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N-methyl-D-aspartate Antagonists Prevent Kainate Neurotoxicity in Rat Retinal Ganglion Cells *in vitro*

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Under defined culture conditions, exogenous glutamate (Glu), NMDA, or an endogenous Glu-related toxin is lethal to rat retinal ganglion cells; these detrimental effects are NMDA receptor mediated because specific NMDA antagonists can prevent cellular injury. In the presence of an endogenous Glu-like toxin, 125 μM kainate (KA) increases the proportion of retinal ganglion cells that die, but the toxicity (due to both KA and the endogenous toxin) is totally prevented by 2-amino-5-phosphonovalerate (APV), a specific NMDA receptor antagonist. These findings indicate that the KA-induced portion of retinal ganglion cell death also appears to be mediated via NMDA receptors. There are at least 2 possible mechanisms for this lethal effect. In addition to KA receptors, KA could directly stimulate NMDA receptors. Alternatively, KA might activate its own specific receptor, which in turn leads to a net increase in the release of an endogenous Glu-related toxin; this endogenous substance would then activate NMDA receptors. Patch-clamp electrophysiology experiments have helped to distinguish between these possibilities. Concentrations of APV that completely block the current elicited by maximal nondesensitizing doses of NMDA exert no detectable inhibition of KA-evoked currents. Hence, at the concentrations used, it appears unlikely that KA directly activates NMDA receptors in this preparation. Furthermore, the fraction of toxicity attributed to the addition of KA can be blocked by the relatively specific non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). This finding is consistent with the hypothesis that KA adds an increment of toxicity in this system by directly interacting with KA receptors. Taken together, the evidence suggests that KA receptor stimulation may lead to the next efflux of additional endogenous Glu-related toxin. In turn, this Glu-related substance activates NMDA receptors and its attendant neurotoxicity.

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Endogenous excitatory amino acids (EAAs) are thought to be responsible for a variety of acute neurological insults, including ischemia/anoxia, hypoglycemia, and trauma, as well as several chronic neurodegenerative diseases (for reviews, see Rothman and Olney, 1987; Choi, 1988a,b; Meldrum and Garthwaite, 1990). In many neuronal tissues, the predominant form of this toxicity is mediated by stimulation of the NMDA subtype of glutamate (Glu) receptor; activation of other types of Glu receptors, the kainate (KA) and quisqualate subtypes, may play a smaller role (Choi, 1987, 1988a,b; but see also Mattson et al., 1989; Sheardown et al., 1990). Nonetheless, in several preparations, the administration of exogenous KA can result in massive neuronal death. While in some cases KA appears to kill neurons directly through intense stimulation of their KA receptors (Seil et al., 1974; Bird and Gulley, 1979), in other systems the mechanism of neurotoxicity is more complex. For example, in many cases the deleterious action of KA requires a functionally intact excitatory input to the vulnerable population of neurons; that is, prior ablation of excitatory inputs greatly attenuates or even eliminates the neurotoxic effect of KA (Biziere and Coyle, 1978, 1979; Köhler et al., 1978; McGeer et al., 1978; Whetsell et al., 1979). These findings have led to the speculation that the direct neurotoxicity of KA may be overestimated because KA is not taken up by cells and, more importantly, because its inherent toxicity is enhanced by the release of endogenous Glu (Johnston et al., 1979; Choi, 1988b). In the current report, we use a model system of retinal cells in culture to show that the toxic effect of KA can be totally overcome with the selective NMDA antagonist 2-amino-5-phosphonovalerate (APV). In addition, patch-clamp recordings have shown that APV has no effect on the electrical response to KA, indicating that KA does not appear to stimulate directly the NMDA receptors of retinal ganglion cells. Together, these findings strengthen the hypothesis that, at least in some preparations, the lethal action of KA is mediated by the release of endogenous Glu-like compounds that subsequently act at the NMDA receptor.

