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MOLECULAR PHARMACOLOGY, 53:564–572 (1998).

MOLECULAR PHARMACOLOGY

Ca^{2+}-Independent Excitotoxic Neurodegeneration in Isolated Retina, an Intact Neural Net: A Role for Cl^- and Inhibitory Transmitters

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Received August 4, 1997; Accepted November 26, 1997 This paper is available online at http://www.molpharm.org

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ABSTRACT

Rapidly triggered excitotoxic cell death is widely thought to be due to excessive influx of extracellular Ca^{2+}, primarily through the *N*-methyl-D-aspartate subtype of glutamate receptor. By devising conditions that permit the maintenance of isolated retina in the absence of Ca^{2+}, it has become technically feasible to test the dependence of excitotoxic neurodegeneration in this intact neural system on extracellular Ca^{2+}. Using biochemical, Ca^{2+} imaging, and electrophysiological techniques, we found that (1) rapidly triggered excitotoxic cell death in this system occurs independently of both extracellular Ca^{2+} and increases in intracellular Ca^{2+}; (2) this cell death is highly dependent on extracellular Cl^-; and (3) lethal Cl^- entry occurs by multiple paths, but a significant fraction occurs through pathologically activated γ-aminobutyric acid and glycine receptors. These results emphasize the importance of Ca^{2+}-independent mechanisms and the role that local transmitter circuitry plays in excitotoxic cell death.

Although early experiments indicated that excitotoxic neurodegeneration was due to Na^+ and Cl^- entry into cells on overactivation of glutamate receptors (Price *et al*., 1985; Rothman, 1985), the role of Ca^{2+} entry has been the focus of much of the work in this field (Choi, 1994). For example, studies by Choi (1985, 1987) with cultured embryonic rodent cortical cultures, Rothman *et al*. (1987) with cultured embryonic hippocampal neurons, and Garthwaite and Garthwaite (1986) and Garthwaite *et al*. (1986) with cerebellar slices have provided evidence that in these systems, neuronal death is triggered by the excessive entry of extracellular Ca^{2+} on stimulation of ionotropic glutamate receptors, particularly the Ca^{2+}-permeant NMDA receptors (McBain and Mayer, 1994). In fact, rapidly triggered neuronal death *in vitro* on activation of non-NMDA receptors has been reported to occur only in those relatively rare cultured cortical cells expressing Ca^{2+}-permeant AMPA receptors (Weiss et *al*., 1994; Gottron et *al*., 1995). This is an attractive mechanism because Ca^{2+} is an important intracellular signaling molecule and plausible hypotheses explaining cellular dysfunction on disruption of intracellular Ca^{2+} homeostasis have been proposed (Coyle and Puttfarcken, 1993; Choi, 1994; Beal, 1995; Mattson et *al*., 1995; Dawson and Dawson, 1996; White and Reynolds, 1996). The excessive entry of Na^+ and Cl^- into neurons with overstimulation of any of the ionotropic glutamate receptors is usually described as contributing to morphological epiphenomena (cell swelling) but not to cell death (Choi, 1994).

Is this true for all neurons? In some systems, it has been difficult to test the role of extracellular Ca^{2+} in excitotoxicity because reductions in the concentration of extracellular Ca^{2+} led to severe neuronal pathology even in the absence of exogenous excitotoxins (Price et *al*., 1985; Freese et *al*., 1990; Lehmann, 1990). This Ca^{2+}-omission injury has been ascribed to membrane destabilization (Goldberg and Choi, 1993) or glial death (Freese et *al*., 1990), but the mechanism remains obscure.

Several studies have indicated that extracellular Cl^- may play an important role in excitotoxic cell death in some neuronal systems. For example, Zeevalk *et al*. (1989) showed that acute excitotoxic damage induced by either NMDA or non-NMDA receptor agonists could be attenuated by removing extracellular Cl^- or including the Cl^- channel blockers DIDS or furosemide. Kato *et al*. (1991), using cultured cerebellar

This work was supported by Grants EY08089, EY08922, EY09370, EY02687, and DA07261 and an unrestricted grant from Research to Prevent Blindness, Inc.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; GABA, γ-aminobutyric acid; KA, kainic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; LDH; lactate dehydrogenase; BSS, balanced salt solution; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

granule cells, demonstrated that KA-induced delayed LDH release was completely prevented in the absence of extracellular Cl^-. These authors also demonstrated that Ca^{2+} omission during the period of KA exposure did not attenuate cell death but instead increased its rate of development.

Although these studies suggested that there may be Ca^{2+}-independent mechanisms of excitotoxic cell death in which extracellular Cl^- may play an important role, they have not been definitive. For example, Cl^- channel-blocking agents are notoriously nonspecific drugs (Cabantchik and Greger, 1992), and some have been shown to directly block glutamate receptors (Lerma and Martin del Rio, 1992). Similarly, impermeant anions used as Cl^- substitutes in Cl^- removal experiments also may be glutamate receptor blockers. Replacement anions are necessarily used at very high concentrations (>100 mM), so they need not be very potent antagonists to provide substantial receptor blockade. Ca^{2+}-omission experiments have not used prolonged periods of Ca^{2+} removal because of the toxicity this causes, and whether manipulations in extracellular Ca^{2+} can affect intracellular free Ca^{2+} levels has not been addressed in any of these studies.

We reexamined the ionic basis of excitotoxicity in an isolated, intact retina preparation. In addition to using biochemical and histological measures of excitotoxicity, we used whole-cell patch-clamping in a retinal slice preparation to assess receptor activation and blockade and Fura-2 Ca^{2+} imaging to examine intracellular free Ca^{2+} during the experimental manipulations. We conclude that excitotoxicity in this preparation is independent of extracellular Ca^{2+} and dependent on extracellular Cl^- and that excitotoxicity depends on Cl^--gating inhibitory transmitters present in retinal circuits.

Materials and Methods

Chick embryo retina preparation. The chick embryo retina preparation protocols have been described previously (Romano et *al*., 1995). Briefly, eyes were isolated from chick embryos (15 ± 1 day old), placed in standard BSS at 0–4°, enucleated, and cut into thirds. The retina segments then were isolated from the rest of the ocular tissues and transferred to a 7-ml glass vial containing 1 ml of the standard BSS saturated with 95% O_2/5% CO_2. The standard BSS contained 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM KH_2PO_4, 22 mM $NaHCO_3$, 20 mM glucose, and 30 μM phenol red, pH 7.4. For experiments in the absence of Ca^{2+}, the BSS was modified so 2 mM $MgCl_2$ was substituted for $CaCl_2$ and 0.1 mM EGTA was added.

