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Glutamate-Induced Destabilization of Intracellular Calcium Concentration Homeostasis in Cultured Cerebellar Granule Cells: Role of Mitochondria in Calcium Buffering

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SUMMARY

The exposure of cultured cerebellar granule cells for 4 min to glutamate (50 μM) in a Mg^{2+}-free medium containing 10 μM glycine elicited a prompt increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to 5 μM, which was followed by a decline to 1.5 μM (as measured using fura-2); both events occurred while the glutamate pulse increased the intracellular sodium concentration ($[Na^+]_i$) to an estimated 60–100 mM. Because under these circumstances the plasma membrane Na^+/Ca^{2+} exchanger cannot extrude Ca^{2+}, other mechanisms should operate in causing the $[Ca^{2+}]_i$ decline. To evaluate a possible role of intracellular Ca^{2+} stores in Ca^{2+} buffering, thapsigargin, ryanodine, and dantrolene were tested. Thapsigargin (1 μM) and ryanodine (10 μM) failed to modify the glutamate-elicited $[Ca^{2+}]_i$ transients; results with dantrolene could not be considered because this drug by itself affected the fura-2 fluorescence. In contrast, carbonyl cyanide *m*-chlorophenylhydrazone (1 μM) and antimycin A1 (1 μM), which dissipate mitochondrial membrane potential by different mechanisms, virtually abolished the $[Ca^{2+}]_i$ decline occurring either during glutamate application or after its removal. Moreover, when the residual $[Na^+]_i$ increase persisting after glutamate removal was artificially abated, the Ca^{2+}-buffering capacity of neurons was significantly improved. These data suggest that most of the Ca^{2+} entering the neurons during excitotoxic glutamate exposure is diverted to mitochondria and that the glutamate-induced increase of $[Na^+]_i$ limits this mitochondrial Ca^{2+}-buffering capacity, presumably via activation of the mitochondrial Na^+/Ca^{2+} exchanger.

In neurons, glutamate-induced Ca^{2+} influx destabilizes $[Ca^{2+}]_i$ homeostasis and activates a number of mechanisms operative in this homeostatic process. These include (i) Ca^{2+} extrusion by the plasma membrane Ca^{2+}-ATPase (1) and Na^+/Ca^{2+} exchanger (2–4), (ii) Ca^{2+} buffering by Ca^{2+}-binding proteins (5), and (iii) Ca^{2+} uptake into endoplasmic reticulum (6) and mitochondria (7–9). The plasma membrane Ca^{2+}-ATPase, because of its high affinity for Ca^{2+} and its relatively low capacity, probably plays a role only in maintaining basal $[Ca^{2+}]_i$ (10), whereas the plasma membrane Na^+/Ca^{2+} exchanger, which has a low affinity for Ca^{2+} but a high capacity, seems to be better suited to extrude Ca^{2+} when $[Ca^{2+}]_i$ reaches micromolar levels (10). During neuronal exposure to excitotoxic glutamate concentrations, while $[Na^+]_i$ increases to 60–100 mM a marked Ca^{2+} buffering is operative in neuronal soma (11, 12). Because effective participation of the plasma membrane Na^+/Ca^{2+} exchanger in $[Ca^{2+}]_i$ homeostasis is virtually impossible when $[Na^+]_i$ is elevated to such levels (10), other mechanisms must contribute to $[Ca^{2+}]_i$ decreases that occur when glutamate is applied. Our experiments were designed to identify these mechanisms. We found that, in cerebellar granule cells exposed to excitotoxic concentrations of glutamate, mitochondrial Ca^{2+} uptake plays a dominant role in Ca^{2+} buffering.

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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 (13), were plated on poly-L-lysine (10 μg/ml)-coated 25-mm coverslips at a density of 2.5×10^6 cells/35-mm dish, and were studied at day 8–9 in culture. Glial cell proliferation was controlled by the addition of 10 μM cytosine arabinofuranoside 24 hr after plating.

Single-cell $[Ca^{2+}]_i$ imaging. For monitoring changes in $[Ca^{2+}]_i$, cells were loaded for 45 min at 37° with fura-2/acetoxymethyl ester (4 μM) applied in culture medium. The cells were then washed three times with 1 ml of BSS (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, 5 mM HEPES, pH 7.4, adjusted with Tris) and were allowed to equilibrate with this solution for 30 min at room temperature. Using an Attofluor digital microscopy system (Atto Instruments, Bethesda, MD), the fluorescence of up to

ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular calcium concentration; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSS, buffered salt solution; $[Na^+]_i$, intracellular sodium concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; NMDA, *N*-methyl-D-aspartate.

