Cellular/Molecular

ATP-Induced Non-Neuronal Cell Permeabilization in the Rat Inner Retina

Barbara Innocenti,[1,2]* **Sylke Pfeiffer,**[1]* **Eberhart Zrenner,**[1] **Konrad Kohler,**[1] **and Elke Guenther**[1,3]

[1]Laboratory of Cell Physiology and Molecular Biology, Department of Experimental Ophthalmology, University Eye Hospital, D-72076 Tuebingen, Germany, [2]Department of Neurobiology and Anatomy, University of Texas Health Science Center at Houston, Houston, Texas 77030, and [3]Natural and Medical Sciences Institute (NMI) at the University Tuebingen, D-72770 Reutlingen, Germany

The P2X[7] subtype holds a special position among P2X receptors because of its ability to act both as a classical, ligand-gated ion channel, and as a permeabilization pore that can induce cell death under prolonged activation by ATP.

We have shown previously that, in rat retina, P2X[7] receptors are located in the inner nuclear layer and ganglion cell layer (GCL). The present study was aimed at finding whether retinal P2X[7] receptors can act as a mediator of cell permeabilization and, if so, at identifying the cellular target(s) of this effect.

As an indicator of cell permeabilization, we used the fluorescent dye YO-PRO-1 (molecular weight, 375 Da), which enters cells only through large pores like those opened by prolonged or sustained stimulation of P2X[7] receptors and binds to DNA, providing a stable labeling of the activated cells.

Different agonists for P2 receptors were tested for their ability to cause cell permeabilization in flat-mounted rat retinas. Among them, only high concentrations of ATP (500 μM) and BzATP (2′,3′-O-(4-benzoyl-benzoyl)-ATP triethylammonium) (100 μM) were able to induce accumulation of YO-PRO-1 in the GCL and in the nerve fiber layer, suggesting that different cell types were responding to P2X[7] stimulation. This effect was blocked by the P2 antagonists suramin and PPADS (pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid) and by the P2X[7]-selective inhibitor Brilliant Blue G.

To identify the retinal cell types affected by ATP-induced permeabilization, we used *in vivo* labeling techniques. Our data clearly reveal that prolonged stimulation of P2X[7] receptors elicits permeabilization exclusively in microglial cells but not in neurons of the inner retina.

Key words: ATP; P2X[7]; purinergic receptors; retinal neurotransmission; microglia; ganglion cells

Introduction

Besides being the universal cellular energy source, ATP is a key extracellular signaling molecule whose role was inferred long before cloning of the receptors that mediate its effects. Nowadays, these receptors have been assigned to a large family, the P2 receptors, comprising P2Ys, G-protein-coupled receptors, and P2Xs, ligand-gated ionic channels (Ralevic and Burnstock, 1998).

Despite the knowledge that ATP is released during physiological and pathological events in the mammalian retina, our understanding of the role of this neurotransmitter and its actions in the retina is still minimal.

Compelling evidence has been accumulated that ATP exerts a modulatory effect on retinal neurotransmission (Neal and Cunningham, 1994; Neal et al., 1998). Moreover, stimulation of retinal glial cells *in situ* evokes an increase in extracellular ATP, accompanied by the spreading of a calcium wave among adjacent glial cells; this in turn affects the firing rate of neighboring neurons (Newman and Zahs, 1997, 1998; Newman, 2001). Primary culture preparations have made it possible to identify cholinergic neurons, retinal ganglion cells (RGCs), astrocytes, Muller cells, and oligodendrocytes as cellular targets of extracellular ATP (Neal and Cunningham, 1994; Kirischuk et al., 1995; Keirstead and Miller, 1997; Newman and Zahs, 1997, 1998; Neal et al., 1998; Taschenberger et al., 1999; Pannicke et al., 2000). We performed a first systematic study of the expression and distribution of different types of ATP receptors in the retina and found that both P2X and P2Y subunits are expressed in identified adult retinal cell types, such as bipolar cells, Muller cells, and RGCs (Braendle et al., 1998a,b; Jabs et al., 2000; Wheeler-Schilling et al., 2000, 2001).

Among these, one specific subunit attracted our attention, namely P2X[7]. P2X[7] is not only a nonselective cationic channel like other P2X receptors, but, under prolonged or sustained stimulation by ATP, it can also expand and form a large pore in the cell membrane. This pore is permeable to molecules up to 900 Da. This P2X[7]-mediated cell permeabilization can lead to cell death, as has been shown for many cell types in the immune system (Di Virgilio et al., 2001).

In our previous studies performed on adult rat retina, P2X[7]

was detected in identified RGCs (Braendle et al., 1998b), and labeling was clearly seen in the inner nuclear layer (INL) and ganglion cell layer (GCL), indicating that both RGCs and subpopulations of amacrine cells express this subunit. Because full activation of P2X$_7$ receptors may have a deleterious effect on the complex retinal electrical circuits, the aim of the present study was to assess whether these receptors were functional and endowed with the ability to induce permeabilization of retinal cells. We were especially interested in learning whether the massive RGC death found during normal retinal development can be attributed to the permeabilizing ability of the P2X$_7$ receptor.