For experiments on the toxic effect of glutamate receptor agonists, the retina was first incubated in agonist-containing BSS for 30 min. At the end of the 30-min incubation, an aliquot of the incubation medium was taken for LDH assay to assess acute toxicity. The retina then was washed twice with agonist-free BSS and incubated in this medium for ≈22 hr. An aliquot of the medium was taken at the end of the 22-hr incubation for measuring delayed toxicity. For the measurement of residual LDH remaining in the retinal tissue (as part of the total LDH in a retinal segment), the retina was treated with Triton X-100 (0.2%) and lysed by freezing and thawing. The lysate was centrifuged, and an aliquot of the supernatant was taken for LDH assay.

LDH assay. The cocktail for LDH assay contained 0.1 mg/ml NADH, 2 mM sodium pyruvate, 0.1 M potassium phosphate, pH 7.4, and an aliquot of the BSS medium containing LDH released from the retina. LDH activities were measured spectrophotometrically by the rate of NADH disappearance. The assay has a >20-fold linear range. Levels of LDH in the aliquots taken for the acute and delayed releases are expressed as a percentage of the total LDH in a retinal segment, which is the sum of the LDH from the aliquots of the two releases and the retinal lysate. All the data in this report reflect total LDH release as a fraction of total retinal LDH content.

Within each experiment, each condition was tested on triplicate retinal segments, and all experiments were repeated at least three times. All reported differences are statistically significant (Student's *t* test or analysis of variance and multiple-comparison Tukey's test) but values for *p* are shown only in cases in which the differences were not apparent by simple inspection.

Ca^{2+} imaging. Intracellular Ca^{2+} concentrations in the retina were measured with digital fluorescence microscopy (Wong, 1995b). The retinal dissection was performed as described above. After the retina was isolated, a segment of the retina was cut and mounted (ganglion cell side up) onto a filter (HABP 045; Millipore, Bedford, MA). For whole mounts, the retinal segment was transferred to a chamber containing the incubation medium (BSS), described above, with the addition of Fura-2 AM (2 μM) and pluronic acid (0.001%), and incubated for 1 hr at 30–35°. For retinal slices, the retinal segment on a piece of Millipore filter was sliced with a tissue chopper at 150-μm intervals before Fura-2 incubation. The tissue then was washed and transferred to a temperature-controlled recording chamber on the stage of a fluorescence microscope. Imaging was performed at 25° to mimic the standard conditions of the toxicity experiments.

The retina in the recording chamber was maintained with superfusion of the O_2/CO_2-equilibrated BSS and alternately illuminated with 340/380-nm excitation. The paired images of the retina, thus generated, were acquired sequentially by computer (Universal Imaging, West Chester, PA) using a low-light-level camera (Hamamatsu Photonics, Hamamatsu, Japan), a Lambda-10 filter wheel (Sutter Instruments, Novato, CA), and a shutter system (Uniblitz, Rochester, NY). The images were stored on optical disk every 5 or 10 sec and played back later in a form of a movie for fluorescence analysis. Values of the intracellular Ca^{2+} concentration were measured by comparing the ratio of cell fluorescence at 340 and 380 nm with ratios calibrated previously against known Ca^{2+} values (calibration was performed under cell-free conditions because ionophore calibration is inappropriate in a slice preparation; for details, see Wong, 1995a, 1995b).

Electrophysiology. Membrane currents were measured by whole-cell patch recording of ganglion cells in the salamander retinal slice. The procedures for preparation and recording of the retinal slices have been described in detail previously (Lukasiewicz and Werblin, 1994). Briefly, a small square was cut from isolated retina, placed photoreceptor side down on a piece of Millipore filter, and sliced with a tissue chopper at 150-μm intervals. The sliced retina/filter complex was transferred to the recording chamber and immobilized by embedding the ends of the filter into two rails of vacuum grease that had been laid down in the chamber. The retinal slice was viewed with a Nikon Optiphot 2 microscope modified to have a fixed stage. A Nikon 40× long working-distance water-immersion objective with Hoffman Modulation contrast (Modulation Optics, Greenvale, NY) was used for visualization of cells on the surface of the slice.

The recording chamber was superfused continually with the bath medium containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES, pH 7.8. The control bath medium also contained 10 μM MK-801 [(5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate] to block NMDA receptor-mediated responses. The intracellular/electrode solution contained 92.25 mM Cs-gluconate, 8 mM TEA-Cl, 0.4 mM $MgCl_2$, 1 mM EGTA, and 10 mM Na-HEPES, pH 7.5.

KA (300 μM pipette concentration) was puffed onto the cell bodies and/or dendrites of ganglion cells in the slice preparation with a Picospritzer (General Valve, Fairfield, NJ). The puff pipette solution consisted of KA in control bath medium. The details of this methodology were described by Lukasiewicz and Werblin (1994).

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Recordings were obtained with a Dagan 3900A (Minneapolis, MN) patch-clamp amplifier. The data were digitized and stored with a 33-MHz 386 PC using a Labmaster DMA data acquisition board (Scientific Solutions, Solon, OH). Responses were filtered at 2 kHz with the four-pole Bessel low-pass filter and sampled at 0.7–2 kHz. Electrodes of <5 MΩ resistance were pulled from borosilicate glass (TW150F-4; World Precision Instruments, Sarasota, FL) with a Sachs-Flaming puller (Sutter Instruments, Novato, CA). The measured series resistances were typically 15–25 MΩ. The magnitude of the series resistance compensation, read from the Dagan 3900A compensation counting dial, was 5–10 MΩ. Patchit software (Geo. Grant, Sommerville, MA) was used to generate voltage command outputs, acquire data, gate the drug perfusion valves, and trigger the Picospritzer. Data were analyzed using Tack (Geo. Grant, Sommerville, MA) and expressed as mean ± standard deviation.

Fertilized chick eggs were purchased from SPAFAS (Roanoke, IL). KA and MK-801 were obtained from Research Biochemicals (Natick, MA). GYKI 53655 [1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5*H*-2,3-benzodiazepine] was kindly provided by Eli Lilly and Co. (Indianapolis, IN). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

Results

KA-induced excitotoxicity in isolated retina is independent of extracellular Ca^{2+}. If extracellular Ca^{2+} is important for excitotoxic neurodegeneration, the application of an excitotoxic agonist in the absence of extracellular Ca^{2+} should eliminate, or attenuate, the damage. However, as has been observed in many other neuronal preparations, the incubation of E14 chick retinas in the absence of Ca^{2+} leads to massive neuronal degeneration (Fig. 1B), precluding the experimental use of this simple manipulation. Two different strategies were used to overcome this limitation.

