Short-term block of Na^+/K^+-ATPase in neuro-glial cell cultures of cerebellum induces glutamate dependent damage of granule cells

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Abstract Granule cells in a dissociated neuro-glial cell culture of cerebellum when exposed to ouabain (10^{-3} M) for 25 min apparently swell, increase their $[Ca^{2+}]_i$ with obvious depolarization of the mitochondrial membrane. In 3 h after ouabain was omitted from the solution, 62 ± 3% of granule cells had pycnotic nuclei. The supplement of a solution with competitive specific antagonist of NMDA receptors, L-2-amino-7-phosphonoheptanoate (10^{-4} M, APH) together with ouabain prevented cells from swelling, mitochondrial deenergization, neuronal death and increase of $[Ca^{2+}]_i$. These data suggest that cellular Na^+/K^+-ATPase inactivation in neuro-glial cell cultures of cerebellum leads to glutamate (Glu) accumulation, hyperstimulation of glutamate receptors, higher Ca^{2+} and Na^+ influxes into the cells through the channels activated by Glu. This process leads to cell swelling, mitochondrial deenergization and death of granule cells. Possibly, the decrease of Na^+/K^+-ATPase activity in brain cells can lead to the onset of at least some chronic neurological disorders.

Key words: Ouabain; Glutamate; Na^+/K^+-ATPase; Mitochondria; Membrane potential

1. Introduction

The excess of excitotoxic amino acids is though to be one of the pathogenic factors resulting from the neuronal lesion under brain damage [1], ischemia and hypoxia [2,3], as well as under chronic neurological disorders such as Hantington's chorea [4,5], epilepsy [6], side amyotrophic sclerosis [7]. The elevation in the brain of the major excitatory neuromediator, glutamate (Glu) occurring under neurological disorders may be due to defects in mitochondrial energy metabolism [8]. Disruption of energy metabolism results in a lowering of the Na^+/K^+-ATPase activity [9], cell depolarization and rising of intracellular Na^+. All these processes induce the distortion of Glu trapping by the cells [10]. The lower Glu trapping results in its accumulation, hyperactivation of Glu receptors and finally, the cell death, the so-called secondary excitotoxicity. The existing link between the rise of endogenous Glu and metabolic distortions was supported by data showing that in neuronal cells the toxic effect of metabolic inhibitors could be prevented by NMDA receptor blockers [11–13]. However, the accumulation of endogenous Glu may not occur as a sequence of impaired cellular energetics, but also as a result of a slower glutamate uptake through the high-affinity sites. The latter can be a result of either damage of the transport system or be mediated by the suppressed activity of Na^+/K^+-ATPase [14]. In this study we were able to demonstrate that in primary culture of cerebellar granule cells the Na^+/K^+-ATPase blocker, ouabain induced the neuronal death through the secondary excitotoxicity and hence, the impaired energy metabolism may be explained by a toxicity manifested by endogenous Glu.

2. Materials and methods

Primary cerebellar cultures were prepared from the cerebella of 7–8 day old Wistar rats using procedure described earlier [15]. On the second day of cultivation, the potassium concentration was risen up to 25 mM. Control cells were exposed for 25 min to a balanced salt solution (BSS) of the following content (in mM): NaCl 154, KCl 5, Na_2HPO_4 0.035, $CaCl_2$ 2.3, $NaHCO_3$ 3.6, glucose 5.6, HEPES 5, pH 7.6–7.8, 20°C. Ouabain (Ou, 1 mM) was used to block Na^+/K^+-ATPase and APH (D,L-2-amino-7-phosphonoheptanoate, 10^{-4} M) as competitive specific antagonist of NMDA glutamate receptors.

Confocal Laser Scanning Microscope (Bio-Rad MRC 600 CLSM) was used to evaluate Ca_i^{2+} changes by monitoring Fluo-3 fluorescence after intracellular cleavage of Fluo-3 AM (10^{-5} M, Molecular Probes, with excitation at 488 nm and emission at >510 nm). Fluorescence data were obtained by evaluating the fluorescence from selected areas within a cell, with the following subtraction of the background fluorescence, and division by the fluorescence intensity before application of drugs (F_0) and expressed as F/F_0. Since Fluo-3 does not allow direct quantitative rationing, we also used a semiquantitative method to estimate $[Ca^{2+}]_I$ changes as described before [16]. The values of $[Ca^{2+}]_i$ were calculated by the equation:

$$[Ca^{2+}]_i = K * F/F_0/[(K/[Ca^{2+}]_{rest} + 1) - F/F_0],$$

where $K = 400$ nM (dissociation constant of the complex Ca-Fluo-3), basal calcium $[Ca^{2+}]_{rest} = 41$ nM [17]. Confocal images were stored every 5 min.

