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On the origin of a sustained increase in cytosolic Ca^{2+} concentration after a toxic glutamate treatment of the nerve cell culture

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A sustained increase of cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, (Ca^{2+} plateau) was induced by a 15-min treatment with 50 μM glutamate of cultured cerebellar granule cells and hippocampal neurons in a Mg^{2+}-free solution. Plateau proved to be insensitive to inhibition of Na^+_o/Ca^{2+}_i exchange caused by removal external Na^+ in the post-glutamate period. A $\sim 10^5$-fold reduction of $[Ca^{2+}]_o$ (from 1.5 mM to 20 nM) in the post-glutamate period caused in most cells only a slow and small decrease in $[Ca^{2+}]_i$, although the same low-Ca^{2+} trial before glutamate treatment caused in hippocampal cells very quick blockade of spontaneous $[Ca^{2+}]_i$ oscillation and a decrease in the basal $[Ca^{2+}]_i$. The results suggest that the Ca^{2+} plateau is due to a suppression of the Ca^{2+} extrusion from the cell (in particular via Na^+/Ca^{2+} exchange) rather than from a persistent increase in Ca^{2+} permeability of neuronal membrane.

Glutamate; Neurotoxicity; Cytosolic Ca^{2+}; Na^+/Ca^{2+} exchange; Nerve cell culture

1. INTRODUCTION

Delayed neuronal death induced by a protracted (minutes) glutamate treatment of nerve cells [1–3] is known to depend on a stable elevation of cytoplasmic concentration of free Ca^{2+} ($[Ca^{2+}]_i$) which persists far beyond the removal of glutamate from the medium [3–8]. The origin of this sustained elevation of $[Ca^{2+}]_i$ (so-called $[Ca^{2+}]_i$ plateau) after the termination of glutamate application has not yet been elucidated. Until recently most of investigators [3,5–9] assumed that the post-glutamate $[Ca^{2+}]_i$ plateau is mainly due to an enhanced Ca^{2+} influx of external Ca^{2+} via some pathway modulated by protein kinase C [6]. However, the results of the present study suggest that the major reason for a development of $[Ca^{2+}]_i$ plateau in the post-glutamate period is an impairment of Ca^{2+} extrusion systems, in particular the transmembrane Na^+/Ca^{2+} antiporter.

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Abbreviations: $[Ca^{2+}]_i$, cytosolic free calcium; $[Ca^{2+}]_o$, extracellular free calcium; pH_i, cytosolic pH; Fura-2/AM, acetoxymethyl ester of fura-2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid, NMDA, *N*-methyl-D-aspartate; TMA, tetramethylammonium; DCB, dichlorbenzamil; BS, buffered salt solution; CS, control solution

2. MATERIALS AND METHODS

Primary cerebellar cell cultures were prepared from the cerebella of 7-to 8-day-old Wistar rats by the use of the procedure described earlier [10]. Experiments were carried out on 7-to 8-day cultures. The hippocampi from C57/Bl mouse embryos of 17–19 days gestational age were dissected and dissociated as previously described [11]. Experiments with cultured hippocampal neurons were performed on days 18–22 of cultivation.

Changes in cytosolic free calcium in the single cultured neurons were monitored by the use of the Ca^{2+} sensitive fluorescent probe, fura-2 acetoxymethyl ester (fura-2/AM) [12]. The cerebellar neurons were exposed for 30 min at 35°C to 3 μM fura-2/AM in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered salt solution (BS) containing (mM): 145 NaCl, 5 KCl, 1 Na_2HPO_4, 0.5 $CaCl_2$, 0.5 $MgCl_2$, 20 HEPES, 10 glucose, pH 7.4. The bovine serum albumin (1%) and pluronic acid (0.02%) were added to this solution. The hippocampal neurons were loaded with 5 μM fura-2/AM during 45 min in the same solution. Before the beginning of the experiments, the cells were washed by the BS solution and left for 30 min at room temperature to allow fura-2 de-esterification and equilibration between the bound and free forms To measure $[Ca^{2+}]_i$ the coverslip with the cell culture was put in a special perfusion chamber and the perfusion of the cell (0.2 ml/min) with the control solution, CS (modified BS solution, with 1.5 mM $CaCl_2$ and 1.0 mM $MgCl_2$) was performed. The experimental chamber was mounted on a Nikon inverted-stage microscope linked to spectrofluorimeter 'Spex' (USA) equipped with a beam splitter, two excitation monochromators and a dual mirror chopping mechanism in a specialized optical configuration to allow the rapid alternating (100 Hz) excitation of fura-2 at two wavelengths 340 and 380 nm. Excitation bandwidths were set at 3 5 nm. The emitted fluorescence was filtered through a cut-off filter at 505 nm. The $[Ca^{2+}]_i$ was measured by the ratio of fura-2 fluorescence excited by 340 nm to that excited by 380 nm and calibrated according to external standards [13].

