0026-895X/94/051050-05$03.00/0

MOLECULAR PHARMACOLOGY, 45:1050-1054

Intracellular Sodium Concentration in Cultured Cerebellar Granule Cells Challenged with Glutamate

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Received January 3, 1994; Accepted February 17, 1994

SUMMARY

We monitored simultaneously the changes in the intracellular sodium concentration ($[Na^+]_i$) and intracellular calcium concentration ($[Ca^{2+}]_i$) in individual neurons from primary cultures of cerebellar granule cells loaded with sodium-binding benzofuran isophthalate and fluo-3. An application of glutamate (50 μM) in Mg^{2+}-free medium containing 10 μM glycine evoked $[Na^+]_i$ and $[Ca^{2+}]_i$ increases that exceeded 60 mM and 1 μM, respectively. The kinetics of $[Na^+]_i$ and $[Ca^{2+}]_i$ decreases after the termination of the glutamate pulse were different. $[Na^+]_i$ failed to decrease immediately after glutamate withdrawal and the delay in the onset of $[Na^+]_i$ decrease after the glutamate pulse termination was proportional to the glutamate dose, the glutamate pulse duration, and the extent of $[Ca^{2+}]_i$ elevation elicited by glutamate. The kinetics of $[Ca^{2+}]_i$ decrease were biphasic, with the first phase occurring immediately after glutamate withdrawal and the second phase being correlated in time with a $[Na^+]_i$ value lower than 15–20 mM. These results were interpreted to indicate that the glutamate-evoked calcium influx may lead to sodium homeostasis destabilization. The delay in the restoration of the sodium gradient may in turn prolong the neuronal exposure to toxic $[Ca^{2+}]_i$ values, due to the decrease in the efficiency of the Na^+/Ca^{2+} exchanger to extrude calcium. The glutamate effects on $[Na^+]_i$ and $[Ca^{2+}]_i$ were potentiated by glycine. Glycine (10 μM) added alone also evoked $[Na^+]_i$ and $[Ca^{2+}]_i$ increases; this effect was inhibited by a competitive inhibitor of the *N*-methyl-D-aspartate receptor, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, indicating an involvement of endogenous glutamate.

The sodium gradient established as a result of the activity of Na^+/K^+-ATPase is one of the most fundamental features of cell viability; it establishes the molecular basis for neuronal excitability, provides energy for uptake of vital substances, and also maintains calcium and pH homeostasis via the operation of Na^+/Ca^{2+} and Na^+/H^+ exchangers. In a previous report (1), we showed that excessive activation of cerebellar granule cell NMDA receptors with glutamate leads to the decline of the sodium gradient and impairment of calcium extrusion. Thus, the increase of $[Na^+]_i$ produced by prolonged cell exposure to an elevated $[Ca^{2+}]_i$ may contribute to excitotoxicity and to delayed neuronal death, which depends on the destabilization of calcium homeostasis (2, 3). The glutamate-induced calcium homeostasis destabilization leads to activation of a cascade of calcium-dependent events, such as persistent protein kinase C activation (4, 5), protracted protein phosphorylation (6), DNA fragmentation due to endonuclease activation (7), and depolymerization of microtubules (8), leading to neuronal death.

Because glutamate exposure causes a more pronounced $[Ca^{2+}]_i$ increase and more severe excitotoxicity in some neurons than in others (9), we wondered whether this greater $[Ca^{2+}]_i$ response was correlated with an analogous $[Na^+]_i$ response. Thus, we decided to monitor $[Ca^{2+}]_i$ and $[Na^+]_i$ simultaneously in the same neurons. To achieve this goal we loaded neuronal cultures with fluorescent sodium (SBFI) and calcium (fluo-3) indicators simultaneously. The present paper examines whether, in the cascade of events leading to glutamate-evoked neurotoxicity in primary cultures of cerebellar granule cells, in addition to the role of Ca^{2+} influx (10) there is also a role for Na^+ influx.