50 cells was monitored simultaneously at room temperature. The fluorescence emitted at >500 nm after excitation at 334 nm (F_{334}) or 380 nm (F_{380}) was averaged from 3.5-μm^2 squares positioned in the center of each cell. Cerebellar granule cells were identified by their characteristic size and morphology. Because the fluorescent properties of fura-2 are strongly affected by the intracellular milieu (14), calibration of $[Ca^{2+}]_i$ was performed *in situ*. At the end of each experiment the cells were treated for 5 min with 10 μM ionomycin dissolved in a buffer containing 5 mM $CaCl_2$, 154 mM KCl, 5.6 mM NaCl, 3.6 mM $KHCO_3$, 1 mM $MgCl_2$, 5.6 mM glucose, and 5 mM HEPES, pH 7.2 (adjusted with Tris), to saturate fura-2 with Ca^{2+} for measurement of the maximal F_{334}/F_{380} ratio (R_{max}) (see representative examples in Fig. 1, A and B). The minimal F_{334}/F_{380} ratio (R_{min}) was measured in a separate calibrating experiment with sister cultures incubated in a buffer containing 5 mM EGTA, 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM glucose, and 5 mM HEPES, pH 7.4 (adjusted with Tris), until a stable plateau for the F_{334}/F_{380} ratio was reached. About 8 min of incubation were sufficient to reach this plateau but in our standard procedure we prolonged the incubation for another 2–4 min to ensure that the R_{min} value was obtained. Ionomycin was purposely omitted from the R_{min} calibration procedure because in preliminary experiments we established that the addition of ionomycin (10 μM) during R_{min} calibration evoked a small increase of the F_{334}/F_{380} ratio; the F_{334}/F_{380} ratio then decreased but only to the value that had been reached before the ionomycin addition. After the R_{min} signal was established, the R_{max} was measured using the protocol described above. In cerebellar granule cells we found a linear relationship, with a correlation coefficient of 0.979, between the basal F_{334}/F_{380} ratio (R_{basal}) and R_{min} (data not shown). This indicates that the variability in R_{basal} in cerebellar granule cells is very likely due to differences in the shading (15), rather than differences in the basal $[Ca^{2+}]_i$. Therefore, by using the slope of this regression line, which was about 0.87, and the R_{basal} measured in each neuron, the individual R_{min} for each neuron could be approximated. The mean F_{380} values for fura-2 free from Ca^{2+} (S_{f2}) and saturated with Ca^{2+} (S_{b2}) in all cerebellar granule cells in the calibrating experiment were used to calculate the S_{f2}/S_{b2} ratio. The calibrating experiment determining the S_{f2}/S_{b2} value and the slope of the regression line to correlate R_{basal} with R_{min} was carried out in each experimental session. This procedure eliminated the need to correct for shading and background; however, it could be used only with a morphologically uniform population of neurons, such as cerebellar granule cells, which show good linear correlation between R_{basal} and R_{min}; for example, we did not observe such correlation in rat fetal cortical neurons, with a basal $[Ca^{2+}]_i$ of about 120 nM,[1] much higher than the $[Ca^{2+}]_i$ of 20–25 nM present in cerebellar granule cells (11). The mean R_{min} obtained from cerebellar granule cells treated with the Ca^{2+}-free medium was used for $[Ca^{2+}]_i$ calibration in astrocytes, which occasionally were present in the recorded field and had a significantly higher basal $[Ca^{2+}]_i$ than did the cerebellar granule cells; R_{max} was measured in the astrocytes individually with ionomycin at the end of each experiment and was comparable to that found in the neurons in the same field. The $[Ca^{2+}]_i$ was calculated from the F_{334}/F_{380} ratio (R) as described by Grynkiewicz *et al.* (16), using the K_d of fura-2 for Ca^{2+} (264 nM) after correction for room temperature (17). The experimental chamber volume was 0.5 ml, and the drug solutions were added by medium replacement. To remove a previously applied drug, the chamber was washed four times with 0.5 ml of BSS. In the experiments where the $[Na^+]_i$ was manipulated, the 5 mM and 100 mM Na^+ solutions were prepared with the appropriate mixture of highly concentrated solutions of Na^+ and K^+. The Na^+ solution contained 5 μM gramicidin, 134.2 mM sodium gluconate, 25.4 mM NaCl, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 5.6 mM glucose, and 5 mM HEPES, pH 7.2 (adjusted with Tris). The high K^+ solution was identical except for complete replacement of Na^+ by K^+. The partial chloride replacement with gluconate in these solutions prevented cell swelling due to the rapid Na^+ and Cl^- fluxes (18).

[1] L. Kiedrowski, unpublished observations.

Materials. Culture media were purchased from GIBCO. Fura-2/acetoxymethyl ester and fura-2 pentapotassium salt were obtained from Molecular Probes (Eugene, OR) and ryanodine from Research Biochemicals (Natick, MA). All other drugs were purchased from Sigma.

Results

To establish the role of different intracellular calcium stores in $[Ca^{2+}]_i$ homeostasis during and after application of a glutamate pulse, we measured glutamate-elicited $[Ca^{2+}]_i$ transients in the presence and absence of thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase (19, 20). A control experiment (Fig. 1A) showed that glutamate (50 μM), applied in a Mg^{2+}-free medium containing 10 μM glycine, evoked a rapid increase of $[Ca^{2+}]_i$ from 21 ± 2 nM to >5 μM; then, while glutamate was still present, $[Ca^{2+}]_i$ decreased and within 4 min stabilized at 1.5 ± 0.1 μM. After glutamate removal, in a medium containing 1 mM Mg^{2+}, the $[Ca^{2+}]_i$ decrease was biphasic (Fig. 1A). In the first phase, which occurred immediately after glutamate removal and lasted about 1 min, $[Ca^{2+}]_i$ decreased to 360 ± 16 nM, whereas in the second phase, which began 6–12 min after glutamate removal and lasted about 2–4 min, $[Ca^{2+}]_i$ reached 70 ± 9 nM (Fig. 1A). This delayed $[Ca^{2+}]_i$ decrease was temporally correlated with a decrease of $[Na^+]_i$ below 15–20 mM (11, 12). The $[Ca^{2+}]_i$ transient profile failed to change if the glutamate pulse was applied in the continuous presence of thapsigargin (1 μM) (Fig. 1B). In cerebellar granule cell culture thapsigargin (1 μM), added under basal conditions for 30 min, failed to modify $[Ca^{2+}]_i$ homeostasis (Fig. 1C); however, in astrocytes present in the same microscopic field thapsigargin, within 2 min, increased $[Ca^{2+}]_i$ from 130–180 nM to about 1 μM in 100% of these cells (see representative example in Fig. 1C). In our cultures, astrocytes were identified by the cell diameter (3–5 times larger than that of cerebellar granule cells), flat appearance, presence of four or more processes, and high basal $[Ca^{2+}]_i$. In two experiments analogous to that illustrated in Fig. 1B, we also tested whether ryanodine, which blocks the Ca^{2+}-induced Ca^{2+} release in cerebellar granule neurons (21), affects $[Ca^{2+}]_i$. In all 70 neurons tested (the astrocytes were not monitored in these experiments), ryanodine (10 μM) added alone failed to change $[Ca^{2+}]_i$ during the 4 min preceding the glutamate addition; moreover, this drug also failed to modify the glutamate-elicited $[Ca^{2+}]_i$ transients. Dantrolene (30 μM), which is known to inhibit Ca^{2+} release from neuronal intracellular stores (22), when added to cerebellar granule cells in which fura-2 had been saturated with Ca^{2+} during the R_{max} calibration (see Materials and Methods) decreased the F_{334}/F_{380} ratio from 3.04 ± 0.10 to 1.87 ± 0.11 ($p < 0.01$, paired Student's t test). To check whether this result was due to a direct effect of dantrolene on fura-2 fluorescence, we measured the F_{334}/F_{380} ratio of fura-2 pentapotassium salt (5 μM) dissolved in BSS with and without dantrolene (30 μM) and found that dantrolene decreased the F_{334}/F_{380} ratio by about 40%.

