ISSN 1990-7478, Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology, 2012, Vol. 6, No. 4, pp. 294–299. © Pleiades Publishing, Ltd., 2012.
Original Russian Text © A.E. Bolshakov, D.A. Sibarov, P.A. Abushik, I.I. Krivoi, S.M. Antonov, 2012, published in Biologicheskie Membrany, 2012, Vol. 29, No. 6, pp. 422–428.

ARTICLES

Dose-Dependence of Antiapoptotic and Toxic Action of Ouabain in Neurons of Primary Cultures of Rat Cortex[1]

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Received April 17, 2012

Abstract—Effects of 0.01 nM–1 nM ouabain on neuronal survival in excitotoxic stress and ouabain self toxic action in concentrations from 10 nM to 30 μM were studied. Neuronal viability was evaluated by measuring Bcl-2 protein expression and using vital staining test allowing recognition of live, necrotic and apoptotic cells. Excitotoxic stress was induced by 240-min treatment with agonists of ionotropic glutamate receptors (NMDA or kainate). Experiments were performed on rat primary neuronal cultures of 7–14 DIV (days in vitro). Thirty μM NMDA induced apoptosis in 45 ± 9% ($n = 5$), and 30 μM kainate, in 52 ± 5% ($n = 5$) of neurons. An antiapoptotic effect of ultra low (0.01 nM–1 nM) ouabain concentrations was found to restore Bcl-2 expression and to bring apoptosis level back to control values (about 10%, $n = 5$). Since in this concentration range ouabain is not able to inhibit NKA, we conclude that neuroprotection discloses the signaling function of NKA. Whereas ouabain self toxic action in higher concentrations (10 nM–30 μM, during 240 min) resulted in necrotic death of 45% neurons (apoptosis remained as under the control conditions), the large portion of neurons were unaffected. The relatively low threshold concentration of ouabain toxic action (10 nM) is consistent with the sensitivity to ouabain of α3-isoform of NKA. Thus, ouabain was found to have a bimodal effect, including antiapoptotic action in excitotoxic stress in the concentration range from 0.01 nM to 1 nM, and self toxic action at larger concentrations. Self toxicity of ouabain is initiated through inhibition of NKA pumping function. Neuronal heterogeneity with respect to ouabain toxic action is probably related with the different expression of α1 and α3-isoforms of NKA in pyramid neurons and interneurons.

Keywords: Na,K-ATPase, ouabain, apoptosis, cortical neurons, glutamate receptors.

DOI: 10.1134/S1990747812050029

Na^+,K^+-ATPase (NKA) is an integral membrane enzyme. Its main function is maintaining transmembrane Na^+ and K^+ gradients by an active transport of these ions. Cardiotonic steroids (CS), in particular ouabain, are highly specific inhibitors of NKA. These ligands when in relatively high doses could cause membrane depolarization because of Na^+ and K^+ transmembrane gradient depletion, which also affects other important cell transport mechanisms including Na-dependent glutamate uptake [1]. Similar NKA dysfunctions take place during ischemia caused hypoxia in neurons, which results in diminished ATP production. The accompanied accumulation of glutamate in synaptic cleft and extracellular space because of increased nonquantum mediator secretion [2] leads to glutamate receptors hyperactivation and uncompensated Na^+ and Ca^{2+} entry into cytoplasm. High level of free intracellular Ca^{2+} promotes glutamate secretion, forming the positive feedback forcing excitotoxic state of neurons. The popular model to study excitotoxicity is primary cortical culture treated with saturating concentrations of ionotropic glutamate receptor agonists like N-methyl-*D*-aspartate (NMDA) or kainic acid (KA) [3–5]. Regardless of the difference in excitotoxicity mechanisms triggered by hyperactivation of NMDA or AMPA/KA receptors, the main cell death mechanism in both cases is apoptosis [6].

It has been shown that in cerebellar neuronal culture, inhibiting NKA with ouabain starts from 10 nM, while at 100 nM and 1 μM NKA activity decreases by 80% and 50% respectively [7]. Further elevation of CS concentration increases cell death because of the excessive membrane depolarization. Nevertheless, in ultra low doses CS have different properties and can, in particular, increase NKA activity [8]. In vivo experiments revealed ouabain injected into striatum to significantly increase neuronal viability and antiapoptotic Bcl-2 protein expression after treatment with KA in neurotoxic doses [9]. Similar results were obtained in vitro in primary cortical culture after toxic action of

[1] The article was translated by the authors.

selective NMDA receptors agonist resulting in suppressed Bcl-2 expression and increased cell death, preferably by apoptosis. Adding 0.1 nM of 1 nM ouabain to the cell medium restored Bcl-2 expression and neuronal viability to control values [10].

