Neuropharmacology 55 (2008) 677–686

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Chloride-dependent acute excitotoxicity in adult rat retinal ganglion cells

Yasuhiro Hama [a], Hiroshi Katsuki [b], Chihiro Suminaka [a], Toshiaki Kume [a], Akinori Akaike [a,*]

[a] Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan
[b] Department of Chemico-Pharmacological Sciences, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan

ARTICLE INFO

Article history:
Received 21 May 2007
Received in revised form 26 February 2008
Accepted 2 June 2008

Keywords:
Ganglion cell
Excitotoxicity
Chloride
Calcium
Niflumic acid
Retina

ABSTRACT

Mechanisms of excitotoxic degeneration of retinal ganglion cells (RGCs) remain controversial, due to the lack of suitable in vitro experimental systems for evaluation of RGC death. In this study, we investigated acute excitotoxicity in RGCs using eyecup preparations obtained from adult rats, with special reference to ionic dependence of *N*-methyl-D-aspartate (NMDA) and kainate toxicity. Retrograde labeling of RGCs with a fluorescent tracer diamidino yellow, combined with labeling of dead cells by propidium iodide, enabled us to discriminate dead RGCs from other cells in the ganglion cell layer. Exposure of eyecups to NMDA or kainate for 30 min followed by 6 h post-incubation caused cell death in a subpopulation of RGCs as well as other (presumably displaced amacrine) cells. RGCs in the peripheral area of the retina were less sensitive to NMDA toxicity than those in the central area. Death of RGCs and other retinal cells by NMDA or kainate was largely abolished by substitution of extracellular Cl^-, whereas chelation of extracellular Ca^{2+} did not inhibit NMDA or kainate toxicity in RGCs. Strychnine but not bicuculline partially inhibited NMDA-induced RGC death, although these drugs were not effective against kainate-induced RGC death. On the other hand, niflumic acid, a Cl^- channel blocker, markedly inhibited RGC death induced by kainate as well as by NMDA. These results underscore the important role of Cl^- in acute excitotoxicity in adult rat RGCs.

1. Introduction

Retinal ganglion cells (RGCs) transmit visual information to the brain via their axons that comprise the optic nerve. Degeneration and loss of RGCs constitute a prominent feature of retinal disorders leading to vision loss. Retinal disorders such as glaucoma, retinal ischemia, diabetic retinopathy and traumatic optic nerve injury may share common properties in the sense that excitotoxic injury participates in their pathogenic processes. For example, retinal ischemia (Adachi et al., 1998), diabetic retinopathy (Kowluru et al., 2001) and optic nerve injury (Vorwerk et al., 2004) are accompanied by increases in intravitreal concentrations of glutamate, and administration of glutamate receptor antagonists prevents degeneration of RGCs in experimental models of glaucoma (Nucci et al., 2005), retinal ischemia (Adachi et al., 1998) and optic nerve injury (Schuettauf et al., 2000).

Ionotropic glutamate receptors including *N*-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors are thought to mediate excitotoxicity in the retina. However, whether or not RGCs are vulnerable to NMDA and kainate is controversial. For example, we and other investigators have shown that intravitreal injection of NMDA induces RGC death (Sun et al., 2001; Manabe et al., 2005; Hama et al., 2006). NMDA also exerts toxicity in dissociated rat RGCs (Kitano et al., 1996; Pang et al., 1999). In sharp contrast, several studies showed that RGCs in dissociated cultures and isolated retinas were resistant to NMDA toxicity (Luo et al., 2004; Ullian et al., 2004). Moreover, some reports have shown that exposure to kainate induces RGC death (Otori et al., 1998; Luo et al., 2001), whereas others demonstrated invulnerability of RGCs to kainate toxicity (Pang et al., 1999; Ullian et al., 2004).

Even if excitotoxicity occurs in RGCs, its mechanisms concerning ionic dependence are another unresolved issue. Although Ca^{2+} plays an important role in excitotoxic degeneration of various types of neurons (Sapolsky, 2001), a considerable amount of evidence indicates that Cl^- influx also makes a significant contribution (Rothman, 1985; Choi, 1987). Recent studies demonstrated that Cl^- influx plays a crucial role in excitotoxic injury in cultured mouse cerebellar granule cells (Babot et al., 2005) and cortical neurons (Inoue and Okada, 2007). In embryonic chick retina, Cl^- seems to play a dominant role in acute excitotoxic injury as assessed by lactate dehydrogenase release (Chen et al., 1998), whereas excessive intracellular Na^+ and Ca^{2+} are the proximal causes of excitotoxic amacrine cell death in adult mouse retina (Luo et al., 2004). No study has directly investigated ionic dependence of excitotoxicity in RGCs in adult rodents.

