Ouabain Modulates the Toxic Action of Glutamate in Dissociated Cultures of Rat Cerebellar Granule Cells

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It is shown that treatment of cultured cerebellar granule cells with ouabain (10 μM, 90 min) before their exposure to the toxic action of glutamate (100 μM, 15 min) results in a significant reduction of neuronal mortality caused by this neurotransmitter, whereas treatment with ouabain after exposure to glutamate increases the toxicity of ouabain.

Key Words: *glutamate; cerebellar granule cells; ouabain*

Glutamate (GLU) is a major excitatory neurotransmitter in the central nervous system; a sharp rise of its concentration in the brain or impairment of its reuptake may cause damage to neurons carrying receptors for this neurotransmitter, which is observed in ischemia, hypoxia, and a number of other states [6,12]. Processes leading to neuronal degeneration have been shown to result from overstimulation of GLU receptors, to begin as Ca^{2+} and Na^+ enter the cell via GLU-activated ion channels [5,6], and to be accompanied by disturbances of energy balance in the cell [7]. A large role in the GLU-induced cell death may be played by membrane systems of ion exchange. We found in our previous studies with cultures of cerebellar granule cells that blockade of Na^+/Ca^{2+} exchange during or after their treatment with GLU increases the neuronal death rate [3], but that inhibition of Na^2/H^+ exchange during the post-glutamate period reduces the number of cells dying in such cultures [4].

In the study reported here we explored the impact of partial blockade of Na^+,K^+-ATPase by ouabain on the survival of cultured cells before and after their exposure to GLU.

MATERIALS AND METHODS

Suspensions of cerebellar granule cells were obtained from 7- to 8-day-old Wistar rats by enzymatic dissociation and cultured on poly-L-lysine-covered slides in plastic dishes for 7-8 days [2] in a medium composed of 10% fetal calf serum, 90% minimal Eagle medium, 0.8% glucose, 0.1 U/ml insulin, 2 mM glutamine, and 10 mM HEPES. The K^+ concentration in the culture medium was 5.6 mM initially and raised to 25 mM on the 2nd day of cultivation.

Cells were treated with GLU (100 μM) for 15 min in a balanced salt solution (BSS) containing (mM): 137 NaCl, 3.6 KCl, 0.035 Na_2HPO_4, 12 $NaHCO_3$, 2.3 $CaCl_2$, and 11 glucose (pH 7.6-7.8, 20°C). Test cultures were treated with ouabain (10 μM) for 1.5 h before being exposed to GLU or for 3 h after their exposure to it (in a CO_2 incubator at 35°C), while control cultures were not exposed to GLU and were incubated in the BSS for 3 h in the presence of ouabain.

Three hours after GLU treatment, the cultures were fixed with a mixture of alcohol, formalin, and acetic acid (7:2:1), and the fixed cells were stained with vanadium hematoxylin [1]. In the histological slides that were prepared, intact granule cells and pyknotic nuclei were counted in 20 randomly selected fields of vision per slide. The significance of differences was estimated by Student's *t* test.

RESULTS

The neuronal population in the dissociated cultures of postnatal cerebellum was mainly composed of granule cells that could be readily identified by the round or oval shape of their body most of which