We expect the neuroprotective properties of CS, which emerge at a nanomolar concentration range, to be the result of two independent effects mediated by NKA: inhibition of pumping function and signal function activating intracellular cascades [10–13]. The great potential of CS as neuroprotective agents forces us to investigate also the mechanisms of the CS toxic effect at high doses. The wide range of ouabain concentrations reported to have cytoprotective propertied by different authors [12, 14, 15], which raises a question of optimal CS doses, and in particular, of ouabain effective concentrations.

The aim of this work was to study the mechanisms of ouabain action on neuronal functional state within the concentration range from 0.01 nM to 30 μM. Neuronal viability staining test allowing discrimination of live, necrotic, and apoptotic cells [16] and Bcl-2 protein expression were used as markers of cell functional state.

MATERIALS AND METHODS

The study was performed on neurons in rat primary cortical culture, obtained from embryos at 16th day of prenatal development, the period when the cortex contains nearly no glial cells. Rats were anesthetized with CO_2 inhalation in compliance with IEPHB RAS local ethics committee rules. Cells were incubated in culture medium on glasses covered with poly-*D*-lysine. Experiments were performed on 7–14 DIV (days in vitro). The following physiological solution was used (mM) NaCl 140, KCl 2.8, $CaC1_2$ 1, $MgCl_2$ 1, HEPES 10 (pH = 7.2–7.4). An absence of glucose in this solution, as it has been shown [17], does not result in decrease of neuronal viability during 5 h of incubation. The magnesium-free solution was used in order to avoid the neuroprotective effect of magnesium ions, which block inward currents through the channels of NMDA receptors [17]. The agonists of ionotropic glutamate receptors NMDA and KA was added to perfusion at the final concentration of 30 μM. NMDA was always coapplied with an equal quantity of glycine, being coagonist for NMDA receptors.

Neurons were tested in control and after the treatment with agonists of glutamate receptors alone and together with ouabain in subnanomolar concentrations (0.01 nM–1 nM), or with ouabain alone in the concentration range from 1 nM to 30 μM. The quantitative evaluation of neuronal death was made using vital assay, by staining of neurons with acridine orange (0.001%) and ethidium bromide (0.001%) consequently for 1 min each [16]. This allows us to separate cells into three groups: live, nonviable necrotic, and apoptotic neurons. Ethidium bromide, a red fluorescent indicator, stains only necrotic cells with damaged membrane but does not enter live or apoptotic cells. Acridine orange passes though cell membrane making neuronal nuclei of live cells to have green fluorescence. Apoptosis is accompanied by cytosole acidification shifting acridine fluorescence to orange region of the emission spectrum. The proportion of live, apoptotic, and necrotic cells was estimated according to fluorescent images obtained in red and green spectral regions. Each pair of images was used to make 2D diagram, where axes presented pixels intensity for green and red images. Dots on the diagram show nonzero fluorescence intensity pixels. The noise thresholds cutting off pixels with too weak intensity splits the diagram into 4 areas: 1) pixels with red/orange fluorescence (600–800 nm) of necrotic cells; 2) pixels with green fluorescence (500–560 nm) corresponding to live neurons; 3) pixels with colocalized green and red fluorescence revealing apoptotic cells, and 4) pixels with intensities below both thresholds, excluded from statistics. The number of pixels in each area was proportional to the total area occupied by nuclei of live, apoptotic, and necrotic neurons.

Before vital assay or fixation, the coverslips with neurons were incubated for 240 min in physiological solution (control), or in solutions containing agonists of glutamate receptors agonists (NMDA or KA) alone or together with ouabain, or in solutions with ouabain alone in different concentrations.

For immunocytochemical detection of Bcl-2, cells were fixed in 4% paraphormaldehyde solution in phosphate-buffered saline (PBS) for 30 min. After fixation, cells were washed twice with PBS (15 min). Before treatment with BSA (bovine serum albumin, 2%), the cells were incubated with Triton X-100 (0.2%) for 15 min, washed with PBS, and exposed to primary antibodies at 4°C for 12 h. After washing to remove primary antibodies (dilution 1 : 150), fluorochrome-conjugated secondary antibodies were added, where phycoerythrin (Phr) was used as a fluorochrome. Reactions with secondary antibodies lasted for 40 min at room temperature (23°C). Antibodies manufactured by Abcam plc (UK) were used. Other reagents were obtained from Sigma– Aldrich Co.