Using fluorescence assays of cell permeabilization, our study clearly showed that, although RGCs are endowed with P2X$_7$ receptors, the latter never promotes the opening of permeabilizing pores. Instead, prolonged P2X$_7$ stimulation induced permeabilization of microglial cells located in the vicinity of RGCs.

Figure 1. P2X$_7$ expression during retinal development. An antibody against P2X$_7$ was applied to vertical slices from P2 (*A*), P7 (*B*), and P14 (*C*) animals. The arrows indicate immunoreactive cell bodies located on one row at the outer limit of the IPL. Scale bar, 50 μm.

Materials and Methods
Animals
Pigmented brown Norway rats between postnatal day 2 (P2) and P17 were used for all of the experiments. The animals were killed by decapitation under ether anesthesia. Their eyes were removed, the anterior poles were dissected, and the retinas were isolated.

For physiological experiments, the retinas were stored in oxygenated (95% O$_2$–5% CO$_2$) Ames' medium, at 32°C, until use.

Fluorescence imaging
Experiments were performed with an upright microscope (BX50WI Olympus Optical, Tokyo, Japan) equipped for epifluorescence microscopy. Excitation light (480 nm) was provided by a monochromator (T.I.L.L. Photonics, Martinsried, Germany) and delivered to the sample through a quartz fiber and a 40× water immersion objective. Fluorescence emission was collected through a dichroic mirror (500 dichroic long-pass) and filtered with a 515 long-pass filter. Light was detected using a CCD Camera (TILL IMAGO; T.I.L.L. Photonics, Martinsried, Germany).

The retinas were continuously perfused with saline containing the following (in mM): 117 NaCl, 3 KCl, 0.3 CaCl$_2$, 15 glucose, 0.5 NaH$_2$PO$_4$, and 26 NaHCO$_3$ (bubbled with 5% CO$_2$).

ATP, 2',3'-O-(4-benzoyl-benzoyl)-ATP triethylammonium (BzATP) (100 μM), YO-PRO-1 (1.5 μM), and all the other agonists (1 mM) and antagonists (100 μM) were applied from a pipette (0.1 mm) connected to a valve-controlled superfusion system (DAD-12; ALA Scientific Instruments, Westbury, NY).

In vivo *retinal cell labeling*
Nuclei. The nuclei were labeled *in vivo* by incubating the intact retina at room temperature with the cell membrane-permeable dye Hoechst 33342 (10 μg/ml) for 10 min after enzymatic treatment with collagenase–dispase (2 mg/ml) plus DNase (100 mg/ml) (5 min).

Retinal ganglion cells. Crystals of micro-ruby were deposited on the optic nerve stump of isolated eyeballs. After 15 min, the crystals were washed out, and the retinas were dissected and stored for 2 hr at 32°C. This time interval allowed retrograde transport of the dye along the nerve fibers to the cell bodies in the GCL. Alternatively, red fluorescent beads (Lumafluor, Naples, FL) were injected into the superior colliculus of P1 animals.

Muller cells. Muller cell end feet were labeled by perfusing for 5 min with the dye Mitotracker Red dissolved in extracellular saline (10 mg/ml).

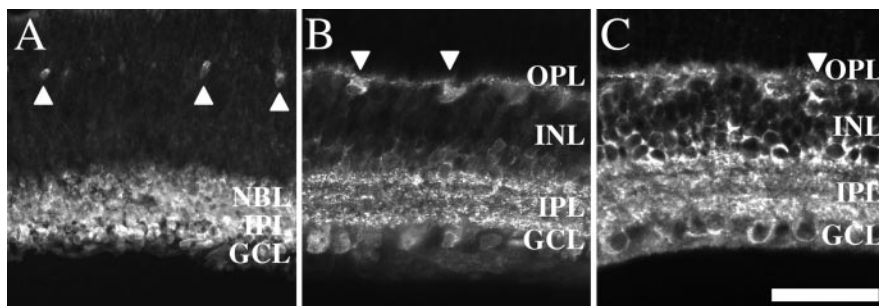*Microglia.* Microglia were labeled by incubation for 1 hr in Cy3-conjugated isolectin B4 (ILB-4) (20 μg/ml; generous gift from Prof. A. Reichenbach, University of Leipzig, Leipzig, Germany) after enzymatic treatment with collagenase–dispase (2 mg/ml) plus DNase (100 mg/ml) (5 min).

Immunohistochemistry
Retinas from Brown Norway rats at different developmental stages between P2 and P14 were used. Retinas were fixed for 20 min in 4% (w/v) paraformaldehyde in phosphate buffer (PB) (0.1 M), pH 7.4, at 4°C. After washing in PB, tissues were cryoprotected by successive immersions for 10 min in 10% (w/v) and 20% sucrose in PB and finally stored overnight at 4°C in 30% sucrose. Samples were then embedded in a tissue-freezing medium (Jung; Leica, Heidelberg, Germany), sectioned vertically into 14 μm slices with a cryostat, and collected on gelatin-coated slides.

