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# Delayed Increase of $Ca^{2+}$ Influx Elicited by Glutamate: Role in Neuronal Death

HARI MANEV, MARCO FAVARON, ALESSANDRO GUIDOTTI, and ERMINIO COSTA
*Fidia-Georgetown Institute for the Neurosciences, Georgetown University, Washington, DC 20007*
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**SUMMARY**

The mechanism of delayed neurotoxicity, triggered by glutamate, was studied in 7–8-day-old primary cultures of rat cerebellar granule cells. Treatment of cultures for 15 min with 50 μM glutamate in $Mg^{2+}$-free medium, followed by removal of the excitotoxin, resulted in neuronal death, which started to appear 2–3 hr after the termination of glutamate treatment. The number of dead neurons increased gradually in the next few hours and 80–85% of neurons were found dead 24 hr later. Antagonists of *N*-methyl-D-aspartate-sensitive glutamate receptors (phencyclidine, dibenzocycloheptenеimine, DL-2-amino-5-phosphonovalerate) or 1.2 mM $MgCl_2$, but not the antagonist of *N*-methyl-D-aspartate-insensitive glutamate receptors (6-cyano-7-nitroquinoxaline-2,3-dione), abolished glutamate neurotoxicity when applied to cultures together with glutamate. 6-cyano-7-nitroquinoxaline-2,3-dione abolished the neurotoxic effect of kainate. Development of glutamate-induced neuronal death depends strongly on $Ca^{2+}$. Removal of extracellular $Ca^{2+}$ (with 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) immediately after the termination of glutamate exposure and before the appearance of the early signs of neuronal death (post-glutamate period) dramatically reduced neuronal degeneration. Neurotoxic concentrations of glutamate induced sustained increase of $^{45}Ca^{2+}$ uptake in the post-glutamate period. The delayed increase of $^{45}Ca^{2+}$ uptake, as well as the delayed neurotoxicity, were not affected by post-glutamate treatment with phencyclidine, dibenzocycloheptenеimine; DL-2-amino-5-phosphonovalerate, or $MgCl_2$ or with voltage-dependent $Ca^{2+}$ channel blockers (nitrendipine, verapamil, diltiazem). Neurotoxic concentrations of glutamate also induced a delayed sustained increase of [$^3$H]phorbol-12,13-dibutyrate binding, reflecting an increased translocation of protein kinase C (PKC) from cytosol to the cell membrane during the post-glutamate period. Pretreatment of neurons with the ganglioside GT1b (trisialosylgangliotetraglycosylceramide), followed by removal of free GT1b from the incubation medium, prevented PKC translocation, the sustained increase of $^{45}Ca^{2+}$ uptake in the post-glutamate period, and the delayed neuronal death. We suggest that the sustained activation and translocation of PKC primed by glutamate receptor stimulation may be the triggering event causing the protracted increase of neuronal $Ca^{2+}$ influx. This influx is insensitive to voltage-dependent $Ca^{2+}$ channel blockers and glutamate receptor antagonists. It appears that this delayed increase of $Ca^{2+}$ influx may be important in causing neuronal death.

Evidence is accumulating that brain damage associated with anoxia, stroke, trauma, and perhaps neurodegenerative illness (Huntington chorea) may be partially mediated by a sustained increase of neuronal free cytosolic $Ca^{2+}$ caused by unregulated and paroxysmal activation of glutamate receptors (1–4). At least two events may play a major role in glutamate-induced neurotoxicity. The first is an acute neuronal swelling caused by depolarization-mediated influx of $Na^+$, $Cl^-$, and water, which is reversible if glutamate is removed. Hence, swelling is not per se the cause of neuronal death unless it is protracted, when it culminates in cell lysis (3, 5). The second event (perhaps the most significant for the extension of neurotoxicity to tissue perifocal to the anoxic area) is characterized by a slowly occurring neuronal degeneration that, depending on the concentration and time of exposure to glutamate, can become resistant to noncompetitive antagonists of excitatory amino acid receptors (6) or glutamate withdrawal (5). This secondary neurotoxic component is absolutely dependent on levels of free cytosolic $Ca^{2+}$ and does not need to be preceded by swelling (5, 7–10).

Although high conductance cationic channels regulated by NMDA-sensitive glutamate recognition sites are the predominant cause of $Ca^{2+}$ entry and neuronal death, the sequence of molecular events intervening between glutamate withdrawal and the time at which a delayed neuronal death is an irreversible endpoint still remains unknown (1, 2).

We have recently reported that a protracted exposure of cultured neurons to glutamate increases $Ca^{2+}$ influx and triggers PKC activation and translocation from the cytosol to the

**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; MK-801, dibenzocycloheptenеimine; APV, DL-2-amino-5-phosphonovalerate; CNQX, 6-cyano-7-nitroquinoxaline-2,3 dione; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $P(BtO)_2$, phorbol 12,13-dibutyrate; PKC, protein kinase C (2.7.1.37); GT1b, trisialosylgangliotetraglycosylceramide; EAA, excitatory amino acid; FDA, fluorescein diacetate; PI, propidium iodide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

neuronal membrane (11, 12). Inhibitors of PKC, such as sphingolipids (gangliosides and sphingosine) (13), prevent the glutamate receptor-mediated activation and translocation of PKC (by a mechanism that does not involve a direct blockade of the opening of glutamate-regulated $Ca^{2+}$ channels) and at the same time protect neurons in culture from glutamate- and kainate-induced neuronal death (14). These data support the view that translocation of PKC elicited by EAA receptor stimulation participates in the sequence of events leading to delayed neuronal death.