Fluorescence images were captured using scanning confocal microscope Leica SP5 MF (Leica Microsystems, Germany). Fluorochromes were excited with 488 nm Argon laser line (focal plane light intensity was less than 3 mW). Green fluorescence was captured in the range of 500–560 nm and red, in the range of 600–800 nm. The emission of Phr was detected in red spectral area (maximum, 620 nm). The quantity of Bcl-2-free and Bcl-2-immunopositive cells was calculated on the images. The colocalization diagram for acridine orange and ethidium bromide staining was automati-

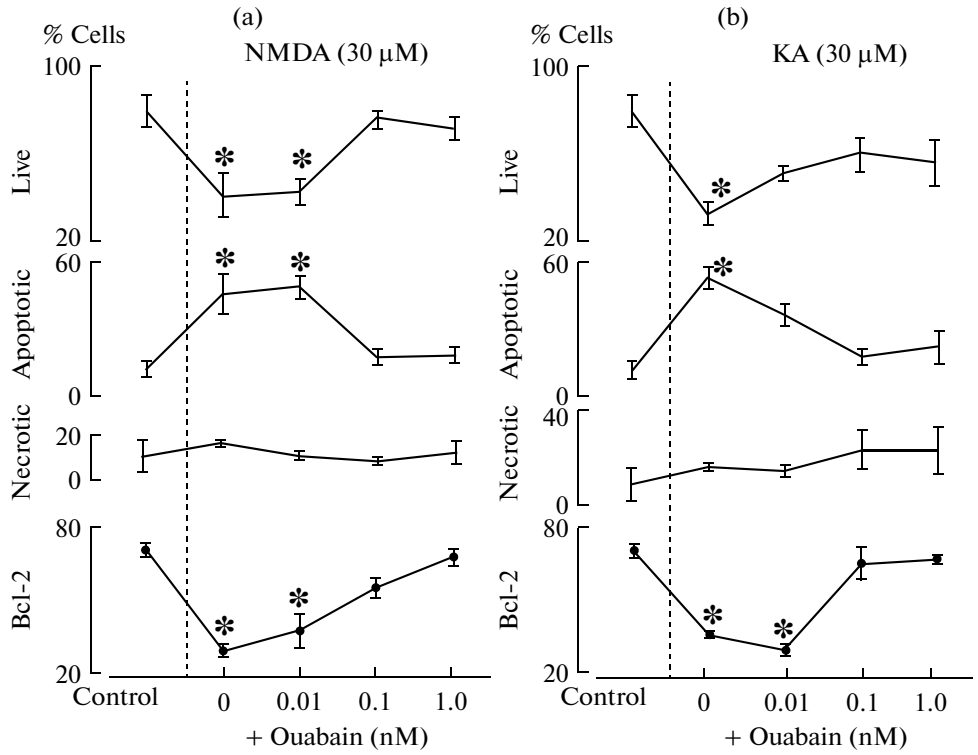

Fig. 1. The dose-dependence of ouabain antiapoptotic action in excitotoxic stress. (a) 240-min treatment with 30 μM NMDA. (b) The same treatment with 30 μM KA. The proportion of live, apoptotic, and necrotic neurons and the number of cells expressing Bcl-2 protein is shown. Each point represents an averaged data of 5 experiments (600–700 cells). *The value differs significantly ($p < 0.001$, $n = 5$) from both control values and values corresponding to application of agonists together with 0.1 nM or 1 nM ouabain.

cally calculated with self-developed plug-in (http://sibarov.ru/index.php?slab=software) for ImageJ image analysis software (rsbweb.nih.gov/ij/).

The difference between groups was evaluated using two-way ANOVA with Bonferroni post-test. The statistical significance of the differences was determined at the confidence level $p < 0.05$. In the text and images, mean values with standard errors are presented.