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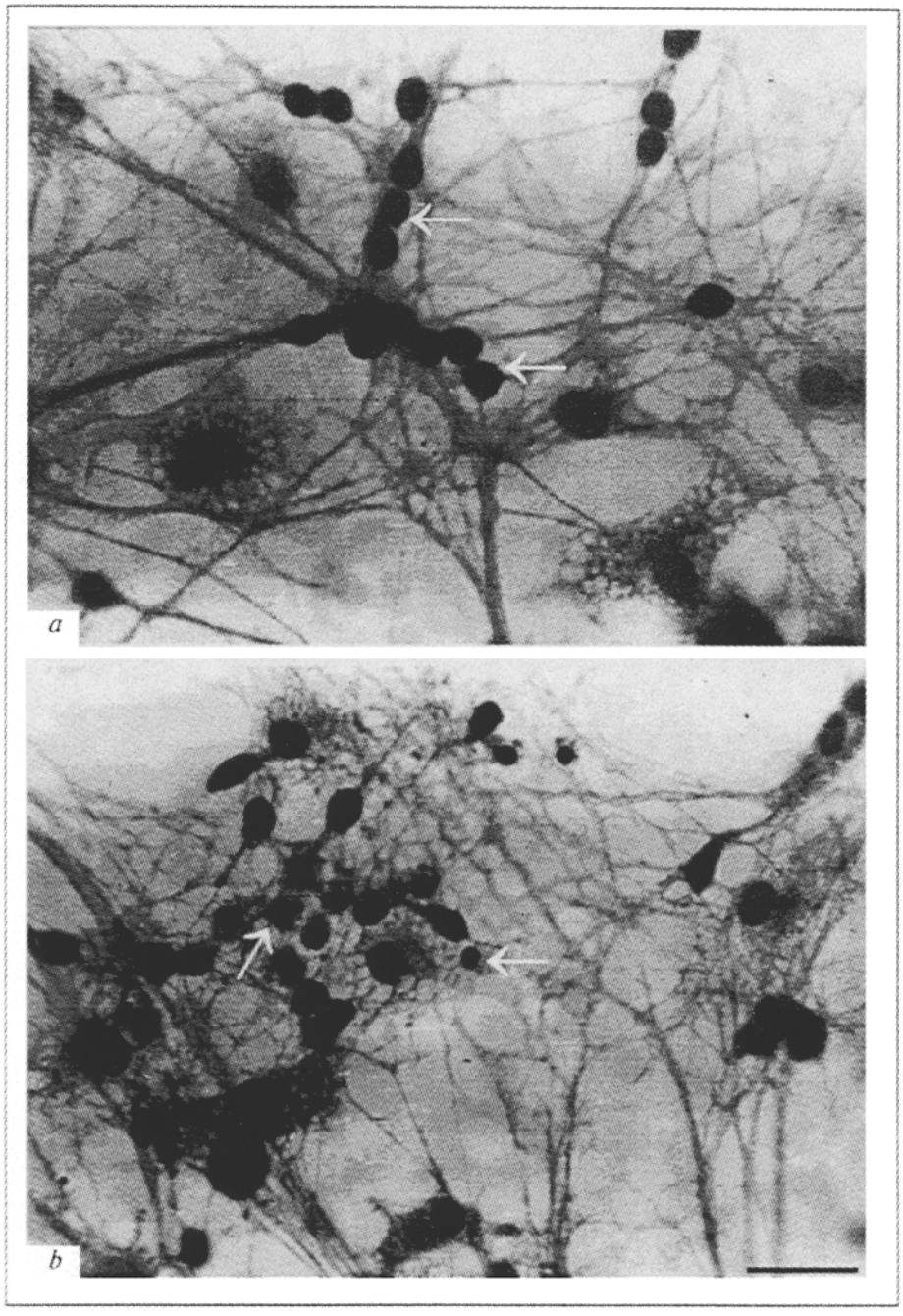

Fig. 1. Cerebellar granule cells from 7- to 8-day-old rats in a monolayer dissociated culture after 7 days of cultivation. Vanadium hematoxylin staining. Scale 20 μ. *a*) control culture with stained nuclei of normal cells (arrows); *b*) granule cells altered by GLU (100 μM), with the arrows showing darkly stained shrunken nuclei of degenerated neurons.

occupied by the nucleus (Fig. 1, *a*). Numerous dead granule cells differing from live cells by having small shrunken pyknotic nuclei appeared in the cultures 2-3 h after their treatment with GLU (Fig. 1, *b*). The proportion of pyknotic nuclei relative to the total number of granule cells in the GLU-treated cultures amounted to 36-56% *vs.* 3-9% in the control cultures incubated in the BSS. The 3-hour treatment with ouabain did not lead to any appreciable increase in the number of pyknotic nuclei in comparison with the control cultures incubated in this solution over the same period.

