Neurotoxic glutamate treatment of cultured cerebellar granule cells induces Ca^{2+}-dependent collapse of mitochondrial membrane potential and ultrastructural alterations of mitochondria

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Abstract Rhodamine 123 staining and electron microscopy were used to reveal a correlation between the ultrastructural and functional state of cultured cerebellar granule cells after short glutamate treatment. Glutamate exposure (15 min, 100 μM) in Mg^{2+}-free solution caused considerable ultrastructural alterations in a granule cell: clumping of the chromatin, swelling of the endoplasmic reticulum and mitochondria, and disruption of the mitochondrial cristae. After glutamate treatment, the mitochondria of the neurons lost their ability to sequester rhodamine 123. Both the *N*-methyl-D-aspartate receptor channel blocker MK-801 (30 μM) and cobalt chloride (2 mM) prevented the deteriorative effects of glutamate. These data suggest that glutamate-induced Ca^{2+} overload of the neurons can lead to non-specific permeability of the inner mitochondrial membrane, resulting in neuronal death.

Key words: Mitochondria; Glutamate; Neurotoxicity; Membrane potential; Ultrastructure; Cytosolic Ca^{2+}

1. Introduction

Glutamate (GLU) appears to be one of the major excitatory neuromediators in the CNS, however, a sharp rise in glutamate content in the brain or an imbalance between its release and re-uptake may cause damage to glutamate receptive neurons, which happens under hypoxia, ischemia and a number of other pathological events in CNS [1–3]. It has been found that processes inducing neuronal degeneration after the hyperstimulation of glutamate receptors result in Ca^{2+} and Na^{+} influx into the cell through ion channels activated by GLU [4,5]. This, in turn, results in overloading of neuronal cytosol with these cations [6,7] and in subsequent distortion of the cellular energetics [8,9] and activation of Ca^{2+}-dependent proteolytic and lipolytic enzymes. Many drugs causing cellular damage are known to use mitochondria as a primary target [10,11]. As for GLU, used at concentrations that are toxic for a cell, little is known about its effects on mitochondrial structure and functions, thus providing the impetus for the present study.

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Abbreviations: GLU, glutamate; R123, rhodamine 123; BSM, balanced salt medium; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo(*a*,*d*)cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate

2. Materials and methods

Primary cerebellar cultures were prepared from the cerebella of 7–8-day-old Wistar rats using a procedure described earlier [12]. The initial potassium concentration in the medium was 5 mM; on the second day of cultivation the potassium concentration was increased to 25 mM. Cells were exposed to GLU (100 μM) for 15 min in a balanced salt medium (BSM) of the following composition (in mM): NaCl (137), KCl (5), Na_2HPO_4 (0.035), $NaHCO_3$ (12), $CaCl_2$ (2.3), glucose (11), pH 7.6–7.8, $t = 20°C$. Cells incubated for 15 min in GLU-free BSM were chosen as a control. $CoCl_2$ (2 mM) was used to block Ca^{2+} channels and MK-801 (30 μM) as a non-competitive specific antagonist of NMDA channels, which were added to BSM simultaneously with the addition of GLU. Exposure to the calcium ionophore A23187 was initiated by addition of A23187 to the culture dish (20 μM, in BSM, 5 min). Mitochondrial energization in a cell was monitored by rhodamine 123 (R123) accumulation after 10 min incubation (5 μg/ml) in BSM following exposure to the drugs. Cellular fluorescence was monitored using a Univar fluorescence microscope, Reichert. For estimation of viability, treated cells were washed twice with BSM and incubated in this medium for 2 h in a CO_2 incubator for the development of delayed neuronal death. After incubation cells were fixed with an ethanol-formaldehyde-acetic acid (7:2:1) mixture and stained with vanadium hematoxylin. The percentage of damaged neurons was determined by counting the intact and pyknotic nuclei of the granule cells in 9-view fields (average number of cells about 10^4). For electron microscopy, cells were fixed using 2.5% glutaraldehyde prepared on phosphate buffer (pH 7.2), postfixation being with 1% osmium tetroxide, dehydration in ethanol, and embedding in Epon 812. Ultrathin sections were prepared on an LKB-3 ultramicrotome and examined at 75 kV in a Hitachi HU-11 electron microscope.

