

Identification of P2Y Receptor Subtypes in Human Müller Glial Cells by Physiology, Single Cell RT-PCR, and Immunohistochemistry

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PURPOSE. Retinal Müller glial cells are known to express metabotropic P2Y receptors. The present study was conducted to identify certain subtypes of P2Y receptors in human Müller cells.

METHODS. The patch-clamp technique was used to measure increases of Ca²⁺-dependent K⁺ currents mediated by the activation of P2Y receptors in freshly isolated human Müller cells. Several P2 agonists were used. Subsequently, the cells were harvested into the patch pipette and a single cell RT-PCR was performed. Moreover, retinal tissue from organ donors was used for immunohistochemistry.

RESULTS. The electrophysiological data were consistent with the expression of P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptor subtypes. RT-PCR revealed that mRNA for all these subtypes was present in Müller cells. However, the incidence of P2Y₂ receptor mRNA was significantly lower than that of the other subtypes. Immunoreactivity for all four subtypes was found in retinal tissue, partly colocalized with immunoreactivity for vimentin.

CONCLUSIONS. The presented data obtained by different techniques revealed that human Müller cells express P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors. The specific roles of these receptor subtypes in retinal physiology and/or pathophysiology remain to be investigated in future studies. (*Invest Ophthalmol Vis Sci.* 2005;46:3000–3007) DOI:10.1167/iovs.05-0043

Adenosine 5'-triphosphate (ATP) and other purines and pyrimidines have been demonstrated to act as signaling molecules by activation of nucleotide (P2) receptors. Two

groups of P2 receptors have been described: the ionotropic P2X receptors and the metabotropic P2Y receptors, which belong to the superfamily of G-protein-coupled receptors.¹ Activation of P2Y receptors results in the release of Ca²⁺ from intracellular stores and thus in an increase in the intracellular Ca²⁺ concentration ([Ca²⁺]_i), which may modify a variety of cellular functions.² Moreover, a coupling to adenylate cyclase has been demonstrated for certain P2Y receptors.³ Eight different subtypes of P2Y receptors have been identified in mammals: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄.^{4,5}

A physiological role of ATP as a neurotransmitter in the retina was proposed 10 years ago⁶ and has been demonstrated since then in several studies.^{7,8} Moreover, ATP can act as a vasoactive signal in the retina.⁹ Different subtypes of P2 receptors have been found to be expressed in retinal neurons and glial cells. These studies have been focused on P2X receptors for several years.^{10–18} There is only limited evidence of the functional expression of P2X receptors in Müller cells; however, Müller cells from virtually all species studied so far have been shown to react to extracellular nucleotides with an increase of [Ca²⁺]_i. This was shown in cells from salamander,¹⁹ rat,^{20,21} rabbit,²² and human,²³ as well as in cultured Müller cells from rabbit²⁴ and guinea pig.²⁵ Responses of retinal cells may change during postnatal development, as has been shown in the early embryonic chick retina²⁶ and in Müller cells in the rabbit retina.²² Moreover, alterations of P2Y receptor-mediated Müller cell responses were observed under pathologic conditions.²⁷

However, the unambiguous characterization of receptor subtype expression was hampered by the facts, that (1) the Müller cells responded to application of different agonists, pointing to the expression of more than one subtype, and that (2) most pharmacologic tools for P2 receptors are not very subtype specific. Only recently, P2Y receptor subtypes have been identified in the whole rat retina and in individual retinal cells by polymerase chain reaction (PCR), in situ hybridization, and immunohistochemistry,^{28–30} and in Müller glial cells from the salamander retina by pharmacologic means.³¹ The identification of P2Y receptor subtypes expressed in the retina may improve the understanding of retinal physiology, because certain receptor subtypes are likely to fulfill different functional roles. For example, the P2Y₂ receptor has been described to regulate the fluid transport through the retinal pigment epithelium^{32,33} and the active transport across the blood–retinal barrier.³⁴ The DNA synthesis of cultured Müller cells is modified by ATP and uridine 5'-triphosphate (UTP), which activate the P2Y₂ and P2Y₄ receptors, but not by the P2Y₁-specific agonist 2-methylthioATP (MeSATP).²⁵ Moreover, the activation of the P2Y₆ but not of the P2Y₄ receptor prevents externally induced apoptosis in cultured astrocytes.³⁵ In brain tissue, the P2Y₆ receptor plays a prominent role in mediating the contraction of cerebral arteries.³⁶ A selectivity of putative physiological agonists (ADP activates P2Y₁ and P2Y₁₃ receptors, UDP

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preferentially activates the P2Y₆ receptor)^{1,37} may result in a selective receptor activation *in vivo* under specific conditions.

