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Glutamate Receptor–induced $^{45}Ca^{2+}$ Accumulation in Cortical Cell Culture Correlates with Subsequent Neuronal Degeneration

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Murine neuronal and glial cell cultures exposed briefly to glutamate accumulated large amounts of $^{45}Ca^{2+}$ from the extracellular medium during the exposure. Most of the accumulation likely reflected influx into neurons, as little accumulation was observed in similarly treated glial cultures. When the concentration of glutamate was varied between 10 and 1000 μM, or exposure duration was varied between 0 and 10 min, the amount of $^{45}Ca^{2+}$ accumulation correlated closely with the amount of neuronal death 24 hr later. Both $^{45}Ca^{2+}$ accumulation and cell death could be attenuated in a dose-dependent manner by the competitive NMDA antagonist D-aminophosphonovalerate or the noncompetitive antagonist dextrorphan, with IC_{50} values of approximately 100 μM and 15 μM, respectively. In contrast, neither $^{45}Ca^{2+}$ accumulation nor cell death was blocked by the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) in the presence of high glycine. With brief exposure, high concentrations of AMPA, kainate, or K^+ produced much less death or $^{45}Ca^{2+}$ accumulation than produced by glutamate, especially if 10 μM MK-801 was included in the exposure medium to block NMDA receptor activation. Kainate- or AMPA-induced $^{45}Ca^{2+}$ accumulation or neuronal cell death was blocked with CNQX. However, high K^+–triggered $^{45}Ca^{2+}$ accumulation was only partially blocked with CNQX plus MK-801, consistent with mediation by voltage-gated Ca^{2+} channels.

In addition to measuring the accumulation of $^{45}Ca^{2+}$ occurring during agonist exposure, we also assessed accumulation during the 30 min immediately following completion of a 3–5 min exposure to 500 μM NMDA. We found this late $^{45}Ca^{2+}$ accumulation to be approximately half that accumulated during the initial exposure period. Delayed addition of MK-801 blocked this late accumulation and produced a corresponding partial reduction in cell death. Our results support the hypothesis that excess net Ca^{2+} influx through NMDA receptor–gated channels is a key step in triggering the neuronal death induced by brief, intense glutamate exposure.

[Key words: glutamate, neurotoxicity, cell death, cell culture, cerebral cortex, calcium, MK-801, dextrorphan]

Brief (3–5 min) exposures to high concentrations of glutamate can induce neuronal degeneration, a process that may contribute to the CNS injury induced by hypoxia–ischemia, hypoglycemia, trauma, and epilepsy (Meldrum, 1985; Rothman and Olney, 1987; Choi, 1988). The mechanisms underlying this rapidly triggered excitotoxicity are not fully understood, but loss of cellular Ca^{2+} homeostasis probably plays an important role. In several cell culture systems, injury depends upon both extracellular Ca^{2+}, and the activation of NMDA receptors (Choi, 1985; Garthwaite and Garthwaite, 1986; Garthwaite et al., 1986; Kudo and Ogura, 1986; Choi et al., 1987; Michaels and Rothman, 1990). Since NMDA receptors are linked to channels with high Ca^{2+} permeability (MacDermott et al., 1986; Mayer and Westbrook, 1987), a key initial event may be the excessive influx of extracellular Ca^{2+}.

Supporting this hypothesis, several investigators have found that exposure to lethal levels of glutamate increases intracellular free Ca^{2+}, $[Ca^{2+}]_i$ (Murphy et al., 1987; Ogura et al., 1988; Mattson et al., 1989; Abele et al., 1990; De Erausquin et al., 1990; Mills and Kater, 1990; Randall and Thayer, 1992). However, Michaels and Rothman (1990) reported that the correlation between elevation in $[Ca^{2+}]_i$ and glutamate-induced neuronal cell death was less than complete; specifically, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Honore et al., 1988) blocked the rise in neuronal somatic $[Ca^{2+}]_i$ induced by glutamate exposure, but did not block subsequent cell death. In addition, McMillian et al. (1990) and Ogura et al. (1990) have reported that kainate is similar to glutamate or NMDA in its ability to induce an increase in somatic $[Ca^{2+}]_i$, although kainate only produces widespread neuronal degeneration in cortical cultures with prolonged exposure times exceeding several hours (Koh et al., 1990). Recently, Dubinsky and Rothman (1991) have reported that NaCN can elevate $[Ca^{2+}]_i$ in hippocampal neurons without inducing neuronal death.

While apparently discrepant, these observations do not necessarily weaken the Ca^{2+} influx hypothesis. Levels of average somatic $[Ca^{2+}]_i$ may only loosely reflect the amounts of Ca^{2+} influx. Ca^{2+} entering a neuron may become bound and thus may not contribute to $[Ca^{2+}]_i$; conversely, only small quantities of Ca^{2+} influx may be required to elevate $[Ca^{2+}]_i$ if sequestration

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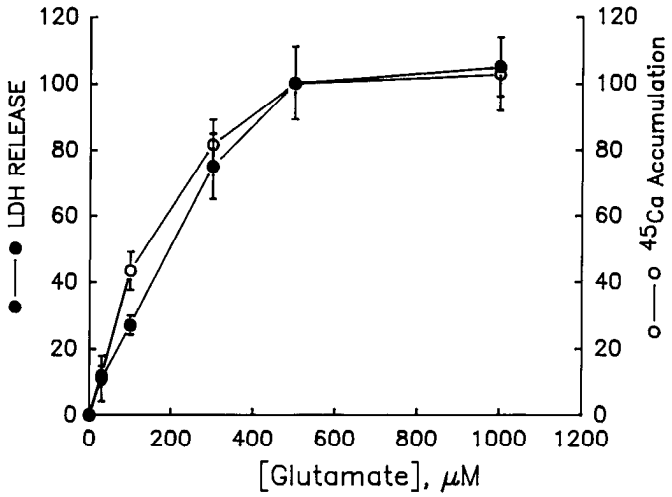

