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Central mammalian neurons normally resistant to glutamate toxicity are made sensitive by elevated extracellular Ca^{2+}: Toxicity is blocked by the *N*-methyl-D-aspartate antagonist MK-801

(excitatory amino acid toxicity/kainate/mammalian retinal ganglion cells)

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ABSTRACT **It is widely held that a glutamate-like toxin that resembles *N*-methyl-D-aspartate may be responsible for the death of nerve cells seen after severe neurological insults including stroke, seizures, and degenerative disorders, such as Huntington disease, Alzheimer disease, and the amyotrophic lateral sclerosis–parkinsonism–dementia complex found on Guam. One puzzling fact about these maladies is the differential vulnerability of specific groups of neurons peculiar to each condition. We report here that an identified population of central neurons, rat retinal ganglion cells, are resistant to the neurotoxic effects of millimolar concentrations of glutamate under otherwise normal culture conditions. Patch-clamp experiments show that this resistance is associated with a very small ionic current response to *N*-methyl-D-aspartate. Varying the ionic milieu by increasing the extracellular Ca^{2+} concentration, however, results in a striking increase in glutamate-induced cell death in this population. Under these conditions, Mg^{2+} or the amino acid antagonist MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo-(α,γ)-cyclohepten-5,10-imine maleate], blockers of *N*-methyl-D-aspartate receptor-coupled ion channels, completely abrogate the lethal effects of glutamate. These findings strongly suggest that Ca^{2+} entry through *N*-methyl-D-aspartate-activated channels is responsible for this type of neuronal death and suggest strategies that may be clinically useful in the treatment of various neurological disorders.**

In the mammalian central nervous system glutamate is thought to be the major excitatory neurotransmitter that acts at three major receptor subtypes. Excessive stimulation of one of these subtypes, selective for the glutamate analog *N*-methyl-D-aspartate (*N*-Me-D-Asp), has been implicated in the pathophysiology of neuronal degeneration caused by a variety of conditions; these include anoxia, ischemia, hypoglycemia, seizures, and several neurodegenerative diseases (1–3). Nevertheless, certain groups of neurons appear to be resistant to toxicity mediated through the *N*-Me-D-Asp receptor (3, 4). The mammalian retina was the first preparation in which central neurons were shown to be vulnerable to glutamate toxicity (5). Since then, however, the effect of glutamate analogs on the vertebrate retina has been studied repeatedly but sometimes with conflicting results regarding toxicity to the ganglion cells, the neurons that link the retina to deeper centers of the brain (6–9). These observations gave rise to the hypothesis that variations in the extracellular environment of the neurons might render them vulnerable or resistant to the toxic effects of glutamate. To define a possible ionic basis for the selective toxicity of some populations of central mammalian neurons and to develop a preventative treatment, we examined the effects of glutamate on rat postnatal retinal ganglion cells under the precisely controlled ionic conditions that can only be afforded by tissue culture. Here, we report that this population of central neurons is resistant to glutamate-induced cell death; however, increasing the extracellular Ca^{2+} concentration above normal levels resulted in a strikingly increased vulnerability of the retinal ganglion cells. Even then, only relatively high levels (1 mM) of glutamate produced toxic effects. Furthermore, a central nervous system permeant, *N*-Me-D-Asp open-channel blocker was capable of preventing the death of the neurons. This antagonist, MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo-(α,γ)-cyclohepten-5,10-imine maleate], acts in an agonist-dependent manner (10–13) to become a more effective inhibitor as the concentration of an *N*-Me-D-Asp-like agonist is increased (14). This type of action offers the possible advantage of at least partially sparing the normal effects of lower concentrations of *N*-Me-D-Asp-like neurotransmitters that have been linked to important processes, such as learning and memory (15).

