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NEURONAL EXPRESSION OF $P2X_3$ PURINOCEPTORS IN THE RAT RETINA

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Abstract—$P2X_3$ purinoceptors are involved in fast, excitatory neurotransmission in the nervous system, and are expressed predominantly within sensory neurons. In this study, we examined the cellular and synaptic localization of the $P2X_3$ receptor subunit in the retina of the rat using immunofluorescence immunohistochemistry and pre-embedding immunoelectron microscopy. In addition, we investigated the activity of ecto-ATPases in the inner retina using an enzyme cytochemical method. The $P2X_3$ receptor subunit was expressed in the soma of a subset of GABA immunoreactive amacrine cells, some of which also expressed protein kinase C-α. In addition, punctate immunoreactivity was observed within both the inner and outer plexiform layers of the retina. Double labeling studies showed that $P2X_3$ receptor puncta were associated with both rod and cone bipolar cell axon terminals in the inner plexiform layer. Ultrastructural studies indicated that $P2X_3$ receptor subunits were expressed on putative A17 amacrine cells at sites of reciprocal synaptic input to the rod bipolar cell axon terminal. Moreover, we observed $P2X_3$ immunolabeling on amacrine cell processes that were associated with cone bipolar cell axon terminals and other conventional synapses. In the outer retina, $P2X_3$ immunoreactivity was observed on specialized junctions made by putative interplexiform cells. Ecto-ATPase activity was localized to the inner plexiform layer on the extracellular side of all plasma membranes, but was not apparent in the ganglion cell layer or the inner nuclear layer, suggesting that ATP dephosphorylation occurs exclusively in synaptic regions of the inner retina. These data provide further evidence that purines participate in retinal transmission, particularly within the rod pathway.

Key words: purinergic, extracellular ATP, bipolar cell, electron microscopy, amacrine cell, ectonucleotidase.

The predominant neurotransmitters mediating communication within the retina are glutamate, GABA and glycine. However, there are numerous other neurotransmitters and neuromodulators that are co-expressed by neurons, especially within the inner retina. The involvement of extracellular ATP in retinal neurotransmission has only been considered more recently.

Extracellular ATP mediates fast, excitatory neurotransmission in the nervous system, through activation of cell surface receptors known as P2X receptors (Edwards et al., 1992). Seven receptor subunits ($P2X_1$–$P2X_7$) have been cloned to date (North and Surprenant, 2000) and these are thought to form functional ligand-gated channels through trimeric assembly of receptor subunits (Nicke et al., 1998). P2X receptor ion channels show non-selective cation permeability to K^+, Na^+ and Ca^{2+}, and some subtypes show substantial calcium permeability (Egan and Khakh, 2004). Termination of ATP-mediated neurotransmission is achieved by the activity of surface bound ectonucleotidases that sequentially dephosphorylate ATP to ADP to AMP and finally to adenosine (Zimmermann, 1996).

$P2X_3$ receptors appear to be expressed predominantly on sensory neurons, particularly primary sensory afferents that transmit nociceptive information (North, 2004). However, there is evidence for synaptic $P2X_3$ receptor localization in other brain regions including the cerebellum and midbrain (Hervas et al., 2005). $P2X_3$ receptor subunits can form either homomeric channels or heteromeric channels with the $P2X_2$ receptor subunit (Lewis et al., 1995).

There is growing evidence for the neuronal expression of P2X receptor subunits in the mammalian retina (Brändle et al., 1998a,b; Ishii et al., 2003; Puthussery and Fletcher, 2004, 2006a,b). The gene expression of the $P2X_3$ receptor has been shown by reverse transcriptase polymerase chain reaction (RT-PCR) of whole retinal tissue (Wheeler-Schilling et al., 2001). Moreover, single cell RT-PCR has shown $P2X_3$ receptor mRNA in some bipolar, Müller and ganglion cells (Jabs et al., 2000; Wheeler-Schilling et al., 2000, 2001). To date, immunohistochemical evidence for $P2X_3$ receptors has been demonstrated in putative amacrine cells and a subset of cells in the ganglion cell layer (GCL) (Wheeler-Schilling et al., 2001). However, no description of the specific cell types expressing the $P2X_3$ receptor have been detailed to date, nor is it known if $P2X_3$ receptors are localized to retinal synaptic regions.

The aim of the present investigation was to identify the specific retinal cell types and circuits expressing $P2X_3$ receptors in the retina, in order to gain further insight into the functional role of ATP in retinal signaling.

EXPERIMENTAL PROCEDURES

Retinal tissue preparation

Animal experimentation procedures were conducted in accordance with the University of Melbourne animal experimentation

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Abbreviations: BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NeuN, neuronal nuclear marker; NGS, normal goat serum; OPL, outer plexiform layer; PB, phosphate buffer; PKC-α, protein kinase C-alpha; RT-PCR, reverse transcriptase polymerase chain reaction; VGLUT1, vesicular glutamate transporter 1.

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ethics committee and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to reduce the number of animals used and to minimize suffering.

Adult (>6 weeks) Sprague–Dawley rat retinae were investigated in this study. The animals were deeply anesthetized with an i.m. injection of ketamine hydrochloride (Ketamil, Troy Laboratories, Smithfield, NSW, Australia; 50 mg/kg) and xylazine (illium xylazine; Troy Laboratories; 5 mg/kg), and then killed with a lethal i.p. overdose of sodium pentobarbital (Nembutal; Merial Australia, Parramatta, NSW, Australia; 60 mg/kg). The eyes were enucleated, the anterior segment and vitreous removed, and the posterior eyecups placed in fixative composed of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 30 min. After fixation, eyecups were cryoprotected in graded sucrose solutions (10%, 20%, 30%) and vertical cryostat sections (12 μm) were collected on poly-L-lysine-coated slides.

