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Involvement of $P2X_7$ receptors in retinal ganglion cell death after optic nerve crush injury in rats

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HIGHLIGHTS

- RGC survival in primary culture was examined after treatment with $P2X_7$ antagonists and agonists.
- $P2X_7$ antagonists preserved RGCs in primary culture although $P2X_7$ agonists decreased them.
- $P2X_7$ antagonists were injected into vitreous body after optic nerve crush (ONC) injuries in rats.
- RGCs were preserved when $P2X_7$ antagonists were applied after ONC injury.
- Activation of $P2X_7$ receptor may be involved in RGC death induced by ONC injury.

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ABSTRACT

We investigated whether $P2X_7$ antagonists rescue retinal ganglion cells (RGCs) in culture and after optic nerve crush (ONC) injury. Rats were sacrificed 7 days after retrograde labeling of RGCs with 4′,6-diamidino-2-phenylindole (DAPI), and the retinas were enzymatically dissociated *in vitro* and incubated with $P2X_7$ antagonists or agonists for 3 days. Adenosine triphosphate (ATP) and benzoylbenzoyl ATP were used as $P2X_7$ agonists, and oxidized ATP and brilliant blue G were used as $P2X_7$ antagonists. DAPI-positive and calcein-positive RGCs were counted to determine the number of living cells. We observed that RGCs were preserved when treated with $P2X_7$ antagonists, as compared with the controls. In contrast, $P2X_7$ agonists significantly decreased the number of viable RGCs. *In vivo*, $P2X_7$ antagonists at various doses were injected into the vitreous body immediately after ONC injuries in rats. Surviving RGCs were stained with anti-neuron-specific β-tubulin antibody in flat-mounted retinas. RGCs were observed to decrease to 61% of baseline 7 days after ONC injury, whereas RGCs were significantly preserved when $P2X_7$ antagonists were applied. When $P2X_7$ receptor expression was examined immunohistochemically in rat retinas after ONC, the retinal expression of the $P2X_7$ receptors was observed to be upregulated after ONC and peaked on day 3. Meanwhile, $P2X_7$ antagonists suppressed this upregulation. Collectively, these results suggest that $P2X_7$ antagonists prevent loss of RGCs after ONC, and that this protective effect is possibly mediated through suppressing the upregulation of retinal $P2X_7$ expression.

1. Introduction

The $P2X_7$ receptor is a member of the P2X receptors and is known to affect neuronal cell death during neurodegeneration, chronic inflammation, and chronic pain [16]. $P2X_7$ receptors have also been shown to activate several intracellular signaling pathways, including the c-Jun N-terminal kinases, extracellular signal-regulated kinases, and p38 mitogen-activated protein (MAP) kinases. In particular, $P2X_7$ receptors have been shown to be expressed in macrophages and microglia, and p38 MAP kinases have been reported to be involved in the production of reactive oxygen species, nitric oxide, and tumor necrosis factor α (TNFα) in these cell types, thereby supporting the role of P2X7 receptors in cell death.

Several studies have also revealed that $P2X_7$ receptors are expressed in the retina [1,13]. Immunolabeling of $P2X_7$ has revealed that these receptors are present in amacrine cells and retinal ganglion cells (RGCs), suggesting extracellular ATP may provide both neuromodulatory and trophic influences on visual processing and the neural retina [1]. One report has also shown that $P2X_7$ receptors may be involved in neuronal transmission, rather than in cytolysis, since these receptors are expressed exclusively in neurons in the primate retina [6]. Other studies have suggested that activation of $P2X_7$ receptors might modulate the uptake of neurotransmitters from the extracellular space by Müller cells [13]. A recent

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study using $P2X_7$ knockout mice showed that the $P2X_7$ receptor likely provides excitatory input to photoreceptor terminals or to inhibitory cells that shape both the rod and cone pathway responses [18].

Previously, we showed that hypoxia-induced retinal neuronal cell death is mediated by the activation of $P2X_7$ receptors [17]. Further, others have reported that stimulation of $P2X_7$ receptors by a $P2X_7$ agonist or ATP leads to a large, sustained intracellular calcium increase and the death of purified RGCs [20]. In addition, the intraocular injection of a $P2X_7$ agonist has been reported to significantly decrease the density of RGCs in normal, adult rats [5]. Thus, the activation of $P2X_7$ receptors has been suggested to be largely related to RGC death, although the exact mechanism remains unknown.

A primary focus in neuroprotective therapy for glaucoma patients is to discover mechanisms by which intervention in the neuronal death pathways can prevent RGC damage. Several animal models for the study of neuroprotection in glaucoma have been used including acute and chronic intraocular pressure elevation, optic nerve axotomy, and optic nerve crush (ONC) injuries [3].

In the present study, we investigated whether inhibition of $P2X_7$ receptor activation is involved in the protection of RGCs in both *in vitro* and *in vivo* experiments. To this end, the effects of $P2X_7$ antagonists and agonists on the survival of RGCs in dissociated, primary cultures were examined. Additionally, intraocular injection of $P2X_7$ antagonists immediately after ONC was examined for its effect on RGC viability.

2. Materials and methods

2.1. Animals

Adult (12-week-old) male Wistar rats, weighing 280–320 g, were used. The care of the animals and the experimental procedures used conformed to the Guide for the Care and Use of Laboratory Animals, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and EC Directive 86/609/EEC for animal experiments. Further, the work was performed in accordance with the Uniform Requirements for manuscripts submitted to Biomedical journals. The animals were anesthetized with intraperitoneal pentobarbital (30 mg/kg) for all surgical procedures.