Fura-2/AM was purchased from Molecular Probes (USA). All other compounds were purchased from Sigma Chemical Company (USA).

3. RESULTS AND DISCUSSION

3.1. *Dependence of $[Ca^{2+}]_i$ on external Ca^{2+} concentration ($[Ca^{2+}]_o$)*

In most of the experiments, hippocampal neurons superfused with CS exhibited spontaneous oscillations of $[Ca^{2+}]_i$ that are known to be associated with synchronized epileptiform activity of these cell cultures [14]. Replacement of CS by low Ca^{2+} (~ 20 nM) 50 μM EGTA-containing solution quickly abolished these oscillations and decreased the basal $[Ca^{2+}]_i$ (Fig. 1). After a return of the cells to CS, $[Ca^{2+}]_i$ began to rise and oscillations resumed.

In contrast, in the post-glutamate period the same lowering of external $[Ca^{2+}]_o$ caused only a very slow and small reduction of $[Ca^{2+}]_i$. Qualitatively similar results were obtained in 3 other hippocampal cells.

The high sensitivity of $[Ca^{2+}]_i$ oscillations to $[Ca^{2+}]_o$ is quite understandable: according to [14] each elevation of $[Ca^{2+}]_i$ is resulted of Ca^{2+} influxes through open NMDA- and voltage-sensitive Ca^{2+} channels. Apparently in the post-glutamate period most of the Ca^{2+} permeable ionic channels are closed and Ca^{2+} is extruded from the cell only by some 'slow' ion transport systems.

Unlike hippocampal neurons, the cerebellar granule cells did not exhibit Ca^{2+} oscillations in CS. Correspondingly, the lowering of $[Ca^{2+}]_o$ before GLU treatment causes only a very slow and small reversible decrease in the basal $[Ca^{2+}]_i$ (Fig. 2). The same low-Ca^{2+} trial in the post-glutamate period either did not induce noticeable changes in $[Ca^{2+}]_i$ (Fig. 2a) or caused only its slow and small decrease (Fig. 3). Only once in 13 experiments with cerebellar granule cells we did observe the neuron which in response to lowering of $[Ca^{2+}]_o$ exhibited fast and deep decrease of $[Ca^{2+}]_i$ both before and after glutamate treatment. Apparently in this cell Ca^{2+} leakage through the resting membrane was much larger than in the other cells. This leakage provided fast Ca^{2+} efflux in the Ca^{2+}-free external medium.

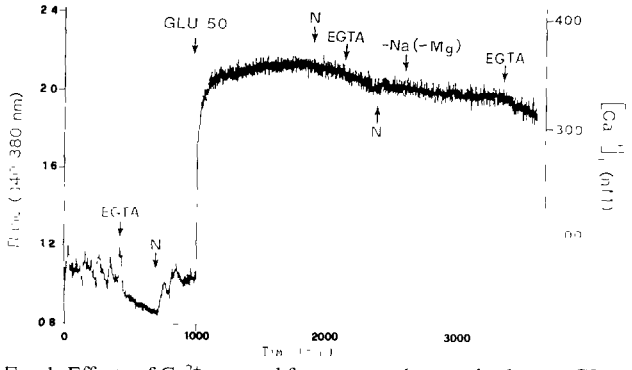

Fig. 1. Effects of Ca^{2+} removal from external control solution (CS) on $[Ca^{2+}]_i$ in the cultured hippocampal nerve cell. The effects EGTA-containing solution were examined: before glutamate application (during $[Ca^{2+}]_i$ oscillations in CS) and two times in the post-glutamate period. Designations: – Na(+ Mg): replacement of CS by the modified CS where NaCl was equimolarly substituted by LiCl. N: washing of the cell by CS; GLU, superfusion of the cells with glutamate (50 μM) containing Mg^{2+}-free solution; – Na(– Mg) additional removal of Mg^{2+} from the Na^+-free solution; EGTA, superfusion of the culture with nominally Ca^{2+}-free solution containing 50 μM EGTA (calculated Ca^{2+} concentration about 20 nM).

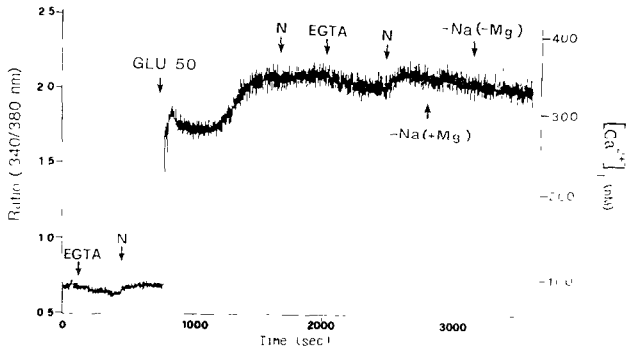

Fig. 2. Small changes in $[Ca^{2+}]_i$ in response to a decrease in external Ca^{2+} concentration (from 1.5 mM to ~ 20 mM) in the cultured cerebellar granule cell before and after glutamate treatment. Designations are the same as in Fig. 1.

