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Growth factors and vitamin E modify neuronal glutamate toxicity

DAVID SCHUBERT*†, HIDEO KIMURA*, AND PAMELA MAHER‡

*The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92186-5800; and ‡Whittier Institute, 9894 Genesee Avenue, La Jolla, CA 92037

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ABSTRACT **The sympathetic nerve cell line PC-12 is killed by glutamate in a concentration-dependent manner. Although glycine and the deletion of magnesium weakly potentiate glutamate toxicity and PC-12 cells express *N*-methyl-D-aspartate-receptor mRNA, most toxicity is mediated by means of a mechanism independent of typical *N*-methyl-D-aspartate receptors. Glutamate toxicity is, however, greatly enhanced by prior exposure to nerve growth factor or basic fibroblast growth factor. Glutamate killing is blocked by epidermal growth factor and, to a lesser extent, by vitamin E. These observations show that synergistic interactions between growth factors and excitotoxic amino acids may play critical roles in the developing nervous system and that antioxidants attenuate this toxicity.**

In addition to its classical role in synaptic transmission, glutamate can regulate several aspects of neuronal development (1), and glutamate receptors may be involved in establishing normal connections within the developing nervous system (2). Several classes of receptors that respond to glutamic acid have been identified (3). One class of these receptors, those that selectively respond to *N*-methyl-D-aspartate (NMDA) in the presence of glycine, is thought to be involved in synaptic modification, whereas both NMDA receptors and members of the other classes mediate information transfer at excitatory synapses (3). Glutamate has also been implicated in the initiation of nerve cell death under conditions of stroke, epilepsy, and other forms of central nervous system insult (4). A form of naturally occurring (programmed) cell death occurs at specific times during the development of the nervous system (5), but little is known about the mechanisms that mediate this form of selective nerve cell destruction. Because some growth factors (GFs), such as basic fibroblast GF (bFGF), are synthesized in large amounts in the central nervous system (for review, see ref. 6) and because there are many synergistic interactions between GFs (for review, see ref. 7), we asked whether specific interactions occur between the excitatory amino acid glutamate and these proteins.

To study the interaction between GFs and glutamate at the biochemical level, it would be advantageous to use clonal neuronal cell lines. Such cell lines have been established from cells transformed spontaneously (8–10), chemically (11), and through the use of viruses (12). To determine whether clonal cell lines can express functional glutamate receptors, 10 nerve and glial lines were screened for their ability to respond to the plant excitotoxic glutamate analogs L-oxalyl-α,β-diaminopropionic acid (BOAA) and β-*N*-methyl-α,β-diaminopropionic acid (BMAA). Several cell lines respond by slowing cell division or by cell death. Our data show that one of these cell lines, PC-12 (10), is killed by glutamate and that this killing is modified by some GFs and vitamin E.

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MATERIALS AND METHODS

Materials. BOAA and BMAA were obtained from Research Biochemicals (Natick, MA). *trans*-1-Aminocyclopentyl-1,3-dicarboxylic acid (ACPD) was from Tocris (Essex, U.K.), and bFGF was from A. Baird (Whittier Institute). All other chemicals were from Sigma. Dialyzed horse and fetal calf sera were from GIBCO/BRL, and Dulbecco's modified Eagle's medium (DMEM) was made according to the original procedure (13). In some cases $MgSO_4$ and $CaCl_2$ were deleted, and the medium was resupplemented with $MgCl_2$ or $CaCl_2$ and, in all cases, with 1 mM Na_2SO_4.

Assays. The following culture conditions were critical for the success of the cytotoxic assays. PC-12 cells were grown in DMEM/10% fetal calf serum/5% horse serum. Exponentially growing cells were dissociated by trituration and plated at 5×10^3 cells per well or 1×10^4 cells per 35-mm culture dish. The growth curves were done in 35-mm tissue culture dishes containing 2 ml of medium with normal serum, and cell number was determined with a Coulter Counter. The cytotoxic assays were done in 96-well microtiter dishes containing 100 μl per well. For the microtiter assays, cells were plated in medium containing dialyzed serum, and the day after plating the medium was replaced with DMEM containing dialyzed serum, and the test reagents were added. The next day, viable cell numbers were determined with a viable stain (14) and an automatic microtiter plate reader. Data are expressed as the mean of triplicate determinations plus or minus the SEM. Northern (RNA) blot analysis was done according to standard procedures (15), and the PCR reactions were done by using a Cetus kit. PCR primers were between amino acid residues 541 and 842 (16) of the rat NMDA receptor and were from J. Boulter (Salk Institute).