Materials and Methods

Cerebellar granule cell cultures. Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rats as described previously (11) and were plated on poly-L-lysine (10 μg/ml)-coated 25-mm coverslips at a density of 3.7×10^6 cells/35-mm plate. Glial cell proliferation was controlled by the addition of 10 μM cytosine arabinofuranoside 24 hr after plating.

Single-cell $[Na^+]_i$ and $[Ca^{2+}]_i$ imaging. For simultaneous $[Na^+]_i$ and $[Ca^{2+}]_i$ monitoring, 9–10-day-old cells were loaded for 1 hr at 37° with SBFI/AM (10 μM) and fluo-3/AM (2 μM) dissolved in BSS containing 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 5 mM HEPES, pH 7.4, supplemented

This work was supported in part by National Institutes of Health Grant NS28130.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; SBFI, sodium-binding benzofuran isophthalate; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); BSS, buffered salt solution; $[Na^+]_i$, intracellular sodium concentration; $[Ca^{2+}]_i$, intracellular calcium concentration.

with 0.1% Pluronic F-125 (BASF Corp., Wyandotte, MI). The loading medium was then washed out three times with fresh BSS and the cells were left to equilibrate for 30 min at room temperature. In the experiments where only $[Ca^{2+}]_i$ was monitored with fura-2, cells were loaded for 30 min at 37° with fura-2/AM (3.5 μM) dissolved in culture medium without addition of Pluronic F-125 (Pluronic F-125 was essential only for SBFI/AM loading) and then the cells were washed with BSS as described above. Digital imaging of fura-2, SBFI, and fluo-3 fluorescence was carried out in individual cells at excitation wavelengths of 334 nm and 380 nm for fura-2 and SBFI and 480 nm for fluo-3, with emission, in all cases, at >520 nm, using an Attofluor digital microscopy system (Atto Instruments, Bethesda, MD). The fluorescence data were averaged from 3.5-μm^2 squares positioned in the center of the cells, and up to 50 cells were monitored simultaneously.

Calibration of $[Na^+]_i$ was performed *in situ* after each experiment. Cells were treated with a calibration medium made from appropriate mixtures of high-concentration solutions of Na^+ and K^+. The high-concentration Na^+ solution contained 134.2 sodium gluconate, 25.4 mM NaCl, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 5.6 mM glucose, 200 μM ouabain, 5 μM gramicidin, and 5 mM HEPES, pH 7.2; the high-K^+ solution was identical except for complete replacement of Na^+ with K^+. The partial chloride replacement with gluconate in the calibration solution prevented cell swelling during calibration (12). The ratios of fluorescence intensities produced by 334-nm (F_{334}) and 380-nm (F_{380}) excitations at three different $[Na^+]_i$ values were used to calculate the sodium calibration curve for each individual cell, as described by Harootunian *et al.* (12). We did not calibrate the fluorescence intensity produced at 480-nm excitation (F_{480}) of fluo-3 for $[Ca^{2+}]_i$ (13). Such calibration in neurons seems to be problematic because it does not allow corrections for the decrease of fluorescence intensity evoked by changes in the cell volume due to glutamate-induced neuronal swelling (data not shown). The recording of the F_{480} of fluo-3 gives only a relative measure of changes in $[Ca^{2+}]_i$ but allows simultaneous monitoring of $[Na^+]_i$ with SBFI. To normalize for variable fluo-3 uptake, F_{480} values were divided by the basal F_{480} value measured at the beginning of each experiment.

The experimental chamber volume was 0.5 ml. Drug solutions were added by medium replacement. To remove previously applied drug, the chamber was washed four times with 0.5 ml of BSS. The experiments were carried out at room temperature (24–26°). The cell viability in the experiments discussed in the text was evaluated as described previously (14).

