

A Non-Excitatory Paradigm of Glutamate Toxicity

WEN SHEN AND MALCOLM M. SLAUGHTER

Department of Physiology and Biophysics and Department of Ophthalmology, School of Medicine, State University of New York, Buffalo, New York 14214

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Shen, Wen and Malcolm M. Slaughter. A non-excitatory paradigm of glutamate toxicity. *J Neurophysiol* 87: 1629–1634, 2002; 10.1152/jn.00532.2000. Retinal ganglion cells are driven by glutamatergic synapses, but they are also very susceptible to glutamate toxicity. Whereas the conventional excitotoxicity model of glutamate-induced cell death requires membrane depolarization, we have found that glutamate toxicity need not be linked with excitation. A large subset of ganglion cells possesses high-affinity kainate receptors that are calcium permeable. At 1–5 μM , kainate produced elevation of internal calcium but did not significantly depolarize ganglion cells. This low concentration of kainate caused ganglion cell death, which could be inhibited by specific kainate receptor antagonists. The toxic effect of kainate may be associated with calcium influx, because toxicity was reduced by polyamines that suppress calcium influx and by an inhibitor of calcium phosphatase. Thus activation of ionotropic glutamate receptors can produce neurotoxicity uncoupled from neuroexcitation.

INTRODUCTION

Glutamate is essential for synaptic communication in the CNS, but inadequate regulation of extracellular glutamate can lead to neurodegenerative disorders. The established model for glutamate-induced neuronal cell death is excitotoxicity (Olney 1982; Olney and Price 1983), a mechanism in which excessive cell depolarization leads to apoptosis or necrosis. Cell death is generally initiated by elevation of intracellular calcium (Choi 1994). The excitotoxicity model might be expected to apply during prolonged and augmented excitation in acute traumatic events such as ischemia (Zhang and Lipton 1999), stroke (Launes et al. 1998), and epilepsy (Prince et al. 1995). However, there are other forms of neurodegeneration that have an imperceptible onset and may not involve neuroexcitation. An example is open-angle glaucoma, a disease of retinal ganglion cells linked to elevated extracellular glutamate (Dreyer 1998). We found that kainate-type glutamate receptors can cause elevated internal calcium and cell death without neuronal excitation.

METHODS

Electrophysiological experiments were performed on acutely isolated neurons from the tiger salamander retina, *Ambystoma tigrinum* (Shen and Slaughter 1998). Ganglion cells were identified based on morphology and voltage clamp profiles (soma > 15 μm , I_{Na} > 1 nA; large outward and no inward rectifying potassium current). Ringer solution contained the following: 111 mM NaCl, 3 mM KCl, 2 mM

CaCl_2 , 1 mM MgCl_2 , 10 mM dextrose, and 5 mM HEPES (pH 7.8). Recording pipettes were filled with 110 mM potassium gluconate, 5 mM NaCl, 0.1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM EGTA, and 5 mM HEPES (pH 7.4).

Calcium imaging

Retinal neurons were loaded with 5 μM Fluo-4AM. An upright Olympus microscope or a laser-scanning confocal BioRad MRC-1024 system detected fluorescent images. Both systems were also used to view stained neurons. Fura-2AM was loaded into cells and used to quantitate changes in internal calcium using a standard 340/380 nm ratioetric imaging procedure and applying the formula:

$$[\text{Ca}] = K_D \left(\frac{F_O}{F_S} \right) \frac{R - R_{\text{MIN}}}{R_{\text{MAX}} - R} \quad R = \frac{F_{340}(\text{Ca}^{2+} - \text{bound fura-2})}{F_{380}(\text{unbound fura-2})}$$

where $[\text{Ca}]$ is the calculated calcium level, K_D of fura-2 = 224 nM, R_{MIN} = 0.106 of calcium-free fura-2, R_{MAX} = 4.112 of saturated fura-2, and F_O/F_S = 10.418, which is the ratio of fluorescence intensity at 380 nm with zero bath calcium over fluorescence in saturated bath calcium.