Sections were incubated for 1 hr with 10% normal goat serum (NGS) (Sigma, Munich, Germany) and 0.3% Triton X-100 in PBS (PBST) to reduce background staining. The P2X$_7$ subunit antibody (Chemicon, Hofheim, Germany) was diluted (1:250) in PBST containing 10% NGS and incubated overnight at 4°C. After being washed with PBS, the samples were incubated for 1 hr with Alexa 488 (dilution 1:1500; Mo Bi Tec, Goettingen, Germany) in PBST with 5% NGS.

Similar immunostaining pattern was obtained with at least two other different antibodies kindly provided by Dr. F. Di Virgilio (University of Ferrara, Ferrara, Italy) and J. A. Barden (University of Sydney, Sydney, Australia).

Reagents and antibodies
All of the reagents used were of analytical grade. ATP, adenosine 5'-O-(3-thiotriphosphate) (ATPγS), 2'-3'-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt, UDP, ADP, suramin, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), 2',3'-dialdehyde ATP [oxidized ATP (OzATP)], and Brilliant Blue G were from Sigma. YO-PRO-1 iodide, micro-ruby, and Mitotracker Red were obtained from Molecular Probes (Mo Bi Tec, Goettingen, Germany).

Results
P2X$_7$ expression in the rat retina
To show that P2X$_7$ is not only expressed in the adult rat retina (Braendle et al., 1998b) but also at the ages when the physiological experiments of the present study were performed, we performed immunocytochemical experiments on vertical retinal slices of P2–P14 animals.

As Figure 1*A* shows, immunoreactivity (IR) for P2X$_7$ was observed as early as P2. At this age, almost all cells in the GCL and in the inner part of the neuroblastic layer (NBL) were clearly labeled. Other immunoreactive cells were located in the outer part of the NBL and seemed to be distributed in one row (arrows).

Many cells in the GCL were labeled at P7 (Fig. 1*B*), suggesting that RGC and/or amacrine cells expressed P2X$_7$ receptors. Labeling in the inner plexiform layer (IPL) was either diffuse or was clustered into three defined layers. In the INL, P2X$_7$-positive cells
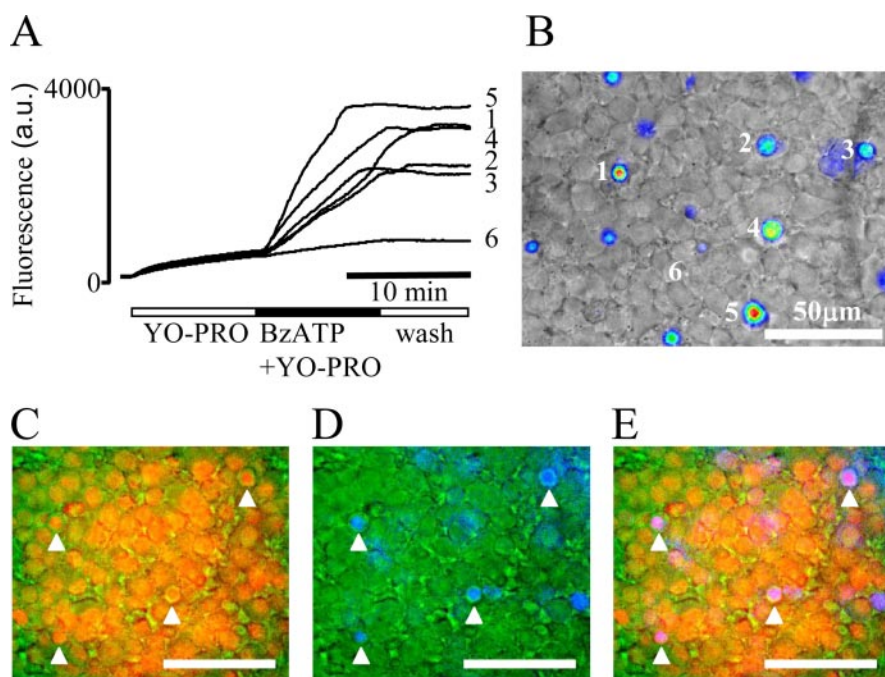
Figure 2. BzATP-induced cell permeabilization in the rat inner retina. A shows the kinetics of YO-PRO-1 (1.5 μM) uptake in rat flat-mount retina exposed to prolonged stimulation with BzATP (10 min, 100 μM). B shows the pattern of YO-PRO-1 labeling in the GCL at the end of the BzATP stimulation. The marked regions correspond to the fluorescence traces shown in A. C, The cell nuclei were labeled with Hoechst 33342 dye (in red) before the experiment. D, YO-PRO-1 accumulation (in blue) was induced by prolonged stimulation with BzATP. E, Merged image of C and D; the white arrows point out the coincidence between YO-PRO-1 staining and labeled cell nuclei in the GCL. Scale bars, 50 μm. a.u., Arbitrary units.

were mostly localized in the two inner rows facing the IPL. As seen already in P2 rats, a row of cells that manifested clear labeling was detected at the outer limit of the INL. In addition, at P7, the receptor was localized in neuronal processes within the outer plexiform layer (OPL). The location and morphology of staining suggested that the labeled cells were horizontal cells.