Materials. Culture media were purchased from GIBCO. SBFI/AM, fluo-3/AM, and fura-2/AM were from Molecular Probes, Inc. (Eugene, OR); CPP was from Research Biochemicals Inc. (Natick, MA). All other chemicals were obtained from Sigma.

Results

To monitor $[Na^+]_i$ and $[Ca^{2+}]_i$ simultaneously, we took advantage of the weak fluorescence emission of fluo-3 and SBFI at excitations below 400 nm (15) and above 400 nm (16), respectively. In agreement with a report by Donoso *et al.* (17), we found that the spectral properties of SBFI *in vitro* and *in situ* are different. During the *in situ* (Fig. 1) but not the *in vitro* (data not shown) $[Na^+]_i$ calibration, only the F_{380} intensity increases with the decrease in $[Na^+]_i$, whereas that of F_{334} remains unchanged. However, only in granule cells loaded simultaneously with SBFI and fluo-3, and not in those loaded with SBFI alone (data not shown), the F_{334} slightly increases upon glutamate-evoked (Fig. 1) or K^+-evoked (data not shown) increases in $[Ca^{2+}]_i$. Most likely, there is interference due to fluo-3-emitted fluorescence. Some contribution of fluo-3 to the fluorescence intensity excited at 334 nm and 380 nm was expected (15), but it failed to affect the $[Na^+]_i$ measurements significantly.

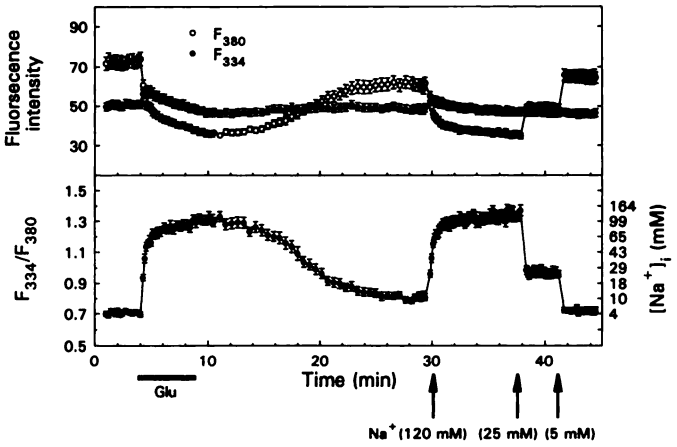

Fig. 1. Calibration of the F_{334}/F_{380} ratio data for $[Na^+]_i$ in cerebellar granule cells in primary cultures. Cells loaded with SBFI and fluo-3 were treated for 5 min with glutamate (*Glu*) (50 μM) and glycine (10 μM) in Mg^{2+}-free medium. The cells were then washed with BSS and after 20 min the $[Na^+]_i$ calibration was performed as described in Materials and Methods. The data are means ± standard errors from 24 cells.

A 5-min glutamate (50 μM) pulse increases $[Na^+]_i$ from the basal value of about 4 mM to >60 mM, and after glutamate removal this elevated $[Na^+]_i$ fails to decrease promptly (Fig. 1). This delayed responsiveness of $[Na^+]_i$ to the glutamate pulse termination is proportional to the duration of the glutamate pulse (Fig. 2) and to the glutamate concentration (Fig. 3, A and B). In fact, the percentage of cells in which the decrease of $[Na^+]_i$ after glutamate removal is delayed by about 25 min increases with the duration of the glutamate pulse (Fig. 2). In the same cells that fail to show a prompt $[Na^+]_i$ decrease, the $[Ca^{2+}]_i$ increase also persists longer after the glutamate removal (Fig. 2A). In contrast, the cells in which $[Na^+]_i$ decreases faster after glutamate removal are those that show the smallest $[Ca^{2+}]_i$ increase in response to the glutamate pulse (Fig. 2B). It seems likely that $[Na^+]_i$ and $[Ca^{2+}]_i$ homeostasis destabilizations are related. The kinetics of the $[Ca^{2+}]_i$ decrease after glutamate removal appear to be biphasic (Fig. 2A). One phase occurs immediately after glutamate removal, when $[Na^+]_i$ is still elevated, and the other phase begins when $[Na^+]_i$ decreases below 15–20 mM (Fig. 2A), suggesting that the return of $[Na^+]_i$ to physiological levels might be linked to calcium extrusion.