First, it was found that less developed (E11) chick retinas are not damaged by incubation in the absence of extracellular Ca^{2+} (Fig. 1A). They do, however, have KA receptors that cause excitotoxic damage. The omission of extracellular Ca^{2+} did not attenuate KA-induced damage to E11 chick retinas; instead, the toxicity was exacerbated (Fig. 1A). These data indicate that KA-induced excitotoxicity in E11 retina is not dependent on the entry of extracellular Ca^{2+}.

Second, it was found that Ca^{2+}-omission toxicity in E14 chick retinas was itself an excitotoxic process, mediated exclusively by NMDA receptors, because it could be completely blocked by the selective NMDA receptor antagonists MK-801 (Fig. 1B) or D-2-amino-5-phosphonovaleric acid (Fig. 6C). GYKI 53655 (Paternain et *al*., 1995), a selective antagonist of AMPA-preferring glutamate receptors, was ineffective against Ca^{2+}-omission toxicity.

Because KA-induced toxicity is not blocked by MK-801, the effect of Ca^{2+} omission on KA-induced excitotoxicity could be examined by including MK-801 in the incubation medium. The omission of extracellular Ca^{2+} did not attenuate KA-induced damage to E14 chick retinas; as with the E11 retinas, the toxicity instead was exacerbated (Fig. 1C). Even in the absence of Ca^{2+}, KA-induced toxicity could be prevented completely by GYKI 53655, indicating that the same receptor mechanisms are operative despite the altered ionic conditions. These data indicate that KA-induced excitotoxicity is not dependent on the entry of extracellular Ca^{2+}.

These experiments demonstrated clearly that AMPA/KA receptor-mediated cell damage can occur in the presence or absence of extracellular Ca^{2+} and, in fact, is more severe in the absence of Ca^{2+}. However, increases in intracellular Ca^{2+} by mobilization of intracellular stores during KA exposure may occur in the absence of Ca^{2+} influx, and this could contribute to the mechanism of toxicity. To explore this possibility, intracellular free Ca^{2+} concentrations in ganglion cells in whole mounted retinas (Fig. 2) or in amacrine and bipolar cells in retinal slices (not shown) were monitored during and after KA treatment, using the ratiometric fluorescent Ca^{2+} indicator Fura-2. As shown in Fig. 2, in the presence of 2 mM extracellular Ca^{2+}, KA caused a substantial and prolonged rise in intracellular Ca^{2+} in these cells. This increase was nearly abolished when Ca^{2+} was removed from the extracellular medium. This was not correlated with a decrease in KA-induced toxicity but with the exacerbation demonstrated above.

The reason for the small rise in intracellular Ca^{2+} observed in the experiment illustrated in Fig. 2 is most likely

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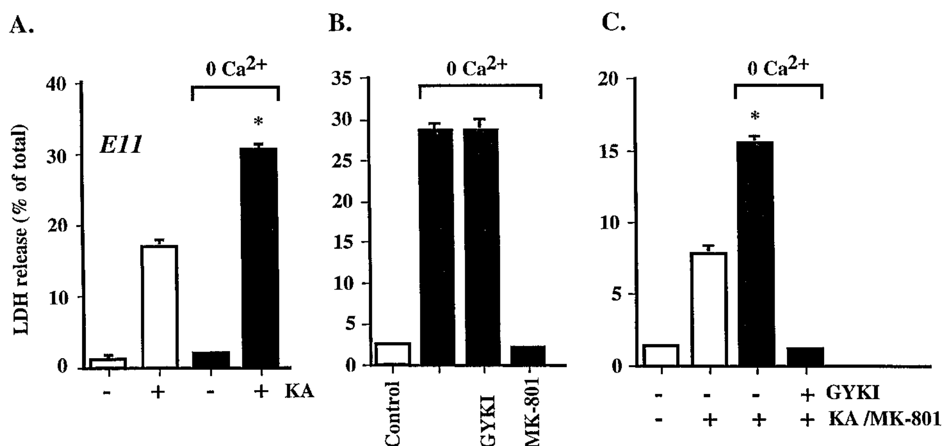

Fig. 1. KA-induced toxicity is augmented by Ca^{2+} omission. In all the experiments in this report, retinas were incubated with excitotoxic agonists for 30 min and washed out, and LDH released over 22–24 hr (as a percentage of total retinal content of LDH) was measured as an index of toxicity. A, In embryonic day 11 (*E11*) retinas, KA (32 μM) toxicity is increased in Ca^{2+}-free media. Note that at this age, Ca^{2+} omission alone is not associated with toxicity. B, In E14 retinas, 22 hr of Ca^{2+} omission caused toxicity. This Ca^{2+}-omission toxicity was completely prevented by MK-801 (10 μM) but not by GYKI-53655 (*GYKI*, 20 μM), an antagonist of AMPA/KA receptors. C, In E14 retinas, KA (32 μM) toxicity is increased in Ca^{2+}-free media. Ca^{2+} was omitted, and MK-801 was present, in the periods during and after KA incubation. GYKI 53655 blocked KA toxicity. Data are mean ± standard error values of at least three experiments. *, $p < 0.05$ compared with KA without Ca^{2+} omission.