The distribution and expression of P2X$_7$ did not change with further retinal development. The IR pattern at P14 (Fig. 1C) resembled that observed at P7 and in adult animals from our previous study (Braendle et al., 1998b).

As mentioned in Materials and Methods, this immunological pattern was obtained using at least three different P2X$_7$ antibodies directed to different epitopes.

P2X$_7$ activation in the intact rat retina

Freshly isolated whole-mount rat retinal preparations with the GCL exposed to flowing saline were used to examine the ability of P2X$_7$ to induce cell permeabilization. As an indicator of cell permeabilization, we used the fluorescent dye YO-PRO-1 (molecular weight, 375 Da). The dye enters the cells only through large pores like those opened by prolonged or sustained stimulation of the P2X$_7$ receptor (Surprenant et al., 1996; Rassendren et al., 1997). After entering a cell, YO-PRO-1 binds to DNA, thus providing stable labeling of the activated cells.

The dye YO-PRO-1 (1.5 μM) was added to the medium, and its fluorescence was recorded (Fig. 2A). A small increase in basal fluorescence was detected and recorded for 10 min, during which a stable steady-state level was reached. At that time, the most potent P2X$_7$ receptor agonist BzATP (100 μM) was applied together with the dye. A few seconds after the beginning of stimulation, hot spots of increased fluorescence were detected at different locations in the tissue (Fig. 2B). Figure 2A shows the time

course of fluorescence increases in different regions during a typical experiment ($n = 32$) compared with the negligible increase in baseline fluorescence (Fig. 2A, trace 6). Figure 2B shows the spatial distribution of the fluorescence hot spots at the end of a 10 min stimulation with BzATP. The fluorescent image was overlaid on a bright-field image of the same preparation that revealed the morphological features of the selected focal plane, in this case the GCL. The intracellular locations of the fluorescence spots were confirmed by two different observations. First, a washout of the agonist and YO-PRO-1 at the end of the experiment had no effect on the intensity or location of the fluorescent hot spots in the tissue, indicating that the dye had not accumulated in the extracellular space (Fig. 2A). Second, retinal cell nuclei were labeled *in vivo* before agonist stimulation, by incubating the intact retina with the cell membrane-permeable dye Hoechst 33342 (10 μg/ml). As illustrated in Figure 2C, the cell nuclei in the GCL were labeled (in red for illustration purposes, to distinguish the fluorescence of Hoechst, excited in the UV range, from that of YO-PRO-1, excited by blue light). After perfusion of YO-PRO-1 and BzATP as just described, many hot spots of fluorescence were identified (Fig. 2D, blue). The overlay of Figure 2C with Figure 2D confirms the localization of the YO-PRO-1 staining within cell nuclei.

Pharmacological characterization of retinal cell permeabilization

To investigate whether a receptor-mediated process induced YO-PRO-1 uptake, we studied the effect of different concentrations of agonist on retinal cell permeabilization. BzATP at concentrations as low as 1 μM ($n = 5$) did not elicit any cell permeabilization on preparations that were fully responsive to 100 μM (Fig. 3A). Moreover, there was no significant increase in fluorescence signals ($n = 5$) (Fig. 3B) with 10 μM BzATP. However, further elevation of BzATP to 100 μM resulted in a significant increase in YO-PRO-1 fluorescence, indicating a cellular uptake of the dye by activation of P2X$_7$ receptors.

To show that the dose of BzATP necessary to induce cell permeabilization does not exert a toxic and thus nonspecific effect on retinal preparations, inhibitors of P2 receptors were used to counteract the BzATP effect.

Suramin (100 μM) was applied 10 min before and during application of BzATP. As shown in Figure 3C, we observed no uptake of YO-PRO-1 in the presence of suramin applied with BzATP. However, many cells were permeabilized after suramin was washed out from the saline and BzATP was applied a second time. The result was reproducible in different retinal preparations ($n = 4$), and only one cell responded to the application of BzATP in the presence of suramin.

