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Stimulation of kainate toxicity by zinc in cultured cerebellar granule neurons and the role of mitochondria in this process

Ekaterina R. Lozier[a], Elena V. Stelmashook[b], Rustem E. Uzbekov[c,d], Svetlana V. Novikova[b], Savva D. Zorov[c], Irina B. Alieva[a], Brigitte Arbeille[d], Dmitry B. Zorov[a], Nickolay K. Isaev[a,b,*]

[a] A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119992 Moscow, Russia
[b] Institute of Neurology, Department of Brain Research, Russian Academy of Medical Sciences, per. Obukha 5, 105064 Moscow, Russia
[c] Faculty of Bioengineering and Bioinformatics, Moscow State University, 119992 Moscow, Russia
[d] Laboratory of Cell Biology and Electron Microscopy, Faculty of Medicine, University Francois Rabelais, 10, Boulevard Tonnelle, 37032 Tours, France

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ABSTRACT

Zinc chloride (0.01 mM kept for 3 h) is not toxic to cultured cerebellar granule neurons (CGNs) while kainate (0.1 mM kept for 3 h) demonstrates some but very low toxicity towards these cells.

Measurements of the relative intraneuronal zinc ion concentration showed that increase in $[Zn^{2+}]_i$ under the simultaneous action of $ZnCl_2$ and kainate was significantly stronger compared to their separate action. Simultaneous treatment of CGNs with kainate and zinc chloride caused the swelling of neuronal mitochondria and consequent intensive neuronal death, which was totally prevented by NBQX (an AMPA/kainate-receptors blocker) or ruthenium red (a mitochondrial Ca^{2+} uniporter blocker). These data imply that Zn^{2+} synergistically to kainate increase their separate toxic effects on mitochondria leading to rapid neuronal death.

1. Introduction

It is widely accepted that hyperstimulation of AMPA/kainate and NMDA receptors by excitatory amino acids is the main pathogenetic factor of neuronal damage during hypoxia/ischemia and some other deleterious conditions of the brain. Activated glutamate receptors initiate opening of associated ion channels, which leads to the disturbance of cellular ion homeostasis (Lipton, 1999). It has been previously thought that excessive entry of Ca^{2+} is one of the main reasons for neuronal damage induced by excitatory amino acids. However, in recent years it has been shown that not only Ca^{2+}, but also Zn^{2+} ions may be considered as secondary messengers that under certain conditions cause cell death. Although intracellular zinc is mainly bound to proteins and entrapped within axonal vesicles together with excitatory neuromediator glutamate, the concentration of free zinc in neurons can be sharply increased under pathological conditions, negatively affecting these cells functioning (Sensi et al., 2009). Synaptically released Zn^{2+} ions are transported through postsynaptic membrane mostly using Zn^{2+}- and Ca^{2+}-permeable channels associated with AMPA receptors (AMPARCa-Zns) (Weiss et al., 1993). The inhibition of AMPA receptors during oxygen–glucose deprivation (OGD) demonstrates a strong neuroprotective effect (Yin et al., 2002; Noh et al., 2005). OGD induces a progressive rise in cytosolic Zn^{2+} levels in neurons, which precedes Ca^{2+} deregulation. Zn^{2+} chelator N,N,N′,N′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) significantly delays both Ca^{2+} deregulation and the increase in plasma membrane permeability, indicating that Zn^{2+} contributes to the degenerative signaling (Medvedeva et al., 2009). Mitochondria are very important targets for calcium and zinc ions, where they interact with both independent and competing sites (Gazaryan et al., 2007). The possibility of zinc transport in mitochondria through Ca^{2+} uniporter has earlier been demonstrated in isolated mitochondria (Saris and Niva, 1994). Probably, zinc and calcium ions can mutually increase their individual toxic effects on mitochondria mediated by AMPA/kainate receptor agonists.