Materials and Methods

Retinal ganglion cell culture system. Rodent retinal ganglion cells, well-characterized central neurons from 1–2-week-old postnatal Long–Evans rats, were identified by retrograde transport of fluorescent labels and enzymatically dissociated from the retina as we have described (Leifer et al., 1984; Lipton and Tauck, 1987). Following dissociation, the cells were rinsed with a physiological saline based on Hanks' salts (composition in mM: NaCl, 138; $NaHCO_3$, 1; Na_2HPO_4, 0.34; KCl, 5.36; KH_2PO_4, 0.44; $CaCl_2$, 1.25; $MgSO_4$, 0.5; $MgCl_2$, 0.5; HEPES, 5; dextrose, 22.2; phenol red, 0.001% v/v; adjusted to pH 7.2 with 0.3 M NaOH). The retinas were then mechanically dispersed, and ~100-μl volumes of the

resulting suspension were plated at an initial density of ~500 cells per mm^2 onto poly-L-lysine-coated glass coverslips previously affixed to the hollow bottom of 35 × 10-mm tissue-culture dishes with Sylgard (Dow Corning, Midland, MI). The cell culture medium was based upon Eagle's minimum essential medium (MEM), except that it was nominally magnesium-free to facilitate the occurrence of NMDA receptor-mediated toxicity (Hahn et al., 1988). The medium was supplemented with 0.7% methylcellulose (w/v), 1 μg/ml gentamicin, 16 mM dextrose, 2 mM glutamine, 5% rat serum (v/v), and a concentration of $CaCl_2$ of 1.8 or 10 mM, with the higher value to enhance excitatory amino acid toxicity in this preparation (Hahn et al., 1988). In some experiments, up to 250 μM glycine was present in the culture medium as measured by HPLC analysis; however, glycine did not affect the results reported here. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Electrophysiology experiments. Whole-cell recordings (Hamill et al., 1981) were performed with patch electrodes on rat retinal ganglion cells as described in detail by this laboratory (Lipton and Tauck, 1987; Aizenman et al., 1988, 1989; Karschin et al., 1988). Prior to recording, the cell cultures were incubated for 4–24 hr in MEM. During recording sessions, coverslips were continuously superfused at ~1 ml/min in nominally magnesium-free Hanks' solution with 2.5 mM $CaCl_2$ and 1 μM glycine, a coagonist at the NMDA receptor. All drugs were diluted in this Hanks' solution and applied by pneumatic pipette. In order to avoid confusion due to the release of endogenous substances from one neuron onto another, only solitary retinal ganglion cells (i.e., cells lacking synaptic connections with other neurons) were recorded from. The patch pipettes contained (in mM) CsCl, 120; tetraethylammonium (TEA)-Cl, 20; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 2.25; HEPES-NaOH, 10 (adjusted to pH 7.2). The temperature was maintained at 35°C, and the whole-cell current responses were filtered at 500 Hz with a Bessel characteristic.

Assessment of neurotoxicity. For the toxicity experiments, the culture medium was composed of the nominally magnesium-free MEM listed above. Under our culture conditions, retinal ganglion cells are susceptible to NMDA receptor-mediated toxicity in the presence of elevated extracellular Ca^{2+} (Hahn et al., 1988). This toxicity manifests itself in the presence of exogenous Glu (10 μM to 1 mM), NMDA (200 μM), or an endogenous Glu-like substance that could be detected in densely plated cultures (Hahn et al., 1988; Levy and Lipton, 1990; Levy et al., 1990). HPLC assays revealed that the endogenous level of Glu in these cultures was about 20 μM, which is itself toxic in this this culture system under these conditions (Rosenberg and Aizenman, 1989; Levy and Lipton, 1990; Levy et al., 1990); thus, the endogenous Glu-like toxin may have been Glu itself.

In the present experiments, the control treatments contained low (1.8 mM) $CaCl_2$ to minimize death, while sibling test cultures contained high (10 mM) $CaCl_2$ to promote toxicity mediated at the NMDA receptor by the endogenous Glu-like compound (Hahn et al., 1988; Levy and Lipton, 1990; Levy et al., 1990). Added to the medium containing high concentrations of Ca^{2+} at the time of plating were the selective NMDA antagonist APV (200 μM), the relatively selective non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), and/or KA (125 μM). The cell cultures were incubated for 18–24 hr after their preparation at 37°C in a humidified atmosphere of 5% CO_2/95% air. Neuronal survival was then scored by counting retinal ganglion cells displaying uptake and cleavage of the fluorescent dye fluorescein diacetate to fluorescein, as described by this laboratory (for color photographs, see Hahn et al., 1988, their Fig. 1). Results are based upon experiments conducted in at least quadruplicate on 3 separate occasions. Prior to statistical analysis, data were normalized so that for any one experiment the mean number of surviving retinal ganglion cells in control low-calcium medium was equal to 100%.