3. Results and discussion

After 15 min incubation of granule cells in BSM, neuronal mitochondria showed very active accumulation of R123 which was indicated by intense green fluorescence after the excitation of a cell with blue light (Fig. 1A). The fluorescence of rhodamine 123 was abolished after treatment of cells with uncoupler (0.1 μM FCCP). Cells treated with 100 μM GLU for 15 min showed swelling of the cytosol within granule cells and only traces of green fluorescence were observed within each granule cell after loading with R123 (Fig. 1B). At the same time, glial cells retain their ability to accumulate this mitochondrial membrane potential-dependent probe (Fig. 2B). To prevent Ca^{2+} entry into the cell as a result of GLU treatment, inhibitors of both Ca^{2+} and NMDA channels were used. $CoCl_2$ (2 mM) completely abolished the effect of GLU on the mitochondrial membrane potential (Fig. 1C). MK-801, a specific antagonist of NMDA receptors, which blocks GLU-activated NMDA channels [13], also prevented mitochondrial deenergization and cytosolic swelling of granule cells. A similar protective effect was observed when cells before glutamate were

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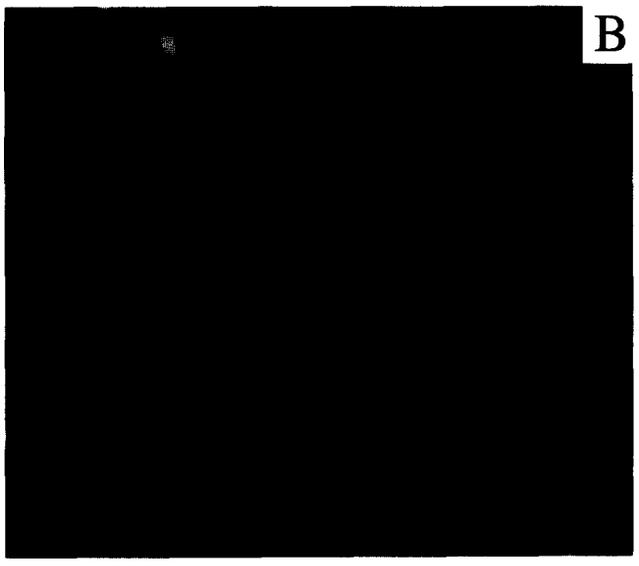
Fig. 1. Photomicrograph of living granule cells in cerebellar dissociated culture demonstrating the mitochondrial localization of rhodamine 123. (A) Brightly fluorescing mitochondria can easily be distinguished inside granule cells (arrows). The cells were exposed to Mg^{2+}-free balanced salt medium for 15 min and then to rhodamine 123 for 10 min. Bar = 10 μm; (B) granule cells after 15 min exposure to 100 μM glutamate in Mg^{2+}-free balanced solution followed by 10 min treatment with rhodamine 123. Faintly fluorescing granule cells (arrows). Mitochondria in these cells do not accumulate rhodamine 123; (C) granule cells after 15 min exposure to 100 μM glutamate and 2 mM $CoCl_2$ in Mg^{2+}-free balanced medium followed by 10 min treatment with rhodamine 123. Brightly fluorescing mitochondria can easily be distinguished inside granule cells (arrows). ←



treated with 10^{-4} M ruthenium red, which is known to block the mitochondrial Ca^{2+} uniporter [14] (Fig. 3A–D). Besides the inability to collapse the mitochondrial membrane potential in the presence of ruthenium red (Fig. 3B,C), the latter also significantly increased cellular viability by abolishing the

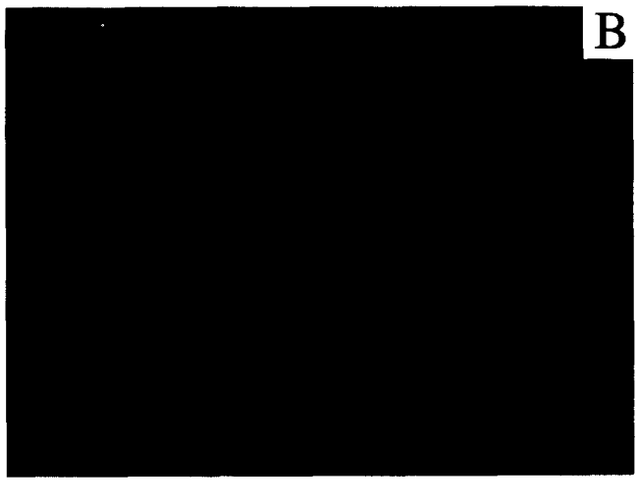


Fig. 2. Photomicrograph of living glial cells in cerebellar dissociated culture demonstrating mitochondrial localization of rhodamine 123. (A) Cells were exposed to Mg^{2+}-free balanced salt solution for 15 min and then to rhodamine 123 for 10 min. Bar = 10 μm. Mitochondria of glial cells accumulate rhodamine and fluoresce intensely. (B) Cells after 15 min exposure to 100 μM glutamate in Mg^{2+}-free balanced solution followed by 10 min treatment with rhodamine 123. Mitochondria of glial cells accumulate rhodamine and are intensely fluorescent.

