Protection by Eliprodil against Excitotoxicity in Cultured Rat Retinal Ganglion Cells

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PURPOSE. To test whether eliprodil (SL 82.0715), a unique antagonist for the N-methyl-D-aspartate (NMDA) receptor, is protective in the glutamate-induced cytotoxicity model in cultured rat retinal ganglion cells (RGCs).

METHODS. Two to four days after a fluorescent dye, DiI, was injected near the superior colliculi, neonatal rats were killed, and retinal cells were dissociated and cultured. Survival of RGCs after drug treatment was assayed by counting DiI fluorescent cells.

RESULTS. In rat RGCs, glutamate-induced toxicity with a mean EC50 of 10.7 μM. Only 47% of RGCs survived after a 3-day treatment with 100 μM glutamate. Studies using selective agonists and antagonists indicated that the glutamate-induced toxicity was mediated largely by the NMDA receptor. Pretreatment with eliprodil protected against such toxicity. Eliprodil exhibited a mean IC50 of 1.0 nM (log [IC50] = −9.00 ± 0.01, mean ± SEM, n = 3; against cell death produced by 100 μM glutamate). At 1 μM, eliprodil was maximally protective; cell survival in the presence of 100 μM glutamate challenge was 100% ± 5% (n = 3). This protective effect of eliprodil may be related to its reduction (by 78%) of NMDA-induced currents recorded under patch-clamp recording in these cells.

CONCLUSIONS. Eliprodil is protective against glutamate cytotoxicity in retinal neurons. It may be a useful novel compound for the treatment of retinopathies including glaucoma in which excitotoxicity has been implicated. (Invest Ophthalmol Vis Sci. 1999;40:1170–1176)
chronic retinoprotective therapy. Therefore, we assessed the effects of eliprodil on EAA-induced neuronal conductance and delayed neuronal death of cultured rat RGCs.

Materials and Methods

RGCs isolated from neonatal Sprague-Dawley rats were used in survival and electrophysiological studies. The treatment of animals and procedures used in these studies conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

RGC Survival Assay

Techniques for the isolation and culture of RGCs were adapted from those reported by Takahashi et al.21 The procedure involved retrograde labeling of ganglion cells by injecting a fluorescent dye, Di-I, into the superior colliculi. Two to 4 days later, retinal cells were dissociated. Cultured RGCs were identified by sufficient Di-I fluorescence to allow them to be observed visually using a fluorescence microscope. The detailed procedures follow.

Neonatal Sprague-Dawley rats 2 to 5 days old were anesthetized by chilling on ice for 10 minutes, after which, a 2 mm midline opening was made in the scalp just caudal to the traverse sinus. The tip of the injection needle (30 gauge) was inserted 6 mm below top of the skull, and a 5 μl Di-I solution, containing 3 mg/ml Di-I (1,1'-dioctadecyl-3,3,3',3'-tetramethylindolo-carbocyanine perchlorate; Molecular Probes, Eugene, OR) in 90% ethanol and 10% dimethyl sulfoxide, was injected. The wound was then covered with a drop of Flexible Collodion (Amend Drug & Chemical, Irvington, NJ). Rats were returned to their mother after warming and recovery from anesthesia.

Two to 4 days after Di-I injection, rats were anesthetized by hypothermia and killed by decapitation. Their eyes were enucleated and placed in Dulbecco's modified Eagle's medium: Nutrient mixture F12 (1:1, DMEM/F12; Gibco, Grand Island, NY). The retina from each eye was detached and isolated. Retinal cells were dissociated by combining 12 retinas with 5 ml of papain solution, containing 10 mg papain (34 units/ml; Sigma, St. Louis, MO), 2 mg dl-cysteine (33 mM; Sigma), and 2 mg bovine serum albumin (0.4 mg/ml; Sigma) in 5 ml of DMEM/F12, for 25 minutes at 37°C, then washed 3 times with 5 ml RGC medium (DMEM [Gibco], supplemented with 10% fetal bovine serum [Hyclone, Logan, UT], 4 mM glutamine [Gibco], 100 U/ml penicillin, and 100 μg/ml streptomycin [Sigma]). Additional RGC medium was added to the retinal pieces to a final total volume of 40 ml. Retinal pieces were triturated by passing through a disposable pipette several times until cells were dispersed. Cell suspension (1.5 ml; containing approximately 4.5 × 10^6 cells) was placed into each of the poly-lysine coated glass-bottomed culture dishes. The cells were cultured for 3 days in 95% air/5% CO₂ at 37°C.