RESULTS

Because one form of the NMDA receptor has recently been cloned (16), a number of cell lines were examined for expression of this mRNA. With PCR primers, a clear PCR product was detected in PC-12 cells and rat brain RNA (data not shown). This 0.9-kilobase (kb) PCR product from rat brain was then isotopically labeled and used in a Northern (RNA) blot analysis for NMDA-receptor mRNA in PC-12 cells grown in the presence of NGF, bFGF, or 50 mM KCl. Fig. 1 shows that the 4.4-kb mRNA for this receptor is abundant but that its synthesis is not modified by these experimental conditions.

To determine whether PC-12 cells respond to glutamic acid, exponentially dividing cells were incubated with 1 mM glutamic acid, 300 μM of the glutamate receptor agonist quisqualic acid, or the plant excitotoxins BOAA and BMAA. Glutamate and BOAA partially inhibit cell division, whereas BMAA has a minimal effect (Fig. 2). Quisqualic acid is the

Abbreviations: NMDA, *N*-methyl-D-aspartate; BOAA, L-oxalyl-α,β-diaminopropionic acid; BMAA, β-*N*-methyl-α,β-diaminopropionic acid; GF, growth factor; NGF, nerve GF; bFGF, basic fibroblast GF; EGF, epidermal GF; ACPD, *trans*-1-aminocyclopentyl-1,3-dicarboxylic acid.

†To whom reprint requests should be addressed.

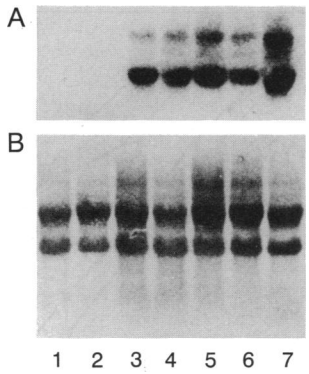

FIG. 1. Northern (RNA) blot analysis. A 1-kb PCR fragment of the rat NMDA receptor was generated from rat brain RNA, isotopically labeled, and used as a probe for a Northern analysis of mRNA from PC-12 cells exposed to the indicated conditions for 3 days. Ten micrograms of total RNA was loaded per lane. (*A*) Hybridization with NMDA-receptor probe. (*B*) Membrane filter stained with methylene blue. Lanes: 1, R14 neural retina cell line; 2, B103 rat central nervous system nerve cell line; 3, PC-12 cells plus 50 mM KCl; 4, control PC-12 cells; 5, PC-12 cells plus NGF at 50 ng per ml; 6, PC-12 cells plus bFGF at 10 ng per ml; 7, total brain RNA. This experiment was repeated three times with no reproducible changes in mRNA levels from the various conditions.

most potent inhibitor of mitosis. Although NMDA alone has no direct effect on the cells, NMDA receptors have the specific property of being inhibited by Mg^{2+} in a membrane potential-dependent manner (17). When PC-12 cells were grown in normal serum with glutamate or BOAA and without added Mg^{2+}, their growth rate was greatly reduced relative to control cultures (Fig. 2). Although NMDA alone does not modify cell growth, NMDA plus elevated glycine significantly decreases the rate of cell division. Quisqualate at 300 μM alone is quite toxic in the absence of Mg^{2+}.