Figure 1. Neuronal cell death and $^{45}Ca^{2+}$ influx as a function of glutamate concentration. Two sets of sister cultures were exposed to the indicated concentrations of glutamate for 5 min. In one set of cultures, neuronal cell death was assessed by measuring the efflux of LDH to the bathing medium 24 hr later (mean ± SEM, $n = 4$ cultures per point). In the second set, glutamate exposure was carried out in the presence of extracellular $^{45}Ca^{2+}$; immediately following the exposure, the cells were washed and lysed, and an aliquot of the lysate was analyzed for $^{45}Ca^{2+}$ (mean ± SEM, $n = 4$ cultures per point). The mean value of LDH or $^{45}Ca^{2+}$ for each condition was scaled to that produced by exposure to 500 μM glutamate for 5 min (=100).

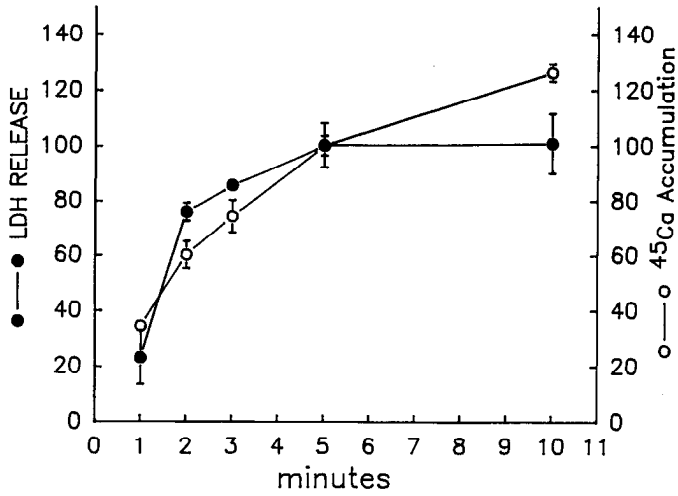

Figure 2. Neuronal cell death and $^{45}Ca^{2+}$ influx as a function of glutamate exposure duration. Two sets of sister cultures were exposed to 500 μM glutamate for the indicated times. In one set, LDH released into the medium 24 hr later was measured (mean ± SEM, $n = 3$–4 cultures per point); in the other set, $^{45}Ca^{2+}$ accumulation was measured (mean ± SEM, $n = 3$–4). The mean value of LDH or $^{45}Ca^{2+}$ for each condition was scaled to that produced by exposure to 500 μM glutamate for 5 min (=100).

into compartments is limited. Release from intracellular stores can elevate $[Ca^{2+}]_i$ independent of Ca^{2+} influx. Further, it is possible that fluorescent probes used to measure $[Ca^{2+}]_i$ may themselves buffer Ca^{2+} and thus alter subsequent events, including neuronal cell death (Scharfman and Schwartzkroin, 1989; Kudo et al., 1990).

For these reasons, direct measurement of total Ca^{2+} influx might provide a better predictor of subsequent neuronal death than provided by measurements of somatic $[Ca^{2+}]_i$. Glutamate and other excitatory amino acids stimulate the accumulation of extracellular $^{45}Ca^{2+}$ in cortical (Berdichevsky et al., 1983), striatal (Retz and Coyle, 1984), or hippocampal (Crowder et al., 1987) brain slices. Marcoux et al. (1988) reported that glutamate induced a concentration-dependent increase in $^{45}Ca^{2+}$ accumulation in rat cortical cell cultures. The purpose of the present study was to extend these observations, specifically examining the hypothesis that agonist-induced $^{45}Ca^{2+}$ accumulation in cortical cell cultures should correlate quantitatively with subsequent neuronal death in several excitotoxicity paradigms. In particular, we sought to test two predictions derived from previous work: (1) NMDA receptors should play a special role in $^{45}Ca^{2+}$ accumulation (Choi et al., 1988), and (2) considerable $^{45}Ca^{2+}$ influx should occur in the 30 min immediately following brief intense glutamate exposure (Hartley and Choi, 1989).

An abstract has appeared (Kurth et al., 1989).

Materials and Methods

Cell cultures. Dissociated neocortical cell suspensions, consisting of both neurons and glia, were prepared from fetal Swiss–Webster mice (embryonic day 14–17) as previously described (Choi et al., 1987). Cells were plated at a density of approximately 2.8×10^5 cells/well in Falcon Primaria 24-well plates, in most cases onto a previously established confluent layer of cortical astrocytes (see below). The initial plating medium was Eagle's Minimum Essential Medium (MEM; Earle's salts, supplied glutamine-free) supplemented with 10% heat-inactivated horse serum, 10% heat-inactivated fetal bovine serum, glutamine (total, 2 mM) and glucose (total, 21 mM). Cultures were kept at 37°C in an incubator containing 5% carbon dioxide. After 7–10 d, non-neuronal cell division was halted by exposure to 10^{-5} M cytosine arabinoside. Subsequently, cultures were maintained in a similar medium lacking fetal serum, with medium exchange twice a week.

Nearly pure glial cultures were prepared using the same procedure, except that neocortices were dissected from postnatal day 1–3 mice. Mixed neuronal and glial cultures were selected for study between 14 and 24 d after plating on glia, a time when extensive synaptic interconnections have developed (Choi et al., 1987).