Differently from thapsigargin and ryanodine, the mitochondrial uncoupler CCCP (1 μM), which dissipates the mitochondrial H^+ gradient (23), virtually abolished the $[Ca^{2+}]_i$ decrease that occurred during the glutamate pulse (Fig. 2A). In only 15 of 212 cells tested, we observed a transient decrease in $[Ca^{2+}]_i$ during the simultaneous exposure of granule cells to CCCP and glutamate (see an example in Fig. 2B). After the simultaneous removal of CCCP and glutamate, a rapid decrease in $[Ca^{2+}]_i$ occurred, but a reapplication of CCCP 2 min later in the

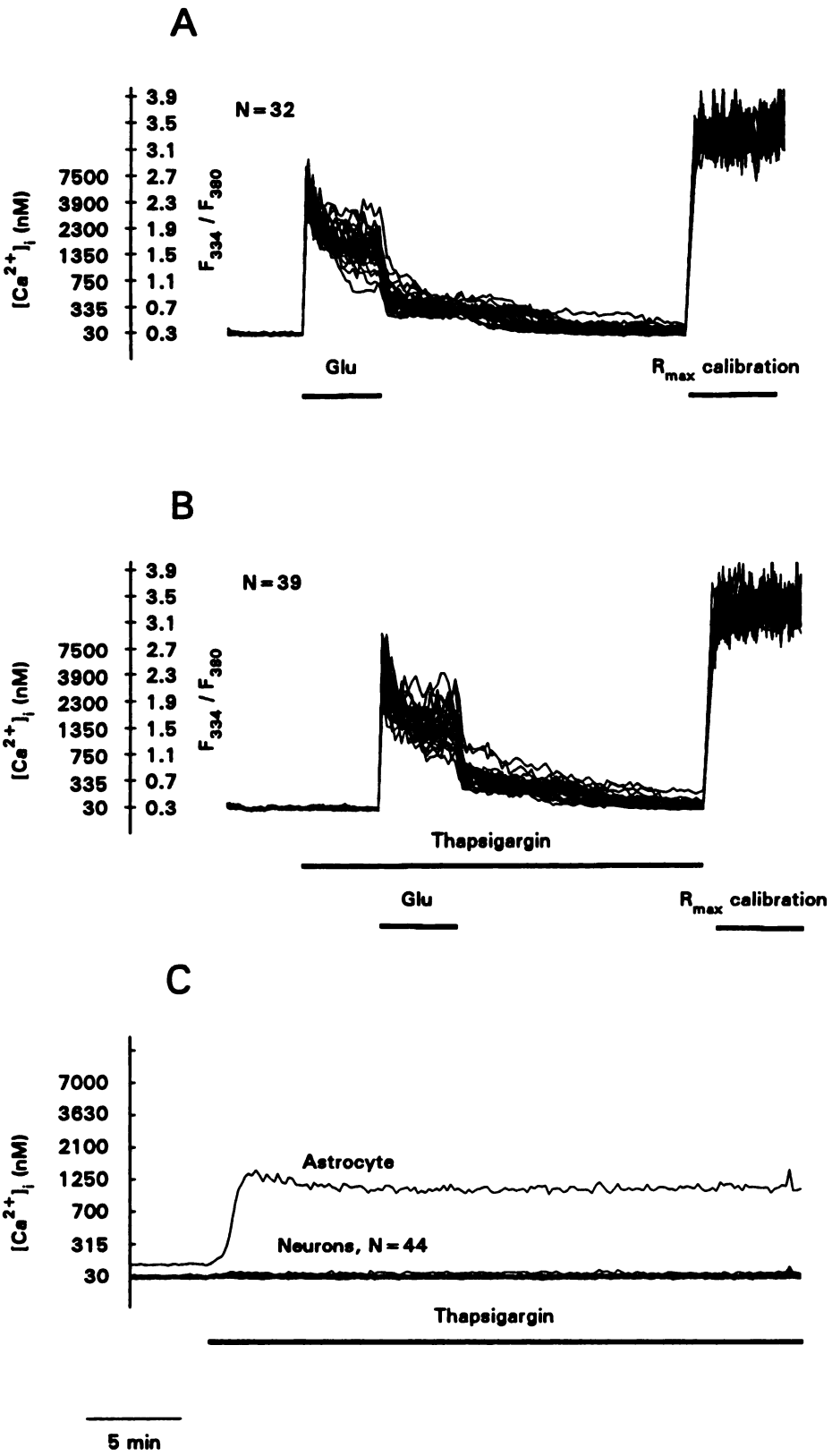

Fig. 1. Thapsigargin fails to affect the glutamate-elicited $[Ca^{2+}]_i$ transients in cerebellar granule cells. A and B, Fura-2-loaded cells were exposed to a glutamate (*Glu*) pulse lasting 4 min (A) or were first treated for 4 min with thapsigargin (1 μM) and then exposed to glutamate (B). Glutamate (50 μM) was applied in a Mg^{2+}-free medium containing glycine (10 μM). Glutamate and glycine were removed from the medium by washing of the cell with BSS (A) or with BSS containing thapsigargin (B). C, Cells were treated for 30 min with thapsigargin (1 μM) applied in BSS. For $[Ca^{2+}]_i$ calibration, at the end of each experiment the cells were treated with ionomycin (10 μM) (examples in A and B) added with the calibrating buffer (see Materials and Methods). Each *line* represents data from a single cell in the same culture dish, and *N* represents the number of neurons. The experiments were repeated three to five times with different batches of cerebellar granule cell cultures, with similar results.