The same stimulation protocol was used with another P2 receptor antagonist, PPADS (100 μM). Again, no BzATP-induced cell permeabilization occurred in the presence of PPADS, as shown in Figure 3D. In contrast to suramin action,
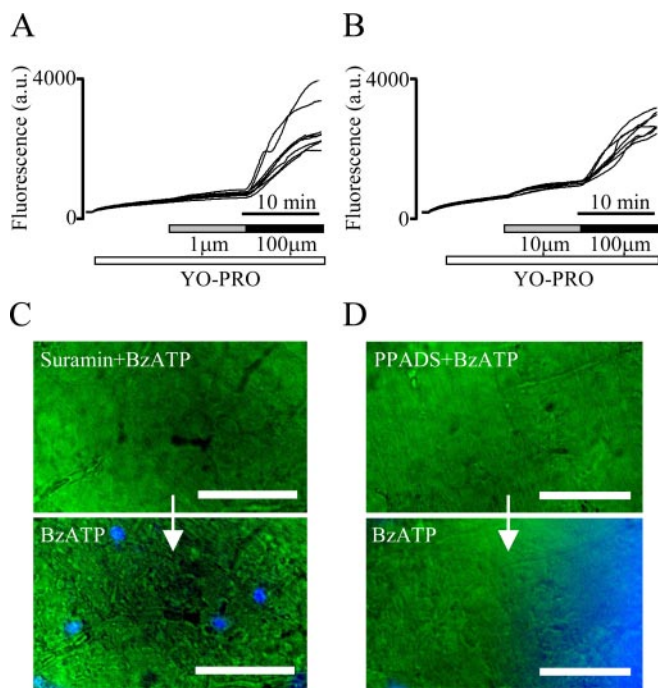
Figure 3. Pharmacology of cell permeabilization. *A, B,* Different doses of BzATP were assessed for their ability to stimulate uptake of the dye YO-PRO-1 in retinal cells. *C, D,* Two different purinergic receptors antagonists, suramin and PPADS (100 μM), inhibited BzATP-induced cell permeabilization. As expected, and in contrast to suramin, the effect of PPADS was irreversible. Scale bars, 50 μm. a.u., Arbitrary units.

Figure 4. BzATP does not permeabilize RGCs and Muller cells. *A,* RGCs were labeled with micro-ruby (red). Crystals of the dye were applied on the nerve stump of dissected eyeballs for 15 min. After washout, the retina was dissected and kept for 2 hr at 32°C to allow retrograde transport of the dye to the RGC bodies. When YO-PRO-1 and BzATP were applied, no micro-ruby-labeled cells allowed the entry of YO-PRO-1 (blue). *B,* Muller cells were labeled by perfusion of red Mitotracker (10 mg/ml) for 2 min in the extracellular saline. Bright-stained Muller cell end feet (red) in the ganglion cell layer do not overlay with the YO-PRO-1 labeling (blue) induced by BzATP stimulation. Scale bars, 50 μm.

however, the PPADS effect was irreversible after up to 30 min of washout ($n = 3$).

Unfortunately, we were unable to verify the action of the most potent antagonist of P2X$_7$ receptor, OzATP (Murgia et al., 1993). Application of this compound to rat retinal preparations induced permeabilization of almost all of the retinal cells in the absence of BzATP. This effect was already evident 5 min after the beginning of perfusion of the antagonist. It has been shown previously that OzATP can exert toxic effects on cultured rat cerebellar neurons, although this effect was manifested after prolonged incubation, approximately hours, with the compound (Craighead et al., 2001).

Altogether, we were able to verify the inhibitory action of another selective blocker of rat P2X$_7$, namely Brilliant Blue G (Jiang et al., 2000). The inhibitor was applied 20 min before application of BzATP (50–100 μM) and was effective at both 0.1 ($n = 3$) and 1 ($n = 5$) μM concentrations. As expected, the inhibition was irreversible after extensive (up to 20 min) washout of the compound.

Once the permeabilizing action of BzATP had definitely been ascribed to a receptor-mediated process, we investigated whether other known P2 receptor agonists were also able to induce cell permeabilization on retinal preparations. ATP, ATPγS, ADP, and UTP were applied respectively with YO-PRO-1. The permeabilizing effect of these compounds was compared in the same preparation with that elicited by BzATP (100 μM) when subsequently applied alone. Even at concentrations of 1 mM, ADP ($n = 3$), UTP ($n = 3$), and ATPγS ($n = 3$) were unable to induce cell permeabilization, indicating that other P2X subunits do not exert a permeabilizing effect on retinal cells (data not shown). Only ATP concentrations of up to 500 μM ($n = 8$) were able to reproduce the fluorescence pattern elicited by BzATP and occlude further activation by BzATP (data not shown). Both of these effects
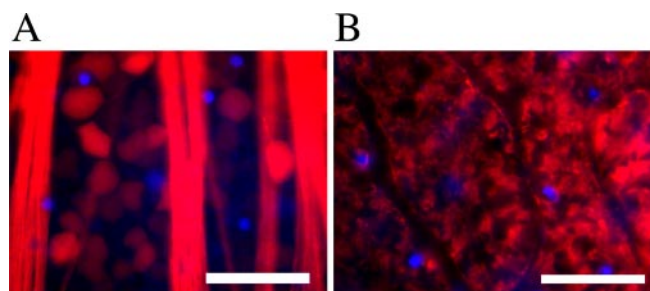
might have been expected from the known pharmacological profile of the P2X$_7$ subunit. Because ADP, UTP, and ATPγS are only weak ligands for the P2X$_7$ subunit, they were not potent enough to induce P2X$_7$-mediated cell permeabilization. In addition, and in contrast to other P2 subunits, P2X$_7$ exhibits only a low affinity for ATP and ATP-like agonists (North and Surprenant, 2000).