Excitatory amino acid exposure. Exposure to glutamate, NMDA, kainate, or AMPA was carried out at room temperature in a HEPES-buffered control salt solution (HCSS), substituted for culture medium by triple exchange; HCSS had the following composition (in mM): NaCl, 120; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; HEPES, 25 (pH 7.4 at 25°C); glucose, 15. Exposure was terminated by washout (>7000-fold dilution) and transfer into Eagle's MEM with augmented glucose (25 mM). High-potassium experiments were conducted in 80 mM K^+ using HCSS modified by substitution of Na^+ with K^+. Control experiments showed that little or no (<10%) cortical cell damage was produced by this protocol if excitatory amino acids or high K^+ was omitted from the exposure solution.

$^{45}Ca^{2+}$ accumulation studies. Cultures were washed with HCSS, and then incubated in the presence of the desired drugs in HCSS containing $^{45}CaCl_2$ (3.75×10^6 dpm/well, approximately 0.025–0.1 mM, a small amount relative to the 1.8 mM cold $CaCl_2$ in HCSS). After 5 or 10 min, depending on the protocol, the exposure solution was quickly washed out with three rinses of HCSS and the cells were lysed by addition of 0.2% sodium dodecyl sulfate (SDS) solution at 37°C. An aliquot of cell lysate was then counted. $^{45}Ca^{2+}$ accumulation in blanks (sister cultures exposed to sham wash) was subtracted from all values to yield the specific $^{45}Ca^{2+}$ accumulation induced by agonist application. Typically 7000–19,000 cpm (1.8–4.8 pmol of $^{45}Ca^{2+}$) represented the maximal level of $^{45}Ca^{2+}$ accumulation induced in single culture wells by exposure to 500 μM to 1 mM glutamate or NMDA.

In some experiments (see figure captions), $^{45}Ca^{2+}$ accumulation and lactate dehydrogenase (LDH; see below) were measured in the same cultures. Cultures were washed immediately after agonist exposure, and placed into Eagle's MEM with augmented glucose (25 mM). After 24 hr, the medium was sampled for LDH; concentrated SDS was then added directly to the cultures (final concentration, 0.2%), and a second aliquot of medium was assayed for $^{45}Ca^{2+}$. This protocol is equivalent

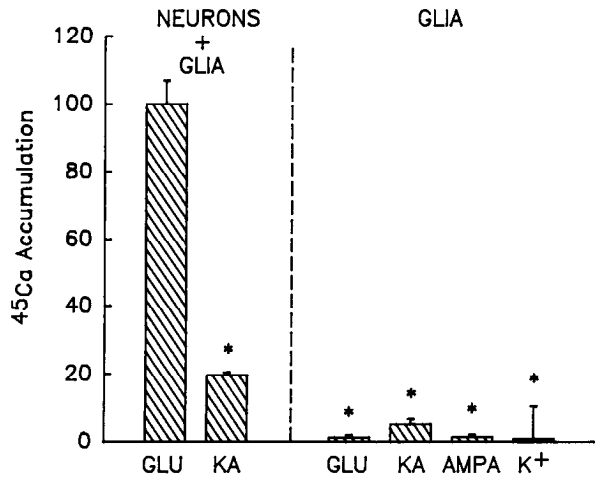

Figure 3. Neuronal versus glial $^{45}Ca^{2+}$ accumulation. Mixed neuronal and glial cell cultures, or near-pure glial cultures, were exposed to the indicated agonists for 10 min. Glutamate (*GLU*), AMPA, and kainate (*KA*) were added at 500 μM; K^+ was 80 mM. $^{45}Ca^{2+}$ (mean ± SEM, n = 4) accumulation for each condition was scaled to that induced in mixed cultures by 500 μM glutamate for 10 min (=100). *Asterisk* (*) indicates those conditions statistically different from $^{45}Ca^{2+}$ accumulation induced in mixed cultures by glutamate at $p < 0.05$ (ANOVA and Student-Newman-Keuls' test).

to the first protocol, as it measures the net amount of $^{45}Ca^{2+}$ retained by the cell layer following washout of toxic agonist.

Assessment of overall neuronal cell injury. Overall neuronal cell injury was estimated in all experiments by examination of cultures with phase-contrast microscopy 24 hr after initiation of excitotoxin exposure, at which point the process of cell death was largely complete (Choi et al., 1987; Koh et al., 1990). Neuronal injury was also quantitatively assessed by the measurement of LDH, released by damaged or destroyed cells, in the extracellular fluid 1 d after excitotoxin exposure (Koh and Choi, 1987; Koh et al., 1990). The small amount of LDH present in the media of sister cultures exposed to sham wash was subtracted from values obtained in cultures exposed to glutamate or NMDA, to yield the signal specifically associated with excitotoxic injury. Control experiments showed that the specific efflux of LDH induced by glutamate exposure (after this background subtraction) was linearly proportional to the number of neurons damaged or destroyed, and that no specific LDH efflux occurred when pure cultures of cortical glia were similarly exposed to glutamate (Koh and Choi, 1987).

The absolute value of the LDH efflux produced by a given toxic exposure was quite consistent within sister cultures of a single plating, but varied between different platings (200–400 conventional units/ml typically corresponded to near complete neuronal degeneration), largely as a function of neuronal density (which varied despite constant original plating densities, presumably reflecting small variations in cell preparation or serum characteristics). Therefore, each observed LDH value was scaled to the mean value obtained by control exposure (high concentration of glutamate, corresponding to near-complete neuronal death, set at 100).

Drugs and reagents. Excitatory amino acid agonists and antagonists were obtained from the following sources: D-2-amino-5-phosphonovalerate (D-APV), CNQX, and AMPA, from Tocris Neuramin (Essex, UK); glutamate, NMDA, and kainate, from Sigma Chemical Co. (St. Louis, MO); $^{45}CaCl_2$, from New England Nuclear (Boston, MA; 0.37–1.5 GBq/mg calcium). Dextrorphan and MK-801 were gifts of Hoffmann-La Roche and Merck, Sharpe and Dohme, respectively.