Recently, it has been shown that, in $CA_1$ hippocampal neurons dissociated *in vitro*, a brief (1–3 sec) application of glutamate not only produces an acute transient peak of free cytosolic $Ca^{2+}$ but also induces a delayed free $Ca^{2+}$ transient that persists after glutamate removal (15). Because this prolongation of the free cytosolic $Ca^{2+}$ transient is blocked by pretreatment of the neurons with sphingosine (a PKC inhibitor), it is suggested that a priming or conditioning stimulus elicited by EAA receptor agonists contributes to a PKC-mediated prolongation in the cytosolic free $Ca^{2+}$ transients. This could be due either to a PKC-mediated protracted opening of preexisting $Ca^{2+}$ channels or to a PKC-mediated phosphorylation of calciosome membrane receptors (16) leading to a decrease in the efficiency of the $Ca^{2+}$-buffering capacity.

In this study we report that, in primary culture of neonatal rat cerebellar granule cells, the development of glutamate-induced delayed neuronal death is associated with prolonged activation and translocation of PKC, which is associated with delayed sustained elevation of $Ca^{2+}$ influx.

## Materials and Methods

**Primary culture of cerebellar granule cells.** Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague Dawley rats (Zivic Miller, Pittsburgh, PA) (17). Neurons were grown on 35-mm culture dishes for 7–8 days *in vitro*, at a density of $2 \times 10^6$ cells/dish. Glial proliferation was prevented by adding cytosine-arabinofuranoside (10 $\mu$M final concentration) 24 hr after plating (17). Immunocytochemical studies of these primary cultures of cerebellar granule cells show that they contain >95% neurons and <5% glia or other contaminating cells (17).

**Culture treatment with glutamate and other drugs.** The initial culture medium (culture-conditioned medium) was collected before the addition of glutamate or kainate, and the cells were washed once with Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.6 mM glucose, 5 mM HEPES, pH 7.4). $MgCl_2$ was routinely omitted from Locke's solution during the exposure to glutamate (15 min, 22°) in order to potentiate the contribution of NMDA receptors to the observed neurotoxicity (14). $MgCl_2$ was present when the cells were exposed to kainate (30 min, 22°) (14). Drugs of interest were added when indicated. For ganglioside treatment, the monolayers were preincubated with GT1b (FIDIA SpA, Abano Terme, Italy) for 2 hr at 37°. Thereafter, the cultures were washed three times with Locke's solution (to remove free GT1b) and then challenged with glutamate.

**Termination of glutamate treatment, removal of extracellular $Ca^{2+}$, and drug treatment.** The incubation with glutamate was terminated by washing the monolayer with Locke's solution three times. Thereafter, the neurons were incubated in Locke's solution, with or without drugs of interest, at 37° for various time intervals; after three additional washes with Locke's solution, they were returned to the culture-conditioned medium and incubated at 37° in 95% air/5% $CO_2$ for 24 hr. The extracellular $Ca^{2+}$ was removed from control or glutamate-treated cultures by one wash with Locke's solution followed by incubation for various times at 37° (95% air/5% $CO_2$) in $Ca^{2+}$-free (no $Ca^{2+}$ plus 1 mM EGTA) Locke's solution.

**Intravital staining of the culture.** Monolayers were washed with Locke's solution and stained for 3 min at 22° with a solution containing 36 $\mu$M FDA and 7 $\mu$M PI (14, 18). The stained cells were examined immediately with a standard epiillumination fluorescence microscope (Vanox, Olympus; 450 nm excitation, 520 nm barrier). FDA, a nonpolar ester, crosses the cell membranes and is hydrolyzed by intracellular esterases to produce a green-yellow fluorescence. Neuronal injury curtails FDA staining (19) and facilitates PI (a polar compound) penetration and interaction with DNA to yield a bright red fluorescent complex (20). After treatment with glutamate, some neurons may degenerate and detach from the dish. Therefore, we estimated the loss of cells by comparing the number of intact or degenerated neurons in one identified field photographed before and 24 hr after application of glutamate. The percentage of surviving neurons in the monolayer was computed by assessing the FDA/PI staining ratio in photomicrographs of four representative fields from each monolayer (14).

**Determination of $^3$H-phorbol ester binding to intact cells.** Granule cells grown on 35-mm diameter culture dishes were washed once with Locke's solution and incubated in 1 ml of Locke's solution that contained [4$\beta$-$^3$H]phorbol-12,13-dibutryate (12.5 Ci/mmol; New England Nuclear, Boston, MA), in 0.1% fatty acid-free bovine serum albumin; $MgCl_2$ was omitted when indicated. The monolayers were incubated with [$^3$H]P(BtO)$_2$ (2 nM) for 15 min at 22° (12). After incubation, the cells were washed three times with ice-cold Locke's solution and were suspended in 0.1 M NaOH. An aliquot of the suspension was used for protein determination according to the method of Lowry *et al.* (21) and another aliquot was used for radioactivity measurements. Nonspecific binding was determined in the presence of 2 $\mu$M phorbol 12-tetradecanoate 13-acetate.