Histochemistry

The cobalt staining technique identified calcium-permeable glutamate receptors (Pruss et al. 1991). The retina was treated with drugs and incubated in cobalt solution, precipitated in 1.2% $(\text{NH}_4)_2\text{S}$, and enhanced with 0.2% AgNO_3 .

Acridine orange staining was used to monitor cell toxicity (Frey 1995; Leite et al. 1999). The retina was treated with drugs and stained with 100 ng/ml acridine orange. Toxicity was quantified by scanning cells and plotting the intensity of staining across the surface area of each neuron. A skewed intensity distribution is indicative of cell toxicity. Alternatively, the APO-BrdU terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay with an Alexa Fluor 488 conjugate (Molecular Probes) was used as a marker of presumptive apoptotic cell death. For either method, the number of dye-positive cells was counted in a number of random $700 \times 700 \mu\text{m}^2$ fields, and the results were expressed as a mean \pm SD.

RESULTS

Low doses of kainate increased intracellular calcium in isolated retinal ganglion cells. In the cell shown in Fig. 1, A and B, 5 μM kainate increased Fluo-4 fluorescence by almost 600% ($419 \pm 244\%$ in 39 cells). On average, 20 μM kainate produced a $650 \pm 160\%$ increase, while 50 and 100 μM

Address for reprint requests: W. Shen, SUNY, Dept. of Physiology and Biophysics, 124 Sherman Hall, Buffalo, NY 14214 (E-mail: wenshen@acsu.buffalo.edu).

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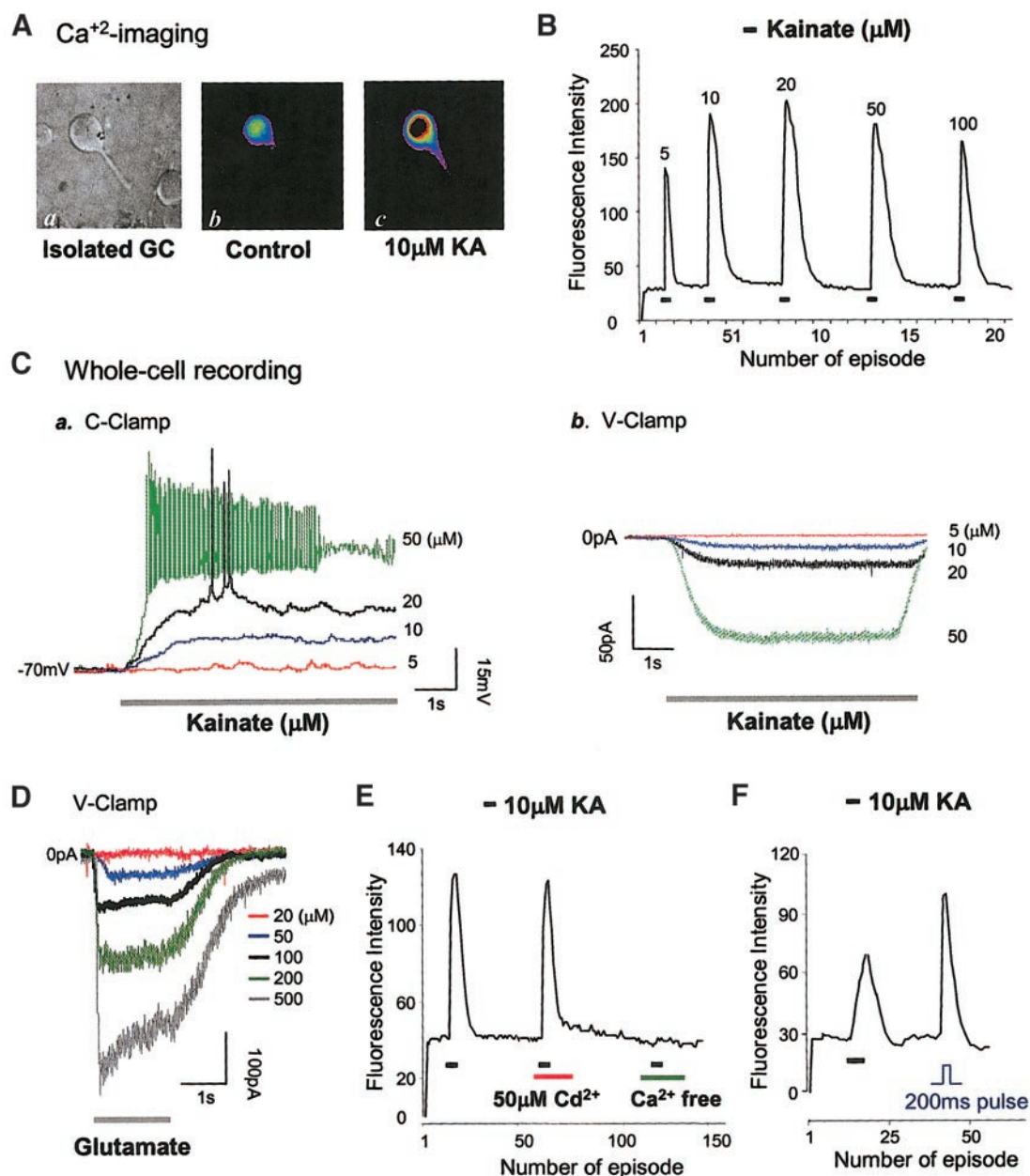