Glutamate-evoked $[Na^+]_i$ and $[Ca^{2+}]_i$ increases are potentiated by glycine (Fig. 3, A and B). Moreover, glycine alone (10 μM) causes $[Na^+]_i$ and $[Ca^{2+}]_i$ to increase (Fig. 3C) by an extent greater than that evoked by 3 μM glutamate (Fig. 3A) or 10 μM glutamate (data not shown) in the absence of glycine. These increases of $[Na^+]_i$ and $[Ca^{2+}]_i$ caused by glycine addition are completely inhibited by both Mg^{2+} (1 mM) and CPP (100 μM) (data not shown), a competitive and specific NMDA inhibitor. Thus, it is likely that activation of NMDA receptors is operative in the action of glycine. Interestingly, in a medium where Na^+ is completely replaced with *N*-methyl-D-glucamine, glycine alone (10 μM) fails to elevate $[Ca^{2+}]_i$ (Fig. 4A), whereas a very large $[Ca^{2+}]_i$ increase is observed when glutamate (1 μM) and glycine (10 μM) are added simultaneously for 15 min (Fig. 4B). Moreover, upon glutamate removal, washing with a medium containing physiological Na^+ and Mg^{2+} concentrations fails to decrease $[Ca^{2+}]_i$ in 23 of 27 of these cells (Fig. 4B), and neuronal death ensues within 12 hr (data not shown). In contrast, in a