MATERIALS AND METHODS

Retinal ganglion cells (RGCs) from postnatal rats were identified with fluorescent labels, as developed in this laboratory (16). The fluorescent dye granular blue was injected as a ≈2% (wt/vol) suspension in saline into the superior colliculus of 4- to 7-day-old Long Evans rats. Two days after injection, the animals were killed by decapitation and enucleated, and the retinas were quickly removed. The retinas were dissociated and cultured as reported in ref. 16. The cells were plated onto glass coverslips coated with poly(L-lysine). The ganglion cells could be recognized by the continued presence of fluorescent blue dye; double-labeling studies with Thy-1 antibody, which is specific in retinal cultures for the ganglion cells, have verified that granular blue labels only these specific neurons under these conditions (16). The retinal cells were cultured in Eagle's minimum essential medium (MEM) with the exclusion of NaH_2PO_4 to avoid precipitation in high concentrations of calcium (composition of salts: 116 mM NaCl, 26 mM $NaHCO_3$, 5.3 mM KCl, 1.8 mM $CaCl_2$, and 0.8 mM $MgCl_2$, pH 7.2), supplemented with 0.7% (wt/vol) methylcellulose, 2 mM glutamine, gentamicin (1 μg/ml), 16 mM dextrose, and 5% (vol/vol) rat serum. For the high-Ca^{2+} experiments, the Ca^{2+} concentration in the culture medium was increased from 1.8 to 10 mM. Nominally Mg^{2+}-free culture medium was used in some experiments.

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Abbreviations: MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-(α,γ)-cyclohepten-5,10-imine maleate; *N*-Me-D-Asp, *N*-methyl-D-aspartate; RGC, retinal ganglion cell.

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Glutamate (0.5, 1.0, or 5.0 mM) or other agonists were added to the cell-culture medium prior to plating. A set of sibling cultures was treated in the same manner, but in the absence of glutamate. In the protection experiments, 1.0 mM glutamate and 20 μM MK-801 were added to the cell-culture medium. This concentration of MK-801 was chosen since, in RGCs, it totally blocks the current induced by *N*-Me-D-Asp at concentrations of 200 μM or higher (14). A concentrated stock of MK-801 was dissolved in absolute ethanol prior to addition in small volumes to the culture medium. The final concentration of ethanol was 0.03%, which was also added to all other dishes in these experiments. Compared to control cultures without ethanol, this concentration did not affect the longevity of the RGCs. Incubation with these drugs lasted for 16–20 hr at 37°C in an atmosphere of 5% CO_2/95% air. We chose to score the death of the cells by an improved method that relies on a positive action of the living cells, rather than dye exclusion, a commonly used method. Dye exclusion may be misleading for several reasons (e.g., even fixed cells can exclude some vital dyes, although they are obviously dead). The ability of the RGCs to take up and cleave fluorescein diacetate to fluorescein was used as an index of their viability (17). Dye uptake and cleavage correlates with the normal electrophysiological properties assayed with patch electrodes (16, 18, 19). To perform this test, the cell-culture medium was exchanged for physiological saline containing 0.0005% fluorescein diacetate for 15–30 sec and then cells were rinsed. RGCs that did not contain the fluorescein dye (and thus were not living) often remained visible under UV fluorescence optics because of the continued presence of the marker dye granular blue; other dead RGCs had simply disintegrated and only debris remained. In contrast, the viable RGCs displayed not only a blue color in the UV light but also a yellow-green fluorescence with filters appropriate for fluorescein (Fig. 1). Other viable nonganglion retinal cells that had taken up and cleaved fluorescein diacetate also appeared yellow-green when the fluorescein filters were used but were not blue when the UV filters were used. Thus, the use of two exchangeable fluorescence filter sets permitted the rapid determination of viable ganglion cells in these cultures. Our physiological saline was based upon Hanks' salts containing 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 1 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 1.25 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM $MgCl_2$, 5 mM Hepes·NaOH, 22.2 mM glucose, and 0.001% phenol red, pH 7.2.

RESULTS

Glutamate Is Not Toxic in Normal Extracellular Ca^{2+} Concentrations. Treatment of retinal cultures (at a final density of 100–450 RGCs per cm^2) with glutamate (0.5, 1.0, or 5.0 mM) in the presence of 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+} did not produce a significant increase in RGC death when compared to controls (Fig. 2*A*). Relatively high doses of glutamate analogs (125 μM kainate or 200 μM *N*-Me-D-Asp) that produced maximal or near maximal nondesensitizing ionic currents in these cells, when measured with patch electrodes (14, 20), also did not produce death (Fig. 2*B*). Extracellular Mg^{2+} blocks the current induced by excitatory amino acid agonists binding to the *N*-Me-D-Asp receptor (20–23). Exclusion of Mg^{2+} from the cell-culture medium (in the presence of 1.8 mM Ca^{2+}), however, still did not produce toxicity in cultures treated with 1 mM glutamate or 200 μM *N*-Me-D-Asp (Fig. 2*C*).