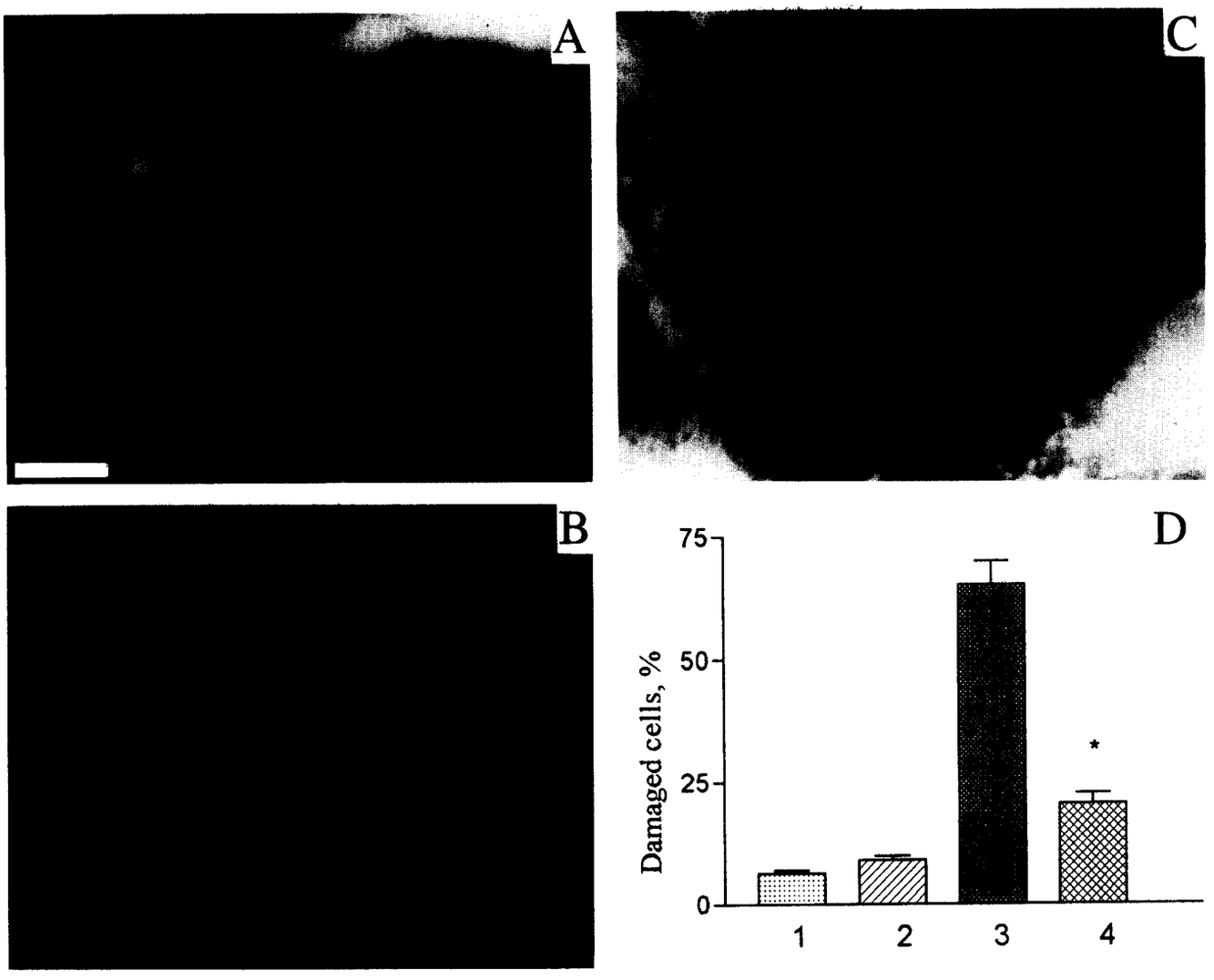


Fig. 3. Protectory effect of ruthenium red on the glutamate-induced neuronal damage. (A–C) Rhodamine 123 neuronal staining; (D) cellular viability (1, control cells incubated for 1 h in BSM; 2, cells incubated for 1 h in BSM with 10^{-4} M ruthenium red; 3, cells incubated for 1 h in BSM, then for 15 min with additional 100 μM glutamate; 4, cells incubated for 1 h in BSM with 10^{-4} M ruthenium red, then for 15 min with additional glutamate. After this all cells stayed in BSM for 2 h and were then stained as described in Section 2. $^{*}P < 0.001$, when compared with bar 3, using Student's *t*-test). (A) Control cells; (B) cells treated with 100 μM glutamate; (C) cells treated with 100 μM glutamate, but pretreated with 10^{-4} M ruthenium red. Conditions as in D (1,3,4) with the exception that they did not stay for 2 h for the development of delayed neuronal death). Arrows in A,C show fluorescing mitochondria inside granule cells; in B arrow points to the granule cell which lost rhodamine 123 as a result of $\Delta\Psi$ collapse on the mitochondrial membrane.