Results

Cultured cortical neurons exposed for 5 min to 10 μM to 1 mM glutamate showed increasing neuronal death without glial death, accompanied by the release of LDH into the culture medium (Fig. 1). A similar concentration–response relationship was observed when sister cultures were assayed for $^{45}Ca^{2+}$ accumulation using the same exposure conditions. The EC_{50} both for neuronal cell death and $^{45}Ca^{2+}$ accumulation was approximately 150–200 μM, and neither event was increased by increasing glutamate concentrations above 500 μM. A close correlation between neuronal death and $^{45}Ca^{2+}$ accumulation was also observed when glutamate concentration was fixed at 500 μM, and the duration of exposure was varied from 1 to 10 min (Fig. 2).

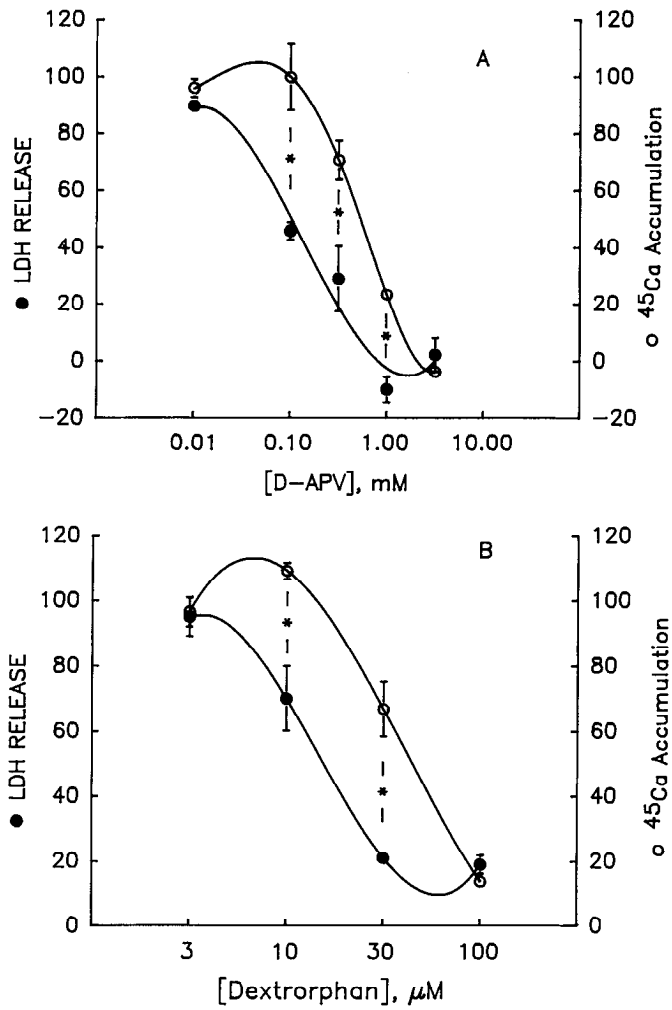

Figure 4. Inhibition of $^{45}Ca^{2+}$ influx and glutamate neurotoxicity by NMDA antagonists. *A*, Two sets of sister cultures were exposed to 500 μM glutamate for 5 min in the presence of the indicated concentration of D-APV. LDH released into the medium after 24 hr and $^{45}Ca^{2+}$ influx were measured in separate cultures as described above (bars indicate SEM, n = 3–4). The mean value of LDH or $^{45}Ca^{2+}$ for each condition was scaled to that induced by 500 μM glutamate for 5 min (=100). *, statistical difference between ^{45}Ca accumulation and LDH release when comparing data at the same drug concentration ($p < 0.05$ level by the ANOVA and Student-Newman-Keuls' test). *B*, Same as in *A*, but with dextrorphan.

Most of this glutamate-induced cellular $^{45}Ca^{2+}$ accumulation likely reflected influx into neurons. When glial monolayer cultures lacking overlying neurons were exposed for 10 min to 500 μM glutamate, kainate, or AMPA, or to 80 mM K^+, they accumulated less than 5% of the $^{45}Ca^{2+}$ that was accumulated in mixed neuronal and glial cultures exposed to 500 μM glutamate (Fig. 3). However, we cannot exclude the possibility that glia will accumulate more Ca^{2+} when cocultured with neurons than when cultured alone.

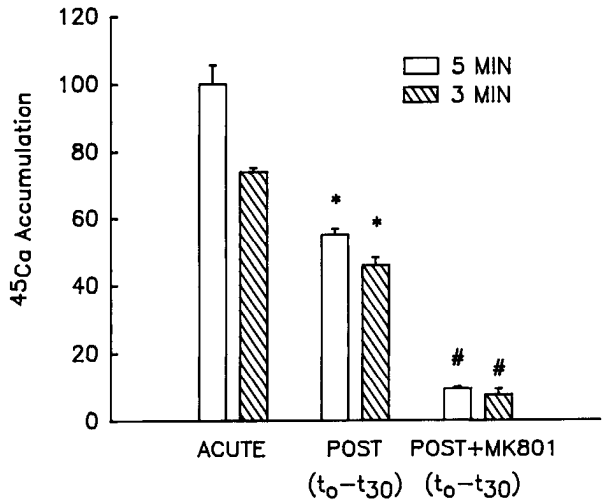

Figure 5. $^{45}Ca^{2+}$ accumulation during and after NMDA exposure. Sister cultures were exposed to 500 μM NMDA for 5 (*open bars*) or 3 (*hatched bars*) min, in the presence of extracellular $^{45}Ca^{2+}$ added only during the NMDA exposure (*ACUTE*) or for 30 min immediately after the NMDA exposure (*POST, POST+MK-801*). Immediately following washout of the $^{45}Ca^{2+}$, $^{45}Ca^{2+}$ accumulation was measured and scaled to the amount of $^{45}Ca^{2+}$ accumulated during the acute exposure to 500 μM glutamate for 5 min (=100). *, statistical difference from respective (3 or 5 min) acute $^{45}Ca^{2+}$ accumulation at $p < 0.05$ (ANOVA and Student-Newman-Keuls' test); #, statistical difference from respective acute or post-$^{45}Ca^{2+}$ accumulation at $p < 0.05$ (ANOVA and Student-Newman-Keuls' test).