In experiments assessing excitotoxicity, glutamate or other excitatory amino acid analogues were added to the cells 30 minutes after isolation and incubated for 3 days at 37°C with 5% CO₂. For experiments involving MK801 or eliprodil, the antagonist was added to the cells 30 minutes before the addition of glutamate. Three days later, the cells were observed with a fluorescence microscope at 200× magnification, and Di-I-labeled fluorescent cells in 20 microscopic fields were counted and averaged.

Electrophysiology of RGCs

The cell-culturing technique used in the electrophysiological studies was slightly different from that involved in the survival studies. Retinas from newborn rats were isolated and incubated for 1 hour in 5 U/ml papain dissolved in DMEM containing 25 mM Hepes and supplemented with 1.5 mM cysteine, 0.5 mM EDTA, and 2 mM Ca²⁺. They were then gently triturated through a fire-polished Pasteur pipette and resuspended in growth medium containing DMEM, 2 mM glutamine, 17 mM KCl, 5% heat-inactivated fetal calf serum, and serum supplement (MITO; Collaborative Research, Grand Island, NY). Cells were grown on glass coverslips coated with poly-lysine in 24-well plates.

Patch pipettes were pulled from hematocrit glass, fire-polished to a resistance of 2 MΩ to 4 MΩ, and filled with a solution containing NaH₂PO₄ 120 mM, NaCl 10 mM, Hepes 10 mM, EGT 10 mM, and MgATP 4 mM. The extracellular solution contained NaCl 150 mM, CaCl₂ 2 mM, and Hepes 5 mM. The pH values of both solutions were adjusted to 7.4 with NaOH. Recordings were obtained using an Axopatch 1D amplifier. Drugs were applied via a series of computer-controlled flow pipes (outer diameter, 358 μm).

Whole-cell recordings were obtained from RGCs cultured for 10 to 14 days. Ganglion cells were distinguished from amacrine cells, the other class of neurons with regenerative sodium currents, by their greater size and larger resting potential, and a more slowly accommodating action potential.22 Virtually every ganglion cell examined at this age responded to NMDA.

Test Compounds

Glutamate, quisqualate, NMDA, and MK801 (dizocilpine) were obtained from Research Biochemicals (Natick, MA). Eliprodil HCl was provided by Synthelabo Recherche (Bagneux, France). All other chemicals were obtained from Sigma Chemical.

Results

Retrograde uptake of the fluorescent dye Di-I after its injection into the superior colliculi should label only ganglion cells in the retina. Thus, the presence of Di-I fluorescence distinguished RGCs from other retinal cells in culture (Fig. 1). After 3 days in culture, the surviving RGCs appeared to be morphologically healthy. Ganglion cells adhered to poly-lysine-coated culture dishes with flat cell bodies and occasional neuritelike extensions (Figs. 1A, 1B). Treatment with glutamate for 3 days was toxic to RGCs. It increased the probability of cells with an aberrant appearance, such as the presence of many vacuole-like structures in the cytoplasm (Figs. 1E, 1F), and cell fragments (Figs. 1C, 1D). Additionally, glutamate treatment also decreased the total number of fluorescent cells. Under control conditions, there were 11.4 ± 0.9 Di-I-labeled cells/microscopic field (mean ± SEM of 30 studies, 20 individual fields were counted in each study). Glutamate decreased the number of surviving cells in a concentration-dependent fashion. A representative concentration-response curve for glutamate-induced excitotoxicity is shown in Figure 2A. The mean EC₅₀ for
glutamate was calculated to be 10.7 μM (log \( [EC_{50}] \) = −4.97 ± 0.13; mean ± SEM, \( n = 3 \)). Compared to controls, only 47% ± 2% (\( n = 14 \)) of cells survived after a 3-day treatment of 100 μM glutamate. Currently, we do not know whether cells that survived the glutamate treatment were different from those that did not. However, treating the cells with glutamate for 5 days did not further decrease the survival rate (data not shown). In initial pilot studies in which retinal cells were cocultured with additional Müller cells, glutamate was much less potent: its apparent \( EC_{50} \) decreased by 10- to 100-fold (data not shown).