FIG. 1. Effects of glutamate receptor activation on internal calcium and electrical activity in ganglion cells. *A*: differential interference contrast image and Fluo-4 fluorescence before and during treatment with kainic acid. *B*: Fluo-4 signal induced by various kainate concentrations. *C*: effect of 5–50 μM kainate on whole cell current and voltage responses of a ganglion cell. *D*: ganglion cell current responses to 20–200 μM glutamate. *E*: Fluo-4 signal in a ganglion cell treated with 10 μM kainate alone (dark bar below trace), with cadmium, or in the absence of extracellular calcium. *F*: Fluo-4 signal in a ganglion cell produced by either kainate application (dark bar) to a voltage clamped ganglion cell or brief (200 ms) depolarization of the cell to 0 mV. Holding potential was -70 mV.

kainate were slightly less effective. Measurements using fura-2 indicated that 5 μM kainate increased average internal calcium in isolated cells by 198 nM, whereas 20 μM kainate increased average calcium by 642 nM. However, 5 μM kainate had a negligible effect on membrane voltage or current (Fig. 1C). A slight depolarization and small inward current was produced by 10 μM kainate, but this failed to induce spike activity. A few spikes were initiated by 20 μM kainate, whereas 50 μM kainate caused a pronounced depolarization and vigorous spike activity leading to accommodation. Thus there is a disparity between kainate-induced calcium loading and neuronal excita-

tion. Glutamate had a similar effect. Low concentrations of glutamate produced an insignificant current (Fig. 1D), yet produced a notable increase in internal calcium (Fig. 2E).

The source of this calcium is influx through calcium-permeable kainate receptor channels. Kainate produced an equivalent increase in internal calcium when voltage-gated calcium channels were blocked by cadmium but no effect when extracellular calcium was removed (Fig. 1E). Kainate increased internal calcium when neurons were voltage clamped to -70 mV, although a voltage pulse to 0 mV could increase internal calcium. Collectively, these results indicate that kainate stim-