Mitochondrial energization within a cell was monitored by the rise of rhodamine 123 (R123) fluorescence monitored by Univar fluorescence microsope, Reichert, after 10 min of incubation (5×10^{-6} g/ml) in BSS. For evaluation of viability, treated cells (25 min, 1 mM Ou or 25 min, 154 mM LiCl) were washed twice with BSS and incubated in this medium for 3 h in CO_2 incubator at 35.5°C for the development of a delayed neuronal death. After incubation, cells were fixed with an ethanol-formaldehyde-acetic acid (7:2:1) mixture and stained with vanadium hematoxhline. The percentage of damaged neurons was determined by counting the intact and pycnotic nuclei of the granule cells in 81-view fields. Mann-Whitney test was used for statistical evaluations.

3. Results and discussion

Granule cells in the dissociated neuro-glial cell cultures had normal morphology past 3 h incubation in BSS (Fig. 1A). Ou

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Abbreviations: Glu, glutamate; R123, rhodamine 123; Ou, ouabain; BSS, balanced salt solution; LiBSS, balanced salt solution where potassium ions were substituted for lithium; APH, D,L-2-amino-7-phosphonoheptanoate; NMDA, *N*-methyl-D-aspartate

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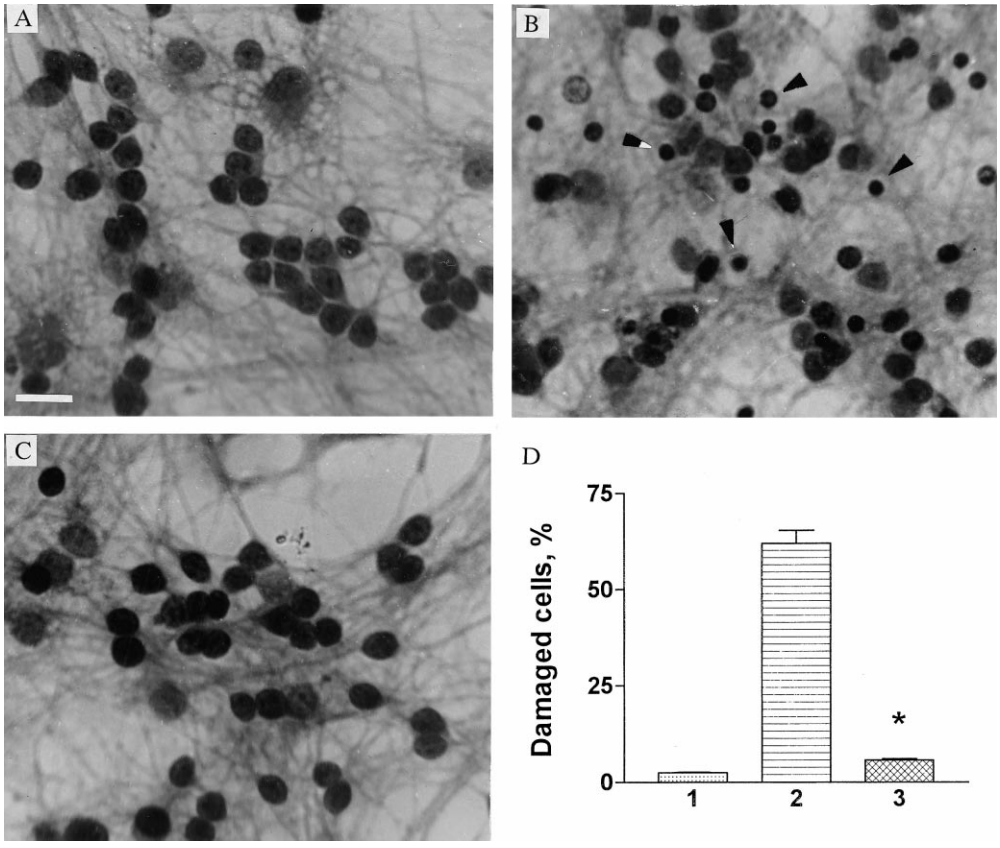