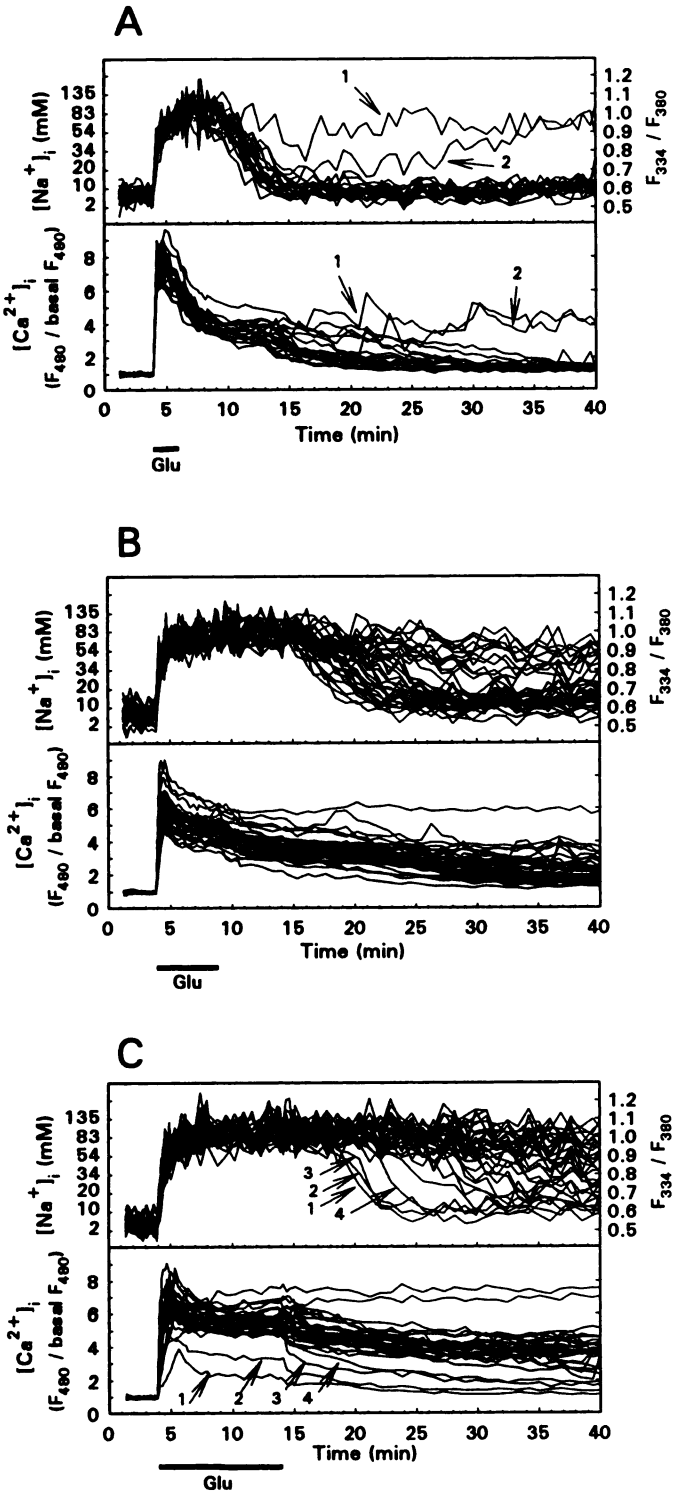

Fig. 2. Effect of the duration of the glutamate pulse on $[Na^+]_i$ and $[Ca^{2+}]_i$ in cerebellar granule cells. Cells loaded simultaneously with SBFI and fluo-3 were incubated with glutamate (*Glu*) (50 μM) and glycine (10 μM) in Mg^{2+}-free medium for 2 (A), 5 (B), or 10 (C) min. The agonists were then washed out with BSS and $[Na^+]_i$ and $[Ca^{2+}]_i$ were measured for another 25–35 min. *Lines*, individual data for 20 (A), 36 (B), and 32 (C) cells. *Upper*, $[Na^+]_i$; *lower*, $[Ca^{2+}]_i$. *Numbered arrows*, lanes representing the same cells in both panels. The experiments were repeated twice on different preparations of cerebellar granule cells, with similar results.