Glutamate-Induced RGC Death in High Concentrations of Extracellular Ca^{2+} Is Antagonized by Extracellular Mg^{2+} or MK-801. Fig. 3 shows that when the concentration of Ca^{2+} was increased to 10 mM and Mg^{2+} was excluded, 1 mM glutamate produced significant cell death after 16 hr when compared to sibling cultures under identical conditions but not treated with glutamate. Incubation with glutamate for shorter periods of time resulted in variable results with many more retinal ganglion cells surviving. In addition, toxicity did not appear to be acute—i.e., little death was produced after only 6–8 hr. The high concentration of Ca^{2+} in this experiment did not in itself affect the viability of the RGCs since in sibling cultures the same percentage of neurons survived in either 10 mM or 1.8 mM Ca^{2+} in the absence of glutamate (data not shown). Furthermore, the inclusion of 0.8 mM Mg^{2+} prevented the glutamate-induced RGC death in a high concentration of Ca^{2+} (10 mM). The prevention of death by Mg^{2+} in this protocol supported the hypothesis that glutamate neurotoxicity might be mediated by activation of *N*-Me-D-Asp receptors and subsequent ionic influx, as discussed below. Moreover, in another experiment in Mg^{2+}-free medium containing 10 mM Ca^{2+}, 200 μM *N*-Me-D-Asp appeared to exert a lethal effect itself. The protective effect of Mg^{2+} suggested to us that we examine the effect of an *N*-Me-D-Asp-channel blocker, such as MK-801, that could readily penetrate the blood–brain barrier (10–12) and thus be of potential clinical benefit. In the high-Ca^{2+}, Mg^{2+}-free medium, cell cultures were treated with 1 mM glutamate in the presence of 20 μM MK-801. The addition of MK-801 to the cultures totally prevented glutamate-induced RGC death (Fig. 4). In fact, in three out of four experiments (including the one illustrated in Fig. 4), MK-801 actually produced a significant increase in the survival of RGCs compared to the controls in Mg^{2+}-free medium containing 10 mM Ca^{2+} in the absence of exogenous glutamate. This finding suggested that an endogenous glutamate-like agent might be present in some retinal cultures; one potential source is the rat serum, since some batches of serum contain significant amounts of glutamate. A second source of glutamate might be from cellular leakage or release. This hypothesis was strengthened by the finding that the protective effect of MK-801 alone (10 mM Ca^{2+} and no added Mg^{2+} or glutamate) was similar to that of MK-801 plus glutamate in the three or four experiments that produced "higher-than-control" values for survival. Also consistent with this interpretation, in the one of four experiments presumptively conducted in the absence of significant levels of endogenous glutamate, MK-801 alone did not exert a protective effect above the control values (obtained in cultures with 10 mM Ca^{2+} but lacking exogenous Mg^{2+}, glutamate, and MK-801). Furthermore, in two of four other experiments with 0.8 mM Mg^{2+} added (as in Fig. 3) and in an additional experiment in which the high-Ca^{2+}, Mg^{2+}-free culture medium contained the specific *N*-Me-D-Asp-receptor antagonist 2-amino-5-phosphovalerate (100 μM), similar "higher-than-control" values for the survival of RGCs were observed. Like the results with MK-801, these findings are also compatible with the notion that some cultures had an endogenous glutamate-like drug contributing to cell death in the "control" cultures.

DISCUSSION

Excitatory amino acids that mediate neuronal depolarization have been implicated in neurotoxicity. Although the precise mechanism of their neurotoxicity is still unknown, originally one of the critical steps in the toxicity was thought to be their ability to depolarize neurons. In the rat RGCs, glutamate produces depolarization in essentially all RGCs in culture (20). Yet despite the presence of their electrophysiologic responses, treatment with up to 5 mM glutamate for several hours did not produce significant RGC death. This indicates that the mere presence of electrophysiological responses to glutamate is not sufficient for neurotoxicity.