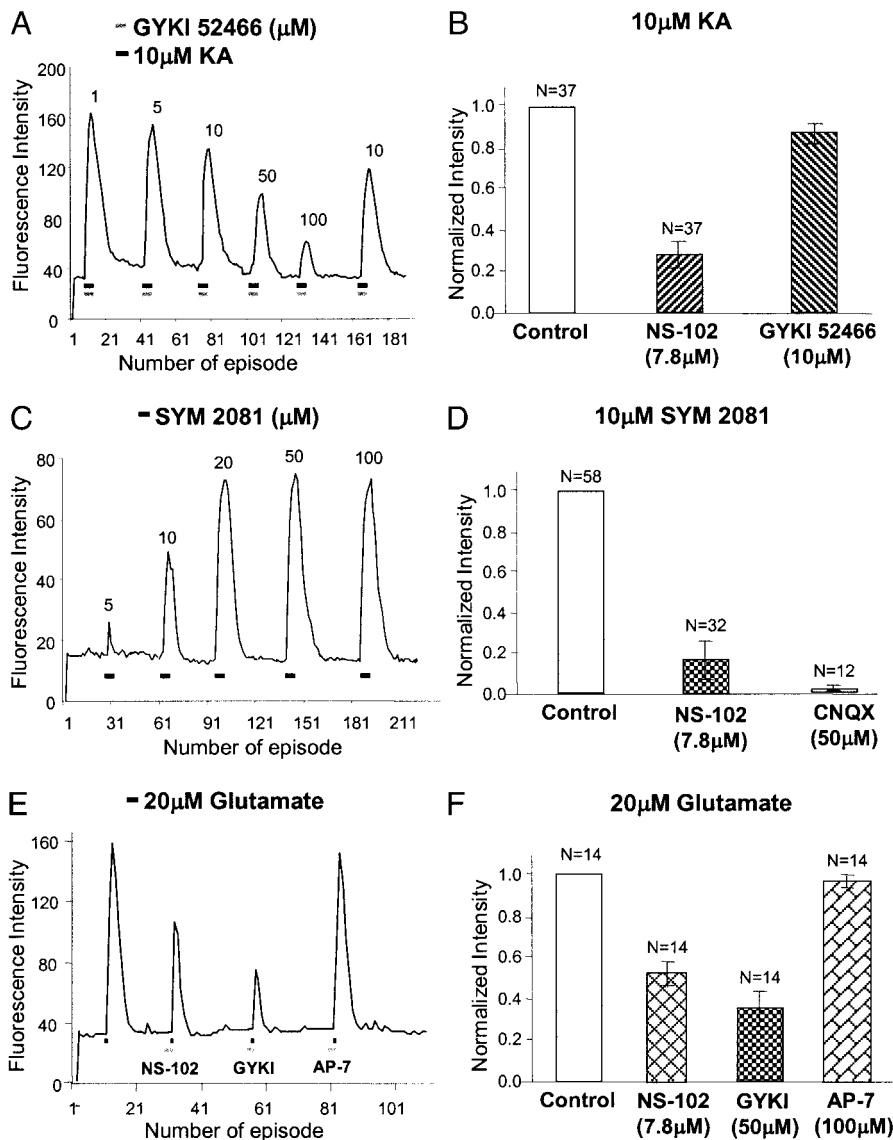


FIG. 2. Kainate receptors elevate internal calcium in isolated ganglion cells. *A*: effect of several GYKI 52466 concentrations on kainate-stimulated internal free calcium. *B*: effectiveness of kainate (NS-102) and AMPA (GYKI 52466) receptor antagonists on kainate-stimulated increases in internal calcium. *C*: SYM 2081 stimulated increases in internal calcium. *D*: effects of glutamate receptor antagonists on action of SYM 2081. *E* and *F*: effects of receptor antagonists on glutamate-induced elevation of internal calcium.

ulates calcium influx that is independent of voltage-activated channels.