Reagents. Tissue culture reagents were purchased from Sigma Chemical Company and from Gibco. D-(−)-2-Amino-5-phosphonovalerate (APV) was obtained from Cambridge Research Biochemicals; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was obtained from Tocris Neuramin (Essex, UK). All other reagents were purchased from Sigma.

Results

Patch-clamp recordings of EAA agonists and antagonists

Electrophysiological experiments were performed to ensure that the drugs used in the subsequent toxicity studies were truly specific in this preparation. Figure 1*A* shows that the peak current evoked by 200 μM NMDA is only partially (~20%) inhibited by 10 μM CNQX; the steady-state current is not inhibited by CNQX at all (n = 5 retinal ganglion cells). However, as illustrated in Figure 1*B*, this NMDA response can be completely blocked by 200 μM APV. In contrast, the response to 125 μM KA, which patch-clamp recording in this preparation has shown to be totally insensitive to APV (Aizenman et al., 1988), is nearly completely (>90%) abrogated by 10 μM CNQX (Fig. 1*C*). Figure 1*D* shows that CNQX antagonizes the response to Glu to a substantial degree, while the addition of APV blocks only a small remaining fraction of the current; these findings confirm other reports that the non-NMDA component of the response in this preparation is considerably larger than the NMDA contribution (Aizenman et al., 1988; Karschin et al., 1988). Finally, one could argue that the ability of KA to elicit NMDA receptor-activated current would be observed only in the absence of extracellular Mg^{2+}, and the presence of Mg^{2+} in the standard physiological saline used in previous experiments with KA (Aizenman et al., 1988) would have blocked the NMDA receptor-operated channels. Therefore, the absence of antagonism of the KA-activated current by APV could be due to prior blockade by extracellular Mg^{2+} of the NMDA component of the response to KA. However, even with extracellular Mg^{2+} removed from the bath, APV still had no effect on the KA-evoked current (Fig. 1*E*). Thus, at least in this preparation, 125 μM KA does not appear to evoke directly an NMDA receptor-activated current.

Toxicity of KA and NMDA agonists

The data of Figure 2, columns 1 and 2, confirm the finding that an endogenous Glu-like compound in these cultures is toxic to retinal ganglion cells in the presence of elevated extracellular Ca^{2+} (Hahn et al., 1988). This form of endogenous Glu toxicity is mediated at the NMDA subtype of receptor because 200 μM APV completely prevents the death (Hahn et al., 1988; Levy and Lipton, 1990; Levy et al., 1990). Figure 2, column 3, shows that the addition of 125 μM KA produces an incremental degree of retinal ganglion cell killing. In these experiments, the inclusion of 200 μM APV in the culture medium completely blocks the toxicity of the endogenous Glu-like substance as well as the additional KA-induced death (column 4). In contrast, only the KA contribution to toxicity is blocked by 10 μM CNQX (cf. columns 2, 5). These results show that the KA component of excitotoxicity can be prevented by the non-NMDA antagonist CNQX while both the KA and the endogenous components can be completely eliminated by the NMDA-specific antagonist APV.

Discussion

In postnatal rat retinal cultures, KA sets into motion a series of calcium-dependent steps that leads in a delayed fashion to the neurotoxicity of the ganglion cells. In a simple model, KA could in theory exert a toxic action on retinal ganglion cells in this preparation by a variety of alternative pathways. Let us consider in turn the evidence for and against each pathway. KA might activate KA-specific receptors to initiate neuronal toxicity directly. However, the complete prevention of the deleterious effect of KA by specific NMDA antagonists suggests that KA must somehow activate NMDA receptors in order to precipitate a lethal outcome. Alternatively, then, KA might directly interact with NMDA receptors, which in turn produce cell death (Hahn et al., 1988). Nevertheless, the patch-clamp electrophysiology data show that the NMDA receptor antagonist APV does not decrease the electrical responses to KA in these neurons. This result suggests, at least in this preparation at the concentrations