It is well known that the activity of P2X$_7$ is partially blocked by divalents such as Ca^{2+} and Mg^{2+}. To maximize receptor activation and thus our ability to detect it, the experiments were generally performed in extracellular saline containing low concentrations of divalent cations (Ca^{2+} at 0.3 mM and Mg^{2+} at 0 mM). However, BzATP was still able to induce cell permeabilization, even in the presence of 2 mM Ca^{2+} and 1 mM Mg^{2+} ($n = 8$).

Identification of the retinal cell types expressing P2X$_7$

To identify the retinal cell types permeabilized by ATP, we labeled each cell type before the imaging experiments with cell-specific markers.

RGCs were labeled by retrograde labeling with micro-ruby. After dissection of the eyeballs, a crystal of micro-ruby was applied to the top of the nerve stump. This operation ensured that only RGCs were exposed to the dye. Figure 4A shows a typical preparation in which both the fibers and numerous RGC bodies were labeled (red). After perfusion of YO-PRO-1 and BzATP, there was no colocalization of YO-PRO-1 fluorescence (blue) with micro-ruby-labeled RGCs, indicating that no permeabilization had taken place. Micro-ruby did not diffuse away from pores opened by BzATP, because the molecular weight of the dye (3000 Da) is higher than the cutoff size of permeating compounds (Surprenant et al., 1996; Rassendren et al., 1997). Moreover, careful comparison of the labeled RGCs before and after the permeabilizing assay revealed no loss of the dye from the cells.

Figure 4B shows that BzATP failed to induce permeabilization of Muller cells, too. Because of their high phagocytic activity, Muller cells were labeled with a dye (Mitotracker) that was simply added to the external medium for few minutes. Avid uptake of the compound by the exposed Muller cell end feet allowed their identification in the whole-mount preparation (Fig. 4B, red). When YO-PRO-1 and BzATP were applied on whole-mount retinas in which Muller cells were labeled, we observed no uptake of YO-PRO-1 by the end feet of Muller cells (Fig. 4B, blue).

Microglial cells were labeled with Cy3-coupled ILB-4, an isolectin that binds to specific terminal D-galactosyl residues present on the plasma membrane of microglial cells (Streit and
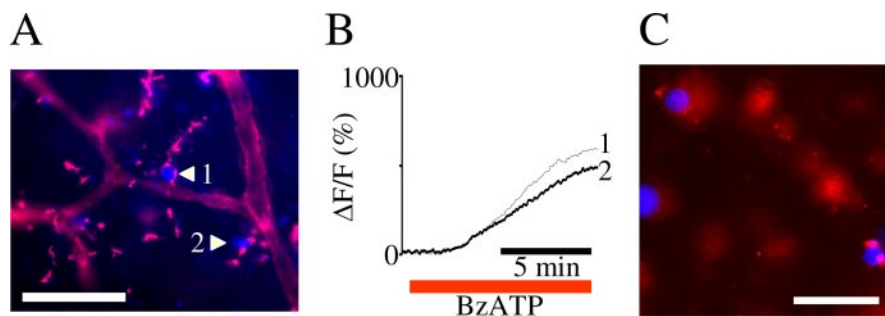
Figure 5. Microglial cells are permeabilized by BzATP stimulation. *A* shows a retinal preparation in which microglial cells were labeled by incubation of flat-mount retinas with Cy3-conjugated ILB-4 (red; 20 mg/ml, 1 hr at 32°C) before the experiment. At the end of the experiment, two ILB-4-labeled cells were labeled by YO-PRO-1 (blue). The graph in *B* shows the kinetics of specific YO-PRO-1 uptake after activation with BzATP at 100 μM. The traces refer to the same two bead-labeled cells shown in *A*. In *C*, several cells in the GCL are labeled by red fluorescent beads. The fluorescent beads were injected in P1 rat superior colliculus, in which the majority of ganglion cell axons terminate. However, in the first week of retinal development, many RGCs die, and microglial cells phagocyte dying RGCs and thus uptake the fluorescent beads as well. Two bead-labeled cells clearly uptook YO-PRO-1 during BzATP stimulation. Because, as we have already shown, RGCs are not permeabilized by BzATP, the two labeled cells were identified as microglial cells that had ingested labeled RGCs. Scale bars, 50 μm.

Kreutzberg, 1987; Streit, 1990). As shown in Figure 5*A*, ILB-4 labeling revealed the ramified morphology typical of resting microglial cells (red). After perfusion of YO-PRO-1 and BzATP, the nuclei of two cells that were delimited by the ILB-4 IR appeared (Fig. 5*A*, blue). The relative spatial localization of the fluorescent signals suggested that YO-PRO-1 entered in two prelabeled microglial cells. Figure 5*B* shows the kinetics of the fluorescence increases, proving the specificity of the signal for the addition of BzATP. The traces refer to the same two microglial cells shown in Figure 5*A*.