To further examine the nature of the PC-12 response to glutamate, a microtiter dish assay was developed to monitor toxicity. Because serum contains Mg^{2+}, antioxidants, glutamate, and other excitotoxins (18), exponentially dividing cells were assayed in medium containing dialyzed sera. Table 1 shows that in this assay glutamate and quisqualate are potent toxins, whereas NMDA is weakly toxic only in the presence of high glycine. In contrast, aspartate, kainate, α-amino-3-hydroxy-5-methyl-4-oxazolepropionic acid, and the selective metabotropic glutamate-receptor agonist ACPD (3) are ineffective. The effects of glutamate and quisqualate were potentiated under Mg^{2+}-free conditions. The quantitative relationship between glutamate toxicity and divalent cation concentrations is presented in Figs. 3 and 4. Fig. 3 shows that the cell death initiated by glutamate is shifted to slightly lower glutamate concentrations by 5 mM glycine and deletion of Mg^{2+} from the culture medium. This result is a characteristic of glutamate killing in hippocampal primary cultures (19). The killing of PC-12 cells by glutamate is also, in part, Ca^{2+} dependent (Fig. 4*A*).

Although PC-12 cells express high levels of NMDA-receptor mRNA and this mRNA directs the synthesis of active NMDA receptors in frog oocytes (data not shown), the cytotoxicity elicited by glutamate appears not to function primarily through the NMDA receptor *per se*. Several observations support this conclusion. (*i*) High concentrations of glutamate are required. (*ii*) NMDA is only a weak agonist. (*iii*) The effects of Mg^{2+} and high concentrations of glycine are small (Fig. 3, Table 1). (*iv*) Neither competitive (DL-amino-5-phosphonovalerate) nor noncompetitive (MK-801) NMDA-receptor antagonists inhibit glutamate-induced killing by >25% (Table 1). (*v*) BOAA is more toxic than BMAA, whereas the latter is more specific for NMDA receptors (3).

An alternative mechanism for glutamate-induced cell death is that glutamate competes for cystine uptake, reducing intracellular glutathione and increasing free-radical generation (20). If this hypothesis was correct, then cells exposed to

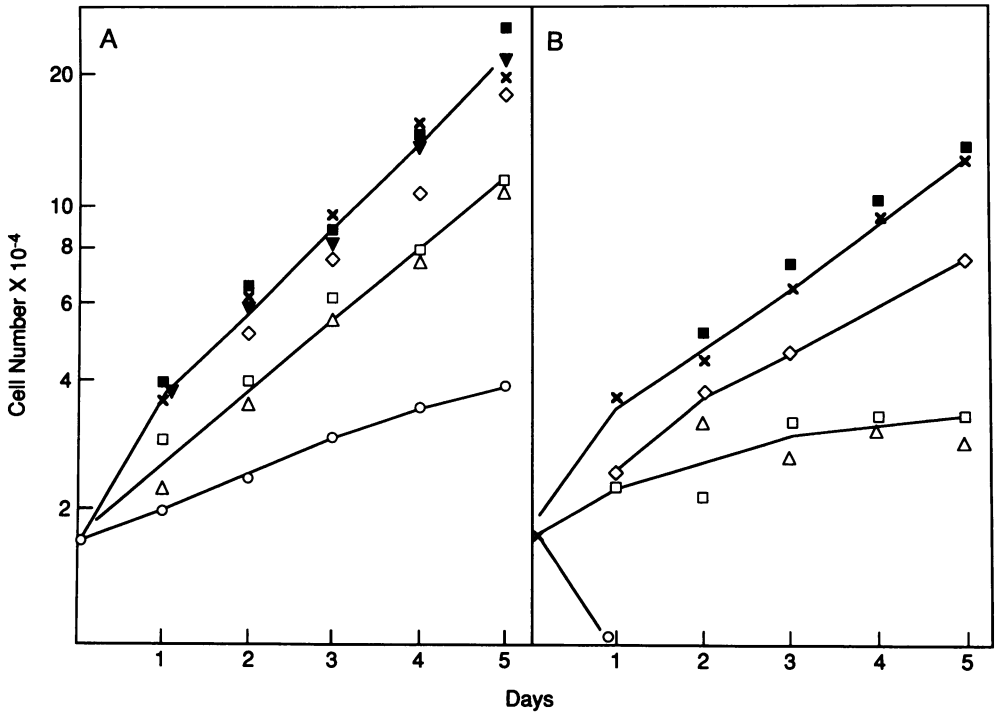

FIG. 2. Effect of glutamate analogs on PC-12 cell division. The following reagents were added to PC-12 cells in complete serum, and cell number was followed over time. ■, Control (without additive); x, 5 mM NMDA; ▼, 2 mM BMAA; ◇, 5 mM NMDA plus 5 mM glycine; □, 1 mM glutamate; △, 2 mM BOAA; and ○, 300 μM quisqualate. Glycine at 5 mM had no effect on cell division. (*A*) Mg^{2+} (0.8 mM). (*B*) Mg^{2+}-free solution.