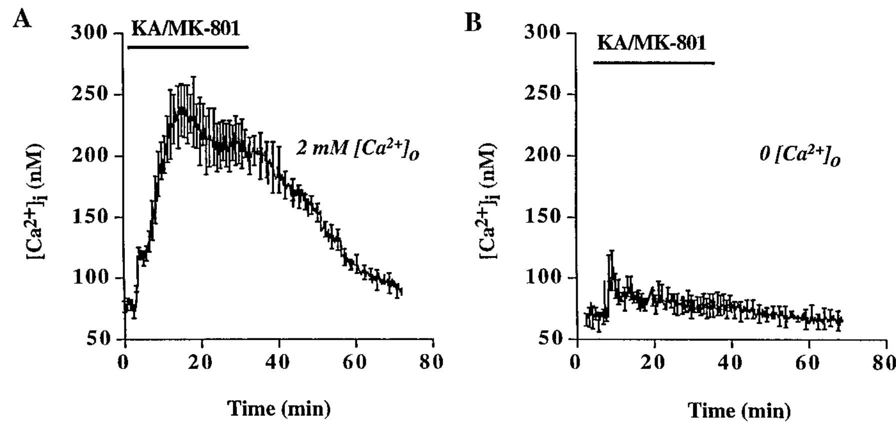

Fig. 2. Ca^{2+} omission blocks the KA-induced elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the retina. A, The whole-mounted retina (E14) was superfused with incubation medium containing 32 μM KA and 10 μM MK-801. $[Ca^{2+}]_i$ was monitored in cells in the ganglion cell layer of the retina by the ratiometric measurements of Fura-2 (see Materials and Methods). KA incubation led to a marked elevation of free Ca^{2+}. B, When $CaCl_2$ in the incubation buffer was replaced by $MgCl_2$ plus 100 μM EGTA, increases in free Ca^{2+} were blocked. Data are mean ± standard deviation values of 31 cells. This experiment was repeated once with a whole-mounted retina and twice with retinal slices (not shown), with similar results. The experiment illustrated had the largest response in the absence of Ca^{2+} of the four repetitions.

that a slight residue of extracellular Ca^{2+} remained, despite several minutes of washing in Ca^{2+}-free, EGTA-containing buffer. The whole-mounted retina is an intact tissue, about a dozen cells and two synaptic layers thick, so the presence of some areas that have not achieved complete buffer exchange seems likely. This slight increase in intracellular Ca^{2+} was not observed when the retinal slice preparation was used (not shown).

KA-induced excitotoxicity in isolated retina is dependent on extracellular Na^+. To clarify further the mechanisms for KA-induced toxicity, we focused our remaining investigations on Ca^{2+}-independent mechanisms. One such mechanism, excessive Na^+ influx, was studied in Na^+-substitution experiments. Choline or *N*-methyl-D-glucamine was substituted for Na^+ in the incubation medium. Unlike Ca^{2+} omission (Fig. 1B), Na^+ omission alone did not cause any cell damage (Fig. 3A). Na^+ omission during exposure to a low concentration of KA (32 μM) blocked toxicity but was ineffective against a higher concentration of KA (320 μM, Fig. 3B). One potential explanation for the failure of Na^+ omission to protect against KA toxicity at high concentrations is that high concentrations of KA activate some Ca^{2+}-permeable channels and that the excessive Ca^{2+} influx through these channels might cause the toxicity. This did not prove to be the case because simultaneous omission of Na^+ and Ca^{2+} did not protect against KA toxicity at 320 μM (Fig. 3B).

KA-induced excitotoxicity in isolated retina is dependent on extracellular Cl^-. Effective protection against KA-induced toxicity in the retina came from Cl^- substitution (Fig. 4). With 75% of the extracellular Cl^- replaced by methylsulfate, toxicity of KA at either of the two concentrations (32 and 320 μM) was nearly blocked. Similar neuroprotection was observed using the impermeant Cl^- substitutes isethionate and gluconate but not the permeant anion bromide (data not shown).

The better protection afforded by Cl^- substitution compared with Na^+ substitution was surprising. To verify that the protection afforded by this manipulation was not due to a direct inhibitory action of the Cl^- substitute methylsulfate on the receptor, the effect of methylsulfate on KA-elicited currents was examined using whole-cell patch-clamp recordings of salamander retinal ganglion cells in an intact retinal slice preparation. We chose to use salamander retina because it provides a robust preparation that survives for long periods of time and is technically amenable to stable, long-duration, whole-cell patch-clamping. Importantly, most of the major

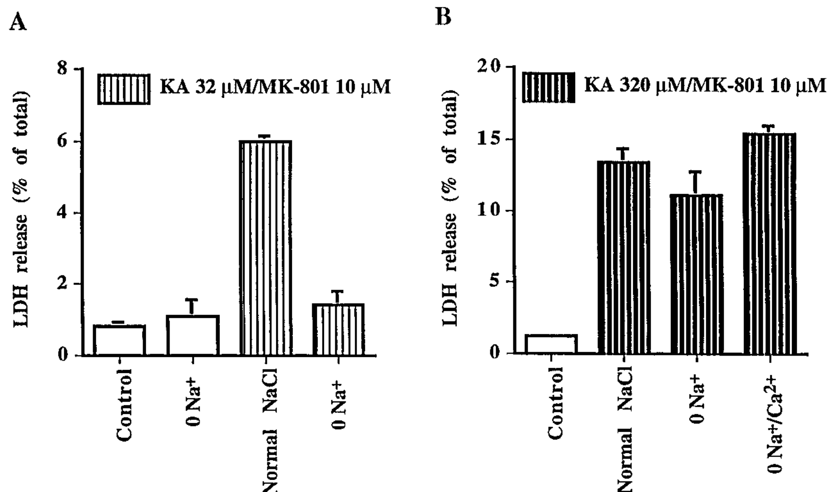

Fig. 3. The effect of Na^+ substitution on KA-induced toxicity. The retina was treated with KA at 32 μM (A) or 320 μM (B) for 30 min, during which the incubation medium contained no added Na^+ (Na^+ replaced by choline) or no added Na^+ and Ca^{2+} (Ca^{2+} replaced by Mg^{2+} and the addition of 100 μM EGTA). The omission of Na^+ prevented toxicity caused by 32 μM, but not 320 μM, KA.

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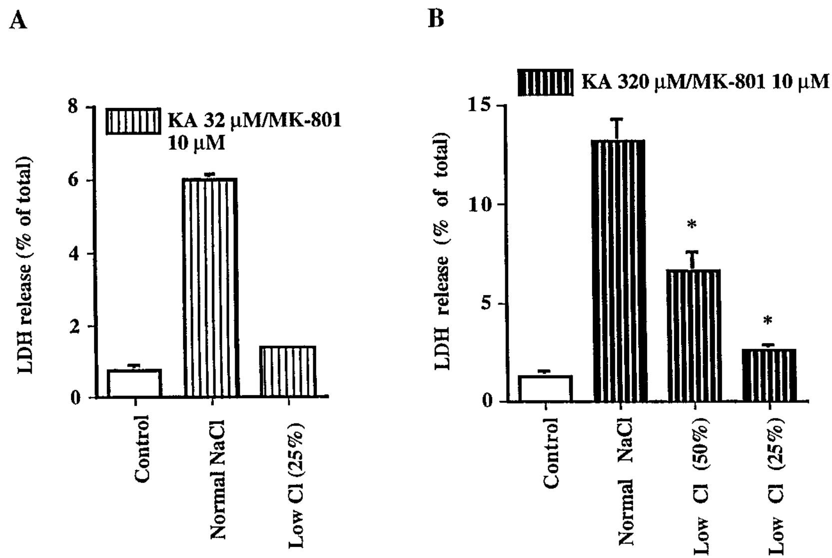