RESULTS

The viability test in rat brain neuronal culture revealed the proportion of live, apoptotic and necrotic cells in control and in experimental conditions. In control, red-green fluorescence intensity diagrams exhibited an absolute majority of live neurons $79 \pm 9\%$ ($n = 5$) (Figs.1a, 1b). Thus, in control conditions there was no colocalization of green and red emission, which indicates unaffected cell membranes. A negligible part of neuronal nuclei emission was shifted towards yellow, showing the apoptosis-related initial acidification of the cytoplasm.

In the model of excitotoxic stress induced by 240-min treatment with 30 μM NMDA or 30 μM KA, a significant ($p < 0.001$, $n = 5$) increase of apoptotic cells quantity was observed (Fig. 1). Addition of ouabain had a neuroprotective effect depending on its concentration. In the presence of NMDA (Fig. 1a) or KA (Fig. 1b) ouabain at 0.1 nM or 1 nM significantly ($p < 0.01$, $n = 4$) elevated the number of live neurons, which became statistically equal to control values. The collation of neuronal viability in the presence of KA alone and together with ouabain revealed a neuroprotective effect of ouabain starting from 0.01 nM ($p < 0.05$, $n = 4$). As for NMDA, the 0.01 nM ouabain concentration did not influence neuronal viability. We suppose that 0.01 nM is the lowest threshold concentration of ouabain for its neuroprotective action on cortical neurons. Despite the difference in cell death mechanisms induced by activation of NMDA or AMPA/KA receptors [6], the ouabain concentration range of 0.01 nM–1 nM was effective to increase neuronal viability, presumably due to the suppression of apoptosis. Notably, the number of necrotic neurons did not change significantly (Fig. 1).

Thus, the addition of agonists of ionotropic glutamate receptors in saturating concentration apparently induces apoptotic neuronal death but not necrosis. Ouabain exhibits a strong antiapoptotic action at concentrations starting from 0.1 nM but does not affect cell necrosis probability.