absence of glutamate evoked a robust increase in $[Ca^{2+}]_i$ (Fig. 2B). Antimycin A1 (1 μM), which inhibits the mitochondrial respiratory chain (24), mimicked the effects of CCCP on glutamate-elicited $[Ca^{2+}]_i$ transients, except that the action of antimycin A1 appeared to be irreversible because the $[Ca^{2+}]_i$ failed to decrease after the removal of antimycin A1 (Fig. 2C). Moreover, CCCP (1 μM) or antimycin A1 (1 μM), dissolved in BSS and applied alone for as long as 30 min, failed to change $[Ca^{2+}]_i$ (each drug was studied in three experiments, each containing 29–35 cerebellar granule cells). CCCP (1 μM) added alone increased $[Ca^{2+}]_i$ in only five of 298 neurons (see examples in Fig. 2, A and B). In 98% of the cerebellar granule cells tested,

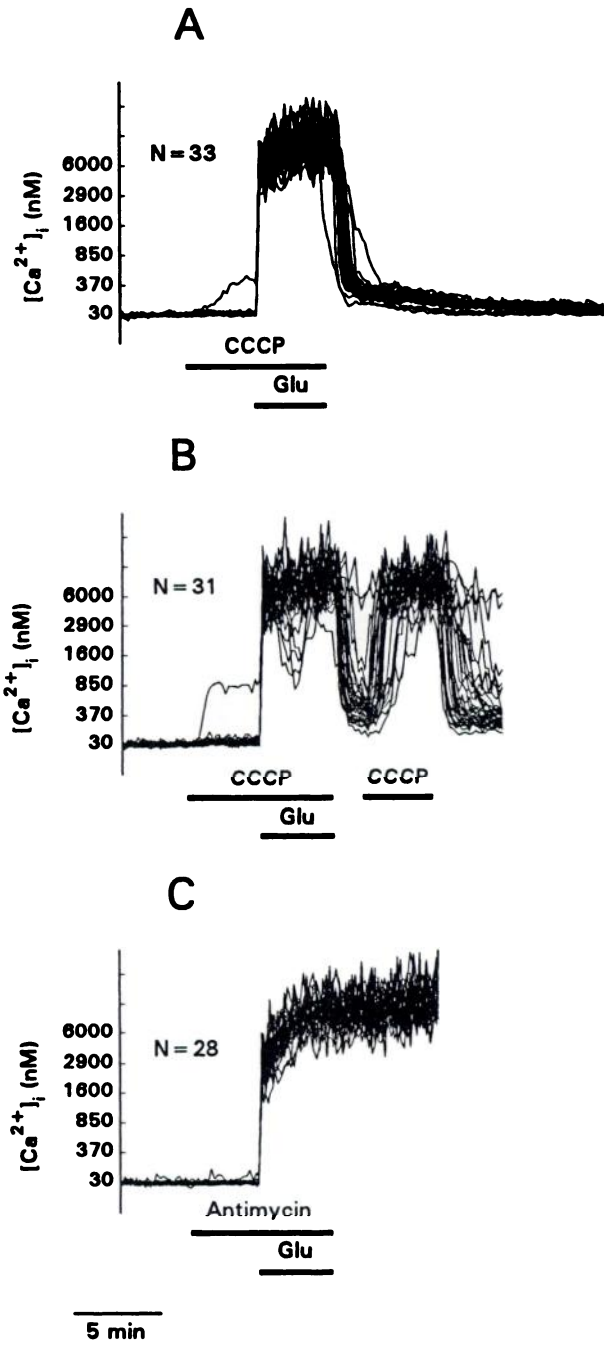

Fig. 2. CCCP and antimycin A1 abolish Ca^{2+} buffering during the glutamate-evoked $[Ca^{2+}]_i$ increase. Fura-2-loaded cells were treated for 4 min with CCCP (1 μM) during (A) or immediately after (B) glutamate (*Glu*) exposure or with antimycin A1 (1 μM) (C), and were then exposed to *Glu*. *Glu* (50 μM) was applied in Mg^{2+}-free medium containing 10 μM glycine. The drugs were removed from the medium by washing of the cell with BSS. Each *line* represents data from a single neuron in the same culture dish; the calibration for R_{max} is not shown. The experiments were repeated three to five times with different batches of cerebellar granule cell cultures, with similar results.