Although dissociated cell cultures provide powerful means to investigate the mechanisms of cell death, they have several drawbacks. Procedures for dissociation and isolation of retinal cells

* Corresponding author. Tel.: +81 75 753 4550; fax: +81 75 753 4579.
E-mail address: aakaike@pharm.kyoto-u.ac.jp (A. Akaike).

0028-3908/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2008.06.006

inevitably include enzymatic digestion of retinal tissues, which may have adverse influences on the functions of membrane-associated receptors and ion channels. In addition, mechanical injury resulting from dissociation procedures causes truncation of neuritic processes, where glutamate receptors are concentrated (Heng et al., 1995). Moreover, low yield of viable isolated cells relative to total neurons may lead to unintended selection of particular subpopulations of RGCs. Finally, loss of cell-to-cell interactions is likely to affect the properties of excitotoxicity. Diffusible substances produced by other cell types may exert substantial influences on the survival of RGCs (Garcia et al., 2002), and cell-to-cell interactions seem to augment the NMDA currents by several-fold (Ullian et al., 2004).

In view of these points, we prepared posterior eyecups from adult rats to study excitotoxic RGC death. In this preparation, cellular architecture of retinal tissues and dendritic processes of RGCs are maintained intact, and the profiles of glutamate receptor expression in retinal cells should match those in vivo because we can analyze excitotoxicity promptly after preparation of eyecups. This preparation also offers important advantages in that we can retain drug levels at a stable concentration and manipulate the extracellular environment easily. In the present study, we observed acute RGC death after exposure of eyecups to NMDA and kainate, and addressed the mechanisms of acute excitotoxic injury in RGCs with special emphasis on ionic dependence.

2. Methods

2.1. Drugs and chemicals

Diamidino yellow dihydrochloride (DY), NMDA, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[*f*]quinoxaline-7-sulfonamide (NBQX), (+)-MK-801, (−)-bicuculline methiodide, niflumic acid and Ames' Medium were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Kainic acid monohydrate, propidium iodide (PI), choline chloride and sodium methanesulfonate were from Wako Chemicals (Osaka, Japan). 4-Di-10-ASP (DiA) was from Invitrogen (Carlsbad, CA, USA). Other drugs and chemicals were obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Retrograde labeling of retinal ganglion cells

Experiments were performed on male Sprague–Dawley rats (7 weeks old; Nihon SLC, Shizuoka, Japan). Animals were housed at 21–23 °C in a 12:12-h light/dark cycle (lights on at 08:00 h). Food and water were freely available. All procedures were approved by our institutional animal experimentation committee and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and also with the National Institutes of Health guide for the care and use of laboratory animals. Each rat was anesthetized with an intraperitoneal injection of sodium pentobarbital (50–70 mg/kg), and the head was placed firmly in a stereotaxic apparatus. To retrogradely label RGCs, we injected 2 μl of 0.3% DY solution bilaterally into four sites in the superior colliculus (5.8 mm posterior to bregma, 1.0 mm lateral to the midline, 3.8 mm from the surface of the skull; 6.8 mm posterior to bregma, 1.0 mm lateral to the midline, 3.4 mm from the surface of the skull). In the experiments to demonstrate cell swelling, we injected DiA, a membrane tracer (2 μl of 0.5% solution in *N,N*-dimethylformamide), in place of DY into the same sites.

2.3. Posterior eyecup preparation

We prepared rat posterior eyecups according to the methods described previously (Izumi et al., 1995a) with modification. Four days after DY injection into the superior colliculus, we euthanized the rats, enucleated their eyes that were then transferred into Petri dishes containing ice-cold artificial cerebrospinal fluid (aCSF) saturated with 95% O_2/5% CO_2. The aCSF consisted of 124 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 22 mM $NaHCO_3$ and 10 mM glucose, with pH adjusted to 7.4. With sharp small surgical scissors and under a dissecting microscope, we removed the adherent tissue, cornea, lens and iris. The posterior eyecups were transferred to a six-well plate, each well filled with 6 ml of aCSF saturated with 95% O_2/5% CO_2, and were maintained at 30 °C for 90 min (Fig. 1A).