2. Materials and methods

2.1. Primary cerebellar cultures

Primary cultures were prepared from the cerebella of 7- to 8-days old Wistar rats. The cerebella were washed with Ca^{2+} and Mg^{2+} free PBS (Dulbecco) and incubated in the same solution containing 0.05% trypsin and 0.02% EDTA (15 min, 37 °C). After incubation, the tissue was washed twice in PBS and dissociated by repeated pipetting in a nutrient medium of the following composition: fetal calf

* Corresponding author at: A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119992 Moscow, Russia. Tel.: +7 495 9395944; fax: +7 495 9390338.
E-mail address: isaev@genebee.msu.su (N.K. Isaev).

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serum (10%), minimum essential medium Eagle (90%), glutamine (2 mM), and HEPES (10 mM). After mild centrifugation (1000 rpm, 1 min) at room temperature, the cells were resuspended in the required volume of the nutrient medium. Cell suspension (0.1 ml) was applied to a poly-L-lysine-coated 96-well plate (3×10^3 cells/mm^2) and then kept in a CO_2-incubator (5% CO_2, 36.5 ± 0.5 °C). On the second day *in vitro*, cultures were supplemented with fresh medium containing 25 mM KCl and maintained up to 7–8 days *in vitro*.

Experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

2.2. Pharmacological treatment

$ZnCl_2$ (0.01 mM), kainate (0.1 mM), and the blockers of ionotropic glutamate receptors – NBQX (0.01 mM), MK-801 (0.01 mM), and ruthenium red (20 μg/ml) – were supplemented directly into the solution.

2.3. Assessment of neuronal viability

Viability was determined as described elsewhere (Stelmashook et al., 2009). Cell cultures were fixed with ethanol–formaldehyde–acetic acid (7:2:1) mixture and stained with trypan blue. The percentage of surviving neurons was estimated by counting the intact nuclei of the CGNs in five fields of view. The viability of untreated control cultures was taken as 100%, and the viability of treated cells was counted as a percentage referred to control values.

2.4. Analysis of intracellular Zn^{2+} concentration

Cells were loaded with 0.005 mM FluoZin-3 for 0.5 h at 36.5 ± 0.5 °C followed by triple washing in BSS (in mM: NaCl 154, KCl 25, $CaCl_2$ 2.3, $MgCl_2$ 1, $NaHCO_3$ 3.6, Na_2HPO_4 0.35, HEPES 10, pH 7.3). The level of intracellular Zn^{2+} or Ca^{2+} concentration was quantified using a Cytofluor plate reader (CytoFluor II, PerSeptive Biosystems, Framingham, MA, USA) for measurements of the fluorescence intensity of FluoZin-3 with excitation at 485 nm and maximal emission at 530 nm.

2.5. Electron microscopy

For electron microscopy we used cell suspension placed on poly-L-lysine-coated coverslips in a 40-mm diameter Petri dish. Cells were fixed using 2.5% glutaraldehyde prepared in phosphate buffer (pH 7.2) followed by postfixation with 1% osmium tetroxide, dehydration in ethanol, and embedding in Epon 812. Ultrathin sections were prepared using a Leica Ultracut UCT ultramicrotome and examined at 100 kV in a JEOL 1011 transmission electron microscope (JEOL, Japan). Totally, 54 cells were examined with comprehensive examination of their mitochondria (13 cells from control samples, 10 cells exposed to 0.1 mM kainate, 10 cells exposed to 0.01 mM zinc chloride, 11 cells exposed to 0.03 mM zinc chloride, 10 cells exposed to both 0.01 mM zinc chloride and 0.1 mM kainate).

2.6. Reagents

Unless otherwise noted, all media and supplements used in cell culture were purchased from Biochrom KG Berlin, Germany. Fluo-4 AM and FluoZin-3 were obtained from Molecular Probes (USA). NBQX and other reagents were from Sigma Chemicals (Germany).

2.7. Statistics

The one-way ANOVA with Newman–Keuls post-test was used for statistical analysis. Levels of $p < 0.05$ were considered as statistically significant. The results are given as means ± SEM. All data were obtained from three independent experiments with at least 12 different cultures in each.