**Determination of $^3$H-phorbol ester binding to cell membranes.** Granular neuron monolayers, washed three times with $Ca^{2+}$-free Locke's solution, were homogenized in buffer that contained 25 mM Tris·HCl, 0.2 mM EGTA, 1 mM dithiothretiol, 3 mM $MgCl_2$, 3 $\mu$g/ml bovine serum albumin, and 0.2 mM phenylmethysulfonyl fluoride, pH 7.5 (12). The homogenate was then centrifuged at 4° (48,000 × *g* for 1 hr). The pellet was resuspended in Tris·HCl buffer (pH 7.4) to reach a protein concentration of 250–300 $\mu$g/ml. Aliquots (200 $\mu$l) were used to determine [$^3$H]P(BtO)$_2$ binding by the addition of 200 $\mu$l of assay buffer (50 mM Tris·HCl, 0.1% fatty acid-free bovine serum albumin, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 2 mM dithiothretiol, pH 7.4) and 50 $\mu$l of [$^3$H]P(BtO)$_2$ in 0.1% fatty acid-free bovine serum albumin. After a 30-min incubation at 22°, the bound ligand was separated from the free ligand by filtration on CF/C glass fiber filters.

**$^{45}$Ca uptake by neurons in monolayer.** Thirty minutes before $^{45}Ca^{2+}$ uptake was measured, cultures were incubated in Locke's solution at 37°. To each dish, 1 $\mu$Ci of $^{45}CaCl_2$ was added (11). The reaction was stopped 30 or 60 sec after the application of $^{45}Ca^{2+}$ solution by rapidly (5–8 sec) washing the cells three times with nonradioactive solution. Cultures were dissolved in 0.1 M NaOH and the radioactivity and protein content were determined.

## Results

**Delayed neuronal death induced by short-lasting glutamate exposure.** As reported in previous experiments (14), the vulnerability of cerebellar granule cells to EAA receptor agonists depends on dose, temperature, pH changes, density, and age of the culture. The conditions adopted in these experiments (7–8 days *in vitro*, $2 \times 10^6$ cells, 22°) were such that 15-min exposure to 50 $\mu$M glutamate in $Mg^{2+}$-free Locke's solution (pH 7.4) consistently induced the death of 80–85% of neurons, when measured 24 hr later. During the period of glutamate exposure, neuronal swelling and the appearance of dark granules in neuronal somata was observed. However, at this time intravital FDA-PI staining (which shows a bright green color

in vital neurons and a red color in dead neurons) revealed only a slight weakening of the green color brightness (Fig. 1B) and no red staining, an index of neuronal death.

If, at the end of the 15-min incubation, glutamate was removed (by washing three times with Locke's solution) and the cells were returned to culture-conditioned medium, within the next 15–30 min the swelling was considerably reduced and the majority of neurons recovered their original FDA staining brightness (Fig. 1C). However, 2 hr after glutamate removal, despite the initial morphological recovery and the disappearance of swelling, the red staining, indicating neuronal death, began to appear (Fig. 1D). In the next few hours, the number of dead neurons increased gradually and, 24 hr after glutamate withdrawal, most of the neurons evinced the bright red staining, index of neuronal death (Fig. 1E).

Both the immediate and delayed effects induced by glutamate were prevented if glutamate was added in the presence of either noncompetitive (PCP, MK-801) or competitive (APV) antagonists of NMDA-sensitive glutamate receptors (Table 1). Moreover, the addition of 1.2 mM $MgCl_2$ to the incubation medium also reduced the neurotoxocity (Fig. 1F). On the other hand, CNQX, a NMDA-insensitive glutamate receptor antagonist (22), failed to prevent glutamate neurotoxicity, although it prevented the neuronal death induced by kainate (Table 2).