Fig. 4. Cl^- substitution prevents KA-induced toxicity. The retina was treated with KA at 32 μM (A) or 320 μM (B) for 30 min, during which Cl^- in the incubation medium was reduced to its 50% or 25% level by the substitution of methylsulfate. *, $p < 0.05$ compared with KA in normal NaCl.

findings that have come out of studies of this preparation have been confirmed in other species, including birds and mammals. For example, the transmitters utilized by distinct subtypes of retinal neurons are similar across species (e.g., photoreceptors and bipolar cells are glutamatergic, amacrine cells are either glycinergic or GABAergic), and the retinal circuitry of salamander is similar to chick as well as to other vertebrate retinas. Fig. 5A shows the response of a ganglion cell to KA in the presence or absence of methylsulfate. With membrane holding potential of −75 mV, substitution of methylsulfate for Cl^- did not inhibit KA-elicited currents. Instead, it enhanced the currents in all 14 cells recorded. This is strong evidence that methylsulfate does not block the receptor mediating the action of KA.

Further examination of the current-voltage relations showed that KA-elicited currents reversed near −37 mV and that replacement of 75% of the Cl^- by methylsulfate shifted the reversal potential to ≈−12 mV (Fig. 5B). These results indicate that KA activates a Cl^- conductance in these ganglion cells. The enhancement of KA-elicited currents by the substitution of methylsulfate for Cl^- can be readily explained in terms of an increase in Cl^- efflux because the equilibrium potential for Cl^- becomes more positive with the reduction of extracellular Cl^-. Because there is no evidence that endogenous retinal ionotropic glutamate receptors are permeable to Cl^-, it seems likely that activation of the Cl^- current must be indirect. One potential explanation for the increased Cl^- conductance in ganglion cells in this slice preparation would be that KA stimulates GABAergic and/or glycinergic amacrine cells that synaptically activate GABA and/or glycine receptors on ganglion cells, leading to the increased Cl^- conductance. Consistent with this explanation, the combined application of bicuculline and strychnine, GABA and glycine receptor antagonists, respectively, blocked the Cl^- current and shifted the reversal potential of KA-elicited currents to near 0 mV, which is expected for the typical cation current gated by AMPA/KA receptors (Fig. 5C).

These results suggest that KA treatment of isolated retina, an intact neural network that contains a diverse population of neurons, leads to pathological release of inhibitory as well as excitatory transmitters. These transmitters then activate their receptor-associated Cl^- channels, thus contributing to excessive Cl^- influx in the neurons. Because the ion-substitution experiments described above indicated that extracellular Cl^- is essential for excitotoxic cell death, block of these receptors should be protective against excitotoxicity. Consistent with this proposal, when both GABA and glycine receptors were blocked by combined application of picrotoxin (which blocks both $GABA_A$ and the retina-selective $GABA_C$ receptors; Lukasiewicz, 1996) and strychnine, the toxicity of 320 μM KA was attenuated partially (Fig. 5D). Interestingly, individual blockade of GABA or glycine receptors did not attenuate KA toxicity.

Extracellular Cl^- also is important for excitotoxic neurodegeneration caused by NMDA receptor overactivation. Are the ionic mechanisms mediating KA-induced toxicity also operative in NMDA receptor-mediated forms of excitotoxic neurodegeneration in the chick retina? When retinas were incubated with NMDA (500 μM) or in the absence of Ca^{2+} for 60 min (which as shown results in NMDA receptor-mediated toxicity), substantial neurodegeneration occurred (Fig. 6, A and B). The toxicity induced by NMDA treatment was almost entirely eliminated when either Na^+ or Cl^- was removed during the NMDA exposure, as was toxicity caused by Ca^{2+} omission. The combination of bicuculline and strychnine also provided significant protection (Fig. 6, C and D), indicating a role for GABA and glycine in both of these forms of toxicity. Bicuculline alone provided complete protection against the toxicity resulting from 1 hr of Ca^{2+} omission, whereas strychnine was without effect (Fig. 6D).

Discussion

The evidence presented here indicates that rapidly triggered excitotoxic cell death is a Ca^{2+}-independent process in some neurons and that it shows a remarkable dependence on extracellular Cl^-. Some of the lethal Cl^- entry occurs through neurotransmitter-gated Cl^- channels activated by pathologically released inhibitory transmitters, pointing to a role for GABA and glycine, as well as glutamate, in excitotoxicity. The differences among neuronal populations in the mechanisms of excitotoxic cell death therefore depend on both the intrinsic properties of the neurons (i.e., which re-