Therefore, we decided to investigate the expression of P2Y receptors in Müller cells from the human retina. Although there are some studies characterizing P2Y subtypes in rat Müller cells,^{21,30} it is possible that the receptor expression differs among mammalian species, as has been shown for other receptors (e.g., the P2X₇ receptor¹⁶ and the GABA_A receptor³⁸). Because the access to human retinal tissue is limited, we confined our study to the subtypes P2Y₁, P2Y₂, P2Y₄, and P2Y₆. These subtypes are widely accepted as functional receptors in mammals, and their structures are well characterized.

MATERIALS AND METHODS

Preparation of Cells

All tissue was used in accordance with applicable laws and with the Declaration of Helsinki. The use of human tissue was approved by the ethics committee of the University of Leipzig Medical School. Retinal tissue from 15 patients with proliferative vitreoretinopathy (PVR) was obtained during vitreoretinal surgery when partial retinectomy was necessary to relieve traction. The tissue was supplied within 2 to 3 hours after surgery. For cell isolation, retinal pieces were incubated for 30 minutes in Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS) containing 0.2 to 0.5 mg/mL papain (Roche Diagnostics, Mannheim, Germany) at 37°C. After it was washed with PBS containing DNase I (150 U/mL; Sigma-Aldrich, Taufkirchen, Germany), the tissue was triturated with a pipette until single cells were dissociated. Suspensions with isolated cells were stored on ice until use within the next 4 hours. No decrease in the efficiency of the PCR reaction was observed during this time. Müller cells were identified by their typical morphology (Fig. 1).

Electrophysiology

Electrophysiological recordings were performed in the whole-cell configuration of the patch-clamp technique at 20°C to 24°C. Cells were suspended in extracellular solution in a recording chamber on the stage of a microscope (Axioskop; Carl Zeiss Meditec, Oberkochen, Germany). Extracellular solution contained (mM) NaCl, 110; KCl, 3; CaCl₂, 2; MgCl₂, 1; Na₂HPO₄, 1; glucose, 11; HEPES-Tris, 10; and NaHCO₃, 25 and was equilibrated to pH 7.4 by continuous bubbling with 95% O₂-5% CO₂. The P2Y agonists ATP (Serva, Heidelberg, Germany), adenosine 5'-O-2-thiodiphosphate (ADPβS), 2-methylthioadenosine 5'-diphosphate (MeSADP) and MeSATP (Tocris Cookson, Bristol, UK), uridine 5'-diphosphate (UDP), and UTP were applied by bath perfusion. Substances were from Sigma-Aldrich, unless indicated otherwise. The recording chamber was continuously perfused (2 mL/min) after the whole-cell configuration was established. Recording electrodes were made from borosilicate glass (Science Products, Hofheim, Germany) and had resistances of 3 to 4 MΩ when filled with 6 μL of a solution containing (mM) KCl, 130; NaCl, 10; MgCl₂, 3; CaCl₂, 1; EGTA, 0.1; and HEPES-Tris, 10 (pH 7.1). For current and voltage recordings, a patch-clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) was used. Currents were low-pass filtered at 1 kHz and digitized at 5 kHz with a 12-bit A/D converter. Voltage command protocols were generated and data analysis was performed on computer (ISO 2 software; MFK, Niedernhausen, Germany; and SigmaPlot; SPSS Inc., Chicago, IL). The membrane potential was recorded in the current clamp mode at *I* = 0.

Results are expressed as the mean ± SD. Student's *t*-test and the Fisher exact test were used for statistical analysis.