Kainate can activate both kainate and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. GYKI 52466 (1-[4-aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine) is a noncompetitive AMPA receptor antagonist. In retinal ganglion cells, GYKI 52466 blocks AMPA receptors with an IC_{50} of 3.6 μM (Lukasiewicz et al. 1997). In our experiments, 1–5 μM GYKI 52466 had little effect on calcium elevation produced by 10 μM kainate. Ten micromolar GYKI 52466 reduced the kainate effect by an average of $16 \pm 2.4\%$ ($n = 18$; Fig. 2, *A* and *B*). NS-102, a selective kainate receptor antagonist (Lerma et al. 1993; Pollard et al. 1993), was a much more potent antagonist of the kainate-induced response (Fig. 2*B*). SYM 2081, a selective kainate receptor agonist (Pollard et al. 1993; Zhou et al. 1997), mimicked the effect of kainate (Fig. 2*C*). SYM 2081 was blocked by NS-102 as well as CNQX, a broad-spectrum glutamate receptor antagonist (Fig. 2*D*). LY382884, another selective kainate receptor antagonist, also blocked the effect of 5 μM kainate on internal calcium. Thus selective activation of high-

affinity kainate receptors elevates internal calcium in retinal ganglion cells.

Low doses of glutamate had a similar effect, which was only partially blocked by 8 μM NS-102 ($51 \pm 4\%$ of control; $n = 14$; Fig. 2, *E* and *F*). Interestingly, none of the effects of 20 μM glutamate could be blocked by AP-7, an *N*-methyl-D-aspartate (NMDA) receptor antagonist ($96 \pm 1\%$ of control). NS-102 blocked only about one-half the glutamate-induced calcium elevation, while it reduced 70% of kainate's effect. Therefore low doses of kainate are fairly selective for kainate receptors, whereas glutamate may also activate AMPA or metabotropic glutamate receptors that can also contribute to elevated internal calcium.

Calcium-imaging experiments demonstrated that kainate had marked effects on internal calcium in isolated ganglion cells. This was evaluated in the intact retina using cobalt staining, which detects calcium permeable ligand-gated, but not voltage-gated, channels. When the retina was treated with 5 μM kainate, many of the ganglion cells were darkly stained, which is indicative of high calcium influx (Fig. 3*Aa*).