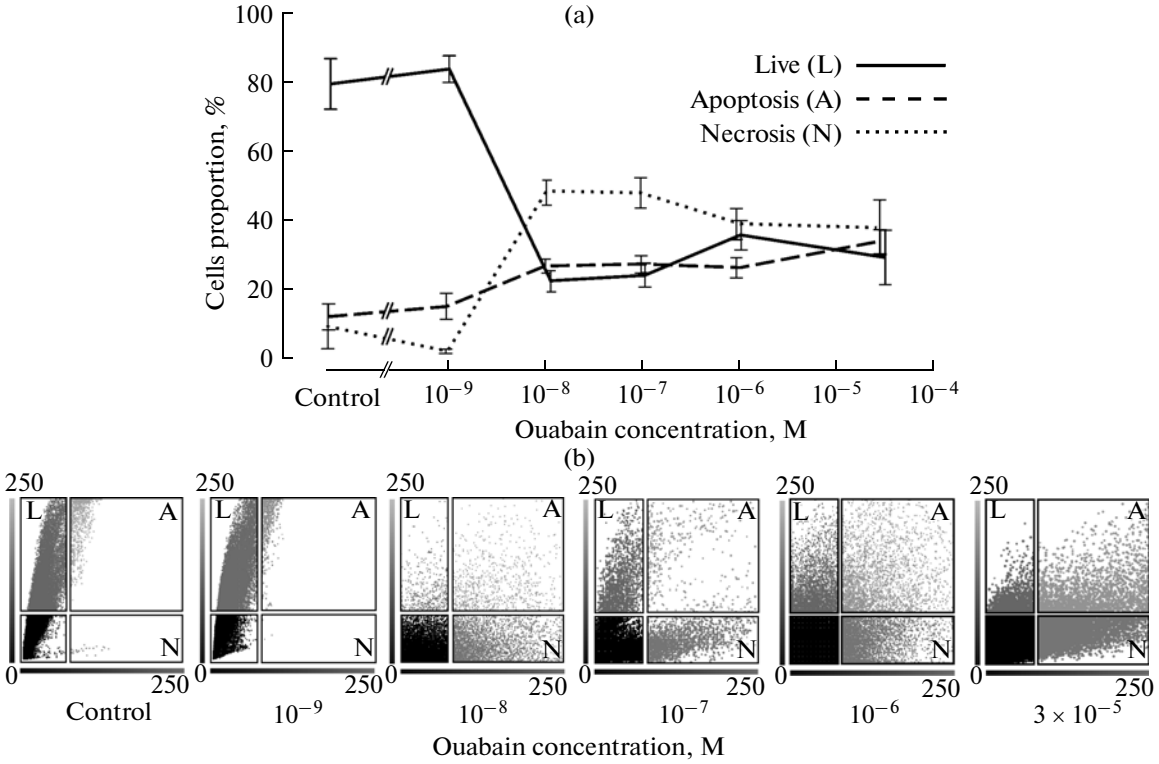

Fig. 2. Dose-dependence of the ouabain toxic effect. (a) The proportion of live, apoptotic, and necrotic neurons after 4-h incubation with ouabain. Each point represents an averaged data of 4–9 experiments. (b) The examples of acridine orange (Y-axis) and ethidium bromide (X-axis) fluorescence colocalization diagrams obtained in individual experiments. Diagrams are splitted into areas corresponding to live (L), apoptotic (A), and necrotic (N) cells. See Matherials and Methods for details.

The neuronal death due to hyperactivation of ionotropic glutamate receptors most commonly occurs as the result of mitochondrial dysfunction [18]. The reliable indicator of damaged mitochondrial functions is an expression of Bcl-2 protein, acting as regulator of cell energy metabolism. Bcl-2 is normally expressed at a high level but decays during apoptosis [19]. In the next series of experiments we studied the influence of ouabain in different concentrations on Bcl-2 expression in the model of NMDA- or KA-induced excitotoxic stress. In control conditions, the majority of neurons expressed Bcl-2 at high level. Incubation in the presence of 30 μM NMDA or 30 μM KA for 4 h significantly reduced the Bcl-2 expression ($p < 0.001$, $n = 5$; Fig. 1). However, concurrent application of the agonists together with 0.1 or 1 nM ouabain did not decrease the Bcl-2 expression. Lower ouabain concentrations (0.01 nM) were not effective. Figure 1 illustrates the coordinated decrease of Bcl-2 expression and neuronal viability after the NMDA or KA treatment and also simultaneous reversion of these parameters to control values in the case of coapplication of the agonists with ouabain. This demonstrates the mutual dependence of neuronal viability and expression of Bcl-2, indicating the efficiency of mitochondrial functions. It also depicts the ability of ouabain to restore mitochondrial functions and to reduce apoptosis during excitotoxic stress.

Both the neuronal viability test and evaluation of Bcl-2 immunopositive cells show that the antiapoptotic effect of ouabain is most pronounced in the concentration range of 0.1 nM–1 nM. At lower concentrations ouabain failed to restore the Bcl-2 expression but turned out to be the threshold level for an increase of the neuronal viability in the used method of neurotoxic stress.

The ouabain upper concentration limit, safe for cortical neurons, should depend on the neurotoxic action of ouabain as an inhibitor of NKA. Subsequent experiments were performed to test the viability of neurons in the presence of 1 nM, 10 nM, 100 nM, 1 μM, and 30 μM of ouabain.

As it was mentioned before, ouabain at 1 nM was most effective to antagonize the neurotoxic effect of ionotropic glutamate receptors agonists. Figure 2a shows that 1 nM ouabain alone has no effect on neuronal viability after 240-min treatment. Fluorescence intensity distribution diagrams (Fig. 2b) indicate nearly identical neuronal survival in control conditions and in the presence of 1 nM ouabain.

The toxic effect of ouabain became apparent starting from 10 nM and was manifested in an increase of

necrotic cells values to 48.3 ± 5.2 %, as compared to 1.8 ± 0.6 % for 1 nM (Fig. 2a; $p < 0.001$, $n = 6$). Fluorescence intensity distribution diagrams (Fig. 2b) show that as far as the ouabain concentration changes from 1 to 10 nM, red fluorescence increases at the expense of green fluorescence. It indicates that 10 nM of ouabain is enough to suppress ouabain-sensitive NKA isoforms [20], which can affect neuronal ion balance and result in elevated necrosis. Further increase of ouabain concentration did not change the ratio of live and dead neurons (Fig. 2a).

Fluorescence intensity distribution diagrams corresponding to 10 nM, 100 nM, 1 μM and 30 μM ouabain (Fig. 2b) reflect the same predominance of red emission of necrotic cells. The gradual elevation of ouabain concentration from 10 nM to 30 μM is also accompanied with the tendency of the increase of apoptotic neurons share, which can reflect the reciprocal conversion of apoptosis and necrosis processes during the loss of Na^+, K^+, and Ca^{2+} gradients [21]. It is possibly related to the fact that increasing of ouabain concentration not only enhances NKA inhibition but also promotes NKA signaling on proapoptotic p42/44 MAP-kinase cascades [7].