Molecular Biology

Subsequent to the electrophysiological recording of the P2Y receptor-mediated responses, cytoplasm from the recorded cell was harvested into the recording electrode by applying negative pressure. The electrode was located at the endfoot of the cell, thereby preventing the

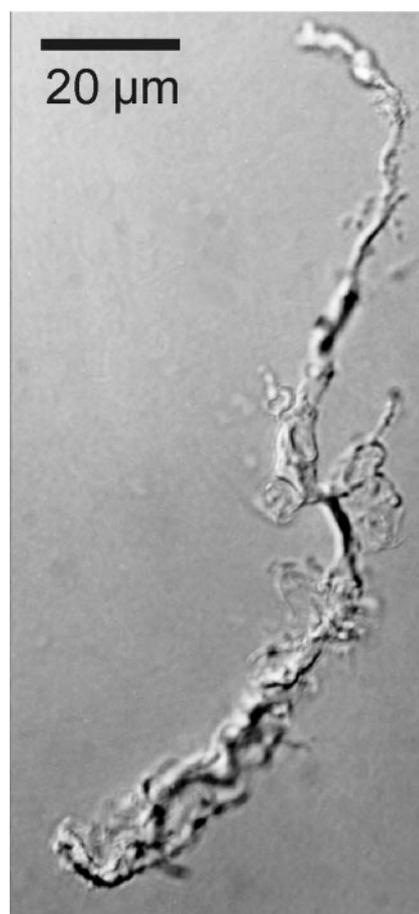


FIGURE 1. Müller cell acutely enzymatically isolated from retinal tissue of patient with PVR. The cell is representative of isolated Müller cells from this tissue that could be clearly identified by their morphology. Only cells without other (neuronal) cells adhering were used.

nucleus from occluding the pipette during aspiration. However, by this method, only a part (approximately 50%) of the cytoplasm could be transferred into the electrode, resulting in a relatively high percentage of negative results. To increase the success rate, we then modified the method. After the patch-clamp experiment, a second pipette with a larger tip diameter (0.5–1 MΩ) was used to harvest the cytoplasm completely into this suction pipette, as was described previously.³⁹ In this case, the recording electrode was located at the soma of the cell, and harvesting of the cell content with the suction pipette started from the endfoot. In some experiments, also the nucleus was included in the pipette. To avoid any false-positive results, special care was taken to use only Müller cells that were not in contact with (parts of) any other cell (Fig. 1). The cell suspension obtained after enzymatic treatment mainly contained such cells. The contents of the recording electrode and of the suction pipette (if used) were expelled into a PCR tube containing deoxyribonucleotide triphosphates (dNTP) and random hexanucleotide primers (N₆; 2 μL dNTP/N₆ mix with a final concentration of 4 × 2.5 mM and 25 μM, respectively, were used; GE Healthcare, Piscataway, NJ), 1 μL dithiothreitol (100 mM), 0.5 μL RNase inhibitor (20 U; Stratagene, La Jolla, CA), and 1 μL 5× first-strand buffer. Subsequently, 0.5 μL reverse transcriptase (200 U/μL Superscript-II; Invitrogen, Karlsruhe, Germany) was added. After an additional 5 minutes at room temperature, reverse transcription was performed at 45°C for 1 hour. Control experiments were performed by advancing the pipette into the bath solution and using its content for reverse transcription (RT)-PCR.

The entire product of the RT reaction from a single cell was split into two samples and used for the first amplification of two different

TABLE 1. Description of Primers and PCR Products

	Forward	Reverse	PCR Product (bp)
P2Y ₁ outer primers	5' CTA CAT CTT GGT ATT CAT ATC GG 3'	5' GAG ACT TGC TAG ACC TCT TGT CAC C 3'	772
P2Y ₁ nested primers	5' GGC CGG CTC AAA AAG AAG AAT GCG 3'	5' AAA TCA AGC CGG GCC CTC AAG TTC A 3'	389
P2Y ₂ outer primers	5' CTC TAC TTT GTC ACC ACC AGC GGG 3'	5' TTC TGC TCC TAC AGC CGA ATG TCC 3'	637
P2Y ₂ nested primers	5' CCG CGT AAC CTG CCA CGA CAC CT 3'	5' CGC CTA GAG TCC TCA CTG CTG CCC A 3'	546
P2Y ₄ outer primers	5' CCA CCT GGC ATT GTC AGA CAC C 3'	5' GAG TGA CCA GGC AGG GCA CGC 3'	425
P2Y ₄ nested primers	5' GCT GTC GCT GCC CAC CCT CAT CTA CTA 3'	5' ATG ACC GCC GAG CTG AAG TGC ACA 3'	359
P2Y ₆ outer primers	5' CGC TTC CTC TTC TAT GCC AAC C 3'	5' CCA TCC TGG GGC CAC AGG CGG C 3'	365
P2Y ₆ nested primers	5' CAC GGC AGC ATC CTC TTC CTC ACC T 3'	5' GCG GTT ACG CTG GAT GCC TGT GG 3'	192