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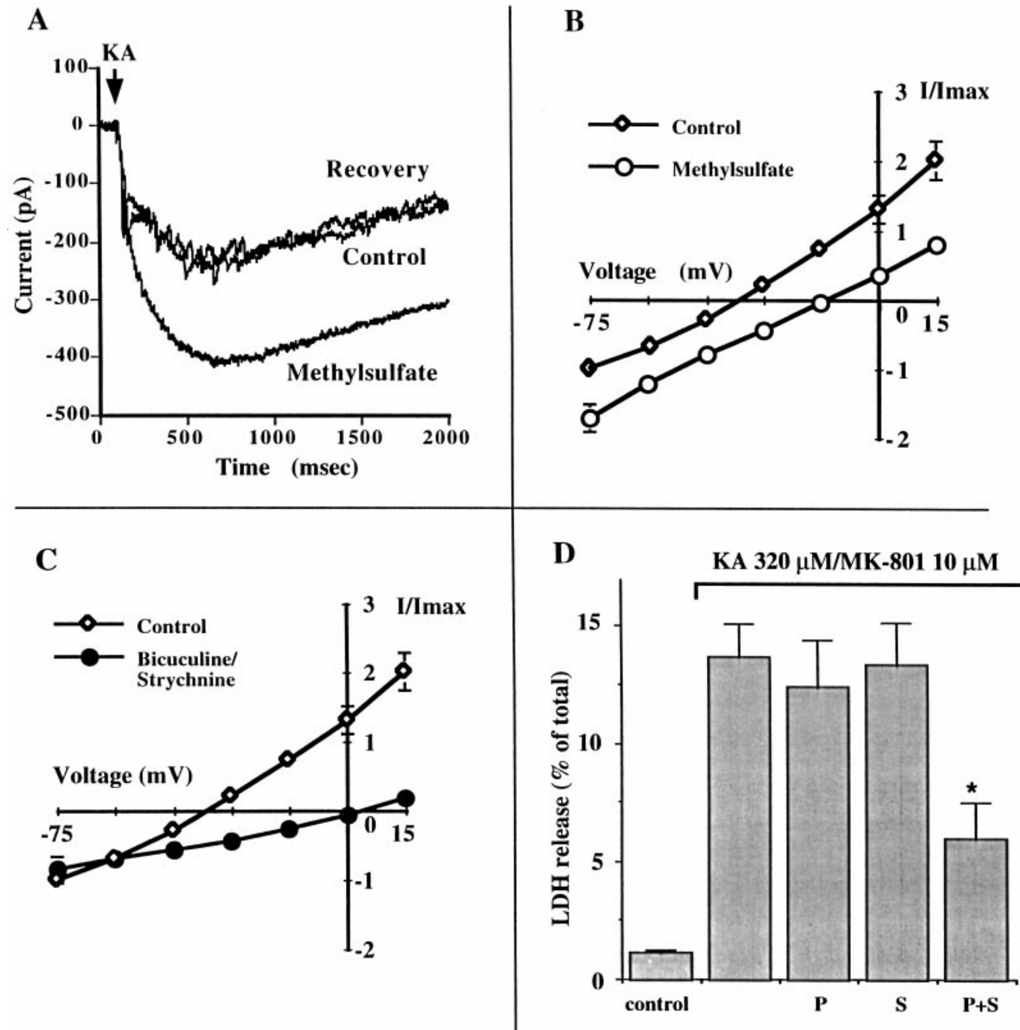

Fig. 5. KA activates Cl^- conductances in retinal ganglion cells via activation of GABA and glycine receptors, and blocking these currents is neuroprotective. KA-elicited whole-cell currents in ganglion cells of the salamander retina (A-C) and protection in the chick retina against KA-induced toxicity by GABA and glycine antagonists (D), A, Whole-cell currents were recorded from a ganglion cell of the salamander retina with the membrane potential held at −75 mV. KA (300 μM pipette concentration) was puffed onto the dendritic area of the cell (*arrow*). KA-elicited currents were increased as Cl^- in the incubation medium was replaced by methylsulfate. B, Current-voltage relations in the presence of Cl^- ($n = 10$) or methylsulfate ($n = 9$). Measurements of KA-elicited currents were normalized against the response at the holding potential of −75 mV in the presence of Cl^-.The calculated reversal potential for Cl^- in ganglion cells of the salamander retina is −65 mV for high extracellular Cl^- (120 mM) and −29 mV for low extracellular Cl^- (30 mM and the addition of 90 mM methylsulfate). C, Current-voltage relations in the presence or absence of bicuculline (150 μM) and strychnine (2 μM). Measurements of KA-elicited currents in ganglion cells of the salamander retina were normalized against the response of control ($n = 9$) at the holding potential of −75 mV. D, Combined application of picrotoxin (*P*, 200 μM) and strychnine (*S*, 2 μM) during the 30-min KA (320 μM) incubation significantly reduced KA-induced toxicity in the chick retina. Data are mean ± standard error values of three or four experiments. *, $p < 0.05$ compared with KA alone.

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ceptors, channels, and so on they express) and the nature of the network in which they are embedded (i.e., the complement of transmitters and other pathological environmental elements they will encounter during an excitotoxic insult).

Ca^{2+} independence of excitotoxicity and Ca^{2+} omission-induced damage. In a previous study providing evidence for Ca^{2+}-independent, Cl^--dependent excitotoxic cell death (Kato et *al*., 1991), Ca^{2+} was absent from the incubation medium only during the period of excitotoxin exposure. It remained possible that Na^+ loading during the agonist exposure led to a later marked increase in intracellular Ca^{2+} due to greatly enhanced Na^+/Ca^{2+} exchange. By using conditions permitting incubation in the absence of Ca^{2+} for the duration of the 24-hr experimental period, the current study does not have this limitation. In addition, Ca^{2+} imaging experiments documented that there was minimal or no increase in free intracellular Ca^{2+} during the agonist exposure, or the period immediately after, when the retinas were maintained in the Ca^{2+}-free medium (Fig. 2); nevertheless, the cells died.

This cell death was not due to rapid osmotic lysis. First, our previous study demonstrated that although pathomorphological changes occur rapidly on agonist exposure, LDH release is a much slower process, requiring hours (Romano et *al*., 1995). Second, if the integrity of the cell membrane were breached, the rapid increase of intracellular Ca^{2+} to extracellular levels would have been immediately apparent during the imaging experiments. This was never observed. It is likely that there are roles for cell swelling and intracellular osmotic/ionic imbalances in cell death, but the precise mech-

