Bifonazole Modulates Death of Cultured Cerebellar Granular Cells Induced by Glutamate and Oxygen-Glucose Deprivation

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Treatment of cultured rat cerebellar granular cells with calmodulin antagonist bifonazole (10 mM) during oxygen-glucose deprivation or exposure to glutamate (75 mM) prevented neuronal death. However, addition of bifonazole after glutamate treatment promoted neuronal death. Calmodulin antagonists trifluoperazine and thioridazine had no protective effects, while thioridazine even potentiated the toxic effect of glutamate.

Key Words: *bifonazole; glutamate; oxygen and glucose deprivation; cerebellar granular cells*

Bifonazole possesses a wide spectrum of biological activity. This compound acts as the calmodulin antagonist [9] and produces antifungal effects [14]. Recent studies demonstrated that bifonazole reduces the content of intermediate glycolytic products and ATP in melanoma cells, decreases their viability [8], and removes hexokinase from mitochondrial membranes in these cells [11]. Bifonazole affects Ca^{2+} transport systems in non-neural cells [4,7,13]. Calcium influx into neurons during hypoxia and ischemia or after exposure to the toxic effect of excitatory amino acids is a key mechanism of neuronal death under these pathological conditions. Calmodulin antagonist bifonazole affects Ca^{2+} transport and intracellular Ca^{2+} content in non-neural cells. Here we evaluated whether bifonazole can modulate toxic effects of glutamate (Glu) on neurons and prevent their ischemic damages.

MATERIALS AND METHODS

Cerebellar granular cells (CGC) were obtained from 8-day-old Wistar rats and cultured for 7-8 days as described elsewhere [5]. Cultures were grown on cover glasses (22×22 mm) coated with poly-L-lysine and placed into 40-mm plastic Petri dishes. The culture medium contained 10% fetal bovine serum, 90% Eagle's minimum essential medium, 0.8% glucose, 2 mM glutamine, 0.2 U/ml insulin, and 10 mM HEPES. On day 2 of culturing KCl concentration in the medium was brought to 25 mM. The neurocytotoxic effect was assayed in a balanced salt solution (135 mM NaCl, 5.0 mM KCl, 0.035 mM Na_2HPO_4, 12 mM $NaHCO_3$, 2.3 mM $CaCl_2$, and 11 mM glucose, pH 7.6-7.8) containing 75 mM sodium glutamate at room temperature for 15 min. For ischemia modeling cultured cells were washed with a modified glucose-free Locke's solution containing 154 mM NaCl, 25 mM KCl, 2.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 0.35 mM Na_2HPO_4, and 5 mM HEPES (pH 7.6) and incubated in the same medium at 36°C for 45 min in a hermetic plastic chamber with pure argon. Bifonazole (10^{-5} M) was added 3 min before treatment. Ischemic damages to cultured CGC were induced by oxygen-glucose deprivation (OGD) in pure argon. After ischemic exposure the cultures were incubated in Locke's solution containing 0.8% glucose in a CO_2 incubator (95% air and 5% CO_2) for 3-4 h. Signs of neurodegeneration (appearance of pyknotic nuclei) were detected using an in-

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verted phase-contrast microscope. Control cultures were incubated in a modified glucose-containing Locke's solution in a CO_2 incubator for 3-4 h. The cultures were fixed in an alcohol-formalin-acetic acid mixture (7:2:1) and stained with trypan blue. Pyknotic nuclei and intact CGC were counted. The ratio between the count of pyknotic nuclei and total number of cells (%) reflected the degree of damages.

The results were analyzed by Wilcoxon and Mann—Whitney tests. The differences were significant at $p<0.05$.

RESULTS

Incubation of cultures for 45 min under conditions of OGD caused death of 50.5±5.9% CGC. Bifonazole (10 mM) decreased the count of damaged neurons to 17.3±6.2% ($p<0.01$, $n=9$, Fig. 1, *a*). Thus, bifonazole protected CGC from ischemic injuries.

Our previous studies showed that blockade of NMDA receptors completely prevents CGC death during OGD [1]. We hypothesized that Glu released from CGC terminals and activating NMDA receptors is responsible for neuronal damages under ischemic conditions. To test this hypothesis, cultured cerebellar neurons were treated with 75 mM Glu for 15 min. After 3-h incubation of cultures with Glu we observed death of 42.5±2.1% CGC. Bifonazole added immediately before treatment with Glu decreased the count of damaged neurons by 21% ($p<0.01$, $n=6$, Fig. 1, *b*). Thus, bifonazole protected CGC from Glu-induced toxic damages. However, the addition of bifonazole after Glu treatment increased the count of damaged neurons to 69.5±3.9% ($p<0.01$, $n=6$, Fig. 1, *b*). In

