Expression Profiles of P2-Receptor Isoforms P2Y₁ and P2Y₂ in the Rat Lens

Rachelle Merriman-Smith, Mark Tunstall, Joerg Kistler, Paul Donaldson, Gary Housley, and Reiner Eckert

PURPOSE. To verify at the molecular level that P2 receptors are expressed in the lens and to determine their expression profiles.

METHODS. The reverse transcription–polymerase chain reaction (RT-PCR) was used to screen rat lens epithelial and fiber cells for the expression of the ionotropic P2X₂ receptor and the G-protein-linked receptor isoforms P2Y₁ and P2Y₂. Northern blot analysis was used to confirm the level of expression of P2Y₁ and P2Y₂. The profile of P2-receptor isoform expression in the lens was identified using an indirect RT-PCR in situ hybridization procedure on paraffin sections of whole rat eyes.

RESULTS. P2X₂-receptor transcripts could not be detected in lens epithelial or fiber cells. P2Y₁- and P2Y₂-receptor transcripts were detected in lens fiber cells but not in the epithelial cells. Their expression profiles were maximal in the lens cortex and mostly overlapped each other, except that transcripts for the P2Y₂-receptor isoform appeared earlier in the not yet fully elongated fiber cells in the lens bow region.

CONCLUSIONS. The molecular data support physiological evidence previously reported by others that P2Y receptors are expressed in the lens and identifies cortical fiber cells as the principal site of expression. (Invest Ophthalmol Vis Sci. 1998;39:2791-2796)

There is accumulating evidence that G-protein-linked receptors and their signaling pathways are active in cultured epithelial cells and the intact lens. For example, it has been shown that activation of lens β-adrenergic receptors, which are coupled to adenylyl cyclase, lead to an increase of intracellular cAMP. Furthermore, the activation of muscarinic acetylcholine receptors results in an increase of intracellular calcium levels either by inducing the release of Ca²⁺ from intracellular stores or by modulating calcium channels in the plasma membrane. The activation of nucleotide receptors coupled to phospholipase C also mediates an intracellular Ca²⁺ mobilization in cultured lens epithelial cells. ATP and UTP both elicited the same response, which was attributed to the P2U receptor because it is the only nucleotide receptor that responds to ATP and UTP equipotently.

To the best of our knowledge, the identification of these G-protein-linked receptors is thus far limited to the β-adrenergic receptors, which have been localized to the lens epithelial and fiber cells by affinity labeling with a receptor antagonist. Our report addresses this lack of molecular data. cDNA encoding for most of these receptors has been sequenced, and together with advanced polymerase chain reaction (PCR)-based technologies, tissue can now be probed for receptor expression by detecting the relevant receptor transcripts. We chose P2 receptors as a test case because P2U receptors have been functionally identified in the lens by two independent research laboratories. We predicted that we should be able to detect the transcript coding for the P2Y₂-receptor isoform in lens RNA preparations because this P2 receptor responds to ATP and UTP equipotently. In addition, we also included two other P2-receptor isoforms for our investigation: P2Y₁, as another example for a metabotropic and P2X₂ as an example for an ionotropic P2 receptor, which are both widely expressed in mammalian tissues. Our results provide a molecular characterization of the UTP-responsive receptor in the rat lens as the P2Y₂-receptor subtype and also identify expression of the UTP-insensitive P2Y₁ receptor in the rat lens. Both receptor isoforms reach maximal expression in the cortical fiber cells. PCR-based screening of the lens is therefore a valuable tool to determine the molecular identity and localization of lens receptors for which physiological evidence exists and to discover new receptors.