Glutamate interacts with many receptor subtypes. To define the contribution of various glutamate receptor subtype(s) to glutamate-induced damage, we compared the excitotoxicity of a variety of subtype-selective glutamate agonists. NMDA (selective to NMDA receptors), kainate (selective to ionotropic AMPA/kainate receptors), and quisqualate (interacts with both the ionotropic AMPA/kainate receptors and metabotropic receptors) were tested on rat RGCs. As shown in Figure 2B, at 100 μM, only NMDA and glutamate caused toxicity. Kainate and quisqualate were ineffective. NMDA-induced toxicity was also concentration-dependent (Fig. 2C), with a mean \( EC_{50} \) of 1.82 μM (log \( [EC_{50}] \) = −5.74 ± 0.35, mean ± SEM, \( n = 5 \)). These results suggest that NMDA receptors are responsible for the majority of glutamate-induced toxicity in these cells. To further confirm this finding, a selective NMDA receptor antagonist, MK801, was tested for its ability to antagonize the excitotoxic effects of glutamate. MK801 was highly protective against the glutamate cytotoxicity, with maximal protection of 100%. A representative concentration-response curve for MK801 protection is shown in Figure 3A. The mean \( IC_{50} \) value of MK801 was 20.9 nM (log \( [IC_{50}] \) = −7.68 ± 0.41, \( n = 3 \)) in the presence of 100 μM glutamate, which translates to an apparent pKi for MK801 of 8.69 ± 0.41. These values agree well with the published affinity of MK801 for NMDA receptors.

Pretreatment of the retinal cells with eliprodil for 30 minutes protected cells against the toxic effects of glutamate. Figure 3B illustrates the concentration-effect relation for eliprodil. The protective effect of eliprodil exhibited a mean \( IC_{50} \) of 1.0 nM (log \( [IC_{50}] \) = −9.00 ± 0.01, mean ± SEM, \( n = 3 \)) against 100 μM glutamate. At 1 μM, eliprodil was maximally protective; cell survival was 100% ± 5% (\( n = 3 \)) despite the presence of glutamate.

To investigate potential mechanism(s) involved in the protective action of eliprodil, we directly measured the effect of eliprodil on EAA-induced inward currents in RGCs. Figure 4 shows inward currents evoked in RGCs by rapid application of

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**Figure 1.** Photomicrographs of cultured rat retinal cells. (A, C, E) Photographs taken under phase optics; (B, D, F) fluorescence photographs of the same cells. (A, B) Cells cultured under control conditions. Scale bar, 50 μm. (C, D, F) Examples of cells with aberrant morphology after they were treated with 100 μM glutamate for 5 days. Scale bar, 15 μm.
FIGURE 2. Effect of excitatory amino acids on cell survival in rat RGCs. Cells were treated with the indicated amounts of compounds, and survival was determined by counting the survived, Di-I-labeled cells. Survival with vehicle alone defines 100%. (A) A representative concentration-response curve of glutamate. Each symbol represents a single datum point. Similar results were obtained in three independent studies each in duplicate. (B) Effect of various compounds (100 μM each) on survival of rat RGCs. Each bar represents mean result of each treatment ± SEM. Numbers of studies are shown at the bottom of each bar. (C) A representative concentration-response curve of NMDA. Each symbol represents a single datum point. Similar results were obtained in 3 independent studies, each in duplicate.

200 μM NMDA plus 1 μM glycine. (This concentration of glycine was added to mimic the saturating concentrations of glycine found in culture media.) Eliprodil reduced the NMDA-evoked response in a dose-dependent fashion. Both the peak and steady state components of the response were reduced. The peak responses versus eliprodil concentrations are plotted in the lower panel of Figure 4. The maximal blockade of the NMDA response by eliprodil was approximately 78%, with a calculated IC50 of 2.3 μM. Incomplete block of NMDA currents by eliprodil has been observed previously in cortical neurons and by ifenprodil, an eliprodil analogue, on hippocampal neurons.

The onset of inhibition of NMDA inward currents by eliprodil was relatively rapid, as is illustrated in Figure 5. The left trace of the upper panel shows a current response to NMDA alone. Starting with the next trace, NMDA was applied for 2 seconds at 16-second intervals, and eliprodil was applied continuously to the cell. The lines above each record indicate NMDA application. Almost all the total blockable current was inhibited with the first application of NMDA (middle versus right traces). In contrast, recovery of...
Eliprodil

NMDA 200 μM

0.2 μM

2.0 μM

20 μM

50 pA

1 sec

FIGURE 4. Current evoked by NMDA is reduced in the presence of eliprodil. Top: four sample records showing response to 200 μM NMDA alone and in the presence of 3 concentrations of eliprodil. Here eliprodil was applied before and during exposure to NMDA, but results were similar when they were coapplied. Holding potential: −50 mV. Bottom: peak NMDA response for 4 cells at 3 concentrations of eliprodil. Data were fitted by the equation: (1 + Max) (1 + ([Eliprodil]/IC_{50}^{N}) + Max, where Max is the fraction of residual current in the presence of saturating concentrations of eliprodil; IC_{50} is the concentration of eliprodil that produced 50% inhibition; Eliprodil is the concentration of eliprodil; and N is the Hill coefficient. The curve is obtained with Max = 0.22, IC_{50} = 2.3 μM, N = 1.16.

