimembrane pools of neurons the ATP level can decrease much more strongly. The maintenance of the perimembrane ATP can promote the Ca^{2+} release outwards by Ca^{2+}-ATPase of PM [36]. ATP can also influence other mechanisms initiating DCD. Thus, there is a hypothesis that the secondary increase in $[Ca^{2+}]_i$ correlating with the depolarization of mitochondria is caused by release of the Ca^{2+} taken up from the depolarized membranes via the uniporter channel or the produced permeability transition pore [8, 30, 32, 52]. Cytosolic ATP inhibits Ca^{2+} release from mitochondria [53-55]. We have observed that ouabain decelerates the protonophore FCCP-stimulated Ca^{2+} release from mitochondria after exposure to glutamate [56]. Similar data on inhibition of the Ca^{2+} release from mitochondria of cerebellar granular cells stimulated with NMDA in the presence of ouabain were obtained in [33]. Because the glucose deprivation considerably enhanced the number of neurons with DCD (table), a decrease in the loss of ATP, e.g. in the presence of ouabain, could increase the resistance of mitochondria to the glutamate-induced stress.

Thus, we conclude that the Na^+/Ca^{2+} exchange is not involved in the Ca^{2+} *release* from the cerebellar neurons during and after exposure to glutamate. Dysfunction and/or reversal of the Na^+/Ca^{2+} exchange facilitates the increase in $[Ca^{2+}]_i$ and has a significant pathogenic significance in the post-glutamate period because of decelera-

tion of recovery of the $[Ca^{2+}]_i$ homeostasis. However, the exchange reversal alone is insufficient for development of delayed calcium deregulation. In addition to Ca^{2+}, other factors associated with the Na^+ entry are required: changes in the activity of Na^+/K^+-ATPase, lowering of ATP level, inhibition of the Na^+/H^+ exchange, intracellular acidosis, generation of free radicals or nuclear factors of apoptosis, etc. [57, 58].

In the present work we studied the contribution of the exchanger to $[Ca^{2+}]_i$ regulation on a model of pathological conditions, i.e. the time of exposure to glutamate was much more longer than the time of nervous impulse conduction. The exchanger is also likely to play a certain role in mechanisms of enhancement of synaptic transmission under physiological conditions due to functioning in a reversed mode upon activation of the glutamate receptors. The delayed recovery of the low level of $[Ca^{2+}]_i$ can increase the release of mediators in synaptic terminals. Prospects for further studies may be associated with elaboration of specific agents capable of selective inhibition of individual isoforms of the exchanger.

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