deleterious effect of glutamate (Fig. 3D, bars 3,4). The effect of ruthenium red was due to its interaction with the mitochondrial calcium uniporter, as it does not interfere with Ca^{2+} transport through the cellular membrane [15]. For testing the possibility that high intracellular Ca^{2+} is responsible for mitochondrial deenergization induced by GLU, similar experiments were performed in the presence of the Ca^{2+} ionophore. A23187 (20 μM, 5 min) alone induced swelling of the cytoplasm and collapse of the mitochondrial membrane potential in a granule cell similar to that with GLU (not shown). We conclude that the GLU-induced mitochondrial membrane potential collapse in granule cells was induced by the rise in intracellular Ca^{2+}. Similar results, albeit after long-term (up to 3 h) GLU exposure of cultivated hippocampal neurons, have been obtained by Mattson et al. [9]. However, Dugan et al. [16] were not able to record the lowered mitochondrial membrane potential when cultivated murine cells were exposed to a number of GLU receptor agonists. These authors initially stained cells with rhodamine and then exposed them to GLU and ionomycin, although we tried the reverse order (GLU exposure first and then R123 staining).

Calcium overload resulting from the neurotoxic action of GLU has been shown to be the main factor inducing cell death [17,18]. It was reasonable to assume that a lower mitochondrial membrane potential is also the result of neuronal calcium overloading. Our data support such an assumption, since both A23187 and GLU induced calcium overload and resulted in mitochondrial deenergization. At the same time, the activation of GLU receptors without subsequent Ca^{2+} entry into the neuron due to the blockade of voltage-dependent calcium channels as well as NMDA channels does not result in lowering of the mitochondrial membrane potential.

For the evaluation of the morphological alterations induced by glutamate treatment of cerebellar granule cells electron