MATERIALS AND METHODS

RNA Preparation and Reverse-Transcription–PCR

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Lenses, dissected free from the extracted eyes of 3- to 5-day old female Wistar rats, were rolled on sterile filter paper to remove any adherent tissue, then gently washed with sterile RNase-free (dimethyl dicarbonate-treated) phosphate-buffered saline (PBS), and incubated at 37°C in minimal essential medium for at least 30 minutes. The lenses were decapsulated under sterile conditions by introducing a tear in the capsule at the anterior pole with a pair of sharpened forceps and pulling off the capsule. Because the epithelial cells adhere largely to the capsule, the lens could be separated into an epithelial and a fiber cell portion that were then processed separately. Total RNA was isolated from epithelial and fiber cell portions from 40 lenses using Trizol reagent as per the manufacturer’s instructions (GIBCO-BRL). Genomic DNA was removed from the total RNA by incubation for 15 minutes at 37°C with 0.1 U/μl DNase I (GIBCO-BRL) before cDNA synthesis. Approximately 300 μg fiber cell total RNA and 25 μg epithelial cell total RNA were obtained before mRNA purification (Quick Prep Micro; Pharmacia).

First-strand cDNA synthesis and PCR amplification were conducted using the GeneAmp RNA PCR kit (Perkin-Elmer) with 2.5 μM oligo(dT)₁₂–₁₅ primers and 1 μg mRNA. A control reaction (no reverse transcription [RT] step) was also conducted with the elimination of Moloney murine leukemia virus reverse transcriptase. Ten microliters of the RT product or the controls was added to separate PCR mixtures, which
TABLE 1. Composition of Primer Sets and Probes

<table>
<thead>
<tr>
<th>GenBank Accession No.</th>
<th>Oligonucleotide*</th>
<th>Expected PCR Product Size (bp)</th>
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<td>CX43 M19317</td>
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<td></td>
<td>Antisense primer (20 bp, position 1349)</td>
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<td>Sense primer (25 bp, position 1413)</td>
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<td>805</td>
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<td></td>
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<td></td>
<td>Antisense probe (38 bp, position 1467)</td>
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<tr>
<td></td>
<td>Sense probe (38 bp, position 1430)</td>
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*Size and position in parentheses; sequence given below. PCR, polymerase chain reaction.

Amplification was performed after a "hot-start" with a two-step thermal cycling program with denaturation at 94°C for 1 minute and annealing and extension at 60°C for 2 minutes for a total of 30 cycles, followed by a 7-minute period for extension at 72°C (Hybaid, Omnigene). After electrophoresis and ethidium bromide staining of the 0.8% agarose gel, the amplified PCR products were analyzed, cloned, and sequenced.

**Northern Blot Analysis**

Ten micrograms of lens epithelial and fiber total RNA was subjected to electrophoresis through a 1%-agarose formaldehyde gel in MOPS buffer and transferred overnight to a nylon membrane. Hybridization was performed for 5 hours and annealing and extension at 60°C for 2 minutes for a total of 30 cycles, followed by a 7-minute period for extension at 72°C (Hybaid, Omnigene). After electrophoresis and ethidium bromide staining of the 0.8% agarose gel, the amplified PCR products were analyzed, cloned, and sequenced.

**RT-PCR In Situ Hybridization**

For RT-PCR in situ hybridization, whole eyes were extracted from 5-day-old female Wistar rats, fixed in 10% buffered formalin for 20 hours, and embedded in paraffin. Sixteen-micrometer-thick axial sections were cut in the central axial position of the eye and attached to silane-coated heat-resistant microscope slides (Perkin-Elmer). Orientation and location of sections in the eye were verified using hematoxylin-eosin staining on representative sections. Tissue sections were dehydrated in xylene (Xylo; Ortho Diagnostics) before fixation, and cells were permeabilized with a 45-minute incubation at room temperature in 1:1 Permeafix (Ortho Diagnostics) in 1X PBS. Treatment with Permeafix was terminated with a brief wash in 1X PBS and alcohol dehydration.

The RT-PCR in situ hybridization procedure was based on that used by Greenwood et al.10 In brief, genomic DNA was removed by an overnight incubation with DNase I (GIBCO-BRL), followed by in situ RT on two of the three sections, leaving a control section without cDNA synthesis. Reagents for both RT and PCRs were from the Cetus GeneAmp in situ PCR core kit (Perkin-Elmer). P2Y1- and P2Y2-receptor-specific RNAs were detected by the nitrobluetetrazolium salt/5-Bromo-4-chloro-3-indoylphosphate (NBT/BCIP) colorimetric reaction (Boehringer-Mannheim).
situ amplification was performed using a two-step thermal cycling program (93°C for 1 minute and 60°C for 2 minutes) for 20 cycles, followed by a 7-minute period for extension at 72°C (GeneAmp in situ PCR system 1000 thermal cycler; Perkin-Elmer). The 50-μL PCR mixture consisted of 4 mM MgCl₂, 1X PCR buffer, 1 mM dNTPs, and 0.5 μM of either the P2Y₁- or P2Y₂-receptor sense primer, 0.5 μM of the appropriate antisense primer, and 10 U Taq DNA polymerase.