Fig. 1. The evidence that the inhibition of Na^+/K^+-ATPase in the glial-neuronal monolayer culture induces the damage of cerebellar granule cells with APH preventing this effect. Bar = 1.4×10^{-5} m. Vanadium hematoxylin staining. A: Control culture exposed to BSS for 205 min. B: Cultures exposed to BSS for 180 min followed by 25 min in OBSS. Arrowheads indicate the pycnotic nuclei of dead granule cells. C: Cultures exposed to BSS for 180 min followed by 25 min exposure to OBSS with 10^{-4} M APH. D: Viability of granule cells after the cease of the Na^+/K^+-ATPase. Lane 1, control culture exposed to BSS for 205 min; lane 2, 180 min in BSS after 25 min exposure to OBSS; lane 3, 180 min in BSS after 25 min exposure to OBSS with 10^{-4} M APH. Lanes 2 and 3, $^*P < 0.001$, $n = 81$–99; U-test.

(10^{-3} M) treatment of granule cells within 25 min caused their obvious swelling. After 3 h Ou was omitted from the solution, pycnotic nuclei have been revealed in many neurons (Fig. 1B). The percentage of the dead cells in treated cultures reached $62 \pm 3\%$ of the total amount of granule cells, while in control only 3% of neurons died (Fig. 1D). Lower Ou concentrations (10^{-4} M or less) were not toxic for these cells.

Since the transmembrane transport of Glu is sodium dependent, this accompanies Na^+ entry into the cell [18]. The inactivation of Na^+/K^+-ATPase or removal of the external sodium from cells caused accumulation of extracellular Glu. Previously, other authors demonstrated that Ou inhibition of Na^+/K^+-ATPase induced the decrease of the glutamate uptake in cultured cortical astrocytes [19]. Possibly in our experiments, the hyperstimulation of Glu receptors by endogenous Glu leads to the death of the neurons. For testing the possibility that neuronal NMDA channels are activated by the Glu, released from internal sources of the cells, the granule cells were incubated in Ou-containing solution in the presence of 10^{-4} M APH, a competitive specific antagonist of NMDA glutamate receptors. This blocker prevented both the ouabain-induced swelling and death of granule cells (Fig. 1C, D). In an attempt to support the idea that the inhibition of Glu transport into the cells may result in an accumulation of Glu to a toxical level, we substituted the NaCl in BSS for LiCl (LiBSS), as it is known that Li^+ competes with Na^+ for binding with Na^+/K^+-ATPase and significantly lowers its functional activity [20]. Also this substitution is known to diminish Glu uptake [21]. The incubation of the cell cultures in LiBSS induced the death of 36% granule cells. This toxic effect has been completely prevented by APH (Fig. 2). In these experiments, the density of granule cells in experimental cell cultures exceeded 5×10^3 cells/mm^2. The toxicity of Ou and Li^+ was not observed in the cell cultures with a lower cell density.

A number of studies demonstrated that hyperstimulation of

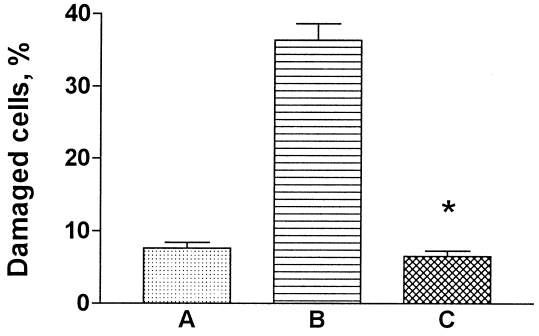

Fig. 2. Viability of granule cells after incubation in a Li^+-containing solution with low Na^+. Lane A, control culture exposed to BSS for 205 min; lane B, 180 min in BSS after 25 min exposure to LiBSS; lane C, 180 min in BSS after 25 min exposure to LiBSS with 0.1 mM APH. Lanes B and C, $^*P < 0.01$, $n = 81$–99; U-test.