P2Y receptor subtype-specific fragments. For a first part of the cell, a PCR with P2Y₁-specific primers was performed in one sample and a P2Y₂-specific reaction in the other. For the second part of the cell, a search for P2Y₄ and P2Y₆ was made in parallel. The sequences used for the different primers are listed in Table 1; outer primers were designed according to Berchtold et al.⁴⁰ For the first step of the PCR reaction, 5 μ L 10 \times reaction buffer, 3 μ L 25 mM MgCl₂, 1 μ L of each outer primer (10 μ M), 34.5 μ L H₂O, and 0.5 μ L DNA polymerase (5 U/ μ L, AmpliTaq; Perkin-Elmer, Weiterstadt, Germany) were added to the RT product. PCR conditions were: 94°C for 3 minutes at the beginning, followed by 35 cycles of denaturation at 94°C for 45 seconds; annealing at 64°C (P2Y₁), 67°C (P2Y₂), or 56.5°C (P2Y₄ and P2Y₆) for 45 seconds; and extension at 72°C for 90 seconds (P2Y₁ and P2Y₂) or 60 seconds (P2Y₄ and P2Y₆), followed by a final extension step at 72°C for 10 minutes. Aliquots of each first PCR were diluted 100-fold and reamplified for 40 cycles in a second PCR step. Five microliters 10 \times reaction buffer, 3 μ L 25 mM MgCl₂, 1 μ L dNTPs (10 mM mix), 1 μ L of each nested primer (10 μ M; see Table 1), 37.5 μ L H₂O, and 0.5 μ L DNA polymerase (5 U/ μ L) were added to the diluted PCR products. PCR conditions were 94°C for 3 minutes at the beginning; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 66°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension step at 72°C for 5 minutes. Human genomic DNA served as the positive control to verify the proper function of the detection method. The PCR products were analyzed by 1.2% agarose gel electrophoresis.

Immunohistochemistry

Methodologic problems, such as the difficulty of identification of Müller cells in PVR membranes, prevented the use of tissue removed during surgery for immunohistochemistry. Therefore, we used retina that had been obtained from four organ donors without any reported eye disease. The tissue was fixed in 4% paraformaldehyde at 4°C for 2 to 4 hours. After the tissue was washed in PBS and embedded in sucrose (30%), it was immersed in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Zoeterwoude, The Netherlands) overnight. Sections (12 μ m) were cut with a cryostat and were mounted on gelatin-coated slides. After unspecific binding was blocked with normal goat serum (10%; Dianova, Hamburg, Germany) and the sections were permeabilized with 0.3% Triton X-100 in PBS for 1 hour, they were incubated with primary antibodies at 4°C for 12 hours. The following primary antibodies were used: anti-P2Y₁, -P2Y₂, -P2Y₄, and -P2Y₆ (host rabbit; Alomone Laboratories, Jerusalem, Israel). For double labeling, antibodies against vimentin (V9 clone, host mouse; Immunotech, Marseille, France) were used. After the sections were washed with PBS, the secondary antibodies carbocyanine (Cy2)-coupled goat anti-mouse IgG antibody (1:750; Dianova), and Cy3-coupled goat anti-rabbit IgG antibody (1:750–1:1000; Dianova) were applied for 2 hours at room temperature. The cell nuclei were stained with Hoechst 33258 (Molecular Probes, Eugene, OR). The labeling was visualized by means of a confocal laser scanning microscope (LSM 510 META; Carl Zeiss Meditec). Omitting of the primary antibodies resulted in a lack of staining. Moreover, we performed a specificity control for the P2Y-specific primary antibodies by preincubation with the respective synthetic control peptides according to the instructions supplied

by the manufacturer. Then, the immunohistochemical procedure was continued as just described.

RESULTS

Tissue removed from patient's eyes during surgery mainly consisted of PVR membranes. In some cases, however, it contained small areas with a rather intact retinal morphology. Isolated Müller cells that could be identified unequivocally (Fig. 1) probably were derived from such areas.