CCCP increased $[Ca^{2+}]_i$ exclusively when applied during the glutamate pulse (Fig. 3A) or shortly after the termination of the glutamate pulse (Fig. 3B). When CCCP was applied 15 min after glutamate withdrawal, an increase in $[Ca^{2+}]_i$ was observed in only three of 37 cells; moreover, also in these three cells, the $[Ca^{2+}]_i$ buffering after glutamate removal appeared to be sluggish (Fig. 3C). In contrast, CCCP failed to increase $[Ca^{2+}]_i$ if after glutamate removal $[Ca^{2+}]_i$ approached basal values; this was observed in one of 32 cells when CCCP was applied 2 min after glutamate removal (Fig. 3B) and in 34 of 37 cells when CCCP was applied 15 min after glutamate removal (Fig. 3C). After glutamate removal, CCCP consistently caused a robust increase in $[Ca^{2+}]_i$ if the basal $[Ca^{2+}]_i$ value was not restored (Figs. 2B and 3B). In 85% of neurons, this $[Ca^{2+}]_i$ increase elicited by CCCP persisted when CCCP was applied in a nominally Ca^{2+}-free medium containing 5 mM EGTA (Fig. 3D) and could be reversed by ionomycin (10 μM) (Fig. 3D). In the remaining neurons (15%), CCCP produced only a modest $[Ca^{2+}]_i$ elevation, and the ionomycin application evoked an increase in $[Ca^{2+}]_i$ followed by a decrease (Fig. 3D).

We then tested whether the residual high $[Na^+]_i$ persisting after glutamate removal (11, 12) interferes with the $[Ca^{2+}]_i$ transients. To manipulate $[Na^+]_i$, we applied medium containing either 100 mM or 5 mM Na^+, in the presence of 5 μM gramicidin to facilitate Na^+ fluxes through the plasma membrane (see Materials and Methods). When glutamate was washed out in the presence of 100 mM Na^+, $[Ca^{2+}]_i$ decreased from 1.4 ± 0.06 μM to 330 ± 20 nM, and the successive application of the medium with 5 mM Na^+ decreased $[Ca^{2+}]_i$ to 140 ± 6 nM (n = 32) (Fig. 4A). When glutamate was washed out with 5 mM Na^+, $[Ca^{2+}]_i$ promptly decreased to 145 ± 4 nM and was increased to 340 ± 16 nM (n = 38) by the successive application of 100 mM Na^+ (Fig. 4B). Analogous $[Na^+]_i$-dependent changes of $[Ca^{2+}]_i$ were also observed in Ca^{2+}-free medium (Fig. 4C). In contrast, when 5 μM gramicidin and various Na^+ concentrations were applied in the same sequence to granule neurons that had not been previously exposed to glutamate, only a very modest and brief $[Ca^{2+}]_i$ transient occurred immediately after the gramicidin application; the $[Ca^{2+}]_i$ then stabilized and remained unchanged despite the application of various Na^+ concentrations (Fig. 4D). The initial brief $[Ca^{2+}]_i$ oscillation might be explained by a depolarization-induced activation of voltage-gated Ca^{2+} channels.

Discussion

In the cytoplasm of cerebellar granule neurons are present Ca^{2+} stores from which inositol-1,4,5-trisphosphate-mediated Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release are well documented (21, 25). In Fig. 1C, we showed that thapsigargin failed to increase $[Ca^{2+}]_i$ in cerebellar granule neurons but not in astrocytes that were present in the same culture dish. The inability of thapsigargin to release Ca^{2+} from cerebellar granule neuron stores is compatible with a report by Irving *et al.* (21) showing that even after a depolarizing pulse of K^+, which loads cytoplasmic Ca^{2+} stores, thapsigargin elicits only a modest increase in $[Ca^{2+}]_i$. This may be tentatively explained by (i) insufficient Ca^{2+} pumping by the endoplasmic reticulum Ca^{2+}-ATPase because the steady state $[Ca^{2+}]_i$ in cerebellar granule cells is 1/13 of the Ca^{2+} affinity constant of thapsigargin-sensitive Ca^{2+}-ATPases (26), (ii) the Ca^{2+} leak from endoplasmic reticulum storage being slow, (iii) the thapsigargin-sensitive Ca^{2+}-ATPase not being abundant in cerebellar granule cells (26), or (iv) additional, thapsigargin-insensitive mechanisms mediating Ca^{2+} uptake into endoplasmic reticulum (26). The failure of ryanodine to modify the glutamate-elicited $[Ca^{2+}]_i$ transient that we report is in keeping with the lack of significant modification by this agent of the K^+-elicited $[Ca^{2+}]_i$ transients (21).