In Figure 5*A*, it is apparent that ILB-4 labeled not only microglial cells but also the endothelium of blood vessels (Streit et al., 1987). To substantiate the identification of microglial cells as targets of cell permeabilization by P2X$_7$ stimulation, we used an additional labeling procedure.

Red fluorescent beads were injected into the superior colliculus of P1 animals. The beads were transported retrogradely to the RGC. During the first 2 weeks of rat retinal development, many RGCs die as a result of natural apoptosis (Cellerino et al., 2000) and are phagocytozed by microglial cells. Thus, microglial cells that ingested bead-labeled dying RGCs become labeled as well (Bodeutsch and Thanos, 2000).

BzATP and YO-PRO-1 were applied to retinas with a mixed population of RGCs and microglial cells labeled with red fluorescent beads. Uptake of YO-PRO-1 was detected in a few bead-labeled cells (Fig. 5*C*). Because we have shown previously that RGCs are not permeabilized by BzATP stimulation, the permeabilized cells can only have been microglial cells that had previously phagocytozed dying RGCs.

In conclusion, our data clearly indicate that, despite the widespread expression of P2X$_7$ subunits in different neuronal types in the inner rat retina, prolonged stimulation of this subunit elicits permeabilization only in microglial cells located in the nerve fiber layer, in close proximity to RGC.

Discussion

Among P2X receptors, the P2X$_7$ subtype holds a special position because of its ability to act as both a classical, ligand-gated ion channel and a permeabilization pore that can induce cell death under prolonged activation by ATP (Ferrari et al., 1997, 1999; Virginio et al., 1999; Di Virgilio et al., 2001). Because we have shown previously that P2X$_7$ receptors are expressed in the inner retina,

including retinal neurons (Braendle et al., 1998b), the aim of the present study was to assess whether the P2X$_7$ subtype can induce permeabilization of retinal cells in response to prolonged activation by ATP and to identify the cell types affected.

The results of this study suggest that the receptor is not only present at developmental stages as early as P2 but is also functionally active. Cell permeabilization was found only in microglial cells that are located close to RGCs in the nerve fiber layer.

Experimental approach

Fluorescence assay of cell permeabilization was chosen for experiments performed on freshly excised retinas in whole-mount preparation with a minimum of manipulation before the experiments. Vertical slices were not suitable because of extended cell damage at the surfaces during preparation. Cells that were dying after damage or that were stimulated by ATP from adjacent dying cells would have taken up the dye, halting our ability to assess the specificity of the uptake by controlled P2X$_7$ stimulation.

We made also a careful choice of the age of the animals to be used. We opted for animals in the first 2 weeks of postnatal development for several reasons. (1) Almost 50% of RGCs die during the first 2 weeks of rat retinal development, before definitive connections to the brain are established. If the P2X$_7$ receptor were indeed involved in mediating RGC death by induction of cell permeabilization, our ability to detect cell activation would have been favored by using early postnatal stages. (2) During early postnatal development, the nerve fiber layer is relatively thin, which would ensure a better penetration of the chemical compounds we were using. At later stages, we should have used enzymatic treatment to penetrate the nerve fiber layer and allow the diffusion of the chemicals deep into the retinal tissue, increasing the possibility of inducing unwanted cell damage.

Pharmacology of P2X$_7$ receptor

Once we had defined the proper experimental model, we applied YO-PRO-1 as a marker of cell permeabilization together with BzATP, the most potent agonist of P2X$_7$ receptors.

BzATP can stimulate P2X$_1$, P2X$_3$, and P2Y receptors as well (Bianchi et al., 1999; Pannicke et al., 2000). However, we have shown previously that the rat retina does not express P2X$_1$ (Braendle et al., 1998a). Moreover, even if other P2 subunits have been shown to induce cell permeabilization, P2X$_1$ and P2X$_3$ are not among them (Khakh et al., 1999; Virginio et al., 1999).

Among the different purinergic receptor agonists we used in this study, only ATP and BzATP elicited YO-PRO-1 uptake. ADP and UTP, agonists of P2Y receptors, were ineffective, suggesting that P2Y receptors were not involved in cell permeabilization.

The generic purinergic antagonists suramin and PPADS inhibited cell permeabilization. This observation was important, because it excluded the possibility that another subtype, P2X$_4$, could be involved in cell permeabilization. P2X$_4$ can induce cell permeabilization in transfected cells under prolonged receptor stimulation. However, the receptor is not sensitive to the action of the aforementioned inhibitors (North and Surprenant, 2000). In addition, YO-PRO-1 entry by P2X$_4$ has much slower kinetics

and less probability to occur than that by P2X$_7$ (Khakh et al., 1999). Instead, the time course of cell permeabilization we observed matches the one expected for P2X$_7$ receptor.

In further support for a P2X$_7$-mediated cell permeabilization, YO-PRO-1 entry was blocked by Brilliant Blue G, a selective inhibitor of rat P2X$_7$ receptor. The effect was observed at nanomolar concentrations of inhibitor, even in the tissue preparations we used in our experiments.