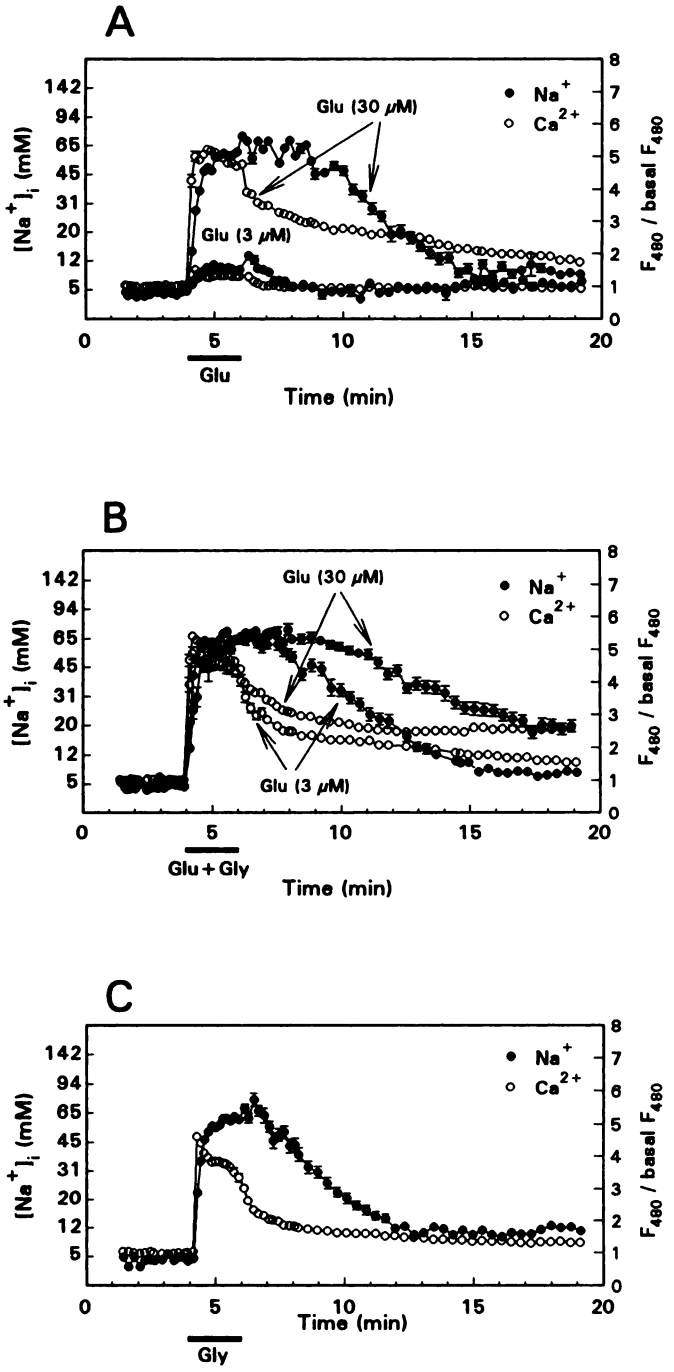

Fig. 3. Potentiation of the glutamate-evoked $[Na^+]_i$ and $[Ca^{2+}]_i$ increases in cerebellar granule cells by glycine (*Gly*) and dependence on the dose of glutamate (*Glu*). The SBFI- and fluo-3-loaded cells were incubated for 2 min with the indicated doses of glutamate in Mg^{2+}-free medium without glycine (A) or with 10 μM glycine (B), or 10 μM glycine was added without glutamate (C). After incubation the agonists were washed out with BSS. The data are means ± standard errors from 18–38 cells. The experiments were repeated with different preparations of cerebellar granule cells, with similar results.

parallel experiment carried out in a medium with the nominal absence of Mg^{2+} but with physiological concentrations of Na^+, glutamate (1 μM) with glycine (10 μM) fails to destabilize calcium homeostasis (Fig. 4C) and to cause neurotoxicity (data not shown). In an experiment analogous to that shown in Fig. 4C, in which 100 μM glutamate was applied instead of 1 μM