After PCR, the tissue sections were washed, and the P2Y₁- or P2Y₂-receptor-specific amplified cDNA was detected at the cellular level after in situ hybridization of a digoxigenin-11-dUTP-tailed sense probe (DIG Oligonucleotide Tailin kit; Boehringer Mannheim). After denaturation of sections at 94°C for 5 minutes, hybridization was performed for 16 hours at 37°C. The 50 μL hybridization solution contained 100 μg/ml herring sperm DNA, 50% formamide, 5X SSC, 1% Tween-20, and 80 ng digoxigenin-11-dUTP-labeled probe. Nonspecifically bound probes were removed by successive washes at 37°C of each of 2X SSC, 1X SSC, and 0.1X SSC. The extent of nonspecific labeling was determined in a separate section using a probe specific for a different receptor isoform than was amplified in the section.

Detection of digoxigenin-labeled nucleic acids was performed using colorimetric detection with NBT BCIP (Boehringer-Mannheim). After equilibration of the sections in buffer 1 (100 mM Tris/HCl, 150 mM NaCl, pH 7.5) and blocking of nonspecific antibody binding in buffer 2 (1% nonfat milk powder in buffer 1), the sections were incubated in the antibody solution (buffer 1 with a 1:100 dilution of anti-DIG antibody conjugated to alkaline phosphatase) for 1 hour. Unbound antibodies were removed in buffer 1 before equilibration in buffer 3 (100 mM Tris/HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5). The colorimetric reaction was performed using the substrates BCIP (175 μg/ml) and NBT (338 μg/ml) to detect P2-receptor-specific amplification products. A deep purple staining was typically obtained within 10 minutes for positive reactions. The color reaction was terminated by a 10-minute wash in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). The slides were mounted with 30% glycerol and viewed by normal bright field light microscopy.

RESULTS

We investigated the extent of P2-receptor expression across the whole lens. In particular, we wished to determine whether P2-receptor expression was limited to the lens epithelial cells in which a P2U-receptor response had already been demonstrated,5,6 or whether the fiber cells also expressed the receptor or receptors. Initially, we used RT-PCR to detect transcripts specific for selected P2-receptor variants separately in lens epithelial cell RNA and in lens fiber cell RNA (Fig. 1A). Amplification of contaminating genomic DNA was excluded by including controls, which had not been reverse-transcribed (data not shown). The separation of epithelial and fiber cells was achieved by harvesting the epithelial cells, which adhered to the capsule, and fiber cells from the decapsulated lens body. The quality of this separation was tested using a primer set specific for the epithelial cell marker Cx4311 and a primer set specific for the fiber cell marker Cx50.12 The differential expression observed with these markers established that complete separation was indeed achieved using this simple method of decapsulation. These RNA preparations were therefore considered suitable for the initial localization of P2-receptor expression. The primer set for the P2X₂ receptor was designed to produce an amplified DNA product with a size of 805 bp. Although the appropriate PCR-amplified product was obtained

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933204/ on 11/25/2018)
Figure 2. Profiles of P2Y<sub>1</sub> and P2Y<sub>2</sub>-receptor expression in the rat eye. Localization of P2Y<sub>1</sub> (A)-receptor- and P2Y<sub>2</sub> (D)-receptor-specific polymerase chain reaction products after in situ hybridization with digoxigenin-labeled probes. The right side of the panel shows the corresponding control sections without reverse transcription (B, E) or with scrambled probes for the in situ hybridization (C, F). Positive staining in the lens appears as a dark purple deposit compared with a light-brown haze in the control sections. (G) and (H) are enlargements of the regions indicated by the frame in (A) and (D), respectively, showing the distribution of labeling at the lens equator and bow region.
in the control tissue (data not shown), no product was obtained in the lens, indicating that this variant was not expressed in either cell type. The expected size for the P2Y,-receptor-specific product was 558 bp, and a single band of this size was obtained with the fiber cell RNA but not the epithelial cell RNA. Similarly, the expected P2Y,-receptor-specific 638-bp product was obtained only with RNA from the fiber cells. All PCR products were sequenced and their identity confirmed.