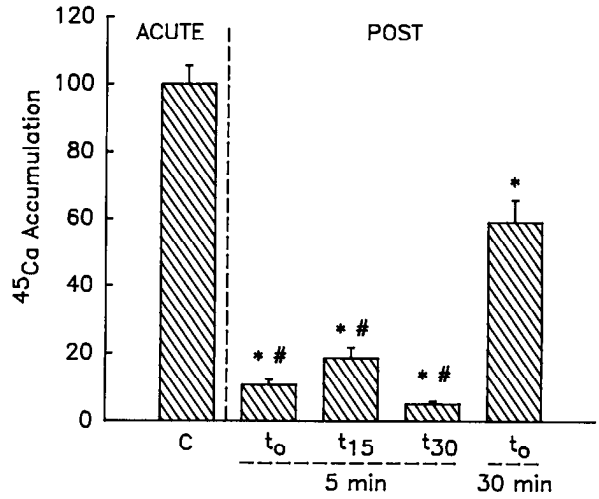

Figure 6. Rate of $^{45}Ca^{2+}$ accumulation after NMDA exposure. Sister cultures were exposed to 500 μM NMDA for 3 min. $^{45}Ca^{2+}$ was added only during the 3 min NMDA exposure (*ACUTE*). Alternatively, $^{45}Ca^{2+}$ was added for 5 min at the following times: (1) immediately after washout of NMDA (t_o, 5 min), (2) after a 15 min delay (t_{15}, 5 min), or (3) after a 30 min delay (t_{30}, 5 min). Accumulation over these chosen 5 min epochs was compared to the total $^{45}Ca^{2+}$ accumulated over the 30 min immediately following the NMDA exposure (t_o, 30 min). The mean value of $^{45}Ca^{2+}$ accumulated for each condition ($n = 4$) was scaled to the mean value in the acute condition (=100). *, statistical difference from acute $^{45}Ca^{2+}$ accumulation at $p < 0.05$ (ANOVA and Student-Newman-Keuls' test); #, statistical difference from the total 30 min accumulation at $p < 0.05$ (ANOVA and Student-Newman-Keuls' test). The $^{45}Ca^{2+}$ accumulation observed at t_o, t_{15}, and t_{30} were not statistically different from each other at the $p < 0.05$ level.

As previously reported, the rapidly triggered excitotoxic injury induced by exposure to 500 μM glutamate for 5 min could be blocked by the selective competitive NMDA antagonist D-APV (Fig. 4*A*). This neuroprotection action was accompanied by a parallel reduction of $^{45}Ca^{2+}$ accumulation, although the $^{45}Ca^{2+}$ curve was somewhat right-shifted relative to the LDH curve (IC_{50} values of approximately 300 μM and 100 μM, respectively). Differences between D-APV effects on neuronal cell death and $^{45}Ca^{2+}$ accumulation were significant ($p < 0.05$, two-tailed *t* test, Bonferonni corrected) at D-APV concentrations of 100 μM, 300 μM, and 1 mM. At high concentrations, D-APV reduced both neuronal degeneration and $^{45}Ca^{2+}$ to near-baseline levels.

Similarly, increasing the concentrations of the noncompetitive NMDA antagonist dextrorphan also produced parallel attenuation of neuronal cell death and $^{45}Ca^{2+}$ accumulation (Fig. 4*B*). The IC_{50} for reducing cell death was approximately 15 μM, while the IC_{50} for reducing $^{45}Ca^{2+}$ accumulation was somewhat right-shifted at 30 μM. Differences between neuronal cell death and $^{45}Ca^{2+}$ accumulation were significant ($p < 0.05$, two-tailed *t* test, Bonferonni corrected) at dextrorphan concentrations of 10 and 30 μM, and high concentrations of dextrorphan blocked both neuronal death and $^{45}Ca^{2+}$ accumulation.

Previous study has suggested that the neuronal degeneration induced by a 3–5 min exposure to 500 μM glutamate or NMDA is mediated in part by continued activation of NMDA receptors for about 30 min after exposure termination. Specifically, application of an NMDA antagonist, or removal of extracellular Ca^{2+}, within 30 min after washout of the exogenously added toxic agonist can significantly reduce resultant neuronal injury (Rothman et al., 1987; Hartley and Choi, 1989; Hartley et al., 1990). We therefore examined the prediction that substantial $^{45}Ca^{2+}$ accumulation should occur over the 30 min following brief overstimulation of NMDA receptors. Substantial $^{45}Ca^{2+}$ accumulated over the 30 min following NMDA receptor overactivation, in fact about half as much as accumulated during a 5 min exposure to 500 μM glutamate (Fig. 5). This delayed $^{45}Ca^{2+}$ accumulation was attenuated by the addition of 10 μM MK-801. The rate of $^{45}Ca^{2+}$ accumulation during this 30 min period appeared relatively constant, measured over 5 min epochs, immediately after, 15 min after, or 30 min after washout of exogenously added NMDA (Fig. 6).