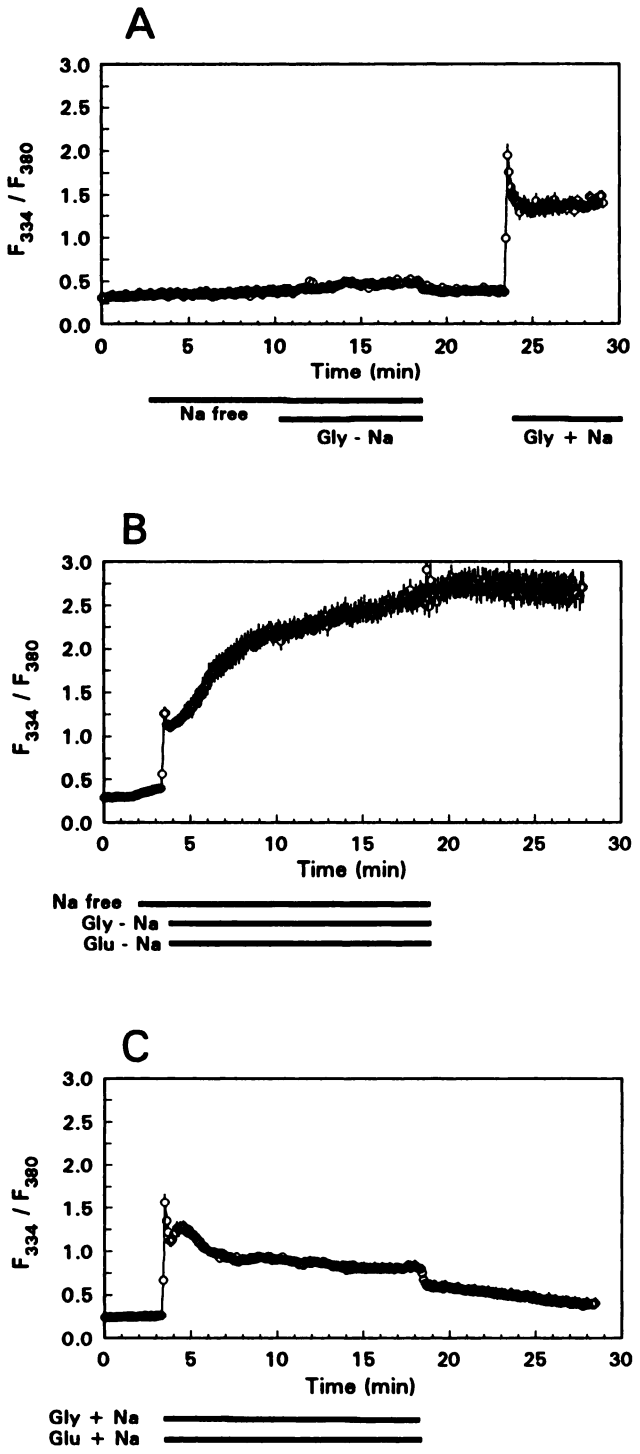

Fig. 4. Effect of Na^+ concentration in the medium on glycine- and glutamate-evoked $[Ca^{2+}]_i$ increase. A, The fura-2-loaded cells were treated with glycine (*Gly*) (10 μM) in Mg^{2+}-free medium containing no Na^+ (*N*-methyl-D-glucamine replacement) and then in medium containing 158 mM Na^+. Glycine was washed out with BSS between applications. B, The fura-2-loaded cells were treated for 15 min with glutamate (*Glu*) (1 μM) and glycine (10 μM) in Mg^{2+}-free medium containing no Na^+ (*N*-methyl-D-glucamine replacement). The agonists were then washed out with BSS. C, The cells were treated with glutamate (1 μM) and glycine (10 μM) in Mg^{2+}-free medium containing 158 mM Na^+. The data are means ± standard errors from 22 (A), 27 (B), and 21 (C) cells. The experiments were repeated with different batches of cerebellar granule cells, with similar results.

glutamate, after the termination of a glutamate pulse the $[Ca^{2+}]_i$ decreased in 39 of 43 cells (data not shown).

Discussion

Recently, it was reported that the elevation of neuronal $[Na^+]_i$ elicited by glutamate contributes to glutamate-induced neuronal death by reducing the ability of the Na^+/Ca^{2+} exchanger to extrude calcium (1). The occurrence of an extended neuronal depolarization that outlasts glutamate removal (18) may be an electrophysiological correlate of the glutamate-induced $[Na^+]_i$ elevation. The delay in the onset of $[Na^+]_i$ decrease after glutamate removal depends not only on the glutamate pulse duration and concentration but also on the characteristics of the glutamate-induced $[Ca^{2+}]_i$ increase. This suggests that the glutamate-evoked destabilizations of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis may be correlated. The high $[Na^+]_i$ may inhibit effective calcium extrusion via the Na^+/Ca^{2+} exchanger (1), leading to potentiation of the calcium-dependent phenomena operative in glutamate-induced neuronal death (see the introduction). Although the molecular mechanism of the Na^+ homeostasis destabilization elicited by glutamate remains to be elucidated, the protracted translocation and activation of protein kinase C elicited by glutamate (4), the phosphorylation-dependent decrease of Na^+/K^+-ATPase activity (19, 20), and the inhibition of Na^+/K^+-ATPase when $[Ca^{2+}]_i$ reaches micromolar levels (21) allow us to speculate that the inhibition of Na^+/K^+-ATPase may be involved in Na^+ homeostasis destabilization. Moreover, Na^+ leakage into neurons due to a change in membrane fluidity and integrity (22) or to the opening of NMDA-gated receptor channels, relieved from Mg^{2+} block by membrane depolarization (23, 24), also must be considered. The glutamate-evoked $[Na^+]_i$ increase may affect mitochondrial function by activating the mitochondrial Na^+/Ca^{2+} and Na^+/H^+ exchangers (25), which in turn may affect mitochondrial membrane potential and ATP supply, thereby depressing the activity of all ATP-dependent enzymes including Na^+/K^+-ATPase and various Ca^{2+}-ATPases.