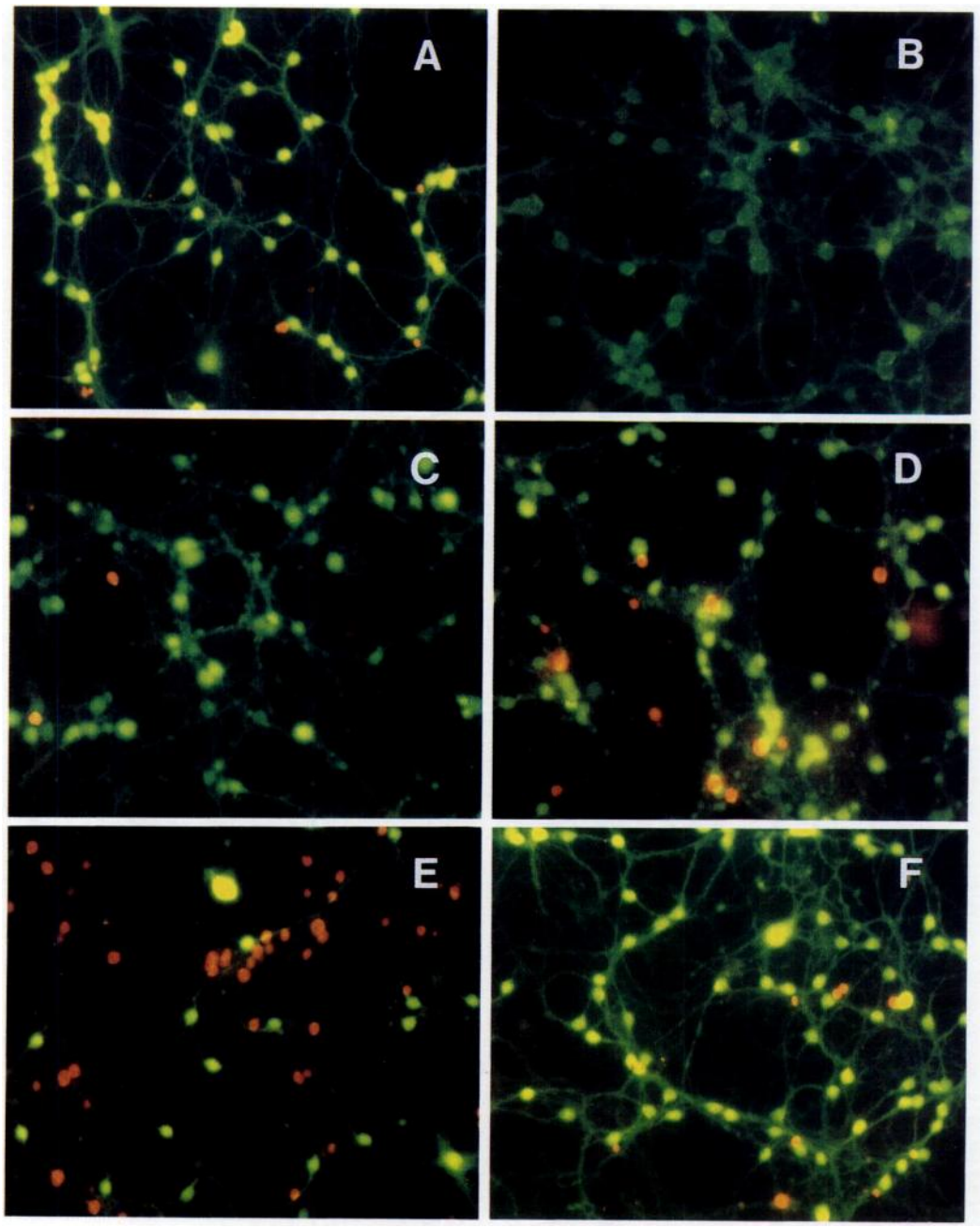


**Fig. 1.** Eight-day-old cerebellar granule cells were incubated at 22° for 15 min in Locke's solution containing 50 μM glutamate, in the absence (B–E) or presence (F) of 1.2 mM $Mg^{2+}$. Control was not exposed to glutamate (A). FDA-PI staining was performed immediately (B), 30 min (C), 2 hr (D), and 24 hr (E and F) after 15-min exposure to glutamate. In the post-glutamate period, the cells were returned to culture-conditioned medium and were incubated at 37° in 95% air/5% $CO_2$. Green color (FDA) shows alive cells, red color (PI) shows dead cells. Data are representative of a typical experiment. Similar results were obtained in four experiments performed in different cell preparations.

TABLE 1

**Effect of glutamate receptor antagonists, $Ca^{2+}$ channel blockers, and removal of extracellular $Ca^{2+}$ on glutamate-induced neuronal death**

Cells were treated for 15 min with 50 μM glutamate (no $Mg^{2+}$) in the absence or presence of different agents. Post-glutamate treatment was conducted for 30 min starting immediately after removal of glutamate. Viability was estimated 24 hr later (mean ± standard error of four to six different monolayers).

| | Viability | |
|---|---|---|
| | Treatment with glutamate | Post-glutamate treatment |
| | % cells alive | |
| None | 19 ± 6.2 | 17 ± 8.0 |
| APV (1 mM) | 86 ± 2.0[a] | 20 ± 1.0 |
| MK-801 (1 μM) | 85 ± 3.5[a] | 19 ± 3.1 |
| PCP (5 μM) | 90 ± 2.0[a] | 21 ± 7.8 |
| CNQX (1 μM) | 21 ± 7.0 | 23 ± 2.4 |
| PCP (5 μM) + CNQX (1 μM) | ND[b] | 28 ± 3.1 |
| No $Ca^{2+}$ + EGTA (1 mM) | 95 ± 2.0[c] | 55 ± 5.0[a] |
| $CoCl_2$ (1 mM) | 94 ± 1.4[a] | 27 ± 6.1 |
| Nitrendipine (1 μM) | 20 ± 3.1 | 25 ± 4.0 |
| Verapamil (1 μM) | 19 ± 2.3 | 21 ± 2.1 |
| Diltiazem (1 μM) | 15 ± 4.8 | 18 ± 4.7 |

[a] $p < 0.01$ when compared with corresponding group treated with glutamate, only. Viability of untreated control was 91 ± 3.4%.

[b] ND, not determined.

[c] $p < 0.01$ in comparison with group treated with glutamate in the presence of $Ca^{2+}$. In this experiment, in order to prevent excessive neuronal swelling due to the simultaneous removal of $Ca^{2+}$ and $Mg^{2+}$, the sodium chloride was replaced with equimolar concentration of choline chloride.

TABLE 2

**Effect of PCP and CNQX on glutamate- and kainate-induced neuronal death**

Cerebellar granule cells were incubated with glutamate (50 μM, 15 min, no $Mg^{2+}$) or kainate (100 μM, 30 min, 1.2 mM $Mg^{2+}$) in the presence or absence of 1 μM PCP or 1 μM CNQX. Viability was estimated 24 hr later (mean ± standard error of six different monolayers). Treatment with 1 μM PCP or CNQX alone fails to change cell viability.

| Glutamate-Induced | | Kainate-Induced | |
|---|---|---|---|
| Treatment | Viability | Treatment | Viability |
| | % cells alive | | % cells alive |
| Control | 95 ± 1.1 | Control | 93 ± 2.0 |
| Glutamate | 18 ± 2.2[a] | Kainate | 20 ± 1.4[a] |
| Glutamate + PCP | 91 ± 3.1 | Kainate + PCP | 54 ± 4.2[a] |
| Glutamate + CNQX | 11 ± 3.5[a] | Kainate + CNQX | 81 ± 8.5 |

[a] $p < 0.01$ when compared with the respective controls.