3.2. *Dependence of $[Ca^{2+}]_i$ on external Na^+ (Na^+_o)*

To reveal a contribution of Na^+/Ca^{2+} exchange system to the mechanism of destabilization of $[Ca^{2+}]_i$ homeostasis caused by glutamate, we have examined the effect of replacement of external Na^+ by Li^+, or tetramethylammonium (TMA), the cations unable to substitute Na^+ in Na^+/Ca^{2+} exchange [15]. In most of cases Li^+ was used since it in contrast to TMA readily replaces Na^+ in Na^+/H^+ exchange [16]. It is necessary, however, to stress that the changes in $[Ca^{2+}]_i$ observed did not depend on whether external Na^+ was replaced by Li^+ or TMA.

In Fig. 3, the removal of Na^+ from CS before glutamate treatment induced a relatively fast and reversible increase in $[Ca^{2+}]_i$ suggesting that in this cerebellar granule cell Na^+/Ca^{2+} exchange was involved in regulation of the basal $[Ca^{2+}]_i$ level. However the same low-Na^+ trial in the post-glutamate period failed to induce any change in the $[Ca^{2+}]_i$ level. Note that the additional removal of Mg^{2+} from the Na^+-free solution also did not affect $[Ca^{2+}]_i$, although this would have promoted the exchange of external Ca^{2+} for internal Na^+ [16]. The subsequent lowering of $[Ca^{2+}]_o$ also did not change noticeably $[Ca^{2+}]_i$. Similar results were obtained in 13 other cells exhibited a high $[Ca^{2+}]_i$ plateau (see Figs. 1 and 2). In 4 cells $[Ca^{2+}]_i$ plateau was absent and after the end of glutamate treatment $[Ca^{2+}]_i$ returned back to the low basal level. In these cases removal of external Na^+ in the post-glutamate period led to a new pronounced elevation of $[Ca^{2+}]_i$ (not shown).

Thus we did not obtain any evidence in favour of the notion that $[Ca^{2+}]_i$ plateau in the post-glutamate period is due either to an enhanced passive Ca^{2+} influx or to a reversal of Na^+/Ca^{2+} exchange. If this were the case

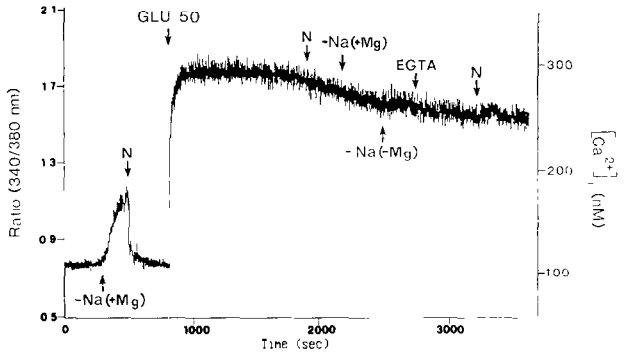

Fig. 3. Toxic glutamate treatment of the cultured cerebellar granule cell eliminates a dependence of $[Ca^{2+}]_i$ on external Na^+. Basal $[Ca^{2+}]_i$ was measured during superfusion of nerve cells by the control solution (CS). Designations are the same as in Fig. 1.

then about 10^5-fold decrease in $[Ca^{2+}]_o$, as performed in our experiments, always would have cause a quick and considerable drop in $[Ca^{2+}]_i$. Such changes were observed, however, only in the cell exhibiting high sensitivity to a decrease in $[Ca^{2+}]_o$ even before glutamate treatment (apparently because of enhanced Ca^{2+} 'leakage' through the membrane).

The other important finding in this report is an insensitivity of post-glutamate $[Ca^{2+}]_i$ plateau to a removal of external Na^+ (see Fig. 3). Apparently the toxic glutamate treatment suppresses the system of Na^+_o-dependent Ca^{2+} extrusion greatly attenuating its contribution to the regulation of $[Ca^{2+}]_i$. This conclusion agrees with the notion on the role of this system in protection of nerve cells against the action of excitotoxines [17]. Indeed as long as this system works effectively $[Ca^{2+}]_i$ can return back after the end of glutamate action (see [18]). This evidently takes place at small and subtoxic glutamate concentrations, which, however, become toxic ones after blockade of Na^+/Ca^{2+} exchanger by the amiloride derivative dichlorbenzamil (DCB) or by a removal of Na^+ in the post-glutamate period [10]. At least two possible reasons for inhibition of Na^+/Ca^{2+} antiport by toxic glutamate treatment were revealed in our recent studies: (i) a stable decrease in cytosolic pH (pH_i) [19] and (ii) a pronounced (about two-fold) reduction of ATP content [20] in cultured nerve cells during a protracted glutamate treatment and in the post-glutamate period.

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