In a previous report we showed that calcium extrusion, after a glutamate pulse (50 μM) lasting 1 min, was faster when the glutamate pulse was applied in Na^+-free medium than in the presence of physiological Na^+ concentrations (1). In contrast, granule cells treated for 15 min with 1 μM glutamate in Na^+-free medium fail to show any $[Ca^{2+}]_i$ decrease after glutamate removal, even though physiological Na^+ concentrations are present in the washing medium. It is unlikely that this excitotoxic effect of 1 μM glutamate applied in Na^+-free medium is due to inhibition of the sodium-dependent glutamate uptake, because in cells treated for 15 min with 100 μM glutamate applied with physiological Na^+ concentrations $[Ca^{2+}]_i$ decreases after glutamate removal. Using the kinetic data for the uptake of glutamate by cerebellar granule cells (26), one can calculate that the glutamate concentration is still about 90 μM at the end of 15-min exposure of neurons to 100 μM glutamate applied with physiological Na^+ concentrations. The potentiation of glutamate-induced neurotoxicity in Na^+-free medium is most likely due to inhibition of the plasmalemmal Na^+/Ca^{2+} exchanger (27). However, the mechanism of the potentiation of glutamate toxicity in Na^+-free medium may also involve other mechanisms. For example, after an application of Na^+-free medium $[Na^+]_i$ decreases below 1 mM (data not shown) and such a drastic decrease of $[Na^+]_i$ may interfere with the function

of the mitochondrial Na^+/Ca^{2+} and Na^+/H^+ exchangers (25). The function of other mechanisms participating in $[Ca^{2+}]_i$ homeostasis in the presence of a destabilization of $[Na^+]_i$ homeostasis has not been studied carefully.

The kinetics whereby $[Ca^{2+}]_i$ returns to physiological levels in the neuronal soma after the termination of a glutamate pulse are biphasic and resemble those occurring after the exposure of cerebellar granule cells to kainate (1). It is likely that the first phase, occurring when $[Na^+]_i$ is still greater than 60 mM, depends on sodium-independent mechanisms such as calcium extrusion by Ca^+-ATPase (28), calcium uptake into endoplasmic reticulum (29, 30), and buffering by calcium-binding proteins expressed in cerebellar granule cells (31). The Na^+/Ca^{2+} exchanger seems to contribute significantly to calcium extrusion from the cell body only during the second phase, when $[Na^+]_i$ is lower than 15–20 mM.

Glycine positively modulates the NMDA receptor (32, 33) and potentiates glutamate-elicited neuronal death (34). In the present report we have shown that in granule cells addition of glycine alone can bring about activation of NMDA receptors, leading to an increase of $[Na^+]_i$ and $[Ca^{2+}]_i$. This effect is probably due to a facilitatory action by glycine on the glutamate that is released from these glutamatergic cells. This possibility is supported by the inhibition of this glycine effect by CPP, a competitive inhibitor of the glutamate recognition site on the NMDA receptor. Because the glycine-evoked $[Ca^{2+}]_i$ increase is observed only at physiological Na^+ concentrations, it is likely that the presence of Na^+ in the medium plays a role in the mechanism of glutamate release from cerebellar granule cells.

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