Activation of P2Y receptors induces an increase of [Ca²⁺]_i via release from intracellular stores.¹ Human Müller cells express Ca²⁺-dependent K⁺ (BK) channels which are activated when [Ca²⁺]_i increases.⁴¹ Therefore, an increase of (outward) BK currents at depolarizing membrane potentials can be recorded by the patch-clamp technique after activation of P2Y receptors.²³ This method was used in the present study to characterize the P2Y-mediated response pharmacologically before harvesting the cytoplasm from the cell. All 61 cells investigated responded to at least one of the used P2Y agonists (concentrations: 10, 50, or 100 μ M). Typical responses of human Müller cells to the application of different P2Y agonists are shown in Figure 2. Two or even more different P2Y agonists were applied to each cell. In most cases, the cells responded to each application with an increase of the BK current amplitude between 186% and 296% (recorded at the end of a 50-ms step to +110 mV). The BK current amplitude differed between individual cells. Thus, the current recorded before agonist application was taken as 100%. No significant differences between the effects of different agonists were found. Detailed results are given in Table 2. In a small number of cells, only one or two reversible physiological responses were recordable. In these few cells the lack of an additional response may have been caused by different effects (increase of BK currents without recovery after removal of the agonist, unspecific BK current increase before agonist application, or receptor desensitization due to binding of agonists to different P2Y subtypes). Nevertheless, cytoplasm from all 61 cells was used for P2Y subtype-specific RT-PCRs, to get a reliable cell count. After the reverse transcription of the RNA harvested from the Müller cell into cDNA, the entire product of the RT reaction was split into two samples and amplified in two different PCR reactions, using P2Y₁-/P2Y₂- or P2Y₄-/P2Y₆-specific primers.

To preclude the amplification of nuclear DNA harvested by nucleus aspiration (which happened in some cells when a second suction pipette was used in our experiments), it has been recommended that the amplified DNA fragment contain an intron at the gene level.⁴² However, this strategy was impossible for the human P2Y₁ and P2Y₄ receptors, because the genes contain no intron in the coding sequence.^{43,44} Moreover, because of problems with self-designed primers in preliminary experiments, we used primer sequences published earlier⁴⁰ that do not span an intron and can amplify genomic DNA (see positive controls in Fig. 3). We did not treat the

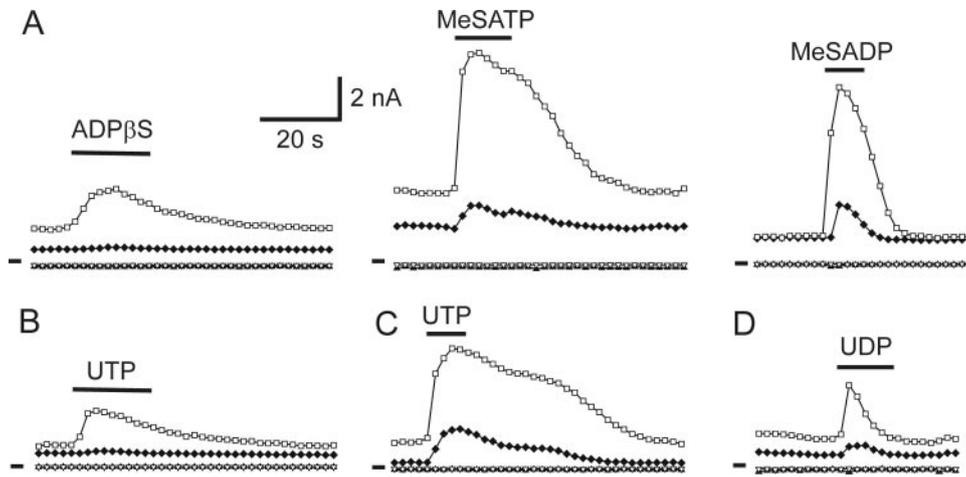


FIGURE 2. Effects of P2Y agonists on the membrane currents of human Müller cells. Agonists interacting with the P2Y₁ (A), P2Y₂ (B), P2Y₄ (C), and P2Y₆ (D) receptor subtypes were applied (indicated by horizontal bars above the traces) onto Müller cells, which were subsequently shown to be positive for the respective PCR product. The agonist concentration was 10 μM, with the exception of MeSATP (50 μM). Cells were voltage clamped at a holding potential of -80 mV, and a series of 50-ms voltage steps was applied in 2-second intervals. Currents evoked at potentials of +110 mV (□), +30 mV (◆), -80 mV (▽), and -100 mV (▲) are shown. Small bars at the left indicate the zero current levels. The increase in [Ca²⁺]_i elicited by activation of P2Y receptors resulted in an activation of BK currents at positive membrane potentials.