In comparison to the large amount of $^{45}Ca^{2+}$ and neuronal death induced by brief exposure to 500 μM glutamate or NMDA, brief exposures to 500 μM AMPA or kainate induced relatively small amounts of either $^{45}Ca^{2+}$ accumulation or neuronal death (Fig. 7). Some of these indices were reduced even further when 10 μM MK-801 was added to the exposure solution to eliminate the secondary activation of NMDA receptors by endogenous glutamate release (Fig. 7). Similarly, only small amounts of neuronal death and $^{45}Ca^{2+}$ accumulation were evoked by exposure to 80 mM K^+, especially in the presence of MK-801.

Consistent with these observations indicating that brief intense stimulation of AMPA/kainate receptors induces only a small amount of $^{45}Ca^{2+}$ accumulation, the $^{45}Ca^{2+}$ accumulation or cell death resulting from submaximal glutamate exposure (sufficient to destroy approximately 70% of the neuronal population) was not attenuated by the addition of the selective AMPA/kainate antagonist CNQX at 300 μM, together with 1 mM glycine (Fig. 8). Glycine was added to eliminate antagonism of NMDA receptors due to CNQX displacement of glycine binding to the NMDA receptor–channel complex (Birch et al., 1988; Hartley et al., 1990; Koh and Choi, 1991). As expected, this

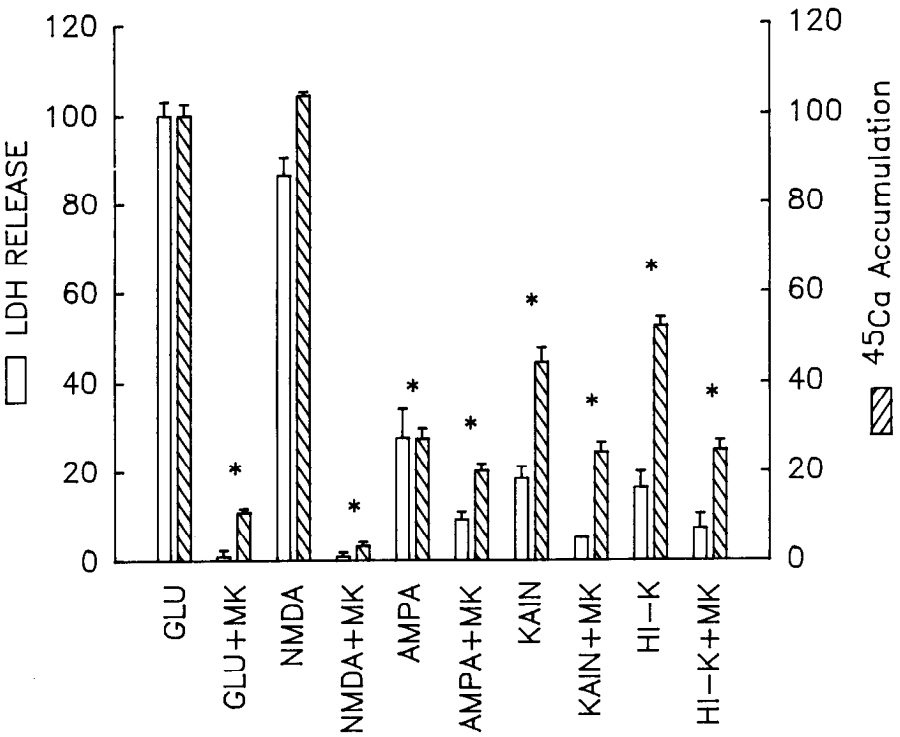

Figure 7. Comparison of NMDA receptor–mediated, AMPA/kainate receptor–mediated, and potassium depolarization–induced $^{45}Ca^{2+}$ accumulation and cell death. Sister cultures were exposed to the indicated drugs for 5 min at the following concentrations: glutamate, NMDA, AMPA, and kainate, 500 μM; K^+, 80 mM; MK-801 (*MK*), 10 μM. LDH released into the medium was assayed after 24 hr to quantify cell death. After sampling for LDH, cells were lysed and the intracellular $^{45}Ca^{2+}$ measured (see Materials and Methods). The mean value of LDH or $^{45}Ca^{2+}$ ($n = 4$) for each condition was scaled relative to the response from 500 μM glutamate for 5 min (=100). LDH values for glutamate and NMDA in the presence of MK-801 were actually slightly less than sham wash background, but are graphed at 1.0 for ease of display. *, statistical difference between *both* LDH efflux and $^{45}Ca^{2+}$ accumulation and that induced by glutamate at $p < 0.05$ (ANOVA and Student-Newman-Keuls' test). Addition of MK-801 produced a statistically significant ($p < 0.05$) reduction in the LDH evoked by glutamate, NMDA, and AMPA, and in the $^{45}Ca^{2+}$ evoked by glutamate, NMDA, kainate, and high K^+.

combination of 300 μM CNQX plus 1 mM glycine was sufficient to block nearly all the $^{45}Ca^{2+}$ accumulation induced by exposure to AMPA or kainate in the presence of MK-801 (Fig. 9). However, CNQX plus glycine only partially blocked the $^{45}Ca^{2+}$ accumulation induced by 80 mM K^+ (Fig. 9).