Table 1. Toxicity of glutamate and its analogs

Reagent	Mg^{2+}	Survival, %
Control	+	100
Glutamate (5 mM)	+	56 ± 3
Glycine (5 mM)	+	96 ± 6
NMDA (10 mM)	+	89 ± 6
NMDA (10 mM) + glycine (5 mM)	+	78 ± 5
Kainate (10 mM)	+	101 ± 10
AMPA (5 mM)	+	92 ± 6
Quisqualate (300 μM)	+	39 ± 2
Aspartate (10 mM)	+	100 ± 10
Cystine (1 mM)	+	95 ± 2
Cystine (1 mM) + glutamate (5 mM)	+	49 ± 10
Cystine (0 mM)	+	96 ± 10
Cystine (0 mM) + glutamate (5 mM)	+	98 ± 6
KCl (25 mM)	+	100 ± 3
KCl (25 mM) + glutamate (5 mM)	+	42 ± 6
ACPD (100 μM)	+	98 ± 5
Control	−	100
Glutamate (5 mM)	−	29 ± 6
Glutamate (5 mM) + APV (10 mM)	−	42 ± 2
Glutamate (5 mM) + MK-801 (10 μM)	−	39 ± 2
Glycine (5 mM)	−	97 ± 3
NMDA (10 mM)	−	100 ± 11
NMDA (10 mM) + glycine (5 mM)	−	86 ± 8
Quisqualate (300 μM)	−	5 ± 1
Aspartate (10 mM)	−	95 ± 6
Cystine (1 mM)	−	92 ± 4
Cystine (1 mM) + glutamate (5 mM)	−	20 ± 5
KCl (25 mM)	−	98 ± 8
KCl (25 mM) + glutamate (5 mM)	−	18 ± 2
ACPD (100 μM)	−	105 ± 11

Control OD_{570} was 1.21 for cells with 0.8 mM $MgCl_2$ and 0.761 for cells without $MgCl_2$. Cell viability after 24 hr was determined by the microtiter plate assay (14). Normal cystine concentration is 0.2 mM, and normal glycine concentration is 0.4 mM. APV, DL-amino-5-phosphonovalerate; AMPA, α-amino-3-hydroxy-5-methyl-4-oxazolepropionic acid.

higher cystine concentrations should be killed less efficiently by glutamate, and cystine deletion should be toxic. In contrast to the predicted results, elevated cystine leads to slightly greater toxicity, and removal of cystine inhibits glutamate toxicity (Table 1). Although these data are not consistent with glutamate inhibition of cystine uptake, cell death from oxidative stress can frequently be prevented by free-radical scavengers, such as vitamin E. Fig. 5 shows that vitamin E at 100 μg/ml can partially protect PC-12 cells from glutamate toxicity. Increasing the vitamin E concentration has no further protective effect on the cells. Glutamate-induced killing of other clonal nerve cell lines is totally blocked by this vitamin E concentration (20). Thus, at least one mechanism by which glutamate kills PC-12 cells involves oxidative stress and generation of free radicals.

Because GFs modulate the energy metabolism of cells, we asked whether NGF, bFGF, or epidermal GF (EGF) could alter the response of PC-12 cells to glutamate. When PC-12 cells are plated in microtiter dishes at a low cell density and exposed to GFs for 1 or 2 days before glutamate addition, NGF and bFGF cause a progressive increase in sensitivity of the cells to glutamate (Fig. 5). In contrast, EGF totally blocks glutamate toxicity at glutamate concentrations <1 mM.