The exceptional sensitivity of the RT-PCR procedure makes it difficult to determine whether positive amplification represents significant P2Y-receptor expression or only background levels. Thus, northern blot analysis was used to detect the respective transcripts in total RNA of lens epithelial and fiber cells (Fig. 1B). With this criterion, P2Y,-receptor expression was judged significant in the fiber cells on the basis of the fact that a strong band was observed with a size similar to that obtained in control tissues (data not shown) and previously reported for the same receptor variant in other tissues. In the case of the P2Y,-receptor isoform, a band of similar size, although weaker, was consistently obtained in the lens fiber cells and the control tissues (data not shown). These results suggest that P2Y-receptor expression in the lens fiber cells is significant. Consistent with the RT-PCR results, no signal for either receptor subtype could be detected by northern blot analysis in lens epithelial cell RNA.

The lens expression profile for P2Y,- and P2Y,-receptor subtypes was investigated in tissue sections through the intact rat eye. An indirect RT-PCR in situ hybridization procedure was used to ensure maximum sensitivity and specificity. Receptor subtype-specific DNA segments were amplified in the sections using the same primer sets as were used in the test tube reactions above. The amplified PCR products were detected by hybridizing them with receptor subtype-specific DNA sense probes that themselves were detected immunocytochemically. Effectively, two levels of specificity were achieved: First, at the level of PCR amplification and, second, at the level of in situ hybridization. Our experiments included two different controls. One control left out the RT reaction. The other control included cDNA synthesis and PCR amplification of one subtype, but the hybridizing probe was specific for the other subtype and, hence, must not detect the PCR product.

The P2Y,-receptor expression profiles are represented as darkly stained tissue portions in Figure 2. Positive staining appeared as a dark purple precipitate and was distinct from the brown haze, an artifact of dewaxing and permeabilization with Permeafix, in the lens interior. P2Y,-receptor expression was maximal in the cortex. It was absent in the lens epithelium and in the bow region but increased in a zone in which the fiber cells reached from pole to pole. The cell nuclei appeared as blank spots, indicating that the DNase treatment was effective and that no genomic DNA was amplified. The lens core region remained unlabeled, although it is unclear whether this indicates the lack of transcript or limited reagent penetration because of the extraordinary tissue compactness in this region. The profile for P2Y,-receptor expression was similar except that the transcript was also detected in the peripheral bow region of the lens. The epithelial cell layer appeared to be unlabeled, which was consistent with our test tube RT-PCR and northern blot analysis results.

**DISCUSSION**

Our results provide the first direct molecular evidence for the expression of P2Y receptors in the lens. The identification of P2Y,-receptor transcripts in cortical fiber cells supports the physiological evidence for such receptor activity in the lens. However, our failure to detect transcript for P2Y in the lens epithelium, in which a UTP-sensitive receptor was first indicated, is somewhat unexpected. Reasons for this apparent discrepancy could include species differences and the possibility that cultured lens epithelial cells have begun differentiating and, hence, could already be expressing fiber-specific transcripts. Alternatively, other P2Y-receptor subtypes with sensitivity to UTP may be expressed in the epithelial cells.

The identification of P2Y,-receptor transcripts in the lens fiber cells is a novel finding. However, the P2Y,-receptor is widely expressed and has been detected in rat heart, brain, spleen, lung, liver, skeletal muscle, and kidney. In this case, the exceptional sensitivity of the RT-PCR procedure, which allows the detection of even a few copies of a specific RNA, requires a careful evaluation of possible artifacts. Most importantly, RT-PCR product could be amplified from RNA representing contaminating non-lens ocular tissues. This is unlikely for several reasons: First, contamination would be expected to affect predominantly RNA prepared from the superficial epithelium and not RNA from the underlying fiber cells. Second, the northern blot analysis signal is very strong for P2Y,-inconsistent with a signal coming from a minor contaminant. Third, the RT-PCR in situ hybridization localization in fiber cells reinforces the result obtained with the crude dissection used for test tube RTPCR and northern blot analysis. Hence, we regard the novel identification of P2Y,-in the rat lens as reliable.