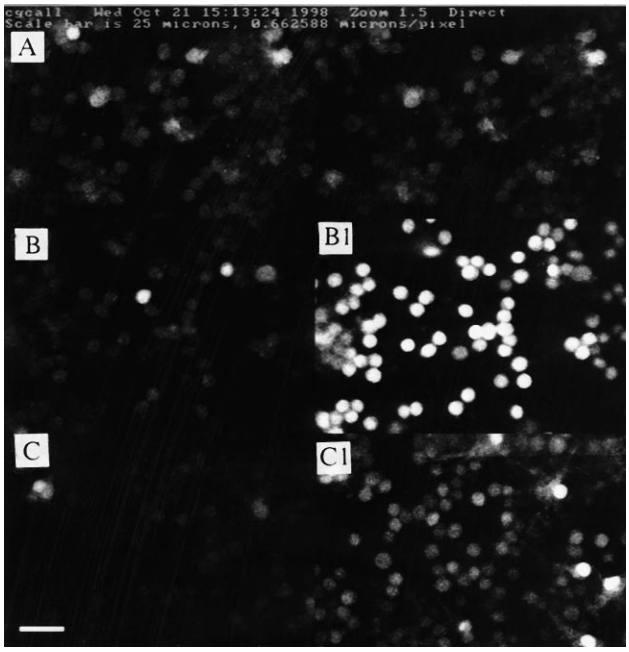

Fig. 3. The measurement of Fluo-3 fluorescence in the cytosol of granule cells shows the increase of Fluo-3 fluorescence in the neurons of Ou treated neuro-glial cell cultures. A: Control, 1 min BSS; A1: control, 30 min BSS; B: 1 min BSS+1 mM ouabain; B1: 30 min BSS+1 mM ouabain; C: 1 min BSS+10^{-3} M ouabain+10^{-4} M APH; C1: 30 min BSS+10^{-3} M ouabain+10^{-4} M APH.

glutamate receptors results in Ca^{2+} overloading of the neuronal cytosol [22,23].

The measurement of Fluo-3 fluorescence in the cytosol of granule cells shows the increase of Fluo-3 fluorescence in the neurons of Ou treated neuro-glial cell cultures (Fig. 3B, B1). By using the semiquantitative methods of evaluation of $[Ca^{2+}]_i$ changes [16] we estimated that after the ouabain exposure of cell cultures for 20 min, $[Ca^{2+}]_i$ of granule cells increased from a basal level (41 nM) to 425 nM and 30 min exposure resulted in the maximal rise of the $[Ca^{2+}]_i$ to 1084 nM (Fig. 4). The supplement of the ouabain-containing solution with the competitive specific antagonist of NMDA receptor, APH (10^{-4} M), significantly abolished the ouabain-induced $[Ca^{2+}]_i$ increase (Figs. 3C, C1, 4). In this case, the maximal increase of the intracellular calcium in granule cells from Ou and APH treated cell cultures was 147 nM.

The $[Ca^{2+}]_i$ was not changing in the control cells for all the

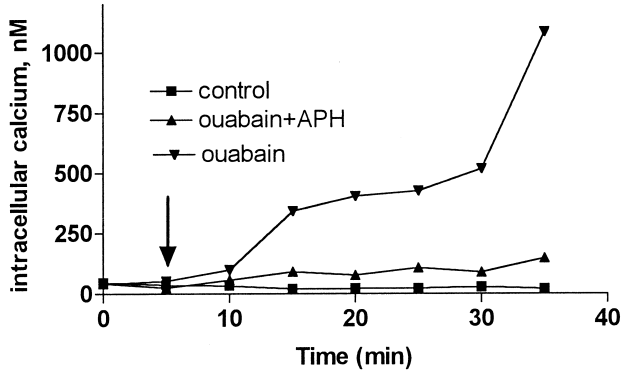

Fig. 4. Increase of intracellular calcium in cerebellar granule cells by ouabain is inhibited by an NMDA receptor antagonist, APH.