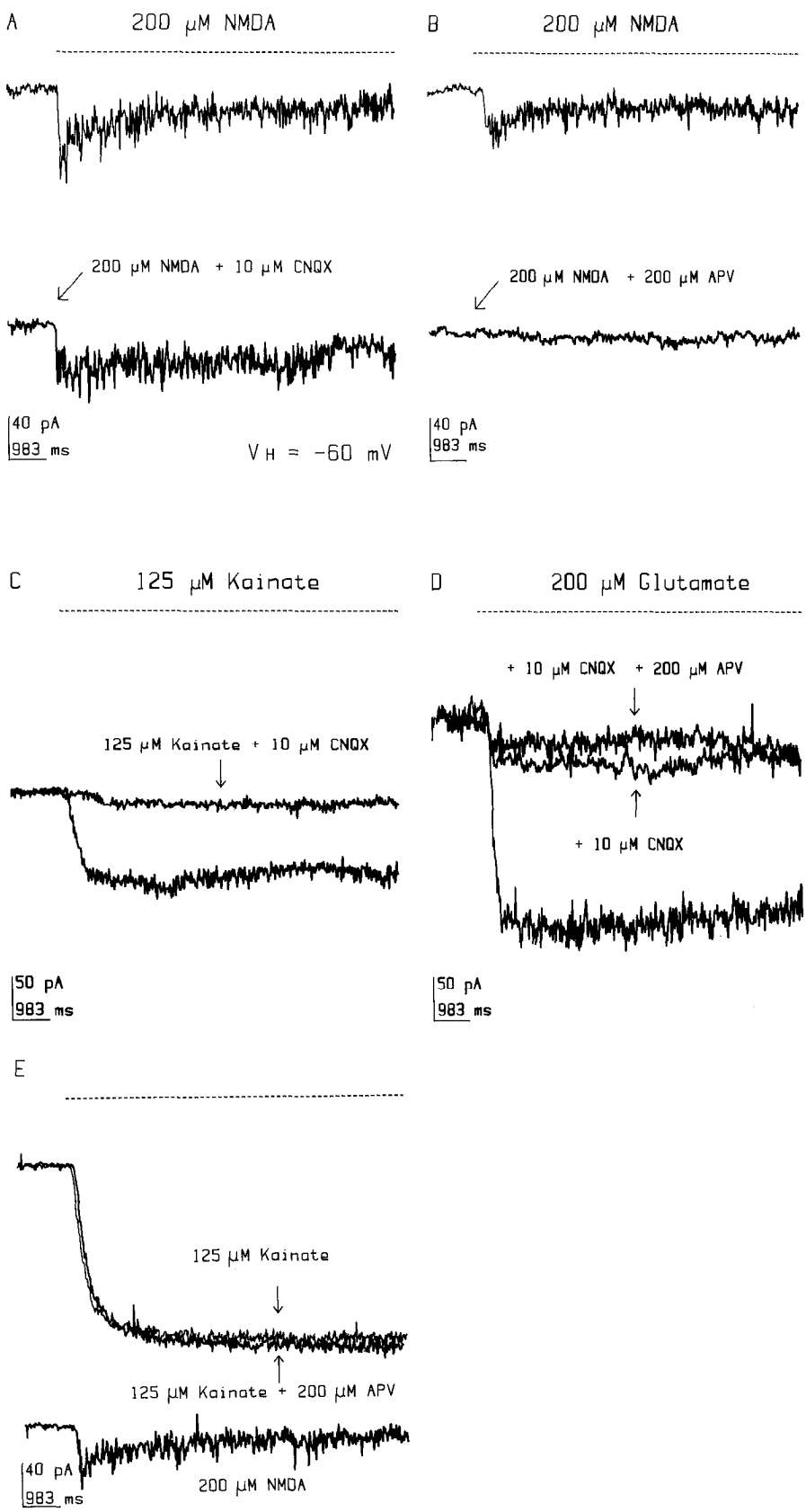

Figure 1. Electrophysiological effects of EAA agonists and antagonists on postnatal rat retinal ganglion cells. Whole-cell current recordings with a patch electrode were obtained at a holding potential of $V_H = -60$ mV. Drugs were applied for the duration indicated by the *broken line* above the traces. Each panel presents a typical recording from a separate ganglion cell. *A*, Responses to NMDA (200 μM) and to NMDA + CNQX (10 μM). *B*, Responses to NMDA (200 μM) and to NMDA + APV (200 μM). *C*, Responses to KA (125 μM) and to KA + CNQX (10 μM). *D*, Responses to Glu (200 μM), to Glu + CNQX (10 μM), and to Glu + CNQX (10 μM) + APV (200 μM). *E*, Responses to KA (125 μM), to KA + APV (200 μM), and to NMDA (200 μM) in the same retinal ganglion cell. See Results for explanation.