harvested material with DNase, because an additional step to remove the enzyme before transcription of RNA into cDNA was supposed to cause the loss of the few mRNA molecules we searched for. Thus, it could be argued, that false-positive results may have been generated by amplified nuclear DNA. However, we assume amplification of nuclear DNA to be unlikely in our experiments, for the following reasons. First, in five of the cells where the nucleus was obviously drawn into the pipette no PCR product was found, demonstrating that the nuclear DNA could not be amplified in these cases. Second, in all experiments using only the recording electrode and in about half of the cases in which a second pipette was used, the nucleus was not harvested, but PCR products were found (discussed later). Third, it was demonstrated that the single-cell RT-PCR method does not amplify genomic DNA when the nucleus is aspirated, even if intronless fragments are used.⁴⁵

At the beginning of the study, the cell content was harvested only by the recording electrode. During this phase, only the P2Y₁/P2Y₂ PCR was performed (*n* = 30 cells). In 12 (40%)

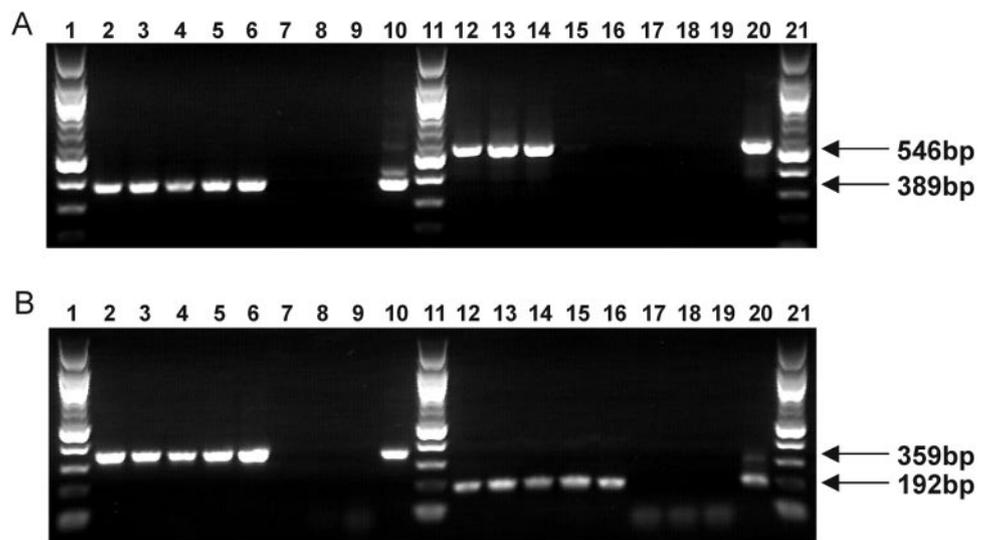
of 30 cells a PCR product of the predicted size for P2Y₁ (389 bp) and in 7 (23%) of 30 cells the PCR product for P2Y₂ (546 bp) were found. Later, we established the method of using a second suction pipette, thereby increasing the efficacy of cell harvesting. Cell content harvested by two pipettes was used for P2Y₁/P2Y₂ PCR (*n* = 14) and for P2Y₄/P2Y₆ PCR (*n* = 17). The number of cells positive for the respective PCR product was *n* = 9 for P2Y₁ (64%), *n* = 3 for P2Y₂ (21%), *n* = 13 for P2Y₄ (76%; 359 bp), and *n* = 14 for P2Y₆ (82%; 192 bp). For statistical comparison of the incidence of mRNA for individual receptor subtypes, only cells investigated under the same conditions were used. Thus, no significant difference was found between the incidence of P2Y₁, P2Y₄, and P2Y₆ receptors, whereas the incidence of P2Y₂ receptors was significantly lower (Fisher exact test, *P* > 0.001 < 0.05). This result is supported by the fact that 12 cells were found to be positive for P2Y₁ but not for P2Y₂, whereas only 1 cell was positive for P2Y₂ but not for P2Y₁. Representative agarose gels with PCR