For pre-embedding immunoelectron microscopy, eyecups were fixed in 4% (w/v) paraformaldehyde in 0.1 M PB (pH 7.4) containing 1 mM $CaCl_2$ for 50 min. Following cryoprotection, the retinae were dissected from the eyecup, and treated with repeated freeze–thaw cycles to improve antibody penetration. Retinal pieces (2×2 mm) were embedded in agar and sectioned at 70 μm with a vibratome and these sections were then immediately processed for pre-embedding immunoelectron microscopy.

Antisera

The primary antibodies that were used in this study are described in detail in Table 1.

Secondary antibodies were raised in goat and directed against mouse, rabbit or guinea-pig IgGs. Secondary antibodies were conjugated to either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA) and used at a dilution of 1:500.

Light microscopic immunohistochemistry

The indirect immunofluorescence method was used for immunohistochemical labeling as described previously (Puthussery and Fletcher, 2004). A blocking buffer containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA), 0.5% Triton X-100 in 0.1 M PB (pH 7.4) was applied for 1 h. Next, primary antibodies were diluted in a buffer containing 3% NGS, 1% BSA, 0.5% Triton X-100 in 0.1 M PB (pH 7.4) and applied to tissues overnight at 25 °C. Secondary antibodies were applied in the same buffer for 1 h at 25 °C. Where multiple antibodies were required for double labeling experiments, sections were incubated in a mixture of primary antibodies, followed by a mixture of secondary antibodies.

For immunohistochemistry on retinal whole mounts, fixed and cryoprotected retinae were dissected from eyecups, cut into quadrants and exposed to repeated freeze–thaw cycles (3×) to improve antibody penetration. Retinal pieces were blocked for 2 h and incubated in primary antibody for 4 days at 4 °C. Secondary antibodies were applied for 4 h in the dark. After rinsing in 0.1 M PB, the retinal pieces were mounted on glass slides, ganglion cell side up, and coverslipped.

The specificity of the $P2X_3$ primary antibody has been confirmed by Western blot analysis in a previous study (Inoue et al., 2003) and was assessed here by performing immunohistochemistry on retinal sections after overnight pre-incubation of the $P2X_3$ antibody in excess control antigen. In this case, no specific immunolabeling was observed. Omission of primary or secondary antibodies also resulted in the loss of specific immunoreactivity.

Pre-embedding immunoelectron microscopy

Pre-embedding immunoelectron microscopy was used to localize the $P2X_3$ at the ultrastructural level. This method has been described in detail previously (Puthussery and Fletcher, 2004). Vibratome sections were blocked for 2 h in 10% NGS, 1% BSA in 0.1 M PB (pH 7.4) then incubated in the $P2X_3$ antibody (1:100) diluted in 3% NGS, 1% BSA in 0.1 M PB (pH 7.4) for 4 days at 4 °C. A biotinylated goat–anti-rabbit secondary antibody (1:100) was applied for 2 h (Zymed Laboratories Inc., South San Francisco, CA, USA). A Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) and 0.05% (w/v) 3,3′-diaminobenzidine (DAB) with 0.01% H_2O_2 was used to visualize the secondary antibody. Post-fixation of sections was with 2.5% (v/v) glutaraldehyde in cacodylate buffer for 2 h at 4 °C. The DAB reaction product was silver intensified and gold toned and sections were then post-fixed in 0.05% OsO_4. After dehydration, tissue sections were embedded in Epon resin. Ultrathin sections were collected on Formvar-coated copper grids, contrasted with uranyl acetate and lead citrate solutions, and viewed with a Phillips CM120 electron microscope.

All pre-embedding immunoelectron microscopy experiments were performed on tissue from two animals in two independent experiments. For control experiments, the primary antibody was omitted and the tissue processed up to the silver intensification step. At this point, no labeling was observed, confirming the specificity of the reaction.

Cytochemical detection of ecto-ATPase activity

The localization of ecto-ATPase activity was achieved using an enzyme cytochemical method. This method was originally described in brain by Zinchuk et al., (1999) and was adapted by our laboratory for ecto-ATPase localization in retina. The detailed protocol and relevant controls of specificity have been described previously (Puthussery and Fletcher, 2006a). Although it is possible that this method may detect low levels of degradation of other endogenous extracellular nucleotides, ATP is the preferred

Table 1. Details of primary antibodies

Antiserum	Description of immunogen	Source/cat. no.	Dilution	Cell type/reference
Guinea-pig anti-GABA	GABA coupled to KLH via glutaraldehyde	Chemicon International (Temecula, CA, USA), #AB175	1:1000	GABAergic amacrine cells (Koulen et al., 1998)
Mouse anti-NeuN	Purified cell nuclei from mouse brain	Chemicon International, #MAB377	1:500	(Franke et al., 2005)
Mouse anti-parvalbumin	Parvalbumin purified from carp muscles	SWant (Bellinzona, Switzerland), #235	1:4000	All amacrine cells (Wässle et al., 1993)
Mouse anti-PKC (clone MC5)	Purified bovine brain PKC (recognizes an epitope within amino acid sequence 296–317)	Sigma-Aldrich (Castle Hill, NSW, Australia), #P5704	1:400	Rod bipolar cells (Greferath et al., 1990)
Rabbit anti-$P2X_3$	Amino acids 383–397 of rat P2X3 receptor	Oncogene Research Products (Merck Biosciences, Kilsyth, VIC, Australia), #PC411	1:100	
Guinea-pig anti-VGLUT1	Synthetic peptide corresponding to amino acids 541–560 from rat VGLUT1	Chemicon International, #AB5905	1:10,000	Bipolar cell axons (Johnson et al., 2003)