under consideration, that KA does not directly elicit an NMDA response. The final possibility is that KA leads to the increase of an endogenous substance that in turn evokes NMDA receptor activation. The findings of the present study are consistent with the latter hypothesis and with other results showing that KA increases the net efflux of an endogenous Glu-related compound in several preparations (see below). Our data show that this endogenous substance contributes to NMDA receptor-mediated neurotoxicity in the retinal ganglion cell neuronal population. Furthermore, the results suggest that it is not KA itself but *only* the endogenous compound that is lethal because APV, which totally prevented neuronal death, should not block a direct toxic effect of KA.

Recently, Michaels and Rothman (1990) and Koh et al. (1990) have raised the alternative hypothesis, outlined above, that a component of the KA (or quisqualate) response could be due to direct activation of NMDA receptors; in this manner, these non-NMDA agonists could directly produce delayed-onset cell death (in the case of KA; Michaels and Rothman, 1990) or acute neuronal swelling (in the case of quisqualate; Koh et al., 1990). However, at least in our preparation, this alternative would appear to be less likely because of the pharmacological evidence gathered in patch-clamp recordings from rat retinal ganglion cells. These recordings have shown that KA (and quisqualate) responses are completely unaffected by NMDA receptor antagonists, such as APV, at concentrations that are sufficient to block totally the electrical response to a maximal nondesensitizing dose of NMDA (Aizenman et al., 1988; Karschin et al., 1988). Given these data, it is difficult to envision a scenario whereby KA would be capable of stimulating NMDA receptors directly, even to a small degree, but the response would be not at all influenced by substantial concentrations of APV. Taken together, the patch-clamp recordings and the toxicity studies of retinal ganglion cells suggest that, rather than acting as a direct toxin, KA increases release of an endogenous substance that in turn leads to neuronal death, at least in this preparation (see also the discussion of CNQX effects, below, and Balázs et al., 1990).

The mechanisms whereby KA might increase Glu or a Glu-like compound have been addressed in other recent reports. In several tissues, KA causes a net release of endogenous Glu and aspartate (Asp; Ferkany et al., 1982; Krespan et al., 1982; Ferkany and Coyle, 1983a,b; Pastuszko et al., 1984; Poli et al., 1985). In some cases, KA may enhance synaptic release of EAAs by stimulating presynaptic receptors (Cox and Bradford, 1978; Ferkany et al., 1982; Krespan et al., 1982; Collins et al., 1983; Coyle, 1983; Ferkany and Coyle, 1983a; Pastuszko et al., 1984; Young et al., 1988). However, there is also evidence that KA-induced blockade of Glu/Asp uptake can be a primary event in producing an increase in extracellular Glu and Asp (Lakshamanan et al., 1974; McGeer et al., 1978; Johnston et al., 1979; Krespan and Padmanaban, 1982; Pastuszko et al., 1984). Along these lines, one recent report in cerebrocortical synaptosomes presented evidence for a slow leak of Glu or Asp and showed that KA could potentiate this Ca^{2+}-independent net efflux of Glu and Asp apparently by inhibiting reuptake; in that system, KA did not affect Ca^{2+}-dependent synaptic release of endogenous Glu (Pocock et al., 1988).

In our preparation, 10 μM CNQX does not inhibit the steady-state NMDA current but nearly totally abolishes the KA-evoked current. Recent reports have shown that the partial inhibition of NMDA-induced current by CNQX in other preparations (and of the peak NMDA current in retinal ganglion cells) is mediated