2.2. Reagents

Unless otherwise noted, chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

2.3. Adult retinal primary culture and effects of $P2X_7$ antagonists and agonists

Primary retinal cultures were prepared as previously described [9]. Seven days after retrograde labeling of RGCs with 4′,6-diamidino-2-phenylindole (DAPI), the animals were sacrificed by an overdose of pentobarbital. Two retinas were dissected in L15 culture medium and incubated at 37 °C for 30 min in a CO_2 incubator in a digestion solution comprising papain (10 U/mL; Worthington, Lakewood, NJ) and L-cysteine (0.3 mg/mL) in L15 medium. Retinas were triturated in L15 containing bovine serum albumin (1 mg/mL) and DNase (0.2 mg/mL). Dissociated cells were passed through a strainer (40-μm nylon net, Falcon, Bedford, MA) and collected by centrifugation. The collected cells were resuspended in 1 mL of pH-adjusted (7.4), filter-sterilized Medium E, containing 20 nM hydrocortisone, 1 mM kainurinate, 100 μM putrescine, 20 μM progesterone, 30 nM selenium, 0.3 nM 3,3′,5-triiodo-L-thyronine, 50 μg/mL transferrin, 150 U/mL catalase, 60 U/mL superoxide dismutase, 1% bovine serum albumin (Type V with free fatty acids), 10 μg/mL gentamicin, 5 μg/mL insulin, and 15 mM HEPES. Cells were seeded into each well of a 4-well chamber (4×10^4 cells/well in a 1 mL volume, Nunc, Naperville, IL) and incubated with adenosine triphosphate (ATP) at 10, 30, 100, or 300 μM or benzoylbenzoyl ATP (BzATP) at 10, 30, or 100 μM as $P2X_7$ agonists, or with oxidized ATP (OxATP) at 3, 30, 100, or 300 μM or brilliant blue G (BBG) at 0.1, 1, 10, or 100 μM as $P2X_7$ antagonists for 3 days in a 5% CO_2 environment at 37 °C. For identifying the living cells, calcein-AM at 0.5 μg/μL was added to the wells and incubated for 30 min, as previously described [14]. RGCs were identified by the presence of DAPI, which appears blue when viewed with appropriate filters under epifluorescence. Cell viability was determined by metabolism of calcein-AM to calcein, which produces a green fluorescence when viewed with fluorescein filters. Viable RGCs were co-stained with DAPI and calcein (Fig. 1A and B). After washing, DAPI- and calcein-positive cells were captured in 20 random images per well using a fluorescent microscope (10× objective) equipped with a digital imaging system. The numbers of viable RGCs were counted using ImageJ software (National Institute of Health, Bethesda, MD), and the numbers were averaged. A standard ratio was calculated by dividing the average number of viable RGCs by the number of cells in the corresponding control wells in the same experiment. In each experiment, a total of 4 standard ratios were obtained from 4 wells, treated with the same dose of antagonist or agonist; the experiment was repeated 3 times. For these *in vitro* experiments, 16 eyes from 8 rats were used.

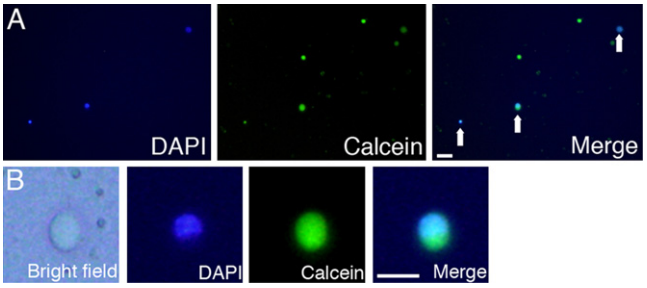

Fig. 1. Retinal ganglion cell (RGC) identification and viability assessment. (A) Low-magnification images and (B) high-magnification images. (A) RGCs were identified in mixed retinal cultures by distinct DAPI staining of the nucleus. Cells staining strongly with calcein, which is cleaved to fluorescent calcein by intracellular esterases, were considered viable. Consequently, cells co-stained with DAPI and calcein were identified as living RGCs. (B) Cultured RGCs possessed a round-shaped soma with a DAPI-labeled nucleus. Calcein homogenously stained throughout cell soma. Scale bar, 50 μm in A and 20 μm in B.

2.4. ONC and drug administration

The left optic nerve was crushed as previously described [8,11]. Briefly, the optic nerve was crushed with fine forceps, approximately 2 mm behind the posterior pole of the eye for 10 s; care was taken not to damage the blood circulation. We excluded animals in which obvious changes in the retina and lens had occurred due to interrupted blood supply after ONC. Immediately after ONC, OxATP at 10 μM, 30 μM, 100 μM, or 300 μM, or BBG at 0.003 μM, 0.03 μM, 0.3 μM, 3 μM, or 30 μM ($n = 4$–5, respectively) dissolved in 5 μL of phosphate-buffered saline (PBS) was injected into the vitreous body with a glass micropipette through a hole made with a 30-gauge needle. The tip of the micropipette was inserted into the peripheral retina just behind the ora serrata, and was deliberately angled to avoid damaging the lens. The right eyes were not used as normal controls because crushing one optic nerve has been demonstrated to affect the morphology of the contralateral retina