As a measure of neuronal cell death, acridine orange was

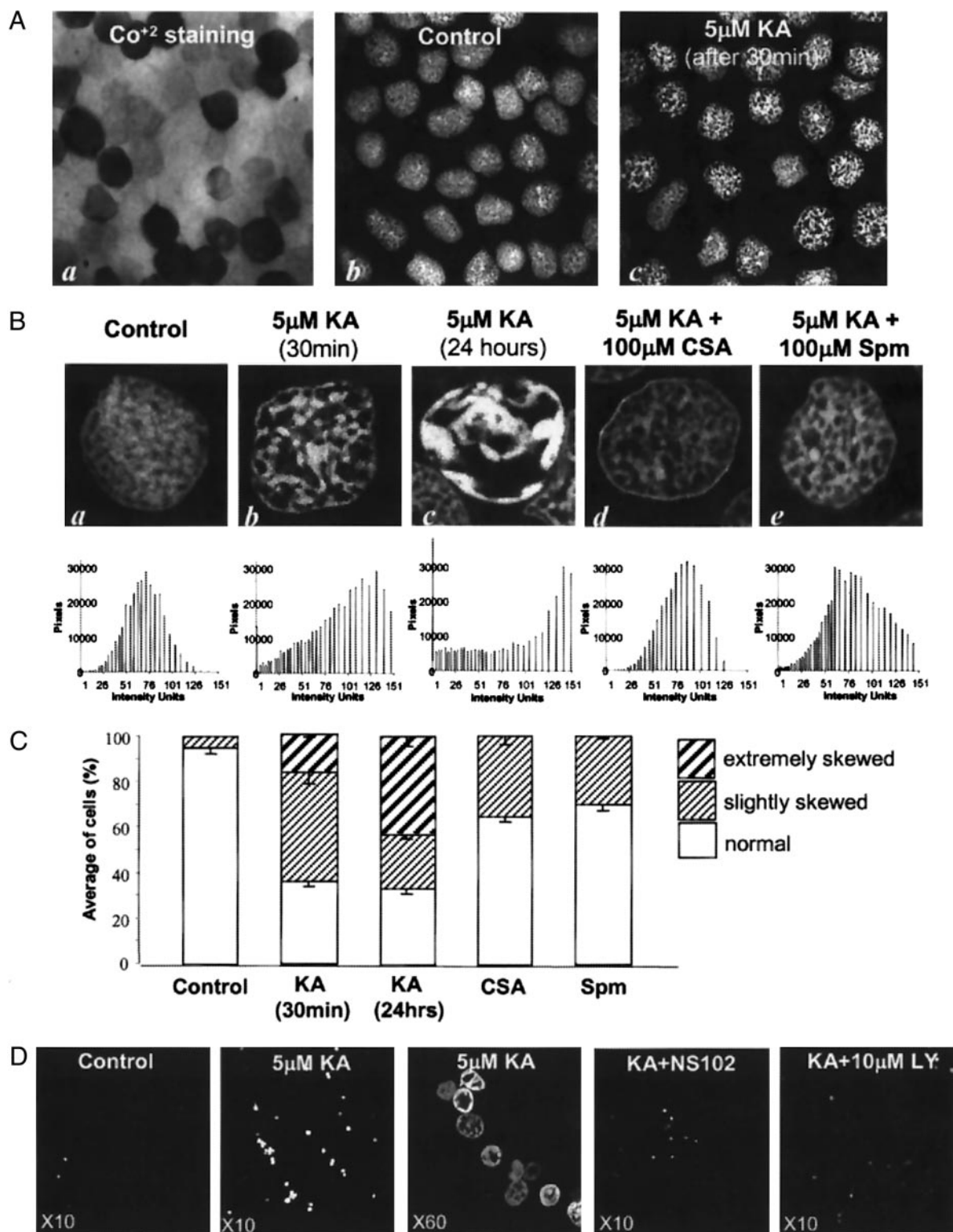


FIG. 3. Kainate-induced toxicity in intact retina. *Aa*: cobalt staining viewed in ganglion cell layer. *Ab*: acridine orange staining of retinal ganglion cell layer under control conditions and (*Ac*) after kainate application for 30 min. *B*: acridine orange staining of single cells in the intact retina and plot of fluorescent intensity distribution across the cell under various conditions: control (*Ba*); 30 min after kainate treatment (*Bb*); 24 h after kainate treatment (*Bc*); after treatment with kainate and cyclosporin A (*Bd*); and after treatment with kainate and spermine (*Be*). *C*: summary analysis of cells from multiple fields in 3–5 retinas under each of these conditions (error bars indicate SD). *D*: TUNEL staining of the ganglion cell layer under control conditions, after kainate alone, or kainate in the presence of the antagonists NS-102 or LY382884. *Middle panel*: portion of second panel at higher magnification. All images were taken in the plane of the ganglion cell layer from flat mounted retinas using a confocal microscope.