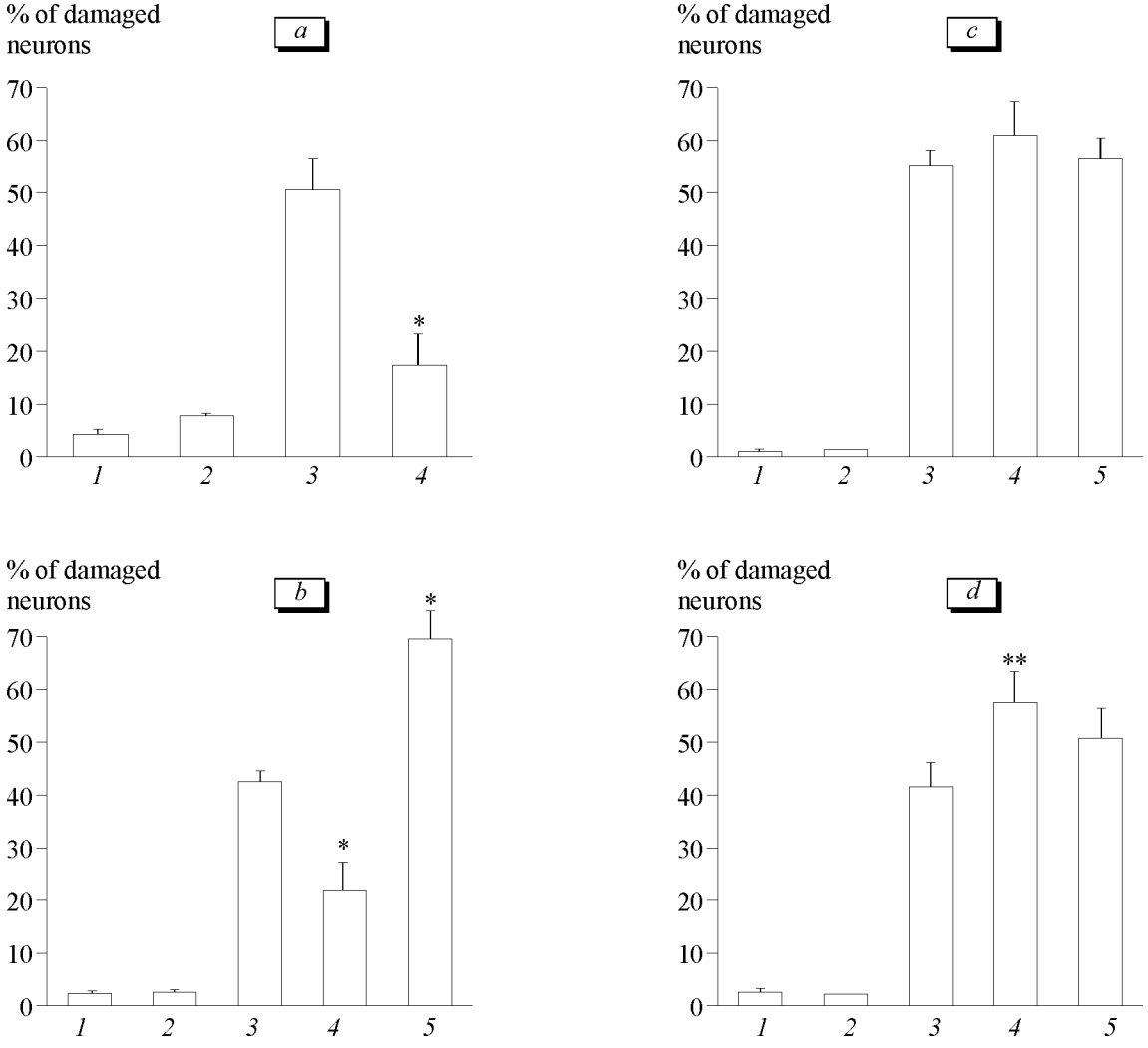

Fig. 1. Effects of calmodulin antagonists (10 mM) on cerebellar granular cell death induced by oxygen-glucose deprivation (*a*) and neurocytotoxic exposure to 75 mM glutamate (*b-d*): *1*) intact cultures, *2*) control cultures in the presence of calmodulin blockers, *3*) injury, *4*) injury in the presence of bifonazole (*a*, *b*), trifluoperazine dihydrochloride (*c*), and thioridazine (*d*), *5*) treatment with bifonazole (*a*, *b*), trifluoperazine (*c*), and thioridazine (*d*) after exposure to glutamate. *$p<0.01$ and **$p<0.05$ compared to cells without correction.

intact cultures spontaneous CGC death did not exceed 4%. Incubation of control cultures with bifonazole for 3 h did not promote CGC death.

Hyperstimulation of Glu receptors induces massive Ca^{2+} entry into neurons and initiates intracellular Ca^{2+}-dependent cascade reactions [6,12], in particular, activation of intracellular protein calmodulin [2]. We hypothesized that bifonazole-induced blockade of calmodulin [9] determines its protective activity. To test this hypothesis, we studied the effects of calmodulin blockers trifluoperazine dihydrochloride (10 mM) and thioridazine (10 mM) differing from bifonazole in their chemical composition. The addition of these compounds into balanced salt solution immediately before and after treatment with Glu did not decrease its toxic effect (Fig. 1, *c*, *d*). Moreover, thioridazine added simultaneously with Glu potentiated its toxic effect (by 16%, $p<0.05$, $n=9$) and added in the postglutamate period promoted CGC death (Fig. 1, *d*).

Thus, calmodulin blockers differing from bifonazole in their chemical structure did not protect CGC from the neurocytotoxic effect of Glu. By contrast, thioridazine potentiated the toxic effects of Glu. Therefore, protective properties of bifonazole are not associated with calmodulin blockade. Bifonazole is an imidazole derivative. Previous studies showed that imidazole derivatives block NMDA receptors-activated ion channels [10] mediating Glu-induced Ca^{2+} and Na^{+} entry. These data suggest that the protective effects of bifonazole during OGD and Glu exposure can be mediated by blockade of NMDA channels.

However, bifonazole added in the postglutamate period potentiates the toxic effects of Glu. Published data show that other imidazole derivative clotrimazole, which is structurally similar to bifonazole, stimulates Ca^{2+} mobilization from the endoplasmic reticulum and, therefore, increases cytoplasmic Ca^{2+} concentration [3]. These changes can contribute to the bifonazole-induced potentiation of Glu toxicity in the postglutamate period.

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