**Role of delayed increase of $Ca^{2+}$ influx on glutamate-induced neuronal death.** Glutamate neurotoxicity can be prevented by the omission of extracellular $Ca^{2+}$ during the exposure of neurons to this EAA (5, 23). Table 1 shows that removal of $Ca^{2+}$ from the cerebellar granule cell culture medium (Locke's solution in which $Na^+$ was substituted by choline) during glutamate exposure prevented the delayed neuronal degeneration. Fig. 2 shows that, in cells incubated with glutamate (in the presence of $Ca^{2+}$), exposure to $Ca^{2+}$-free Locke's solution (no $Ca^{2+}$ plus 1 mM EGTA) during the period between the end of glutamate exposure and the appearance of red staining indicating neuronal death (post-glutamate period) also reduced the neurodegenerative effects of glutamate. Approximately 70% of the neurons survived if $Ca^{2+}$ was omitted for 60 min immediately following glutamate exposure and approximately 50% of the neurons survived if $Ca^{2+}$ was omitted for only 15 min (Fig. 2).

Fig. 3 shows the importance of the time interval between the termination of glutamate exposure and the beginning of exposure to $Ca^{2+}$-free Locke's solution. The omission of $Ca^{2+}$ had a maximal protective effect (approximately 70%) when initiated immediately after glutamate withdrawal. Only marginal or no protection was observed when $Ca^{2+}$ omission was initiated 30 or 60 min after glutamate removal (Fig. 3).

In the post-glutamate period, treatment with the NMDA-sensitive glutamate receptor antagonists APV, MK-801, and PCP, as well as treatment with the non-NMDA glutamate

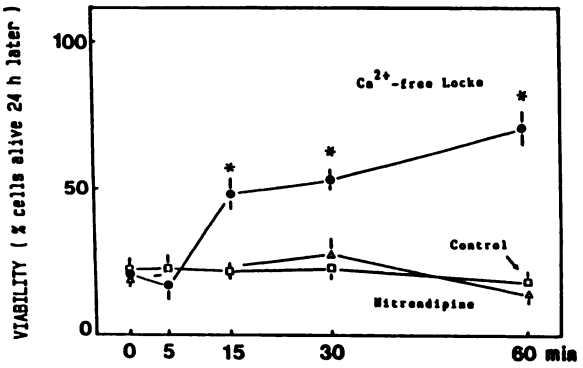


**Fig. 2.** $Ca^{2+}$-free Locke's solution (0 $Ca^{2+}$ plus 1 mM EGTA), but not nitrendipine treatment, in the post-glutamate period counteract glutamate neurotoxicity; effect of the duration of $Ca^{2+}$-free Locke's exposure or nitrendipine (1 μM) treatment. Cerebellar granule cells were treated for 15 min with 50 μM glutamate in $Mg^{2+}$-free Locke's solution containing 2.3 mM $CaCl_2$. Viability was estimated 24 hr later, after returning the cells to culture-conditioned medium at 37° in 95% air/5% $CO_2$. Results (mean ± standard error of at least three experiments) are expressed as percentage of neurons surviving. *$p < 0.01$ when compared with cells incubated in Locke's solution (control). Post-glutamate treatment was initiated immediately (time 0) after removal of glutamate (for further details see Materials and Methods).

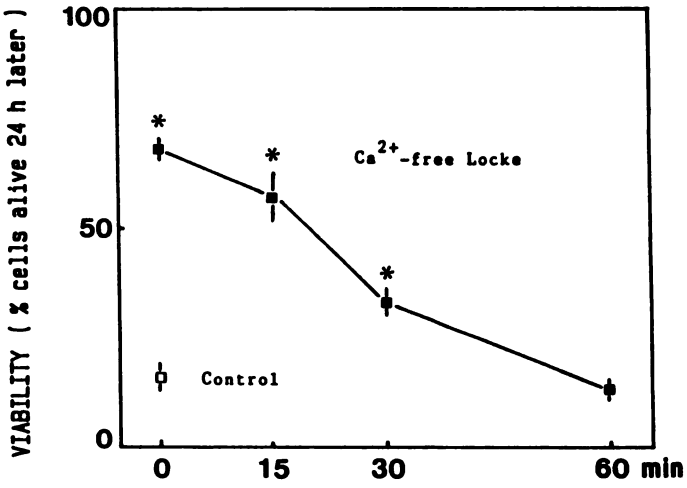


**Fig. 3.** Influence of the time interval between the end of glutamate treatment and the beginning of 60-min exposure to $Ca^{2+}$-free Locke's solution on viability of cerebellar granule cells. Cultures were treated for 15 min with 50 μM glutamate in $Mg^{2+}$-free Locke's solution containing 2.3 mM $CaCl_2$. *Control*, at the end of glutamate exposure, the cells were incubated for 60 min in Locke's solution containing 2.3 mM $CaCl_2$. Viability was estimated 24 hr later, after returning the cells to culture-conditioned medium. Results (mean ± standard error of at least three experiments) are expressed as percentage of neurons surviving. *$p < 0.01$ when compared with control.

receptor antagonist CNQX, failed to prevent neuronal death (Table 1). Even the combination of PCP and CNQX was ineffective (Table 1). Table 1 also shows that, in the post-glutamate period, treatment with voltage-dependent $Ca^{2+}$ channel-blockers (nitrendipine, verapamil, and diltiazem) failed to prevent the development of glutamate-induced neurotoxicity. Also, the combination of glutamate antagonists and the voltage-dependent $Ca^{2+}$ channel-blocker nitrendipine in the post-glutamate period was ineffective (data not shown). However, treatment with 10 $\mu$M verapamil and diltiazem partially (~30%) attenuated neuronal degeneration when they were applied together with glutamate, but not in the post-glutamate period (data not shown).