inhibition after washout of eliprodil occurred much more slowly. The first application of NMDA in the absence of eliprodil revealed only slight recovery (top right versus bottom left), and only approximately 50% of the current was restored after 5 applications (bottom panel). A slow off rate of eliprodil has been reported previously.25

DISCUSSION

We have shown that in cultured rat RGCs, EAA markedly decreased survival. Although functional glutamate receptors of NMDA and non-NMDA subtypes are expressed in these cells,26,27 EAA-induced toxicity appears to be mediated by NMDA receptors, because only glutamate and NMDA, but not quisqualate or kainate, were cytotoxic. Furthermore, the selective NMDA antagonist MK801 was potent and efficacious in preventing the excitotoxic effects of glutamate. Interestingly, with application of glutamate, only approximately half of the RGCs were susceptible to the toxic insult. In our experimental conditions, we never observed a complete loss of the cells. It is currently unclear whether the neurons that survived the insult were phenotypically different from those that did not. One possible explanation is suggested by the report of Dreyer et al.,10 that ganglion cells of larger size are more sensitive to NMDA receptor-mediated death. Studies are being conducted to test this and other hypotheses.

The RGC culture model used in the survival assay was almost identical to that described by Takahashi et al.,21 with one major difference. In their study, a confluent monolayer of glial cells (retinal Müller cells or cortical astrocytes) was present in the retinal cell culture to assist and sustain the growth of RGCs, whereas, in our studies, the feeder cells were eliminated. We found that, by doubling the density of retinal cells in culture to approximately 3 × 10^6 cells/ml, sufficient
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Di-I-prelabeled and morphologically normal cells were available for quantification at the time of assay. More important, in the absence of a feeder layer, the potency of glutamate toxicity increased by at least 10-fold. It is probable that Müller cells and cortical astrocytes can interfere with the actions of glutamate by uptake into glial cells, decreasing its effective concentration for the ganglion cells.

Our electrophysiological results provide direct evidence that ganglion cells in these cultures express NMDA receptors and that currents elicited by activation of these receptors are inhibited by eliprodil. These data provide a potential mechanism for the neuroprotective properties of eliprodil demonstrated here, although a higher concentration of eliprodil is required to block the NMDA current than to provide neuroprotection. One possible explanation is the dramatic difference in exposure times in these two experiments. The 3 days of exposure to eliprodil that are required for the neuroprotection experiments might have induced an additional high-affinity mechanism of NMDA block that is not apparent during the brief exposure used in the electrophysiological experiments. It would be interesting to measure NMDA responses in ganglion cells that have undergone long-term exposure to eliprodil.

Thus, we demonstrated that eliprodil was effective in blocking both glutamate-induced inward currents and cytotoxicity in RGCs. These results agree with previous reports that eliprodil is an antagonist affecting the polyamine site of NMDA receptors (cf. Ref. 19). Eliprodil and its analogues have been suggested to interact selectively with one subtype of NMDA receptor (i.e., one comprised of heteroligomeric complexes of NR1A and NR2B). Pharmacologically, it has been shown that eliprodil blocks NMDA-induced responses in a variety of neuronal cell types. For example, eliprodil inhibits the depolarizing effects of NMDA on cultured mouse spinal neurons, reduces the NMDA-induced inward current in cultured rat cortical neurons, suppresses NMDA-stimulated production of cGMP in cerebellar slices, and blocks acetylcholine release in striatal slices. In addition to excitotoxicity, eliprodil was also effective in reduc-
ing neuronal damage due to ischemia/anoxia, both in vivo\textsuperscript{20,31,32} and in hippocampal slices in vitro.\textsuperscript{33}

The in vitro protective effects of eliprodil on retinal cells we demonstrate here predicted that the compound may be protective against EAA-induced toxicity in the retina in vivo. Indeed, as demonstrated in the accompanying manuscript,\textsuperscript{34} eliprodil is effective in a dose-dependent fashion in protecting the rat retina from damages due to NMDA in vivo. Furthermore, it also minimized retinal functional changes caused by pressure-induced ischemia in the rabbit eye.

Hence, our data show that eliprodil reduces EAA-evoked membrane conductance and ultimate cell loss in cultured rat RGCs. Taken together with our in vivo data,\textsuperscript{34} this suggests that eliprodil may be a useful compound for the treatment of various retinopathies, especially those related to glaucoma, and/or retinal ischemia.

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**References**