used to stain nucleic acids, and the pattern of staining in the ganglion cell layer of the intact retina was viewed using a confocal microscope. Nuclear staining was diffuse under control conditions (Fig. 3, *Ab* and *Ba*). Brief treatment (30 min) with 5 μ M kainic acid produced nuclear aggregates without cell swelling (Fig. 3, *Ac* and *Bb*). More prolonged treatment (24 h) with 5 μ M kainic acid resulted in a pronounced nucleic acid clumping and reduced cell density (Fig. 3*Bc*). To compare different conditions, we scanned each cell body in a field and measured the fluorescence intensity of each pixel over the surface of each cell. Under control conditions most somas had a fairly random distribution of intensities, and therefore a histogram showed an approximate Gaussian intensity distribution (Fig. 3*Ba*). However, after a short treatment with kainic acid, many cells showed patches of fluorescence, and consequently the intensity distribution for those cells was slightly skewed (Fig. 3*Bb*). More prolonged kainate treatment resulted in a very clumped fluorescence and an extremely skewed intensity distribution (Fig. 3*Bc*). We ranked each cell in the field based on this intensity distribution and expressed them as a percentage of all the cells (Fig. 3*C*). This was done for multiple fields in 3–5 retinas for each treatment condition. Without kainate treatment, $95 \pm 3\%$ of the cells had a near-Gaussian distribution; the rest had a slightly skewed distribution. Treatment with 5 μ M kainic acid caused more cells to be skewed, and the effect was much more pronounced after 24 h compared with 30 min (Fig. 3*C*). Spermine is a polyamine that inhibits calcium permeable glutamate receptors (Bowie et al. 1998) and reduced kainate currents in ganglion cells. Spermine suppressed the nuclear accretion produced by kainic acid (Fig. 3*Be*). In the presence of spermine, the number of ganglion cells with a normal distribution of intensities was almost double that in the absence of spermine ($P < 0.01$), although it was still less than control conditions. One mechanism of cell death is calcium-stimulated dephosphorylation, which can be blocked by cyclosporin A. After pretreatment with 100 μ M cyclosporin A, 5 μ M kainate (45 min) produced less aggregation in the nucleus (Fig. 3*Bd*). The number of cells with a normal intensity distribution was significantly greater when cyclosporin A was present ($P < 0.01$). Similar results were obtained with an alternative measure of cell death: the TUNEL stain. An example of this method is illustrated in the evaluation of kainate receptor antagonists in multiple fields from two retinas. Under control conditions, the mean number of TUNEL positive cells per field was 4 ± 1 . Treatment with 5 μ M kainate for 45 min produced 56 ± 6 positive cells per field. In the presence of 8 μ M NS-102 plus 5 μ M kainate this was reduced to 23 ± 8 positive cells per field, and in the presence of 10 μ M LY382884 plus 5 μ M kainate, the number of positive cells per field was 19 ± 4 cells. The effects of both kainate receptor antagonists were statistically significant ($P < 0.01$). These experiments demonstrate that “nonexcitatory” levels of kainate receptor activation can produce cell damage. It suggests that calcium influx might contribute to cell death, and this may be mediated, in part, by activation of calcium phosphatases.

DISCUSSION

A subpopulation of retinal ganglion cells possesses kainate receptors with comparatively high calcium permeability based on cobalt staining and calcium imaging. In rat retina, glutamate

receptors with differing calcium permeabilities are expressed in subpopulations of ganglion cells (Zhang et al. 1995), and rabbit retina is sensitive to low micromolar kainate concentration (Marc 1999a,b). Furthermore, these low concentrations can be toxic to neurons (Carriedo et al. 2000; Cheung et al. 1998). Our experiments reveal that glutamate’s calcium and electrical signals are separable and relay different information about extracellular glutamate concentrations. We focused on the pathological ramifications, but glutamate could also be involved in normal synaptic communication.

Weak activation of excitatory amino acid receptors may be a particularly effective method of elevating intracellular calcium. The driving force for calcium entry is maximal when neurons are near their resting potential. Furthermore, calcium permeable AMPA and kainate receptors are blocked by polyamines in a voltage-dependent manner. Therefore as neurons become more depolarized, both the driving force and polyamine block reduce the calcium influx per channel.

Another distinctive feature of kainate receptors in retinal ganglion cells is that they do not seem to be synaptic (Lukasiewicz et al. 1997). Therefore kainate acid receptors are uniquely positioned to report elevated levels of nonsynaptic glutamate. Dreyer (1998) found that glutamate levels are higher in the vitreous of patients with glaucoma. This is intriguing because it places extracellular glutamate near the ganglion cells, which might explain why these neurons are preferentially damaged in glaucoma. Furthermore, it has been shown that strong stimulation of AMPA receptors rapidly reduces their calcium permeability, possibly through a change in subunit composition (Liu and Cull-Candy 2000). Because kainate receptors are less stimulated, they are likely to have relatively greater calcium permeability. All of these factors may contribute to kainate receptor toxicity in this system.

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