**PKC translocation and $Ca^{2+}$ uptake.** Exposure of cerebellar granule cells to 50 $\mu$M glutamate for 15 min in the absence of $Mg^{2+}$ killed approximately 80% of neurons 24 hr later (Fig. 4B). In contrast, no neuronal death was induced by the application of 1 $\mu$M glutamate. Exposure of cerebellar granule cells to 50 and 1 $\mu$M glutamate was equally efficacious in increasing (up to 2 times control value) the [$^3$H]P(BtO)$_2$ binding to neurons (Fig. 4A). However, when the cells were washed thoroughly at the end of 15 min of glutamate application and [$^3$H]P(BtO)$_2$ binding was measured during the post-glutamate period, only the membranes of neurons treated with neurotoxic doses of glutamate showed a protracted elevation of [$^3$H]P(BtO)$_2$ binding (Fig. 4). This delayed and protracted increase of P(BtO)$_2$ binding in the post-glutamate period was not associated with neuronal swelling (see Fig. 1) and could not be reversed by PCP treatment [glutamate alone increases [$^3$H]P(BtO)$_2$ binding to 250 ± 10% and glutamate plus 5 $\mu$M PCP for 60 min to 240 ± 18% (three experiments); [$^3$H]P(BtO)$_2$ binding in untreated neurons is 330 ± 31 fmol/mg of protein (10 experiments)] but was reversed to control levels by a 30-min exposure to $Ca^{2+}$-free Locke's solution [glutamate without $Ca^{2+}$ is 130 ± 10% (three experiments)].

The binding of [$^3$H]P(BtO)$_2$ was also determined in membranes prepared from neurons harvested 15 min after termination of 50 $\mu$M glutamate exposure. The $B_{max}$ value for [$^3$H]P(BtO)$_2$ binding was 1.5 ± 0.4 pmol/mg of protein in membranes prepared from control neurons (three experiments) and increased to 2.6 ± 0.11 pmol/mg of protein ($p < 0.05$) in membranes of glutamate-treated cells (three experiments). The corresponding $K_d$ values were 4.7 and 4.2 nM, respectively.

Thirty minutes after the termination of the treatment with 50 $\mu$M glutamate, the uptake of $^{45}Ca^{2+}$ measured for 30 and 60 sec was elevated, whereas, after the same time interval following 1 $\mu$M glutamate, the $^{45}Ca^{2+}$ uptake was comparable to that of untreated cells (Table 3). The increase of $Ca^{2+}$ uptake following the termination of 50 $\mu$M glutamate treatment was insensitive to PCP, nitrendipine, and verapamil (Table 3).

In order to establish the importance of the delayed protracted PKC translocation in the development of $Ca^{2+}$-dependent neurotoxicity, we experimented with the ganglioside GT1b, which blocks PKC translocation (12). As previously reported (14), pretreatment for 2 hr with GT1b, followed by complete removal of the sphingoglycolipid from the culture medium, resulted in complete protection against glutamate-induced neurotoxicity. Fig. 5 shows that such a pretreatment with GT1b resulted in a blockade of the sustained PKC translocation and of the increase in $^{45}Ca^{2+}$ uptake during the post-glutamate period.

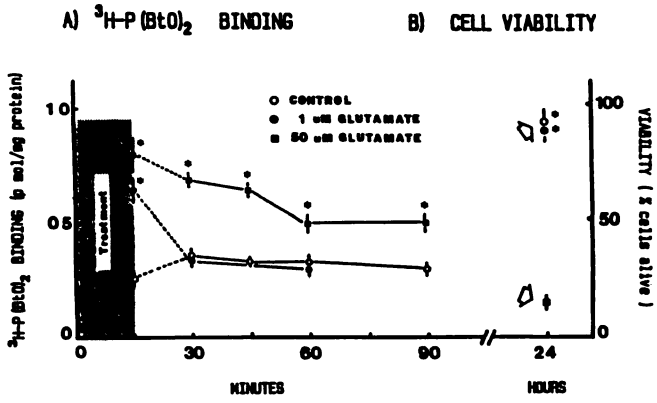


Fig. 4. Relation between glutamate-induced neurotoxicity and protracted translocation of PKC. Protracted (in the post-glutamate period) enhancement of [$^3$H]P(BtO)$_2$ binding in cerebellar granule cells in culture is observed after exposure to neurotoxic doses of glutamate (50 $\mu$M) but not after exposure to nonneurotoxic doses (1 $\mu$M) of glutamate. Cells were exposed to glutamate for 15 min (2.3 mM $Ca^{2+}$, no $Mg^{2+}$). [$^3$H]P(BtO)$_2$ binding was established by incubating the monolayer with 2 nM [$^3$H]P(BtO)$_2$ for 15 min during the exposure to glutamate or at various times thereafter (A). Viability was estimated 24 hr later (B). Results are mean ± standard error of at least three experiments. *$p < 0.05$ when compared with the respective control cells.