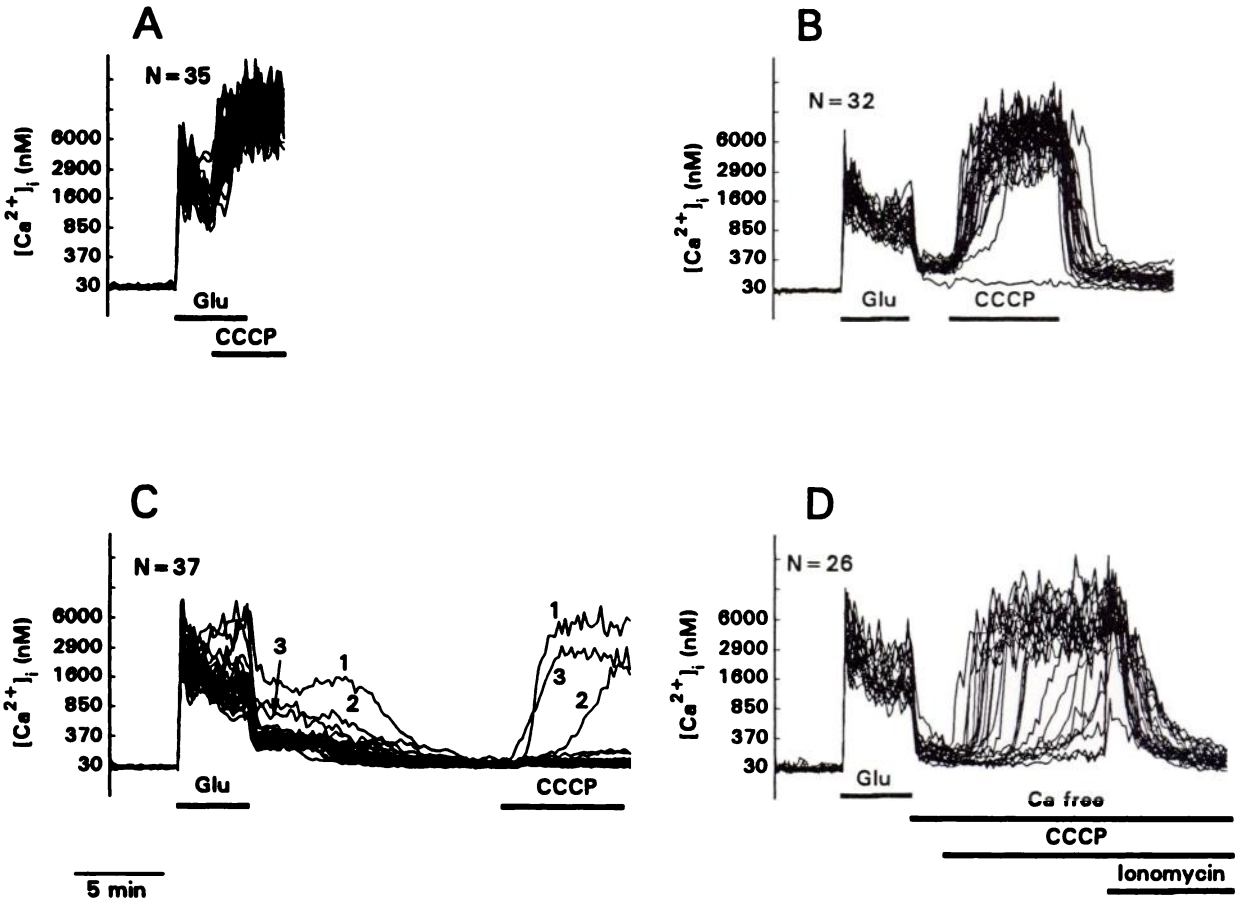

Fig. 3. CCCP increases the glutamate-evoked $[Ca^{2+}]_i$ transients only when applied during the glutamate (*Glu*) pulse or shortly after glutamate removal. Fura-2-loaded cells were exposed to CCCP (1 μM) during the glutamate pulse (A), 2 min after glutamate removal by washing with BSS (B), 15 min after glutamate removal by washing with BSS (C), or 2 min after glutamate removal by washing with a Ca^{2+}-free medium containing 5 mM EGTA (D). In each case glutamate was applied for 4 min in Mg^{2+}-free medium containing 10 μM glycine and was then removed by washing of the cell with the respective Mg^{2+}-containing medium. Each *line* represents data from a single neuron in the same culture dish; the calibration for R_{max} is not shown. The experiments were repeated three times with different batches of cerebellar granule cell cultures, with similar results. *Numbers in C*, $[Ca^{2+}]_i$ profiles recorded from the same cell.

Together, these data indicate that only a small proportion of the total Ca^{2+} that enters cerebellar granule cells after glutamate receptor- or voltage-gated Ca^{2+} channel activation is actually taken up by the endoplasmic reticulum Ca^{2+} stores. However, in another report (27) on cerebellar granule cells, showing that $[Ca^{2+}]_i$ transients elicited by NMDA application were variable and unpredictable, the $[Ca^{2+}]_i$ transients were significantly different when NMDA was applied alone, then withdrawn, and applied again with ryanodine or thapsigargin. The interpretation of these data is complex and escapes critical evaluation because of the cascade of events elicited by the the previous NMDA application.

In the present report, we also confirmed that dantrolene induces a Ca^{2+}-independent decrease of the F_{334}/F_{380} ratio of fura-2 (28); hence, dantrolene could not be used as a research tool to study the intracellular Ca^{2+} store dynamics in cells loaded with fura-2.

In cerebellar granule cells the amount of Ca^{2+} that may be stored in the endoplasmic reticulum appears to be limited. In fact, these stores must be refilled with Ca^{2+} to prevent a response rundown upon repetitive applications of agonist (21, 25). The $[Ca^{2+}]_i$ transients induced by the activation of glutamate and muscarininc metabotropic receptors or by ryanodine receptors are prompt and brief (21, 25). These data indicate that the Ca^{2+} released from endoplasmic reticulum Ca^{2+} stores plays a significant role in signal transduction of glutamate and muscarinic metabotropic receptor activation; however, our data combined with the findings of Irving *et al.* (21, 25) suggest that the capacity of endoplasmic reticulum Ca^{2+} stores of cerebellar granule cells may not be sufficient to buffer Ca^{2+} influx elicited by excitotoxic doses of glutamate.

In attempting to identify processes that have the capacity to buffer the $[Ca^{2+}]_i$ transients during Ca^{2+} homeostasis destabilization elicited by excitotoxic doses of glutamate, we found that CCCP or antimycin A1, which abolish the mitochondrial proton gradient, evoked a large increase in $[Ca^{2+}]_i$ when applied during or shortly after the neuronal exposure to excitotoxic doses of glutamate (Fig. 2). These data imply that a sizable proportion of the Ca^{2+} entering neurons exposed to excitotoxic doses of glutamate is diverted to the mitochondria and may be released from these organelles when their proton gradient collapses upon application of CCCP or antimycin A1. Moreover, because CCCP and antimycin A1, when applied in the absence of glutamate, failed to increase $[Ca^{2+}]_i$, it could be argued that in untreated neurons the releasable Ca^{2+} pool is modest in size. This inference is supported by data in Fig. 4, which show that the modulation of $[Ca^{2+}]_i$ by changes in $[Na^+]_i$ occurred only after mitochondria had been loaded with Ca^{2+} as a result of