Thus, NKA inhibition with ouabain in concentrations of 10 nM or higher forces neurons to die preferentially by necrosis, which happens because of the loss of Na^+ and K^+ gradients, consequent failure of water balance and disruption of cell membrane. Higher ouabain concentrations from 0.1 to 30 μM are equally effective to promote necrosis or apoptosis. It can be possibly due to effective inhibition of neuronal NKA isoforms already with 10 nM ouabain, which conforms to published data concerning the sensitivity of these isoforms to ouabain [7, 20, 22].

DISCUSSION

The excitotoxic stress contributes to the pathogenesis of numerous neurodegenerative disorders (epilepsy, ischemia, stroke, etc.) [1]. It causes neuronal death because of neuronal overexcitation, excessive intracellular calcium accumulation and related failure of energy balance. In the model of excitotoxic stress used here (applying the saturating concentrations of glutamate receptors agonists in primary neuronal culture), ouabain at concentrations of 0.01 nM–1 nM exhibited antiapoptotic dose-dependent effect. The threshold ouabain concentration for the antiapoptotic effect was slightly higher for NMDA (Fig. 2a) than for KA (Fig. 2b). This could be due to the fact that NMDA causes a rapid elevation of the intracellular Ca^{2+} concentration, while KA induces a gradual rise of intracellular Ca^{2+} [5].

It is generally accepted that NKA α-subunit site is the only place for ouabain binding. Neurons are known to express α1- and α3-subunits of NKA, being ouabain-resistant and ouabain-sensitive respectively [22]. The blocking constants for these NKA isoforms differ significantly but are in the range of tens of μM for α1-isoform and of about 10 nM for α3-isoform. Thus, the concentration of ouabain demonstrating the antiapoptotic effect (0.01–1 nM) is less than it is needed to inhibit NKA pumping function. This suggests that ouabain neuroprotective action is realized through another function of NKA, which is the signaling function. This signaling is supposed to happen through NKA conformation changes following ouabain binding, which trigger various intracellular signal pathways [11–13]. The particular mechanism of the NKA signaling that takes place in our experiments is still unknown. However, our data on the elevated expression of antiapoptotic Bcl-2 protein suggest that the antiapoptotic signaling cascades can be involved.

Higher concentrations of ouabain (10 nM and more) cause neuronal death mostly due to necrosis. NKA α3- and α1-isoforms are shown to be unequally expressed in different neuronal types. Interneurons mostly express α3-NKA, which can be inhibited with as little as 10 nM ouabain [20]. Pyramid neurons mostly express α1-NKA requiring 25 μM ouabain to be inhibited [20]. In primary cortical culture neurons are heterogeneous. Cells keep features of interneurons and pyramid cells [5]. The dramatic increase of the number of necrotic cells after the treatment with 10 nM ouabain in our experiments probably reflects the death of interneurons. However, the third part of neurons survived even after the application of 30 μM ouabain, which confirms the heterogeneity of neuronal population by the sensitivity to ouabain. We suppose that ouabain in the concentration range of 1–10 nM inhibits α3-NKA in some neuronal types and triggers cell death mostly via the necrotic pathway. The rest of the neuronal population, in which membrane potential is maintained by α1-NKA, are resistant to ouabain up to 30 μM. Thus, different NKA-subtypes expressed by different types of neurons may account for the observed concentration range (0.01 nM–10 nM) for the ouabain neuroprotective effect.

In summary, the excitotoxic stress induced by ionotropic glutamate receptor agonists (NMDA or KA) in primary cortical culture causes apoptotic cell death. Depending on the concentration, ouabain induces two contradictory effects. At the concentration range of 0.01–1 nM it has an antiapoptotic action manifesting in excitotoxic stress and does not exhibit toxic effect on neurons. At the concentration range from 10 nM to 30 μM, ouabain is toxic and induces neuronal death by necrosis. Notably, even at high doses ouabain does not promote apoptosis. Here we demonstrate the substantial difference in the mechanisms of ouabain antiapoptotic action in excitotoxic stress and its toxic action at high doses.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project nos. 11-04-00397 and 10-04-00970) and by the SPBSU (project no. 1.37.118.2011).

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SPELL: 1. depolarization, 2. coapplied, 3. acridine