2.4. Drug treatment

After incubation for 90 min, we treated eyecups with excitotoxins by transfer to a 12-well plate whose wells were filled with 3 ml of aCSF containing excitotoxins. After 30 min of treatment, eyecups were transferred into a six-well plate, each well filled with 6 ml of fresh aCSF containing 5 μg/ml PI, and incubated for a further 6 h (1 and 3 h in several experiments). All solutions were continuously bubbled with 95% O_2/5% CO_2 and maintained at 30 °C. MK-801 and NBQX were applied only during excitotoxin treatment. Ethyleneglycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), bicuculline, strychnine, and niflumic acid were applied from the pre-incubation period and were also added in the aCSF during excitotoxin treatment and post-incubation. The pH of aCSF was readjusted to 7.4 after addition of EGTA. Where necessary, choline chloride or sodium methanesulfonate was substituted for NaCl in aCSF. In several sets of experiments, we maintained eyecups in Ames' Medium in place of aCSF during the entire course of incubation.

2.5. Cell death assessment

At termination of post-incubation, we fixed eyecups for 30 min in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The retinas were dissected as flattened whole mounts with four radial cuts, mounted with the vitreal side up on slides and covered with Gel/Mount (Biomeda Corp., Foster City, CA, USA).

Under an inverted fluorescence microscope, we observed DY and PI fluorescence of each retina with a UV and a rhodamine filter set, respectively. For each retina, we randomly selected four fields of 230 × 310 μm^2 from the central area (approximately 1 mm from the optic disc) and the peripheral area (more than 3 mm from the optic disc). By focusing on DY-labeled RGCs, we acquired a DY fluorescence image of the ganglion cell layer (GCL), and then acquired a PI fluorescence image from the same area.

Within each area, we counted DY-, PI- and double-labeled (DY^+/PI^+) cells with the aid of Adobe Photoshop® software. For each retina, the number of DY^+/PI^+ cells was expressed as a ratio (%) to that of total DY-labeled cells. In addition, we obtained the number of PI- but not DY-labeled (DY^-/PI^+) cells by subtracting the number of DY^+/PI^+ cells from that of PI-labeled cells.

2.6. Statistics

Data are expressed as means ± S.E.M. Statistical analyses were performed with one-way analysis of variance followed by the Student–Newman–Keuls test. Probability values less than 5% were considered significant.

3. Results

3.1. NMDA and kainate exhibits cytotoxicity in the GCL

PI is widely used as an indicator of cell death, because this compound is excluded from viable cells but can penetrate into dead cells with ruptured plasma membrane and emits bright fluorescence after binding to DNA (Laake et al., 1999). Obviously, PI staining is not specific for dead RGCs, and therefore we identified RGCs by retrograde labeling with a fluorescent tracer, DY. Retinas obtained from eyecups maintained in aCSF containing PI for 6 h exhibited PI fluorescence only in a few, scattered cells (Fig. 1B). In contrast, many small round spots with bright PI fluorescence, which represented dead cells, were observed at the central area when eyecups were exposed to 3 mM NMDA (Fig. 1C) or 1 mM kainate (Fig. 1D) for 30 min and incubated for further 6 h in aCSF containing PI. No distinct difference was observed in the total number of DY^+ cells that represented RGCs, between retinas obtained from control eyecups (Fig. 1B) and eyecups exposed to NMDA (Fig. 1C) or kainate (Fig. 1D). We regarded DY^+/PI^+ cells as dead RGCs and obtained % values of the number of DY^+/PI^+ cells relative to that of total DY-labeled RGCs, as a measure of RGC death. We also obtained the number of DY^-/PI^+ cells in the GCL, which represented dead cells other than RGCs.

Application of NMDA at concentrations ranging from 0.3 to 3 mM caused a significant increase in dead RGCs (DY^+/PI^+ cells) in the central area of the retina (Fig. 2A). In contrast, NMDA even at a concentration of 3 mM did not cause a significant increase in dead RGCs (DY^+/PI^+ cells) in the peripheral area. The number of dead cells other than RGCs (DY^-/PI^+ cells) markedly increased both in the central area and in the peripheral area in response to NMDA (Fig. 2B). Cell death induced by 3 mM NMDA was almost completely abolished by 10 μM MK-801, a non-competitive NMDA receptor antagonist, both in the central area and in the peripheral area. Kainate at concentrations of 0.1 mM or higher caused a concentration-dependent increase in dead RGCs (DY^+/PI^+ cells) and other dead cells (DY^-/PI^+ cells) both in the central area and in the