DISCUSSION

These results show that the PC-12 cell clone used in our laboratory expresses the mRNA for the NMDA receptor and is killed by glutamic acid. Although this result is evidence for

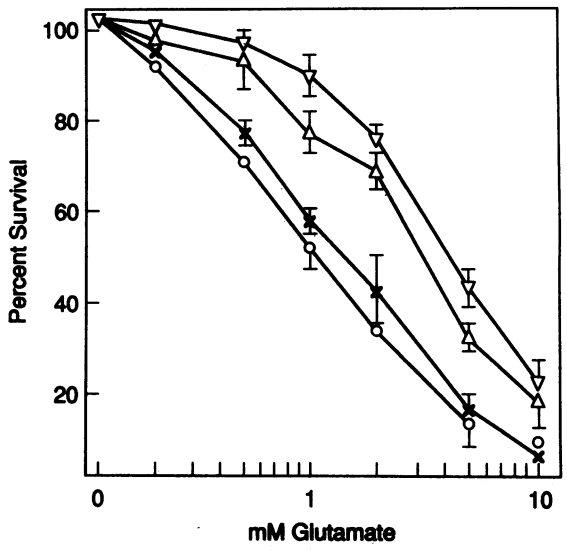

FIG. 3. Concentration dependence for glutamate cytotoxicity. Exponentially dividing cells were plated in microtiter dishes, and 1 day later the medium was replaced by that containing dialyzed serum plus or minus Mg^{2+} and glycine and the indicated glutamate concentrations. Cell viability was determined 24 hr later. ▽, Control [complete medium (dialyzed serum)]; △, medium plus 5 mM glycine; x, medium minus Mg^{2+}; ○, medium minus Mg^{2+} plus 5 mM glycine. DMEM normally contains 0.8 mM Mg^{2+}.

an NMDA-receptor transcript in a clonal cell line that is linked to excitoxicity, most data suggest that the primary mode of glutamate killing in these cells is not via activation of the NMDA receptor. There are a number of significant differences between glutamate toxicity in cortical primary cultures and PC-12 cells. (*i*) The first is the high concentrations of glutamate, quisqualate, and NMDA required to effect a cytotoxic response. This difference may, however, be from the fact that these reagents are more rapidly metabolized in this actively growing cell line than in nondividing primary cultures. (*ii*) The concentration of glutamate required to kill neurons also depends upon the anatomic location from which the cells are derived and the culture age (21). Only older cultures are killed via an NMDA receptor-mediated process,

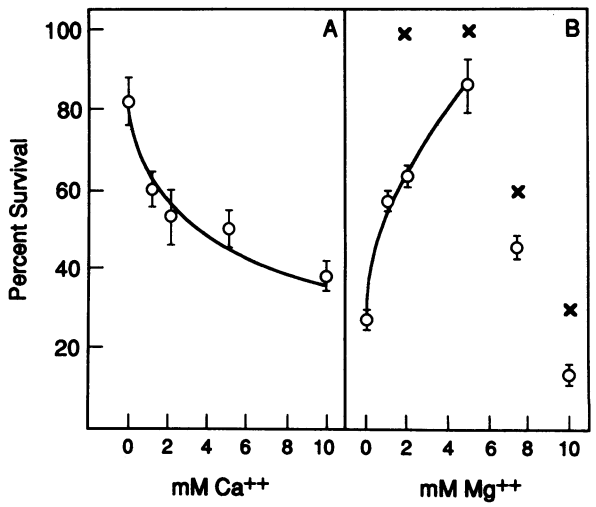

FIG. 4. Ca^{2+} and Mg^{2+} influence excitoxicity. Cells were assayed as described for Fig. 3, except that concentrations of the indicated molecules varied. (*A*) Varied Ca^{2+} concentrations plus 5 mM glutamate and 0.8 mM Mg^{2+}. (*B*) ○, Varied Mg^{2+} concentrations plus 5 mM glutamate and 1.4 mM Ca^{2+}; x, varied Mg^{2+} concentration without glutamate. Ca^{2+} (10 mM) alone was not toxic.