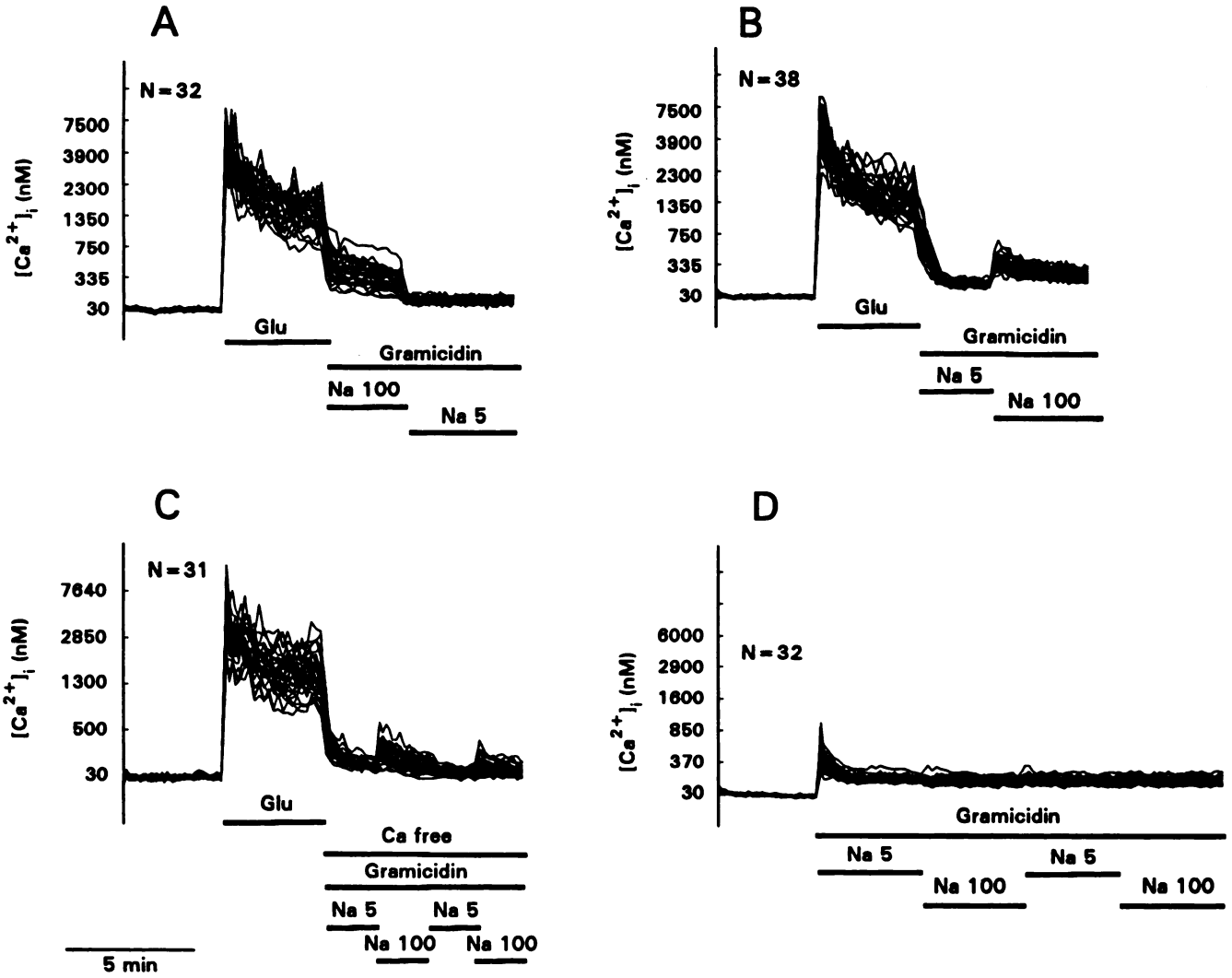

Fig. 4. Effects of different $[Na^+]_i$ on Ca^{2+} buffering in cerebellar granule cells after glutamate (*Glu*) exposure. A, Fura-2-loaded cells were treated for 4 min with glutamate (50 μM) applied in Mg^{2+}-free medium containing 10 μM glycine, then glutamate and glycine were washed out with medium containing 100 mM Na^+ and 5 μM gramicidin (see Materials and Methods), and, after 3 min, 5 mM Na^+ with 5 μM gramicidin was applied. B, The cells were treated with glutamate as described for A but 5 mM Na^+ was applied first and then 100 mM Na^+. C, The cells were treated as described for A and B, but glutamate and glycine were washed out with nominally Ca^{2+}-free medium containing 0.5 mM EGTA and 5 or 100 mM Na^+, as indicated. D, The indicated Na^+ concentrations with 5 μM gramicidin were applied to cerebellar granule cells under basal conditions. Each *line* represents data from a single neuron in the same culture dish; the calibration for R_{max} is not shown. The experiments were repeated three times with different batches of cerebellar granule cell cultures, with similar results.

glutamate exposure. This inference is also in keeping with the low levels of Ca^{2+} detected in liver mitochondria *in situ* (29).

Several lines of independent investigation with isolated mitochondria indicate that these organelles can take up large amounts of Ca^{2+} when they are incubated with micromolar concentrations of Ca^{2+} (7–9). Such a Ca^{2+} uptake by mitochondria was also demonstrated in rat primary neuronal cultures (30, 31) and in mouse dorsal root ganglia (32, 33) during K^+-elicited depolarization. Our data suggest that in cerebellar granule cells the mitochondrial Ca^{2+} uptake plays a major role in buffering micromolar $[Ca^{2+}]_i$ after application of excitotoxic doses of glutamate.