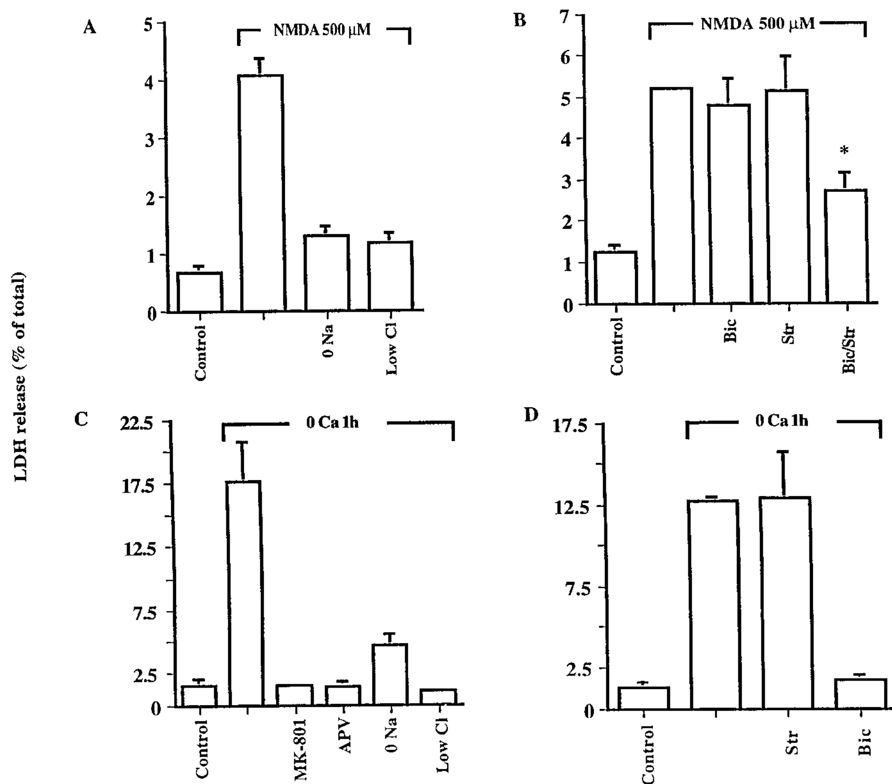

Fig. 6. Na^+ and Cl^- dependence of NMDA and Ca^{2+} omission-induced toxicity. A, NMDA (500 μM, 30 min)-induced toxicity was completely blocked by either Na^+ omission or a 75% reduction in Cl^-. B, Combined application of bicuculline (*Bic*, 150 μM) and strychnine (*Str*, 2 μM) during the NMDA incubation significantly reduced the NMDA-induced toxicity in the retina (*, $p < 0.05$ compared with NMDA alone). C, Toxicity caused by 1 hr of Ca^{2+} omission was prevented by a 75% reduction in Cl^- (*Low Cl*) or omission of Na^+ (*0 Na*), as well as by MK-801 (10 μM) or 2-amino-5-phosphonovaleric acid (500 μM). B, Bicuculline (150 μM, *Bic*), but not strychnine (2 μM, *Str*), also reduced this Ca^{2+}-omission toxicity.

anism involved is neither trivial nor apparent and is not likely to be rapid mechanical failure of the cell membrane.

Ca^{2+} omission triggered excitotoxicity in the E14 retina dependent on NMDA receptor activation (Figs. 1B and 6C). The E11 retina is not susceptible to Ca^{2+}-omission injury (Fig. 1A) or NMDA-mediated excitotoxicity (data not shown), which is consistent with the requirement for NMDA receptor overactivation in Ca^{2+}-omission injury. Similar observations pertaining to Ca^{2+}-omission injury in other types of neurons have been made by other investigators (McCaslin and Smith, 1990; Eimerl and Schramm, 1991). The precise mechanism of this Ca^{2+} omission-induced excitotoxicity is unknown and was not the explicit subject of this study. Nevertheless, the mechanism presumably involves the induction of hypersensitization of NMDA receptors, a pathological release of glutamate, or both, upon lowering extracellular Ca^{2+}. Evidence for both these possibilities has been gathered in other systems (Clark et *al*., 1990; McCaslin and Smith, 1990; Legendre et *al*., 1993; Rosenmund et *al*., 1995). The ability of bicuculline to prevent Ca^{2+} omission-induced (Fig. 6D) damage indicates that overactivation of GABA receptors plays an important role in this form of toxicity.

Ca^{2+} omission increased KA-induced delayed LDH release (Fig. 1C). This probably was due to the increased Na^+ conductance of KA-activated channels in the absence of Ca^{2+} because extracellular Ca^{2+} modulates KA-activated currents by impeding the permeation of monovalent cations (Gu and Huang, 1991).

Because NMDA receptor antagonists must be present to completely block Ca^{2+}-omission injury and they also provide complete or near-complete blockade of NMDA receptor-mediated excitotoxicity, we could not use this method to investigate the Ca^{2+} dependence of this NMDA receptor-mediated injury. However, the marked neuroprotection provided by Cl^- omission against NMDA treatment (Fig. 6A) and the fact that Ca^{2+} omission injury itself is NMDA receptor mediated are not easily explained by postulating a Ca^{2+}-dependent mechanism for NMDA receptor-dependent toxicity in this system.

Another potential means for examining the Ca^{2+} dependence or independence of cell death is by use of the membrane-permeant "prochelator" BAPTA (Tymianski et *al*., 1993). On hydrolysis by intracellular esterases, the acid is formed, which (1) cannot leave the cell and (2) is a Ca^{2+} chelator. Therefore, extracellular application of this substance should lead to a large increase in the Ca^{2+}-buffering capacity of the cytoplasm and theoretically should protect against Ca^{2+}-dependent, but not Ca^{2+}-independent, forms of excitotoxicity. We have found that BAPTA acetoxymethyl ester (20–100 μM) does not protect against KA toxicity, which is consistent with our other findings documenting a Ca^{2+}-independent mechanism for KA-induced cell death (Q. Chen and C. Romano, unpublished). However, because several investigators have reported that BAPTA acetoxymethyl ester is not a very effective neuroprotectant against agonist-induced excitotoxicity (Dubinsky, 1993; Abdel-Hamid and Tymianski, 1997), even in systems in which cell death is known to be Ca^{2+} dependent, this evidence, although consistent, is not compelling.

Na^+, Cl^-, and inhibitory transmitters in excitotoxic cell death. The mechanism for excessive Na^+ influx in excitotoxicity is understood readily in terms of the conductance of Na^+-permeable receptor channels activated by all excitotoxins. The mechanisms for excessive Cl^- influx triggered by excitotoxins are less direct. Rothman and Olney (1987) proposed a general mechanism by which excessive Na^+ influx would draw Cl^- into cells to restore ionic balance. However, they did not identify specific ion channels, transporter sys-

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tems, or receptors that might mediate the influx of Cl^-. Zeevalk *et al*. (1989) reported that the Cl^-/bicarbonate anion channel blocker DIDS protected the chick retina from acute excitotoxicity. We have observed that DIDS also reduced KA-induced delayed LDH release in our retinal preparation (data not shown). However, the effect of DIDS against excitotoxicity is difficult to interpret because we found that DIDS completely inhibited KA-induced currents in ganglion cells of salamander retina, suggesting it is a receptor blocker (data not shown). The blockade of GABA release by DIDS observed by Zeevalk *et al*. 1989) also indicates that DIDS may be operating as a receptor blocker. The poor specificity of other Cl^- antagonists has been described (Cabantchik and Greger, 1992; Lerma and Martin del Rio, 1992).

