# Calcium Reduces Mitochondrial Membrane Potential of Cultured Rat Cerebellum Granule Cells Under Toxic Action of Glutamate

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Glutamate-induced decrease in mitochondrial membrane potential of granule cell is prevented by cobalt ions and the noncompetitive selective antagonist of NMDA-receptors MK-801. Similar to glutamate, the calcium ionophore A23187 reduces this potential.

**Key Words:** *glutamate; granule cells; mitochondria; calcium, A23187, rhodamine 123*

It was reported that hyperactivation of glutamate (GLU) receptors induces $Ca^{2+}$ and $Na^{+}$ influx into the neuron via activated GLU channels [12,13], which leads to subsequent $Ca^{2+}$ influx via potential-operated $Ca^{2+}$ channels after cell depolarization [7]. Impaired $Ca^{2+}$ outflow [4] from neurons results in calcium overload of these cells [11]. Sustained increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) results in activation of calcium-dependent proteolytic and lipolytic enzymes and cell damage [5,14]. Depletion of intracellular ATP was observed in normoxic cultures of GLU-treated cells isolated from the cerebellum and hippocampus [1]. It was demonstrated that neurocytotoxic concentrations of glutamate decrease mitochondrial membrane potential of granule cells and induce ultrastructural changes in the mitochondria [3,8]. However, the causes of GLU-induced changes in neuronal mitochondria remain unclear. It the present study we attempted to elucidate them.

## MATERIALS AND METHODS

Dissociated granule cells from the cerebellum of 7-8-day-old rats were cultured as described previously [2,3]. The culture medium consisted of minimal Eagle medium (90%), 10% fetal serum, 0.8% glucose, 0.1 U/ml insulin, 2 mM glutamine, and 10 mM HEPES. On the second day of culturing the potassium concentration was increased from 5.6 to 25 mM. Cells were incubated with glutamate (10 or 100 μM) for 15 min in medium containing (in mM): NaCl 137, KCl 5.6, $Na_2HPO_4$ 0.35, $NaHCO_3$ 12, $CaCl_2$ 2.3, and glucose 11. Cells incubated for 15 min without GLU served as the control. Calcium channels were blocked with cobalt chloride (2 mM) and MK-801 (30 μM), a noncompetitive selective antagonist of N-methyl-D-aspartate (NDMA) channels, which were added to the incubation medium together with GLU. Cell cultures were treated with the calcium ionophore A23187 (20 μM) for 5 and 30 min. Functional state of the mitochondria was analyzed after a 10-min staining with rhodamine 123 (Rh123) added to the incubation medium to a final concentration of 5 μg/ml after experiment. This method offers visual determination of potential on the mitochondrial membrane [9]. Fluorescence was observed under an MBI-15 microscope.

## RESULTS

After a 15-min incubation of cultured granule cells in balanced salt solution, the mitochondria of neuron and glial cells actively accumulated Rh123 and emitted intense fluorescence after excitation with blue light (λ=450-490 nm). Treatment with GLU (100 μM, 15

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min) led to cytoplasmic edema of granule cells; accumulation of Rh123 was observed in glial cell mitochondria but not in neuronal mitochondria. This effect was concentration-dependent: incubation with 100 μM GLU abolished mitochondrial accumulation of Rh123 practically in all granule cells, while after 10 μM GLU the ability of mitochondria to accumulated Rh123 was partially preserved (Table 1). Calcium increase upon incubation with GLU was prevented with blockers of NDMA and calcium channels. Cobalt chloride (2 mM) abolished the effect of high GLU concentrations on the membrane potential of neuronal mitochondria. The selective antagonist of NDMA-receptors MK-801 that blocks GLU-activated NMDA-channels [10] also prevented mitochondrial de-energizing and cytoplasmic edema in granule cells (Table 1).

In order to find out whether $Ca^{2+}$ influx into neurons reduces mitochondrial potential in granule cells in the absence of GLU, we performed a separate series of experiments with the calcium ionophore A23187 which induces an increase in $[Ca^{2+}]_i$. A 5-min incubation with 20 μM A23187 resulted in pronounced edema of granule cells and prevented accumulation of Rh123 in their mitochondria (Table 1).

It should be noted that neurons and glia responded to A23187 in different ways. After a 5-min incubation with this ionophore, mitochondria in most glial cells accumulated Rh123; this accumulation was prevented by a longer (30 min) incubation.

It was demonstrated that calcium overload of neurons associated with the neurotoxic effect of GLU is the major activator of pathological biochemical processes leading to cell death [5,14]. It is reasonable to suggest that reduction in mitochondrial potential of GLU-treated granule cells is also associated with calcium overload. This suggestion is confirmed by our findings that calcium overload induced with calcium ionophore or toxic doses of GLU leads to mitochondrial de-energizing, while activation of glutamate receptors without $Ca^{2+}$ influx into neurons does not reduce their mitochondrial membrane potential. Our experiments showed that a prolonged and sustained $Ca^{2+}$ increase is necessary for triggering the processes leading to a decrease in mitochondrial membrane potential, i.e., it was shown that transitory $Ca^{2+}$ rise induced by a low (10 μM) GLU dose [6] did not affect the accumulation of Rh123 by granule cells. Presumably, mitochondrial de-energizing in GLU-treated granule cells results from $Ca^{2+}$-induced changes in the permeability of mitochondrial membranes, which may represent the initial stage of mitochondrial destruction [15].

A decrease in the mitochondrial membrane potential in granule cells was always accompanied by cytoplasmic edema (Table 1). Obviously, the calcium-induced mitochondrial de-energizing leads to a marked decrease in intracellular ATP levels, impairs energy-dependent sodium outflow by $Na^+/K^+$ ATPase, and induces cytoplasmic edema.

TABLE 1. Effects of Calcium Modifying Agents on Cultured Granule Cells from Rat Cerebellum

| Substance | No. of experiments | Cytoplasmic edema | Rh123 accumulation |
|---|---|---|---|
| Control solution | 18 | — | + |
| Glutamate, 100 μM | 18 | + | — |
| Glutamate, 10 μM | 6 | — | + |
| $Co^{2+}$, 2 mM+ glutamate, 100 μM | 6 | — | + |
| A23187, 20 μM | 6 | + | — |
| MK-801, 30 μM+ glutamate, 100 μM | 6 | — | + |

Our results indicate that GLU-induced increase in the intracellular $Ca^{2+}$ concentration reduces the energy potential of neuronal mitochondria and, consequently, depletes energy resources of the neuron. From these data it can be suggested that GLU-induced calcium-dependent processes in the mitochondria are one of major causes of neuronal damage and death in brain ischemia and hypoxia.

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