3. Results

3.1. Cytotoxicity of kainate and Zn^{2+}

Application of kainate (0.1–0.35 mM) for 3 h induced strong dose-dependent neuronal death (Fig. 1). We found that incubation with 0.1 mM kainate did not significantly change the viability of CGNs. Counting neurons with normal morphology demonstrated that after exposure to kainate 89 ± 3% of cells survived. Chloride zinc alone (0.01–0.03 mM) did not have any effect on neuronal viability. The addition of kainate to BSS containing Zn^{2+} caused a pronounced increase in the death rate of neurons in culture (Fig. 2). After 3 h incubation with 0.1 mM kainate and 0.01 mM Zn^{2+} only 22% of neurons survived. The addition of NBQX (AMPA/kainate glutamate receptor blocker) or ruthenium red (mitochondrial Ca^{2+} uniporter blocker) protected CGNs from the toxic effect of the simultaneous presence of kainate and Zn^{2+} (Fig. 3A). Supplementation the medium with MK-801 (NMDA glutamate receptor blocker) significantly protected CGNs from the toxic effect of simultaneous presence of kainate and Zn^{2+} (Fig. 3B).

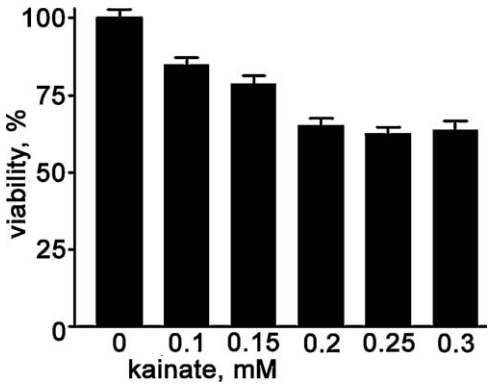

Fig. 1. Relationships between viability of cultured cerebellar granule neurons and kainate concentration in incubation solution.

3.2. Measurements of intracellular Zn^{2+} concentrations

Further, we analyzed the effect of zinc supplementation on FluoZin-3 fluorescence in neurons. We found that supplementation of the incubation medium with zinc chloride for 0.5 h causes a strong dose-dependent increase of FluoZin-3 fluorescence in neurons (Fig. 4). The fluorescence increase comprised 495 ± 23% versus control when 0.03 mM of zinc chloride was added.

Measurements of intracellular Zn^{2+} concentration using the fluorescent probe FluoZin-3 revealed that 1.5 h exposure to Zn^{2+}, kainate, or kainate + Zn^{2+} induced an increase in FluoZin-3 fluorescence in neurons by 309 ± 14%, 134 ± 10%, and 359 ± 25%, respectively (Fig. 5).

3.3. Mitochondrial ultrastructure

Mitochondrial ultrastructure in control rat cerebellar granule neurons displayed features typical for this cell type. Mitochondria carried multiple laminar cristae, and their matrix was more electron-dense compared to surrounding mitochondrial cytoplasm (Fig. 6A). The diameter of mitochondria comprised 0.21 ± 0.007 μm in average ($n = 118$). Zinc chloride or kainate alone did not significantly alter mitochondrial ultrastructure: the diameter of mitochondria after such exposures was 0.21 ± 0.005 μm ($n = 111$) and 0.20 ± 0.004 μm ($n = 132$), correspondingly (Fig. 6B and C). It should be noted that mitochondrial matrix in some neurons was slightly lighter after exposure to kainate. A similar effect was caused by 0.03 mM zinc chloride, when mitochondrial diameter reached 0.23 ± 0.006 μm ($n = 103$) (Fig. 6D). The simultaneous presence of kainate and zinc chloride led to significant disturbances: remarkable mitochondrial swelling and matrix lightening were observed (Fig. 6E and F). The diameter of mitochondria under such combination was 0.29 ± 0.01 μm ($n = 81$).

4. Discussion

Many terminals of the brain glutamatergic neurons are enriched with ionized zinc, which release during neuronal activity is necessary for normal physiological activity of the brain. However, current data reveal that in addition to its contribution to epilepsy and ischemia Zn^{2+} is involved in the development of several