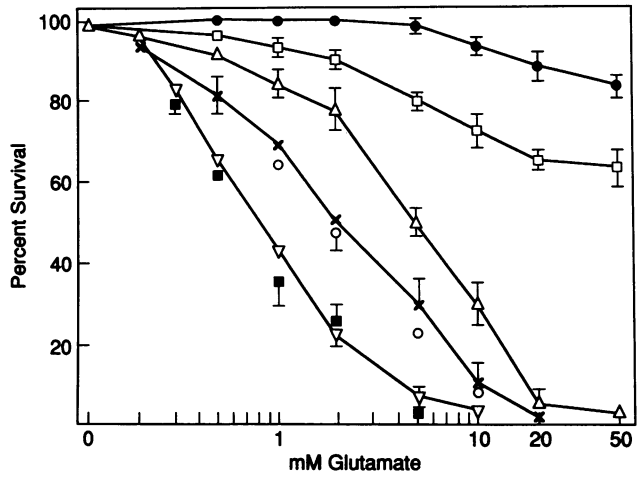

FIG. 5. Effect of bFGF, NGF, EGF, and vitamin E on glutamate toxicity. Cells were grown as described for Figs. 2 and 3, but in some cases, bFGF, NGF, or EGF was added to the cultures 1 or 2 days before adding the indicated glutamate concentrations. Cell death was monitored 24 hr after glutamate addition. △, Control; x, NGF (50 ng/ml) added 1 day before glutamate; ■, NGF added 2 days before glutamate; ○, bFGF (5 ng/ml) added 1 day before glutamate; ▽, bFGF added 2 days before glutamate; □, vitamin E (100 μg/ml) added with glutamate; ●, EGF (10 ng/ml) added 1 day before glutamate.

whereas cells from the developing nervous system may be killed by a different mechanism. (*iii*) The relatively strong Mg^{2+} concentration-dependence curve of Fig. 3*B* may be from the fact that deleting Mg^{2+} from the culture medium weakens the physiological state of the cell and nonspecifically potentiates killing. In smooth muscle cells the removal of extracellular Mg^{2+} leads to large increases in intracellular Ca^{2+} (22), which mediates cytotoxicity (4). (*iv*) Another difference between NMDA-mediated glutamate killing and the glutamate toxicity to PC-12 cells is the relative excitoxic potencies of quisqualate, BOAA, and BMAA relative to NMDA and glutamate. BMAA is thought to activate NMDA receptors in preference to BOAA (23), whereas on PC-12 cells BOAA is more toxic than BMAA. In addition, quisqualate is 10-fold less potent than NMDA on hippocampal neurons (24), and aspartate activates NMDA receptors but is not toxic to PC-12 (3). These discrepancies, plus the facts that glycine only marginally accelerates cell death and that NMDA antagonists do not inhibit glutamate toxicity, suggest that an alternative mechanism is responsible for the death of the PC-12 cells.

Because vitamin E inhibits most of the glutamate-induced cell death (Fig. 5), the cells are probably killed by oxidative stress and free-radical generation. In a neuroblastoma–retina hybrid cell line glutamate has been shown to inhibit cystine uptake, leading to reduced intracellular glutathione and subsequent cell death (20). This conclusion was made because of the ability of cystine to directly compete with glutamate in the toxicity assay. In addition, K^+-induced depolarization blocked the glutamate toxicity (24). The data in Table 1 show that this situation is not so for PC-12 cells because a 5-fold excess of cystine over the normal medium concentration has little effect on glutamate-induced cell death, cystine deletion inhibits toxicity, and K^+ depolarization enhances toxicity. Thus, glutamate kills PC-12 cells by a mechanism involving free-radical generation that is distinct from that described for the hybrid cell line. Free-radical scavengers also protect against kainate-induced toxicity in some central nervous system primary cultures (25).

Enhancement of glutamate killing by GFs is likely to have developmental significance, for massive nerve cell death occurs during embryonic development (5). PC-12 and other clonal nerve cell lines represent immature neuronal cells transformed at an early developmental stage (26). This early stage is a period of extensive GF synthesis, including such GFs as bFGF (6) and NGF (27). One function of bFGF could be to sensitize cells to glutamate, leading to an elimination of some cell populations. In contrast to NGF and bFGF, EGF protects PC-12 cells from killing by intermediate doses of glutamate (Fig. 5). bFGF can also act as a protective agent in old central nervous system cultures against toxicity caused by low glutamate (below 1 mM) (28). Thus, the synergistic interactions between bFGF and glutamate may evolve during development from potentiating toxicity in an immature system to a more protective role in the adult nervous system.

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