At least two mechanisms for glutamate-induced toxicity have been described in the vertebrate nervous system (1, 24). One is an acute process, independent of the extracellular

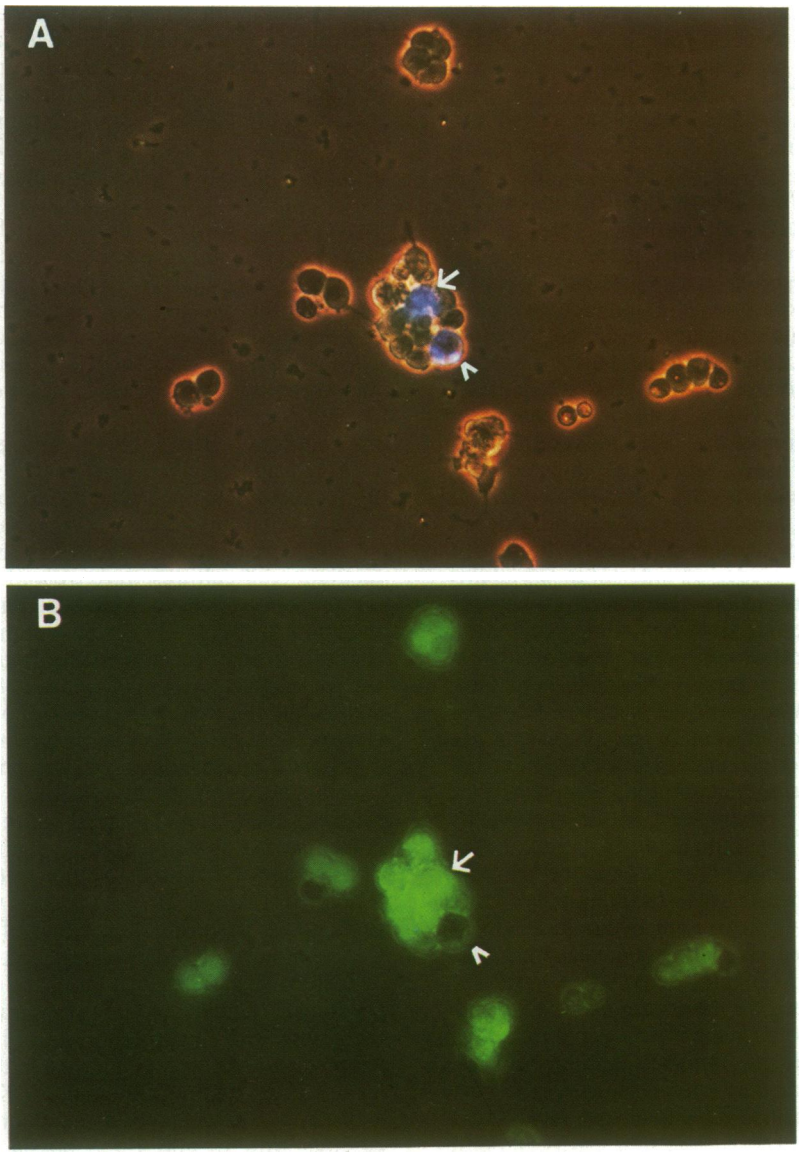

FIG. 1. Photographs of the same field of a retinal culture under a combination of phase-contrast and UV epifluorescence optics (*A*) and under epifluorescence for the visualization of fluorescein (*B*). This culture was treated for ≈20 hr with 1 mM glutamate. The culture was then treated with fluorescein diacetate to determine viability. (*A*) The two ganglion cells appear blue because of labeling prior to dissociation with the fluorescent dye granular blue (marked by an arrow and an arrowhead). Other types of retinal cells are unlabeled. Cellular debris is also evident in the picture due to some cells disintegrating because of glutamate-induced toxicity. (*B*) The arrowhead points to the ganglion cell that did not take up and cleave fluorescein diacetate and is, therefore, not viable. The second ganglion cell (arrow) is fluorescent and thus living. Many of the other types of retinal cells in this field are also alive by this criterion, but not all. Photographs were taken with a Zeiss IM-35 microscope equipped with standard UV and fluorescein filter sets.