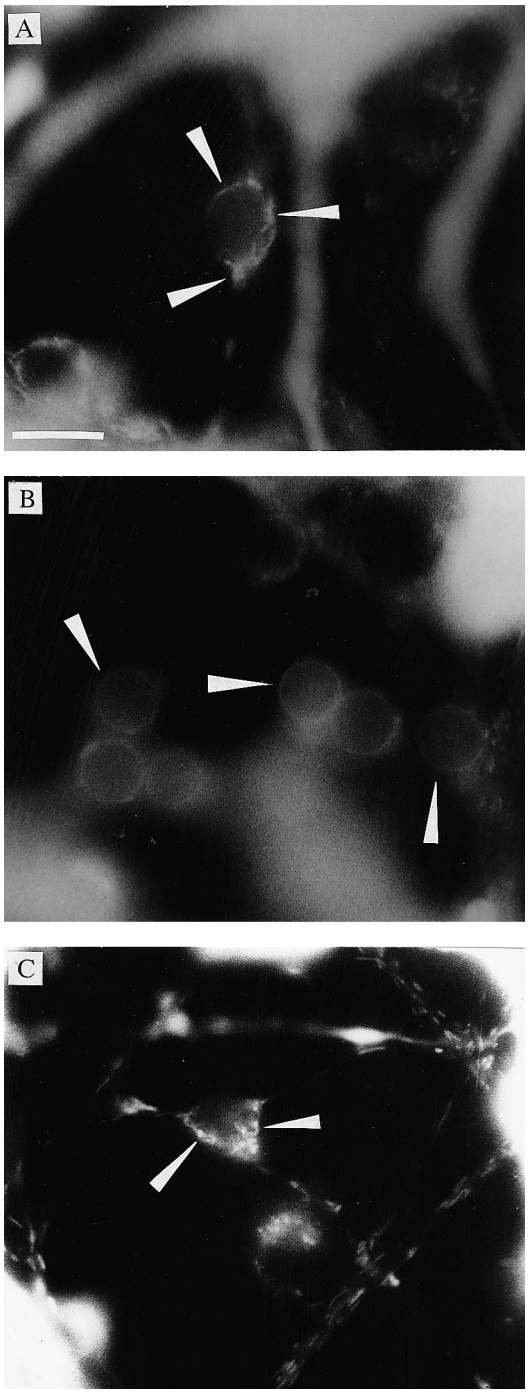

Fig. 5. Photomicrograph of living granule cells in cerebellar dissociated culture stained with mitochondrial membrane potential fluorescent probe, rhodamine 123. Bar = 1.4×10^{-5} m. A: Brightly fluorescent mitochondria in granule cells (arrows) exposed to balanced salt solution for 25 min and then to rhodamine 123. B: Granule cells after 25 min exposure to balanced salt solution with 1 mM ouabain followed by rhodamine 123 staining. Faintly fluorescent mitochondria in granule cells (arrows) demonstrate the loss of the mitochondrial membrane potential. C: Granule cells after 25 min exposure to balanced salt solution with 10^{-3} M ouabain with 10^{-4} M APH followed by rhodamine 123 staining. Note brightly fluorescent mitochondria inside granule cells (arrows) demonstrating high membrane potential.

incubation times in BSS (Figs. 3A, A1, 4). Due to the above shown sensitivity of the intracellular entry of Ca^{2+} to specific blocker of NMDA channel, APH, we conclude that in granule cells, when incubated in the Ou-containing medium, calcium ions enter the neurons preferentially through NMDA channels activated by Glu.

Many drugs causing cellular damage are known to use mitochondria as a primary target [24,25]. In order to evaluate the influence of Ou on the mitochondrial energization in a cell, the mitochondrial membrane potential was monitored by rhodamine 123 (R123) fluorescence due to accumulation in mitochondria. After 25 min of incubation granule cells in BSS, the neuronal mitochondria showed an active accumulation of R123 which followed from intense green fluorescence after the excitation of a cell with a blue light (Fig. 5A). At the same time, very dim fluorescence was observed within granule cells treated with 1 mM Ou for the same period of time after loading with R123 (Fig. 5B). The competitive antagonist of NMDA glutamate receptors, APH prevented mitochondrial deenergization (Fig. 5C). Previously we, as well as other authors, have demonstrated that excessive Ca^{2+} entrance into neurons induced by glutamate resulted in a fast collapse of the mitochondrial membrane potential within these cells [26–30].

The protection by APH from Ou toxicity, as well as the resemblance of Ou-induced cells death in neuro-glial cell cultures of cerebellum with that developed after Glu treatment in some features like cells swelling, increase of $[Ca^{2+}]_i$, mitochondrial deenergization and the appearing of pycnotic nuclei, all allow to suggest that the inactivation of Na^+/K^+-ATPase in neuro-glial cell cultures may lead to cell absorbing the released Glu which is high enough for being toxic for neurons. Possibly, the decrease of Na^+/K^+-ATPase activity in brain cells can lead to the onset of at least some chronic neurological disorders. This idea is supported by the findings that in epileptic cortex the glial Na^+/K^+-ATPase is altered [31,32] and this is accompanied by the increase of Glu level in the epileptic brain. Possibly, in this case the release of endogenous Glu has a strong impact on the energetic metabolism and finally results in neuronal death.

Relatively high effective concentrations of ouabain used may be due to participation of Na^+/K^+-ATPase isozyme ($\alpha 1$) for its known low affinity to ouabain [33,34]. This type of Na^+/K^+-ATPase is known to be present in both neuronal and glial cells. If so, both types of cell may participate in Glu release as well as in Glu capture.

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