Our electrophysiological studies (Fig. 5, A–C) in the salamander retina demonstrated that channels gated by GABA and glycine constituted the major route of Cl^- influx in ganglion cells during brief exposure to KA (i.e., puffs). The straightforward implication of this is that KA treatment induced the release of GABA and glycine receptor agonists from nearby neurons. It has been established that KA treatment of intact chick retina (Zeevalk et *al*., 1989) or chick retinal cells in culture (Ferreira et *al*., 1994) leads to GABA release. Much of this release is independent of extracellular Ca^{2+} (Ferreira et *al*., 1994) and presumably is mediated by the reversed operation of the voltage-dependent GABA transporter. Such Ca^{2+}-independent release of GABA from retinal neurons has been well documented for several classes of retinal neurons (Schwartz, 1987; O'Malley et *al*., 1992). Taurine also is released on KA from intact chick retina (Zeevalk et *al*., 1989), and this amino acid is well known to be an agonist at some subtypes of inhibitory (Cl^- gating) glycine receptors (Schmieden et *al*., 1992).

Consistent with the hypothesis that excitotoxic cell death may depend on overactivation of these ligand-gated Cl^- channels, block of GABA and glycine receptors by picrotoxin and strychnine during KA exposure provided a partial protection against excitotoxicity in the chick retina (Fig. 5D). The fact that protection afforded by the inhibitors was less than that provided by Cl^- omission suggests involvement of other modes of Cl^- entry. The exposure to KA was prolonged (30 min) during the toxicity experiments and very brief during the electrophysiological experiments. Perhaps Cl^- currents (other than the ligand-gated ones we observed) were not seen in the electrophysiological experiments because they develop slowly.

NMDA receptor-mediated toxicity was also Cl^- dependent and blocked by bicuculline and strychnine (Fig. 6), suggesting a more general involvement of GABA and glycine in excitotoxic injury.

We propose the model in Fig. 7 to illustrate mechanisms mediating the Cl^- influx triggered by excitotoxins. Persistent activation of ionotropic glutamate receptors, in addition to causing Na^+ influx, could persistently activate GABA/glycine-gated Cl^- channels (via excess GABA/glycine release), causing excessive Cl^- entry into the neurons already subjected to excessive Na^+ entry. Other pathways for Cl^- entry would be activated, perhaps with slower time courses. This would create osmotic conditions conducive to obligatory water entry and cell swelling. Prolonged and massive NaCl influx and water uptake may eventually lead to cell death.

In the isolated retina, and unlike cultures of dissociated cells, the well preserved synaptic organization permits the study of integrated responses of neurons to excitotoxic insult. The present ion-substitution experiments indicate that the ionic mechanisms that are operative in acute morphologically defined excitotoxic degeneration (Price et *al*., 1985) are also responsible for excitotoxin-induced neurodegeneration defined biochemically in terms of the delayed LDH release. These findings are in contrast to observations in cultured cerebrocortical and hippocampal neurons in which NMDA receptors mediate a Ca^{2+}-dependent delayed toxicity (Choi, 1987; Rothman et *al*., 1987). These apparent differences cannot be readily explained in terms of differences in methodological approach and more likely reflect some intrinsic differences among neurons or neural networks in their ability to cope with a specific ionic overload produced during excitotoxin exposure. These intrinsic differences may be essential for our understanding of multiple mechanisms for excitotoxicity.

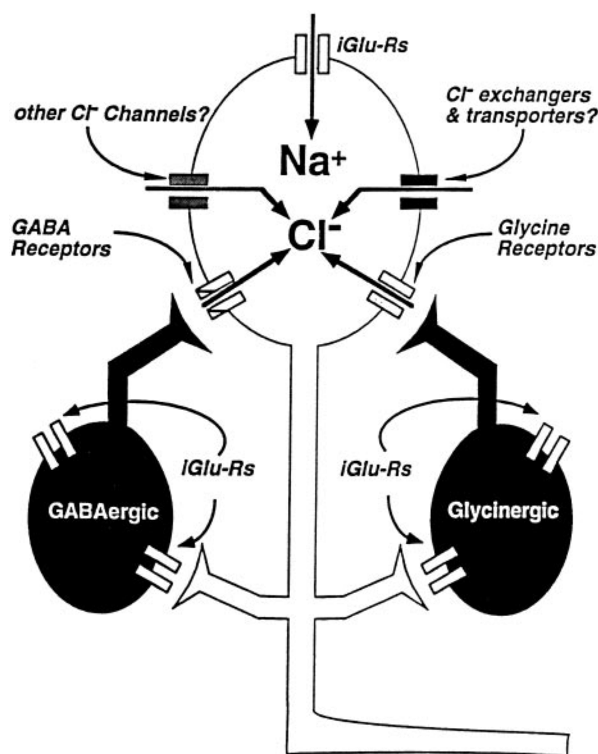

Fig. 7. Potential mechanisms for lethal Na^+ and Cl^- entry. During excitotoxic insults, both excitatory and inhibitory receptors will be overstimulated. Overactivation of ionotropic glutamate receptors will lead to excess Na^+ entry, whereas GABA and glycine receptors stimulated by excessive release of GABA and/or glycine provide paths for Cl^- entry. This will happen in all central nervous system regions with substantial GABA- or glycine-containing neurons. Particular circuits may be involved (e.g., those physiologically mediating recurrent inhibition). A primary ionotropic glutamate receptor activated on a glutamatergic neuron leads to Na^+ entry and increased firing, which activates a recurrent inhibitory feedback loop to drive Cl^- into the same cell via GABA and glycine receptors. Alternatively, other glutamatergic neurons under excitotoxic insult may persistently activate the GABAergic or glycinergic neurons. In addition, there are non-ligand-gated Cl^- channels, Cl^- exchangers, and Cl^- transporters colocalized with ionotropic glutamate receptors in most neurons. These pathways for Cl^- entry may become highly activated after excitotoxin-induced Na^+ influx and membrane depolarization.

The results presented here demonstrate that Cl^- influx plays an essential role in excitotoxic degeneration of the chick retina, but it remains to be determined how the Cl^-

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ions contribute to the toxic process. Cl^- transmembrane movement is important for membrane excitability, intracellular pH regulation, and cell volume control. We can only speculate, at present, that prolonged disruption in any of these functions may be detrimental to the neuron. If excessive Cl^- influx proves to be a mechanism that is generally operative in excitotoxicity, it may be possible to develop therapeutic approaches to the management of neurological disorders based on this principle.

Acknowledgments

We thank Dr. R. O. Wong (Department of Anatomy and Neurobiology, Washington University School of Medicine) for generously providing us with her expertise and device for the Ca^{2+} imaging experiments.

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