Ca^{2+} concentration. It is manifest by reversible neuronal swelling apparently due to the influx of NaCl and water; this acute excitotoxic component can lead to cell death if intense stimulation by glutamate analogues occurs. The second mechanism of glutamate-induced injury is often the predominant cause of cell death (24) and is observed with a delay of many hours after exposure to the excitatory amino acid; the irreversible changes and death of the neurons are dependent upon the presence of Ca^{2+} in the medium bathing the cells (24, 25). The latter mechanism appeared to predominate in the present study since only the delayed onset of cell death was observed among the retinal ganglion cells in culture.

Studies in mammalian culture systems indicate that lowering extracellular Ca^{2+} concentrations can protect cortical neurons normally susceptible to the delayed form of glutamate-induced death (24–26). These findings, and those reported here for high extracellular Ca^{2+}, suggest that Ca^{2+} influx might play a role in glutamate-induced neurotoxicity. Most of the sustained glutamate-induced Ca^{2+} influx is now thought to occur through the *N*-Me-D-Asp receptor-coupled channels rather than through channels coupled to non-*N*-Me-D-Asp glutamate receptors (27–29). Heretofore, however, a different Ca^{2+}-dependent mechanism resulting in cell death could not be ruled out.

These data suggest that robust responses to *N*-Me-D-Asp in a neuron would result in a larger entry of Ca^{2+} than small *N*-Me-D-Asp responses. Interestingly, when measured with patch electrodes, responses to 200 μM *N*-Me-D-Asp in rat RGCs are quite small, usually −5 to −40 pA with the membrane voltage clamped to the resting potential of −60 mV (14, 20); the magnitude of these responses may reflect a relatively low *N*-Me-D-Asp receptor density on rat RGCs. [In contrast, the current responses to 125 μM kainate are substantial under these conditions, ranging from −80 to −750 pA (14, 20), but the channels coupled to the excitatory amino acid receptors activated by kainate are relatively

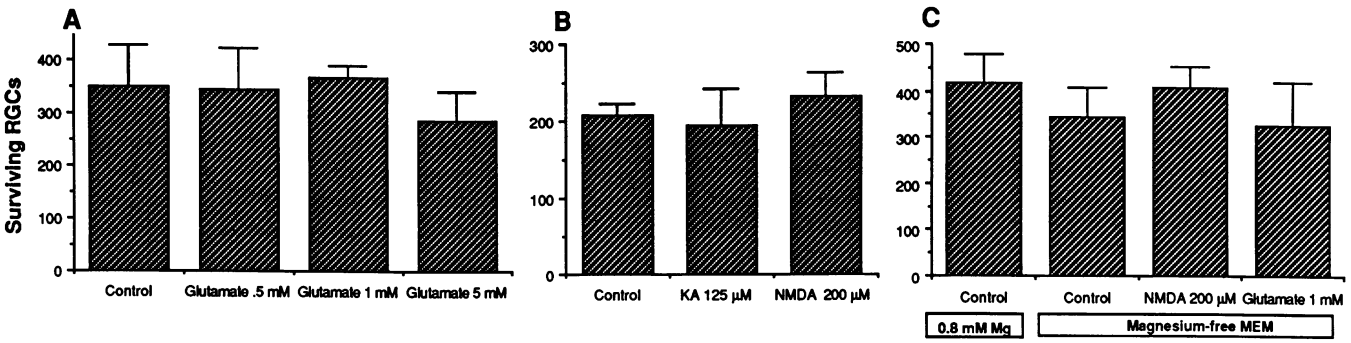

FIG. 2. Survival of RGCs as a function of treatment with glutamate or with glutamate analogs for 20 hr. The controls for each experiment contained normal MEM, as defined in the text, with 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+}, unless otherwise stated. (*A*) Compared to the controls, the addition of up to 5 mM glutamate to sibling cultures did not decrease viability, as assayed with the fluorescein diacetate technique. (*B*) Kainate and *N*-Me-D-Asp also did not affect survival. (*C*) Removing Mg^{2+} from the medium also did not affect viability of the RGCs in the control or in the *N*-Me-D-Asp- or glutamate-treated cultures. For this and subsequent figures, each experiment was conducted in quadruplicate and repeated on 3 or more days with similar results. The absolute number of ganglion cells surviving varied from experiment to experiment because of different plating densities. Since density effects are known to influence the viability of RGCs in culture (15), the density was varied to ensure that the results of the current experiments were true at various densities. Values shown are mean + SEM. An analysis of variance was used to test for significance.