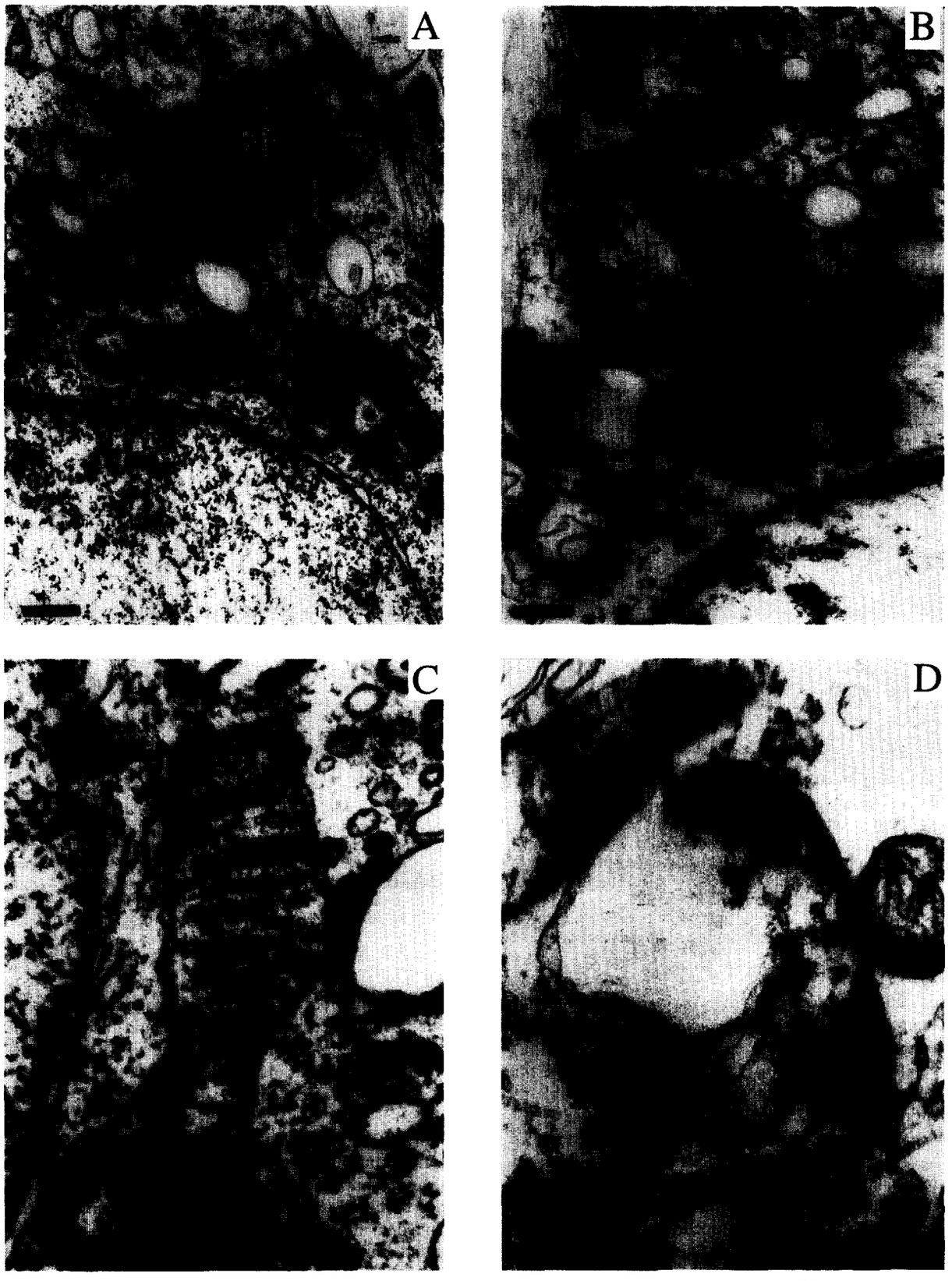


Fig. 4. Transmission electron micrograph of cultured cerebellar granule cell. (A) Control culture was exposed to Mg^{2+}-free balanced salt solution for 15 min. Mitochondria (arrows) appear undamaged. Bar=0.3 mm; (B) cells after 15 min exposure to 100 μM glutamate in Mg^{2+}-free balanced medium. C and D represent parts of A and B, respectively, with higher magnification. Note mitochondrial swelling and disruption of cristae (arrows). Diffuse clumping of heterochromatin (1) and endoplasmic reticulum swelling (2) is seen.

microscopy has been used. Control granule cells had a mitochondrial ultrastructure typical for normal cells: mitochondrial cristae were clearly seen, and the mitochondrial matrix had a higher density than the surrounding cytoplasm (Fig. 4A,C). In glutamate-treated cells, the mitochondria were enlarged, the density of the mitochondrial matrix was much lower, and the majority of cristae were damaged (Fig. 4B,D). Thus, ultrastructural alterations are usually indicative of the organelles' disfunctioning. Some pathological signs of glutamate-treated cells were also seen in some other neuronal compartments: nuclear chromatin was clamped, and significant swelling of Golgi cisternae, and slight swelling of granular reticulum were apparent. Similar neuronal ultrastructural changes were observed previously in cultured cerebrocortical neurons after 5 h of hypoxia or 2.5 h of glucose deprivation [19]. It is impossible to exclude the possibility that, under hypoxic conditions, mitochondrial alterations in neurons are the result of a cytotoxic effect of endogenous GLU. The fact that ultrastructural changes under hypoxia and glucose deprivation are abolished by competitive glutamate antagonists supports this speculation [19]. Thus, our data provide strong evidence that the rise in intracellular Ca^{2+} concentration induced by GLU treatment of neuronal cells results in lowering of the mitochondrial membrane potential, as well as in damage to the mitochondrial ultrastructure. The tentative explanation of such calcium-dependent mitochondrial deenergization of granule cells after GLU treatment is induced nonspecific permeability transition pore in the inner mitochondrial membrane, which might be the first step in mitochondrial destruction [20]. In our experiments, cyclosporin A, a specific inhibitor of mitochondrial non-specific permeability transitions, was without apparent effect on the mitochondrial deenergization induced by GLU. This might be the result of the lack of Mg^{2+} in the incubation medium, since Mg^{2+} significantly depresses the effect of GLU on the mitochondrial membrane potential. At the same time Mg^{2+} is strongly required to manifest the effect of cyclosporin A on permeability transitions [21]. Taken together, these facts lead us to suggest that GLU-induced, calcium-dependent processes of mitochondrial deenergization may be among the major causes of neuronal cell damage and death. This can take place not only under exposure to exogenous GLU, but also during brain hypoxia and ischemia. An exclusive role of mitochondria was recently emphasized in cellular death where the step of mitochondrial permeability transitions seems to be crucial [22] makes the data on the loose mitochondrial coupling in intact cells under pathological conditions [23] very significant.

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