## Discussion

Depending on the experimental conditions, stimulation of NMDA-sensitive glutamate receptors may determine almost instantaneous neuronal death or may cause death after various time periods (1–3, 5). The latter event is referred to as delayed neurodegeneration. Here we have used the delayed neuronal death induced by glutamate in primary culture of cerebellar granule cells as an *in vitro* model (14) of the slowly evolving neuronal damage observed in perifocal tissues of hypoxic/ischemic brain regions (24). Using this model, we have attempted to elucidate the cascade of biochemical events involved in glutamate neurotoxicity. We demonstrate that the development of glutamate-induced neuronal death depends on the presence of extracellular $Ca^{2+}$, not only during the exposure of neurons to glutamate (as has been reported previously) (5–7) but also during a time window of 30 to 90 min immediately following termination of glutamate treatment (post-glutamate

TABLE 3

**Neuronal $^{45}Ca^{2+}$ uptake in the post-glutamate period**

Cerebellar granule neurons were incubated for 15 min at room temperature with glutamate (in absence of $Mg^{2+}$). After glutamate was removed by washing, cultures were incubated for the next 30 min at 37° with or without PCP, nitrendipine, or verapamil. At the end of this period, cultures were incubated with Locke solution containing $^{45}Ca$, with or without PCP, nitrendipine, or verapamil, and neuronal $^{45}Ca^{2+}$ content was measured 30 or 60 sec later. This time (30 or 60 sec) was selected because in preliminary experiments we observed that the difference between control and treated cultures was maximal at these intervals. Results are mean ± standard error of five or six experiments.

| Treatment Schedule | | $^{45}Ca^{2+}$ Uptake 30 min after Glutamate Removal | |
|---|---|---|---|
| Glutamate | Post-glutamate treatment | 30 sec | 60 sec |
| $\mu$M | | *nmol/mg of protein* | |
| 0 | None | 8.8 ± 0.22 | 11 ± 0.56 |
| 1 | None | 10 ± 0.24 | 12 ± 1.6 |
| 50 | None | 22 ± 1.17* | 22 ± 1.6* |
| 50 | PCP (5 $\mu$M) | 22 ± 1.71* | 22 ± 1.3* |
| 50 | Nitrendipine (10 $\mu$M) | 21 ± 1.4* | |
| 50 | Verapamil (10 $\mu$M) | 22 ± 0.4* | |

*$p < 0.01$ in comparison with untreated control.

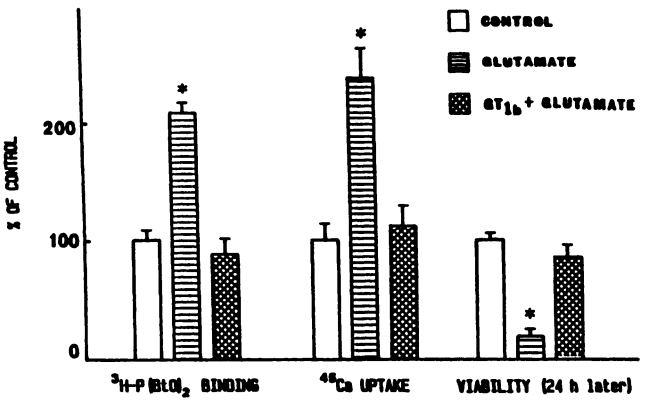


**Fig. 5.** Ganglioside GT1b prevents the delayed glutamate-induced increase of $^{45}Ca^{2+}$ uptake, [$^3$H]P(BtO)$_2$ binding, and neuronal death. Neurons were pretreated with 60 $\mu$M GT1b for 2 hr. Thereafter, cultures were washed three times with Locke's solution and exposed to 50 $\mu$M glutamate for 15 min (when indicated). After washing, cells were incubated in complete Locke's solution at 37° for 30 min. At the end of this period $^{45}Ca^{2+}$ uptake and [$^3$H]P(BtO)$_2$ binding were measured. Results (mean ± standard error of at least three experiments) are expressed as percentage of corresponding control. *$p < 0.001$ when compared with the corresponding control. Viability was estimated 24 hr after glutamate exposure; in the post-glutamate period, cells were returned to culture-conditioned medium.

period). It is well established that stimulation of NMDA-sensitive glutamate receptors gates high conductance cationic channels, allowing an influx of extracellular $Ca^{2+}$ into the neurons (25, 26). Depending on the extent of glutamate receptor stimulation and the abundance of glutamate receptors, the $Ca^{2+}$ influx rates may exceed the buffering capacity of the intracellular mechanisms and an increase in free cytosolic $Ca^{2+}$ ensues (15, 27).