The RT-PCR in situ hybridization identification of P2Y,- and P2Y,-receptor expression in lens fiber cells supports the in vitro mRNA analysis. The in situ RT-PCR findings are further supported by the dual nature of the negative controls: One has no RT step, thus excluding amplification of genomic DNA. The other has all reaction steps, but the probes for the hybridization are scrambled, showing that the stringency of the hybridization step is adequate. Despite the totally different ways in which these controls work, both produced similar negative results.

Finally, we wish to stress that our expression profiles for P2Y,- and P2Y,-represent mRNA transcripts and not the proteins themselves. Thus, we cannot make any claims on receptor levels in the fiber membrane, nor can we judge whether or not receptors survive aging-related modifications in the lens core. However, the molecular techniques used here to screen for functionally identified receptors are powerful, and our detection of transcripts for two P2Y-receptor isoforms in fiber cells adds a new dimension to fiber cells. Further physiological examination is thus required with a special emphasis on lens fiber cells and the roles these P2Y receptors might have in lens homeostasis.

**References**

Twenty-Four-Hour Change in Axial Length in the Rabbit Eye

John H. K. Liu and Hamed Farid

**Purpose.** To study the 24-hour changes in axial length, lens thickness, and anterior chamber depth in rabbits and to examine the role of ocular sympathetic activity on these changes.

**Methods.** Young adult rabbits were entrained to a daily 12-hour light-12-hour dark cycle. Axial length, lens thickness, and anterior chamber depth were measured using ultrasonic techniques. In the first group of 12 rabbits, measurements were taken in the middle light phase and in the early dark phase. In the second group of 12 rabbits, measurements were taken in constant dark every 2 hours for a period of 24 hours. The latter group of rabbits underwent unilateral transection of the cervical sympathetic trunk. Three to four weeks later, axial length, lens thickness, and anterior chamber depth were measured again in constant dark every 2 hours for 24 hours.

**Results.** Under the light-dark condition, axial length and anterior chamber depth were larger in the early dark phase than in the middle light phase. However, magnitudes of nocturnal enlargement were relatively smaller than those in the intact eyes. Although larger in the decentralized eyes, lens thickness appeared unchanged in either eye for 24 hours.

**Conclusions.** Consistent 24-hour changes in axial length and anterior chamber depth occur in young adult rabbits. These changes are driven endogenously. Significant portions of the nocturnal enlargements of axial length and anterior chamber depth are unrelated to ocular sympathetic activity. (Invest Ophthalmol Vis Sci. 1998;39:2796-2799)

Intraocular pressure (IOP) in light-dark entrained rabbits appears in a circadian pattern. Pressure is low during the light period and high during the dark period, with a significant elevation at the onset of dark. An endogenous oscillator or oscillators drive this circadian rhythm; a similar IOP change occurs in a constant-dark environment. Nocturnal increase of ocular sympathetic activity is closely related to the elevation of IOP.

Possibly related to the circadian rhythm of IOP in rabbits, a significant increase of anterior chamber depth from the middle light period to the middle dark period has been reported. It suggests that dimensions of the rabbit eyeball may vary during a 24-hour period. Using ultrasonic biometry, we measured 24-hour changes of axial length, lens thickness, and anterior chamber depth in light-dark entrained young adult rabbits. Measurements were taken under the light-dark and the constant-dark conditions. These structural parameters were also measured in eyes with decentralized ocular sympathetic nerves in constant dark to examine the role of ocular sympathetic activity on these parameters.

**Methods**

Young adult New Zealand albino rabbits (3.0–4.2 kg) were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits were housed in individual cages at constant temperature (21°C) and humidity, under a daily 12-hour light-12-hour dark cycle.