Discussion

The present study demonstrates a close quantitative correlation between the cellular accumulation of extracellular $^{45}Ca^{2+}$ induced by brief glutamate receptor activation in cortical cultures, and subsequent neuronal degeneration. Two arguments additional to this correlation per se suggest that most of the observed $^{45}Ca^{2+}$ accumulation reflected influx into neurons: (1) the accumulation was largely blocked by NMDA antagonists, whereas glia have few or no NMDA receptors (Cornell-Bell et al., 1990;

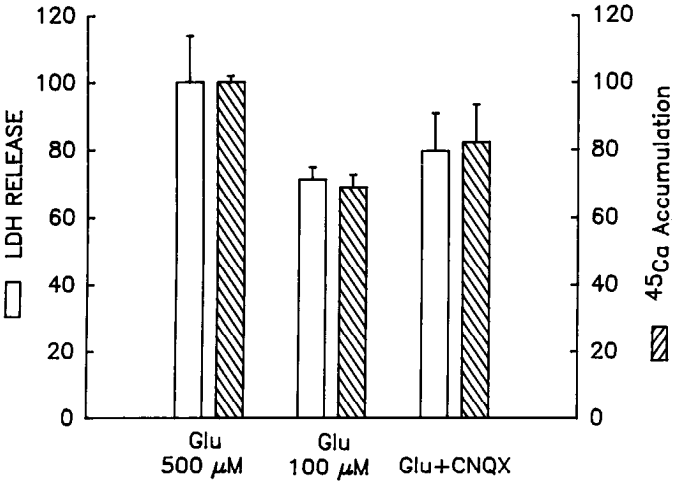

Figure 8. CNQX has little effect on glutamate-induced $^{45}Ca^{2+}$ or cell death. Cultures were exposed to 500 μM or 100 μM glutamate, the latter without or with 300 μM CNQX plus 1 mM glycine. LDH released into the medium was assayed after 24 hr to quantify cell death. After sampling for LDH, cells were lysed and the intracellular $^{45}Ca^{2+}$ measured. The mean value of LDH or $^{45}Ca^{2+}$ ($n = 4$) for each condition was scaled relative to the response from 500 μM glutamate for 5 min (=100). CNQX did not produce any significant difference in the LDH efflux or $^{45}Ca^{2+}$ accumulation induced by 100 μM glutamate ($p > 0.05$).

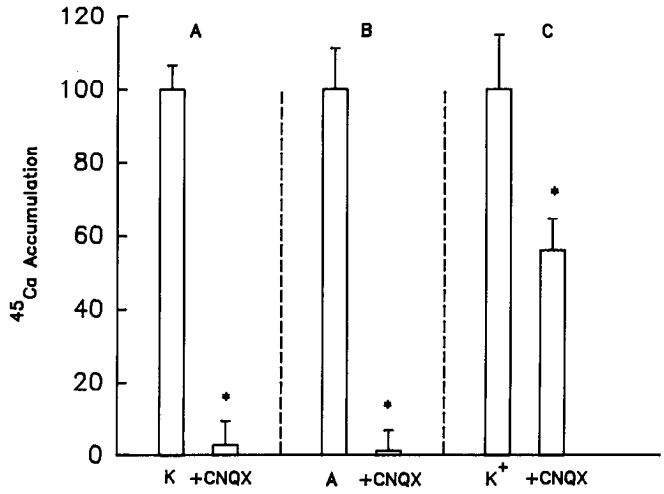

Figure 9. CNQX blocks AMPA- or kainate-induced $^{45}Ca^{2+}$ influx, but only partially blocks high K^+–induced influx. Sister cultures were exposed to the indicated agonists for 10 min at the following concentrations: *A*, kainate (*K*), 300 μM; *B*, AMPA (*A*), 30 μM; and *C*, K^+, 80 mM. All conditions contained 10 μM MK-801. CNQX was used at 300 μM. After 10 min the agonist was removed and the accumulation of intracellular $^{45}Ca^{2+}$ was measured by lysing cells with the addition of SDS to the medium. The mean value ($n = 4$) of $^{45}Ca^{2+}$ accumulation for each individual agonist was scaled separately to 100. *, statistical difference in the CNQX-containing condition from the condition containing the respective agonist alone ($p < 0.05$, ANOVA and Student-Newman-Keuls' test).

Barres, 1991); and (2) glutamate induced little accumulation in cultures containing glia without neurons. $^{45}Ca^{2+}$ accumulation in glia cells, or binding to the exterior membranes, may have contributed to measured $^{45}Ca^{2+}$ influx, but probably at a low level relative to neuronal Ca^{2+} influx. Since some of the $^{45}Ca^{2+}$ that enters a neuron may be extruded prior to the end of the exposure period, measured NMDA receptor–dependent $^{45}Ca^{2+}$ accumulation likely underestimates true neuronal Ca^{2+} influx. The low level of $^{45}Ca^{2+}$ accumulation induced by glutamate in glial cells is interesting in light of work by Cornell-Bell et al. (1990) indicating that glutamate can induce substantial increases in glial intracellular free Ca^{2+} levels. As those authors suggested, some of the Ca^{2+} contributing to this glutamate-induced free Ca^{2+} level in glia might come from intracellular stores rather than from extracellular Ca^{2+}.

The extent of $^{45}Ca^{2+}$ accumulation during a 5 min exposure to glutamate, like the subsequent neuronal degeneration, was dependent on glutamate concentration. EC_{50} for both was approximately 150 μM, a value similar to that reported by Marcoux et al. (1988) for glutamate-induced $^{45}Ca^{2+}$ accumulation in rat cortical cultures. In addition, the exposure duration needed for 500 μM glutamate to induce relatively large amounts of $^{45}Ca^{2+}$ accumulation, 3–5 min, matched the exposure duration needed to trigger widespread neuronal degeneration by the following day.

Consistent with experiments showing that NMDA receptor activation is necessary for most of the cortical neuronal death induced by brief glutamate exposure (Choi et al., 1988), NMDA receptor activation was necessary for most of the associated $^{45}Ca^{2+}$ accumulation. Increasing amounts of the competitive NMDA antagonist D-APV or the noncompetitive NMDA antagonist dextrorphan reduced $^{45}Ca^{2+}$ accumulation and neuronal degeneration in parallel. However, an offset was observed—neuronal death was reduced at lower antagonist concentrations than $^{45}Ca^{2+}$ accumulation. One simple explanation would be a threshold effect, that is, if neuronal injury only occurred when Ca^{2+} entry exceeded a critical threshold. Only a small reduction in NMDA receptor activation may be sufficient to reduce Ca^{2+} influx below this threshold, reducing neuronal cell death. For example, injury might be linked to a small percentage of total Ca^{2+} entry, occurring late in the course of NMDA receptor–induced Ca^{2+} influx but involving some critical compartment, such as a subset of dendrites or a specific intracellular Ca^{2+} binding site. Block of this small but critical influx with low concentrations of an NMDA antagonist might have little effect on total Ca^{2+} influx, but a large effect on resultant neuronal death. More complex explanations can also be considered.