When the effect of Na^+,K^+-ATPase blockade on the GLU-induced cell death was considered, ouabain-pretreated cultures were found to have significantly reduced numbers of degenerate neurons exposed to GLU (Fig. 2, *a*), the average decrease in the cell death rate being 24%. This finding may be explained by the strong depolarizing action of ouabain on cells. It has been shown in recent studies that the depolarization of *in vitro*-developing neurons following the addition of K^+ (up to 50 mM) or veratridine (1 μM) causes the neurons to express the mRNA of growth factors [8,11]. Many growth fac-

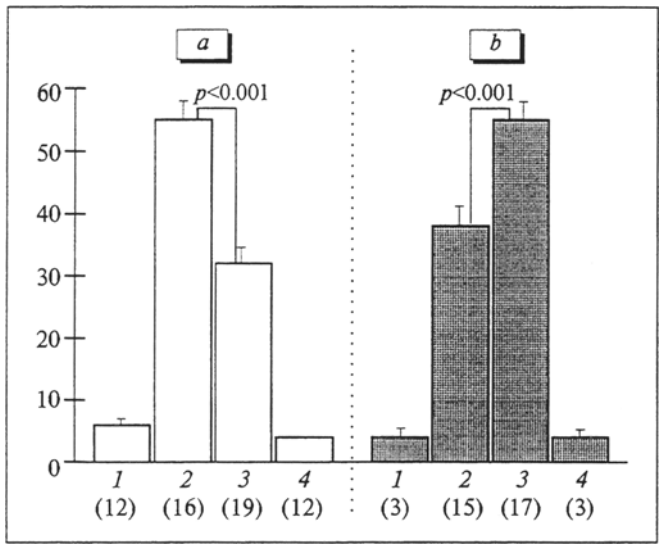

Fig. 2. Effect of ouabain on the destruction of cultured cerebellar granule cells by GLU (100 μM, 15 min). Ordinate: % of degenerated cells relative to the total number of granule cells. Ouabain treatment (10 μM) for 1.5 h before (*a*) and for 3 h after exposure to GLU (*b*). *1*) control; 2) GLU; 3) GLU+ouabain; *4*) ouabain. The number of cultures is shown in parentheses.

tors are known to prolong the survival of various types of neurons in culture [10,13] and to prevent their degeneration after treatment with toxic GLU concentrations [14]. In our tests where cells were pretreated with ouabain in the BSS with a low K^+ concentration (3.6 mM), depolarization caused by this Na^+,K^+-ATPase blocker also probably promoted the expression of growth factors which rendered the neurons more resistant to the toxic GLU treatment.

In subsequent tests, Na^+,K^+-ATPase was blocked in the postglutamate period, which resulted in an opposite effect, namely, a statistically significant increase in the number of dead granule cells in the cultures pretreated with GLU and then incubated with ouabain (Fig. 2, *b*). Such cultures contained, on average, 17% more dead cells than those treated with GLU alone and then incubated in the BSS. This enhancement of GLU toxicity was apparently due to the depolarizing action of ouabain given that, as demonstrated in several studies, neuronal depolarization during exposure to GLU markedly increases its cytotoxicity [9]. When the toxic action of GLU was completed and the neurons remain overloaded with Ca^{2+} [5], cell membrane depolarization appears to bring about a further elevation of intracellular Ca^{2+} concentration as a result of Ca^{2+} entry into cells via voltage-dependent channels and inhibition of its Na^+-dependent exit from the cells. As shown previously, these two processes cause a considerable increase in the GLU-induced cell mortality [3,6].

It should be noted that the number of dead neurons in cultures incubated in the BSS low in K^+ (3.6 mM) before GLU treatment, was appreciably higher than the number of damaged neurons in GLU-treated cultures that had not been preincubated in the salt solution. This difference was most likely due to the fact that the development of neurons in a culture medium under conditions of potassium depolarization (25 mM K^+) is accompanied by expression of growth factors [8], and that their expression may decrease after the incubation of cultures for 90 min in a BSS low in K^+ (3.6 mM).

As evident from the results of the present study, ouabain treatment of cultured granule cells before their treatment with GLU lowers the level of neurocytotoxic damage and the death rate of these neurons as a consequence of their depolarization and subsequent expression of growth factors by them. This study also showed that cell depolarization by ouabain in the postglutamate period in the presence of elevated intracellular Ca^{2+} concentration augments GLU cytotoxicity with a resultant increase in the number of dead neurons.

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