The mitochondrial uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone was reported to release Ca^{2+} from nonmitochondrial stores (34). However, it is unlikely that, in our experiments, CCCP released Ca^{2+} from endoplasmic reticulum storage sites, because antimycin A1, which is not an uncoupler but inhibits the transfer of electrons from cytochrome *b* to cytochrome c_1 (24), increased $[Ca^{2+}]_i$ by an extent (Fig. 2C) comparable to that evoked by CCCP (Fig. 2A). Because both drugs abolish the mitochondrial membrane potential, it is likely that their actions on glutamate-elicited $[Ca^{2+}]_i$ transients reflect an inhibition of Ca^{2+} uptake by mitochondria. The persistent $[Ca^{2+}]_i$ increase seen after antimycin A1 removal indicates that the inhibition of electron transfer evoked by antimycin A1 is not reversed in the time frame of our experiments (24).

The transient decrease in $[Ca^{2+}]_i$ that we occasionally observed in glutamate-treated granule cells when the mitochondrial Ca^{2+} uptake was inhibited by CCCP (Fig. 2B) suggests that some extramitochondrial Ca^{2+}-buffering mechanisms may occur simultaneously with the mitochondrial Ca^{2+} uptake. However, these extramitochondrial mechanisms appear to have a low buffering capacity. In contrast, the Ca^{2+} storage capacity

of mitochondria appears to be high, as suggested by the data of Fig. 3D showing that the CCCP-evoked increase in $[Ca^{2+}]_i$ failed to decline even in neurons kept in medium with the nominal absence of Ca^{2+}; in these neurons the addition of ionomycin could promote a decrease of $[Ca^{2+}]_i$, most probably due to Ca^{2+} efflux from the neurons. In a few neurons in which $[Ca^{2+}]_i$ was low, a brief ionomycin-elicited increase of $[Ca^{2+}]_i$ could be observed (Fig. 3D), confirming that this compound induces Ca^{2+} release from intracellular stores (35, 36). In the remaining neurons this effect of ionomycin was probably masked by already high $[Ca^{2+}]_i$.

It is likely that, during exposure to excitotoxic doses of glutamate, the large pool of Ca^{2+} accumulated in mitochondria is precipitated as calcium phosphate crystals (7, 8, 26). This possibility is in keeping with the visualization, after brief ischemia, of calcium phosphate deposits in brain mitochondria (37). Moreover, when CCCP was added to our cultures 15 min after glutamate removal, the $[Ca^{2+}]_i$ failed to increase in about 92% of the neurons (Fig. 3C), indicating that at that time the releasable Ca^{2+} pool in mitochondria was unavailable.

In cerebellar granule cells, an excitotoxic pulse of glutamate increases $[Na^+]_i$ to 60–100 mM (11); this increase outlasts the presence of glutamate, for a time period that appears to be related to the duration of the glutamate pulse (12). We investigated whether this $[Na^+]_i$ elevation can affect the Ca^{2+}-buffering capacity of mitochondria. Fig. 4A shows that, when $[Na^+]_i$ was artificially decreased to 5 mM immediately after the removal of glutamate, $[Ca^{2+}]_i$ stabilized at a level severalfold lower than when 100 mM $[Na^+]_i$ was present (Fig. 4B). These differences in $[Ca^{2+}]_i$ transient kinetics evoked by changes in $[Na^+]_i$ most probably are unrelated to a putative Ca^{2+} flux through the plasma membrane, because they were observed also when the cells were kept in Ca^{2+}-free medium (Fig. 4C). Because the rate whereby the $[Ca^{2+}]_i$ decreases after glutamate removal in the presence of 100 mM $[Na^+]_i$ (Fig. 4A) fails to deviate significantly from that in a control experiment (Fig. 1A), it is very likely that the glutamate-evoked increase in $[Na^+]_i$ may attenuate mitochondrial Ca^{2+} buffering. Most probably, high $[Na^+]_i$ releases Ca^{2+} from mitochondria via the mitochondrial Na^+/Ca^{2+} exchanger (38). Under physiological conditions, this mechanism may regulate intramitochondrial Ca^{2+} concentrations and thereby participate in the modulation of oxidative phosphorylation rates (39).

Our data show that what happens in the somata of dorsal root ganglion neurons after K^+ depolarization (30, 31) may also occur in the somata of cerebellar granule cells exposed to excitotoxic doses of glutamate; in both cases, the Na^+/Ca^{2+} exchanger located in the plasma membrane appears to play only a marginal role in counteracting depolarization- or glutamate-dependent $[Ca^{2+}]_i$ homeostasis destabilization; in fact, it may even contribute to the $[Ca^{2+}]_i$ increase due to the activation of the reverse mode of exchanger operation by high $[Na^+]_i$ (11). However, the plasma membrane Na^+/Ca^{2+} exchanger may have an important role in maintaining $[Ca^{2+}]_i$ homeostasis in the nerve terminals (2–4), where the Na^+/Ca^{2+} exchanger was visualized by immunohistochemical studies (40).

In conclusion, our results show that mitochondrial Ca^{2+} uptake plays a dominant role in reducing the increase of $[Ca^{2+}]_i$ in cerebellar granule cells exposed to excitotoxic doses of glutamate. However, this mitochondrial Ca^{2+} buffering is limited and probably can be saturated when neurons are flooded with Ca^{2+} for minutes, leading to calcium phosphate precipitation within the mitochondrial matrix (7, 8), which may damage mitochondrial function. It seems pertinent to suggest that such a Ca^{2+}-buffering mechanism, by reducing the mitochondrial membrane potential, inhibits ATP synthesis (7) and thereby participates in the mechanism of glutamate-elicited neurotoxicity.

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