Two recent studies, in which measurements of intracellular free $Ca^{2+}$ were conducted in isolated neurons with microscopic fluorimetric techniques, have indicated that short-lasting application of relatively small, nontoxic, doses of glutamate produces a transient increase in the free cytosolic $Ca^{2+}$ concentration, with a rapid return to prestimulation concentrations upon glutamate removal (15, 17). However, protracted or repeated application of large neurotoxic doses of glutamate resulted in a sustained increase in free cytosolic $Ca^{2+}$ content that extended far beyond the termination of glutamate receptor stimulation. The time required for the recovery of free cytosolic $Ca^{2+}$ to prestimulus levels closely parallels the extent of neuronal death in the cultures (27). In line with these observations, we report that, in the cerebellar granule cell culture model, the sustained increase of $^{45}Ca^{2+}$ uptake during the post-glutamate period occurs only when neurotoxic doses of glutamate are applied. This and the observation that the removal of $Ca^{2+}$ from the extracellular medium during the post-glutamate period protects neurons from death in the following 24 hr suggest that protracted activation of $Ca^{2+}$ uptake is the primary event that is operative in glutamate-induced neurotoxicity. There are several possible mechanisms by which, during the post-glutamate period, a sustained uptake of $Ca^{2+}$ through the cell membrane might occur. This could happen directly through sustained stimulation of NMDA- or non-NMDA-gated cationic channels (due to the persistent presence of excitotoxins after glutamate removal from the medium) or, alternatively, it could occur through indirect activation (phosphorylation by PKC translocation?) of voltage-sensitive or voltage-insensitive $Ca^{2+}$-carrying channels. To decide which mechanism is operative in inducing the increase in $Ca^{2+}$ uptake in the post-glutamate period, we treated the cultures with different drugs and conditions known to block selectively the different subtypes of glutamate-activated cationic channels or voltage-dependent $Ca^{2+}$ channels. However, $Mg^{2+}$ ions (which negatively modulate NMDA-sensitive glutamate receptors), APV (an isosteric antagonist of glutamate), PCP or MK-801 (two allosteric noncompetitive blockers of NMDA-sensitive glutamate receptor) (for reviews see Refs. 28 and 29), and CNQX (an isosteric antagonist for the NMDA-insensitive glutamate receptor subtypes) (22), while being potent blockers of $Ca^{2+}$ influx in these cells (30) and delaying the appearance of neuronal degeneration when applied together with glutamate or kainate, fail to prevent neuronal death or the sustained increase of $Ca^{2+}$ uptake when applied in the post-glutamate period. Furthermore, nitrendipine and verapamil, in doses known to block the voltage-dependent $Ca^{2+}$ channels in granule cells (31), failed to inhibit the sustained $Ca^{2+}$ uptake and to protect neurons from glutamate-induced neurotoxicity, when administered in the post-glutamate period for a time interval equal to that of extracellular $Ca^{2+}$ removal that protects neurons from glutamate-induced neurotoxicity.

Other possible routes that could be responsible for the increase in $^{45}Ca^{2+}$ uptake in the post-glutamate period are 1) the impairment of $Ca^{2+}$ transporters capable of pumping out cytosolic $Ca^{2+}$ across the cell membranes, 2) $Ca^{2+}$ entry via the membrane $Na^+/Ca^{2+}$ exchange system, and 3) $Ca^{2+}$ entry via nonspecific leakage across the cell membrane. The possible contribution of these mechanisms to the increase in $Ca^{2+}$ uptake during the post-glutamate period remains to be elucidated.

Connor *et al.* (15) have proposed that the long-lasting activation of the normally transient increase in free cytosolic $Ca^{2+}$ due to the priming or conditioning stimulus with EAA agonists, in hippocampal cells, is expressed through a glutamate-induced PKC-dependent phosphorylation of $Ca^{2+}$-carrying channels. Enhancement of voltage-gated $Ca^{2+}$ channels or recruitment of dormant, "covert," $Ca^{2+}$ channels by cAMP-dependent protein kinases or PKC-dependent phosphorylation processes has been demonstrated in neurons and in a vascular smooth muscle cell line (32–34).

We have previously reported that one of the consequences of glutamate application to the cerebellar granule cells is the $Ca^{2+}$-mediated translocation of PKC from the cytosol to the neuronal membrane (12). The membrane-translocated enzyme is fully activated by the membrane content of 1,2-diacylglycerol, which increases as a consequence of glutamate receptor stimulation (28). In the present report, we have demonstrated that a difference between neurotoxic and nonneurotoxic concentrations of glutamate is the duration of PKC translocation following removal of glutamate, which is longer lasting with neurotoxic concentrations of glutamate. Such a sustained translocation of PKC is abolished by removal of $Ca^{2+}$ from the extracellular medium, a condition that reduces dramatically glutamate neurotoxicity. If the glutamate-primed activation of PKC translocation were to be responsible for the sustained $Ca^{2+}$ transient and the consequent $Ca^{2+}$-mediated neurotoxicity, then both effects should be attenuated by inhibition of PKC. Having this in mind, we used as a PKC inhibitor the ganglioside GT1b (12, 35, 36). We have already reported that GT1b, as well as other gangliosides (GM1, GD1b, and GD1a), incorporates into the neuronal membranes and inhibits both PKC translocation (12)

and neuronal death induced by glutamate (14). Although GT1b fails to affect the opening of glutamate-operated high conductance cationic channels (14), it potently inhibits the glutamate-induced sustained increase of $[^3H]P(BtO)_2$ binding, the delayed increase of $^{45}Ca^{2+}$ uptake, and the subsequent appearance of neuronal death.

It is tempting to conclude that translocation and activation of PKC, triggered by the persistent stimulation of glutamate receptors, to phosphorylate membrane proteins, is directly or indirectly operative in controling $Ca^{2+}$ influx. An increase in free cytosolic $Ca^{2+}$ may act as a priming event in causing neuronal death. However, the successive activation of enzymes such as calpains, phospholipases, and various other $Ca^{2+}$-dependent enzymes may ultimately be the immediate cause of neuronal death. A study of these enzymatic activities may uncover additional biochemical mechanisms that can be used as a site of action for new drugs for the treatment of brain ischemia. Within the limits of our experimental conditions, we exclude the possibility that PCP analogues or voltage-dependent $Ca^{2+}$ channel blockers can be used to control the chain of biochemical events, leading to neuronal death, that is triggered by excitotoxins. Moreover, we suggest that gangliosides may prevent post-glutamate and perhaps post-ischemic (37) neuronal death by inhibiting the PKC translocation and the delayed increase of $Ca^{2+}$ influx during the post-glutamate period.

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**Send reprint requests to:** Erminio Costa, Fidia-Georgetown Institute for the Neurosciences, Georgetown University, 3900 Reservoir Rd., N.W., Washington, DC 20007.