impermeable to Ca^{2+} (29)]. Therefore, even with prolonged treatments of glutamate to activate the *N*-Me-D-Asp receptors, there may have been insufficient Ca^{2+} influx to induce cell death in the presence of physiologic extracellular Ca^{2+} concentrations. By using a higher concentration of Ca^{2+} (10 mM), glutamate produced significant toxicity to RGCs. Therefore, the resistance of the RGCs to glutamate neurotoxicity could be overcome by increasing the Ca^{2+} concentration in the cell-culture medium. The supposition that Ca^{2+} entered through the *N*-Me-D-Asp-activated channels is greatly strengthened by our finding that 0.8 mM extracellular Mg^{2+}, which is known to block these channels but not the voltage-dependent Ca^{2+} channels, antagonized the lethal effect of glutamate in this system. In addition, MK-801, a blocker of *N*-Me-D-Asp- but not of kainate-activated channels in this system (14), produced similar protective effects; these findings strongly suggest that Ca^{2+} influx through the *N*-Me-D-Asp-activated channel is the critical factor in glutamate-induced neurotoxicity. Although voltage-dependent Ca^{2+} channels might be activated by depolarization induced by glutamate or its analogues (30), it would appear far less likely that Ca^{2+} entering through these channels is essential for toxicity in this system for two reasons: (*i*) Ca^{2+} channels inactivate within a period of seconds (19) whereas several hours of glutamate treatment were required to obtain consistent killing of the RGCs; and (*ii*) in the presence of MK-801 or Mg^{2+}, glutamate still depolarized the retinal ganglion cells through kainate and quisqualate receptor sites (presumably activating voltage-dependent Ca^{2+} channels), yet no increase in cell death was observed even when the concentration of extracellular Ca^{2+} was increased. Nevertheless, these data do not preclude the possibility that the voltage-dependent Ca^{2+} channels also contribute to an increase in intracellular Ca^{2+} concentration and, thus, to neuronal toxicity; but blockade of Ca^{2+} entry through *N*-Me-D-Asp-activated channels alone is sufficient to prevent death.

Drugs such as phencyclidine that interact at the MK-801 binding site may, in addition, exert nonspecific effects at other locations—e.g., blocking K^+ or Na^+ channels (31–33). Nevertheless, this explanation for the action of MK-801 is unlikely in the present experiments for two reasons: (*i*) generally, higher doses of antagonist are required to block these other channels; and (*ii*) unlike the action of MK-801

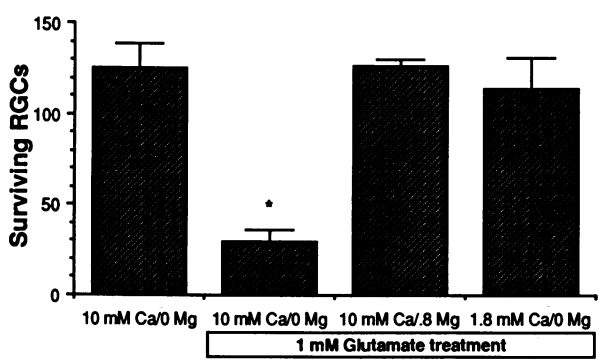

FIG. 3. Survival of RGCs treated with glutamate for 20 hr as a function of Ca^{2+} and Mg^{2+} concentrations in the medium. In this experiment treatment with high (10 mM) Ca^{2+} in Mg^{2+}-free medium resulted in survival of RGCs similar to that found in cultures with normal (1.8 mM) Ca^{2+}, Mg^{2+}-free medium containing 1 mM glutamate. (Fig. 2*C* shows that the addition of 1 mM glutamate to Mg^{2+}-free, 1.8 mM Ca^{2+} medium did not increase cell death when compared to cultures growing in this ionic milieu without glutamate.) When glutamate was added to the high-Ca^{2+}, Mg^{2+}-free medium, however, RGC survival was markedly decreased. In contrast, increasing the extracellular Mg^{2+} concentration to normal levels (0.8 mM) protected the RGCs from the neurotoxic effects of glutamate. The value of the column with the asterisk was significantly less ($P < 0.01$) than the other values by a one-way analysis of variance and then by a Scheffé multiple comparison of means.