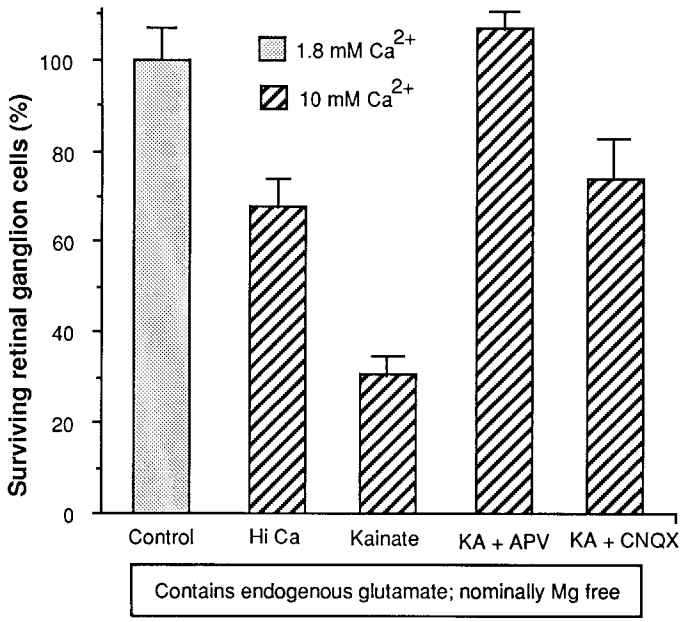

Figure 2. Relative contributions to neurotoxicity of an endogenous Glu-like toxin and of exogenous KA. Mean and SEM of surviving retinal ganglion cells after 1 d in culture are shown for the following groups: control (*column 1*; extracellular $[Ca^{2+}]$ = 1.8 mM); high calcium (*column 2*; extracellular $[Ca^{2+}]$ = 10 mM); 125 μM KA in the presence of high Ca^{2+} (*column 3*); 125 μM KA + APV (200 μM) in the presence of high Ca^{2+} (*column 4*); and 125 μM KA + CNQX (10 μM) in the presence of high Ca^{2+} (*column 5*). Statistical testing with an ANOVA followed by a Scheffé multiple comparison of means revealed the following rank order of results ($P < 0.01$ for each comparison): [Control] = [KA + APV] > [Hi Ca] = [KA + CNQX] > [KA].

at the glycine binding site of the NMDA receptor (Yamada et al., 1989). However, the presence of up to 250 μM glycine in our culture medium did not detract from the protective effect of CNQX against KA toxicity. Under these conditions, CNQX does not block NMDA responses at all. Taken together, these findings suggest that, in our culture system, CNQX prevents the toxic effect of exogenously added KA by antagonism at the KA receptor. These results also make it unlikely that KA toxicity is mediated by direct activation of NMDA receptors because CNQX, acting at the KA receptor and not at the NMDA receptor, can block KA-induced cell death. Moreover, in retinal cultures it would appear more likely that KA-enhanced release of an endogenous Glu-like molecule, rather than block of reuptake, is responsible for the NMDA receptor-mediated toxicity because CNQX might not be expected to affect the reuptake mechanism.

The importance of the present study is its clear demonstration under the relatively well-controlled conditions of cell culture that KA-induced neurotoxicity can result, at least in one preparation, from an indirect effect on the NMDA receptor; the lethal action of KA appears to be mediated by the release of an endogenous Glu-related compound that subsequently activates NMDA receptors. Although the effects of KA may vary from preparation to preparation, evidence that our findings may possibly be generalized to other systems is beginning to mount. For example, in dissociated neonatal hippocampal cultures, KA damage was blocked by dibenzocycloheptenimine (MK-801; Michaels and Rothman, 1990), a relatively selective antagonist of NMDA receptor-operated channels (Huettner and Bean, 1988;

Karschin et al., 1988). Also, in cultured cerebellar granule cells, NMDA antagonists blocked a deleterious component of KA action (Balázs et al., 1990). In addition, in the intact chick retina maintained *in vitro,* APV partially blocked KA-induced toxicity (Olney et al., 1986). In a sense, the indirect toxic action of KA is somewhat surprising in light of the fact that the KA-evoked current is much larger than the NMDA-activated ionic current in our cultured retinal ganglion cells (cf. Fig. 1). However, one explanation for this apparent paradox involves the calcium-dependent nature of the predominant form of excitotoxicity (Choi, 1985, 1987; Garthwaite et al., 1986; Hahn et al., 1988): because only NMDA receptor-operated channels, and not the channels directly activated by KA, are permeable to Ca^{2+} (MacDermott et al., 1986; Mayer and Westbrook, 1987; Mayer et al., 1987), the NMDA contribution to toxicity might be expected to outweigh that of KA. Although KA would be expected to depolarize retinal ganglion cells and thus trigger Ca^{2+} entry via voltage-sensitive calcium channels (Sucher et al., 1990; Weiss et al., 1990), activation of this pathway alone may not be sufficient to elicit neuronal cell death, at least in this preparation. Hence, an additional component of EAA neurotoxicity mediated directly by NMDA receptor activation appears to be necessary for retinal ganglion cell death *in vitro.*

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