Unexpectedly, we were unable to examine the action of the best-known P2X$_7$ antagonist, OzATP. The compound had a fast and wide neurotoxic effect on retinal preparations, inducing nonspecific permeabilization and thus YO-PRO-1 uptake in all cells in the inner retina in <5 min of stimulation. Whether this phenomenon is attributable to a tissue-specific effect needs to be explored further.

Identification of retinal cell types expressing permeabilizing P2X$_7$

To find which retinal cell types expressed functional P2X$_7$ receptors, we used live staining for the different cell types.

Muller cells did not manifest any phagocytic activity for YO-PRO-1 under resting or ATP/BzATP-stimulated conditions. It might be objected here that Muller cell nuclei lie deep in the INL, so that the binding of the dye to DNA could pass undetected in experiments like ours in which the focal plane is located in the ganglion cell layer. However, we have already shown that rat Muller cells do not express the P2X$_7$ subunit (Jabs et al., 2000), and preliminary experiments of double immunostaining with antibodies against P2X$_7$ and glutamine synthethase fully support the observation that P2X$_7$ is not expressed by rat Muller cells.

Regarding the second glial cell type in the retina, astrocytes, our study found no evidence of the expression of "pore-forming" P2X$_7$. There is evidence that astrocytes express P2X$_7$ both in culture and *in situ* (Ballerini et al., 1996; Kukley et al., 2001). However the size and shape of the nuclei that took up YO-PRO-1 in our study did not match with the large and oval shape of the nuclei belonging to astrocytes. In addition, no colocalization between GFAP and P2X$_7$ was detected in preliminary immunofluorescence experiments.

In a previous study, using single-cell reverse transcription-PCR and immunohistochemical techniques, we found evidence for the expression of P2X$_7$ receptors in at least a subpopulation of RGCs (Braendle et al., 1998b). In the present study, receptor activation failed to induce pore permeabilization in RGS. The two sets of experiments are not in opposition to each other. The fluorescence imaging approach of the present study aimed at detecting only a specific operating mode of P2X$_7$, namely the permeabilizing pore state induced by prolonged agonist stimulation rather than its function as a ligand-gated ion channel. It is thus possible that RGCs or a subpopulation of them express functional P2X$_7$ receptors acting as "classical ionic channels." To verify this hypothesis, we are performing electrophysiological patch-clamp recordings from identified RGCs in intact tissue.

Several research groups have shown that P2X$_7$ is expressed and functionally active in the RGCs of different animal species (Davies and Baldridge, 2001; Zhang et al., 2002; Ishii et al., 2003). Independently of the lack of permeabilizing activity, P2X$_7$ subunit activation may induce cytotoxicity through prolonged calcium influx (Zhang et al., 2002) or through stimulation of release of neurotoxic compounds. In the CNS, P2X$_7$ receptor activation has been reported to enhance glutamate release from presynaptic terminals and cultured astrocytes (Deuchars et al., 2001; Ye et al.,

2003). Additional studies will be aimed at identifying the role of P2X$_7$ in RGCs.

Our study indicated that cell permeabilization was induced by P2X$_7$ activation only in microglial cells. It is known that ATP activates P2X$_7$ receptors in microglial cells in which it mediates cytotoxic responses and cytokine release. This suggests that it may have an important role in immunomodulation (Collo et al., 1997; Di Virgilio et al., 1998; Ferrari et al., 2000; Inoue, 2002). There is increasing evidence that microglial cells are related to a number of physio-pathological events in the retina when massive cell death occurs (Schnitzer and Scherer, 1990; Milligan et al., 1991; Thanos, 1991; Frade and Barde, 1998). Their role is not restricted to a mere removal of damaged and dying neurons. Microglia contribute actively to the degradation of neurons, because it has been shown that suppression of microglial activity results in a retardation of tissue cell death (Thanos et al., 1993; Frade and Barde, 1998).

Because P2X$_7$ activation is the major trigger of microglial activation, P2X$_7$ may indeed play a role in retinal cell death. In response to stimulation, microglia release ATP, nitric oxide, tumor necrosis factor-α, interleukin-1β, and other compounds (Chao et al., 1992; Hide et al., 2000; Sanz and Di Virgilio, 2000) which may contribute to neuronal cell death. In addition, microglial cells interact with various other cell types, which in turn can affect neurons, including astrocytes (Verderio and Matteoli, 2001; John et al., 2003) and Muller cells (Harada et al., 2002).

Recently, another group provided evidence that P2X$_7$ activation induces permeabilization of retinal pericytes in microvessels freshly isolated from the adult rat retina (Kawamura et al., 2003). Difference in the kinetics and intensity of the uptake of YO-PRO-1 between microglia and pericytes could explain why YO-PRO-1 uptake in pericytes was undetected in our experiments.

In conclusion, we demonstrated that P2X$_7$ receptors are expressed and functionally active during development of the mammalian retina. A discrepancy between the distribution of the receptor subunit and its permeabilizing activity suggests that the receptor has different activation properties in different retinal cell types, and thus P2X$_7$ may be involved in multiple physiological and pathological states of the mammalian retina.

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