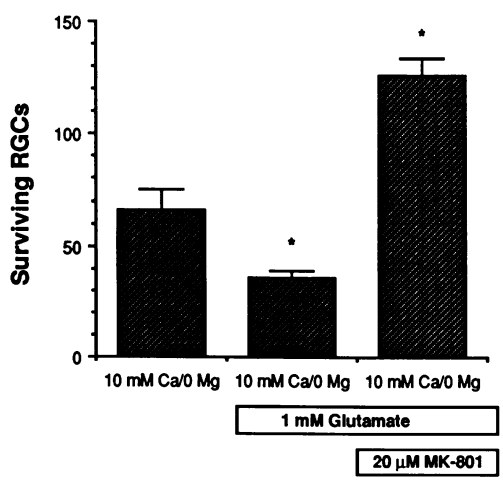

FIG. 4. Prevention of glutamate-induced neurotoxicity in RGCs by MK-801. As shown in Fig. 3, in the presence of high-Ca^{2+}, Mg^{2+}-free medium, 1 mM glutamate produced a significant decrease in the viability of RGCs. The addition of MK-801 reversed this effect.

reported here, nearly complete blockade of Na^+ or K^+ channels with 1 μM tetrodotoxin or 20 mM tetraethylammonium ions ± 5 mM 4-aminopyridine did not increase the longevity of retinal ganglion cells in these cultures (refs. 18 and 19, and S.A.L., unpublished observations).

Several workers have demonstrated the potential of relatively selective *N*-Me-D-Asp-receptor antagonists, such as 2-amino-5-phosphonovalerate, to prevent glutamate neurotoxicity (e.g., refs. 34 and 35). 2-Amino-5-phosphonovalerate and related drugs prevent *N*-Me-D-Asp from binding to its receptor but do not affect the ionic channels directly. Thus, from these previous results it was not possible to determine if the glutamate-induced neuronal death was due to an effect at the *N*-Me-D-Asp receptor or an ionic current flowing through its channels. In the present study, however, the prophylactic effects of ionic channel blockers clearly implicate an ionic current, carried by Ca^{2+}, in the lethal effects induced by glutamate. In addition, the previously utilized *N*-Me-D-Asp-receptor blockers affect low-dose, possibly normal, stimulation of *N*-Me-D-Asp receptors as well as more-intense pathological activation. Here, we report the neuronal life-saving effect of an antagonist, MK-801. MK-801 preferentially blocks excessive, moreso than low-level, stimulation of *N*-Me-D-Asp receptors, and it inhibits these responses equally at all physiological transmembrane potentials, at least in our preparation (14); therefore, this antagonist may be clinically useful in the setting of abnormally high levels of *N*-Me-D-Asp receptor activation, such as those seen in several neurological maladies.

In summary, our findings prove that a relatively resistant population of central neurons can be made susceptible to glutamate-induced cell death by increasing the extracellular Ca^{2+}. Thus, neurons with even small numbers of *N*-Me-D-Asp receptor sites may become vulnerable to glutamate-like toxins, depending on environmental conditions, specifically the ionic milieu. Along these lines, the ionic content of brain tissue is known to change under certain pathological conditions; for example, brain Ca^{2+} content increases during ischemic infarction (36). These findings are relevant here since RGCs, like other central neurons, suffer stroke-like episodes clinically after thromboembolic events in the retinal circulation. In our cultures, the fact that MK-801 can block *N*-Me-D-Asp-activated currents with increasing efficiency as the dose of agonist increases (14) suggests that this antagonist may prove useful under conditions of escalating levels of glutamate, such as those found in anoxia or stroke. In fact, the protective effect against ischemia of MK-801 appears to have been borne out in the intact gerbil (37) and rabbit (38) central nervous systems. Also, MK-801 can prevent *N*-Me-D-Asp-induced toxicity in the intact embryonic chicken retina (39). Nevertheless, further work is necessary to determine if this or related drugs will eventually be clinically useful.

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