Ca^{2+} influx occurring during brief agonist exposure is probably only part of the total influx load involved in cell injury. $^{45}Ca^{2+}$ continued to accumulate over 30 min following glutamate exposure, reaching about half the level occurring during the agonist exposure itself. The magnitude of this late accumulation, and its observed dependence on NMDA receptor activation, provides good correlates for previous findings that either removing extracellular Ca^{2+}, or adding an NMDA antagonist, after brief glutamate exposure can prevent about half of the subsequent neuronal degeneration (Rothman et al., 1987; Hartley and Choi, 1989).

In contrast to protection provided with NMDA antagonists, high concentrations of the AMPA/kainate receptor antagonist CNQX had little effect on either neuronal death or $^{45}Ca^{2+}$ accumulation induced by glutamate. Glycine was added with CNQX to reduce CNQX displacement of glycine from its binding site on the NMDA receptor–channel complex (Birch et al., 1988; Hartley et al., 1990; Koh and Choi, 1991). CNQX did block the small $^{45}Ca^{2+}$ accumulation induced by exposure to AMPA or kainate, and it partially reduced the small $^{45}Ca^{2+}$ accumulation induced by 80 mM K^+. These small $^{45}Ca^{2+}$ signals likely reflected Ca^{2+} influx through voltage-gated Ca^{2+} channels, perhaps modestly augmented by Ca^{2+} influx through NMDA receptor–gated channels activated by endogenously released glutamate. Consistent with this latter possibility, both kainate-induced and high K^+–induced $^{45}Ca^{2+}$ accumulation were reduced by the addition of MK-801. Crowder et al. (1987) reported that 2-amino-7-phosphonoheptanoate reduced high K^+–induced $^{45}Ca^{2+}$ accumulation in hippocampal slices, and previous cell culture studies have noted reduction of kainate toxicity by an NMDA antagonist (Manev et al., 1989; Michaels and Rothman, 1990).

Most likely, NMDA receptor activation leads rapidly to Ca^{2+} influx and the triggering of lethal cascades because of the especially high Ca^{2+} permeability of the NMDA receptor–gated channel (MacDermott et al., 1986; Mayer and Westbrook, 1987). Some Ca^{2+} has been shown to enter through AMPA/kainate receptor–gated channels on hippocampal neurons (Iino et al., 1990; Ozawa et al., 1991) and retinal bipolar cells (Gilbertson et al., 1991), and several cloned AMPA/kainate receptor subunits activate Ca^{2+}-permeable channels when expressed in homomeric configuration (Hollmann et al., 1991; Verdoorn et al., 1991). However, the channels activated by native adult forebrain AMPA/kainate receptors are usually not Ca^{2+} permeable, perhaps due to the presence of a GluR2/GluR-B subunit containing a critical arginine residue in the putative TM2 region of the peptide (Hollmann et al., 1991; Verdoorn et al., 1991; Burnashev et al., 1992). The relatively small $^{45}Ca^{2+}$ accumulation induced by brief exposure to AMPA or kainate may explain why these agonists produce widespread cortical neuronal death only after prolonged exposure times of several hours or more (Koh et al., 1990).

The present observations add support to the idea that excessive accumulation of extracellular Ca^{2+} is a key initial step in triggering cortical neuronal degeneration after exposure to glutamate agonists. The rate of Ca^{2+} accumulation may determine the speed with which lethal injury is attained, rapid accumulation with high levels of NMDA receptor activation correlating with rapidly triggered excitotoxicity (Choi et al., 1987), or slow accumulation even with high levels of AMPA or kainate receptor activation correlating with slowly triggered excitotoxicity (Koh et al., 1990).

As discussed above, our observations need not conflict with prior studies of neuronal $[Ca^{2+}]_i$ measured with Ca^{2+}-sensitive dyes. A given amount of Ca^{2+} entry may or may not lead to elevation of $[Ca^{2+}]_i$, depending on buffering or sequestration. Furthermore, full understanding of $[Ca^{2+}]_i$ changes will probably require greater temporal and spatial resolution than that used in many previous studies. Randall and Thayer (1992) found that the initial rise in $[Ca^{2+}]_i$ induced in hippocampal neurons by brief glutamate exposure can recover to basal levels, before eventually rising again in neurons that exhibit delayed cell death. The delayed rise in $[Ca^{2+}]_i$—which, those authors pointed out, might be missed with brief recording times—depended on the presence of extracellular Ca^{2+} during the period of glutamate exposure.

Dumuis et al. (1988) found that NMDA receptor activation,

but not kainate receptor activation or K^+-induced depolarization, stimulated the release of arachidonic acid from striatal neurons. Although these different stimuli can all produce large increases in $[Ca^{2+}]_i$, their different abilities to activate phospholipase A_2 are consistent with present data indicating different abilities to induce Ca^{2+} influx. Work by both Murphy et al. (1989) and us (Rose et al., 1990) has suggested that arachidonic acid metabolism may contribute importantly to excitotoxic injury, perhaps due to the associated generation of free radicals (Monyer et al., 1990). Many other processes activated by cellular Ca^{2+} overload might also contribute to injury (Choi, 1988), for example, the activation of proteases or the excess production of nitric oxide (Dawson et al., 1991).

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