TABLE 2. Physiological Responses to P2Y Agonists and Results of PCR Reactions with P2Y-Specific Primers in Human Müller Cells

P2Y Subtype	Tested Agonist (Concentration, μM)	Recorded BK Current Increase (%) (Responding Cells/Total Cells, <i>n</i>)	Cells with Detection of PCR Product, (<i>n</i>) (Total Tested Cells, <i>n</i>)
P2Y ₁ *	ADPβS (10)	258 ± 128 (8/8)	7 (8)
	MeSATP (50)	256 ± 162 (9/9)	3 (9)
	MeSATP (100)	228 ± 96 (9/9)	2 (9)
	MeSADP (10)	247 ± 128 (5/5)	2 (5)
	MeSADP (50)	186 ± 63 (6/7)	4 (7)
	ATP (100)	296 ± 82 (6/6)	3 (6)
Total P2Y ₁			21 (44)
P2Y ₂ *	UTP (10)	285 ± 117 (11/12)	3 (12)
	UTP (50)	223 ± 80 (16/16)	3 (16)
	UTP (100)	234 ± 80 (9/9)	2 (9)
	ATP (100)	284 ± 80 (7/7)	2 (7)
Total P2Y ₂			10 (44)
P2Y ₄	UTP (10)	250 ± 116 (16/17)	13 (17)
P2Y ₆	UDP (10)	243 ± 104 (14/17)	14 (17)

* Pooled data from experiments in which only the recording electrode or a second suction electrode was used for cell harvesting.

FIGURE 3. Detection of mRNA for P2Y receptors in individual human Müller cells. Amplification products of the predicted size were found in nested PCR in a certain percentage of single Müller cells, thereby demonstrating the existence of the respective mRNA. Representative agarose gels demonstrating the products of P2Y₁ (A, lanes 2–10), P2Y₂ (A, lanes 12–20), P2Y₄ (B, lanes 2–10), and P2Y₆ (B, lanes 12–20) subtype-specific PCRs. Lanes 1, 11, 21: 100-bp molecular weight standard (Invitrogen-Gibco; Karlsruhe, Germany); lanes 2 to 7 and 12 to 17: examples of positive and negative amplification results from individual Müller cells; lanes 8 and 18: negative control of cell harvesting (i.e., negative pressure was applied to a recording electrode without touching a cell); lanes 9 and 19: negative PCR control (i.e., the PCR reaction was performed as described without adding the RT product); lanes 10 and 20: positive control for PCR using human genomic DNA for amplification. Material from the same cell was used for lanes 2 and 12, 3 and 13, 4 and 14, 5 and 15, 6 and 16, and 7 and 17. The 1.2% agarose gels were stained with ethidium bromide.



products of the respective size for the four investigated P2Y subtypes are shown in Figure 3.

Antibodies are commercially available for all four P2Y subtypes investigated in the present study. Because of methodical problems when using PVR membranes for immunohistochemistry, we decided to expand the immunohistochemical study to retinal tissue from healthy organ donors. In a previous physiological study, no differences were found in the expression of P2Y receptors between Müller cells from diseased retinas and from human donor retinas.²³ In slices of retinas from organ donors, Müller cells were unequivocally identified because of the expression of vimentin (Figs. 4A2, 4B2, 4C2, 4D2) in combination with their distinct morphologic appearance and

localization. Vimentin-positive structures labeled with the antibodies against all four P2Y subtypes were found, indicating the existence of the receptor proteins in Müller cells (Fig. 4A3, 4B3, 4C3, 4D3). Control experiments performed after preadsorption of the antibodies with the respective peptides resulted in a lack of staining, thereby verifying the specificity of the antibodies (data not shown). It is clearly documented in the micrographs that the P2Y receptors were not expressed over the whole Müller cell surface, but only in distinct regions. It should be kept in mind that vimentin is an intracellular filament protein, whereas functional P2Y receptors are located in the cell membrane, possibly on very fine processes that may contain no vimentin. Moreover, other cell types in the retina may

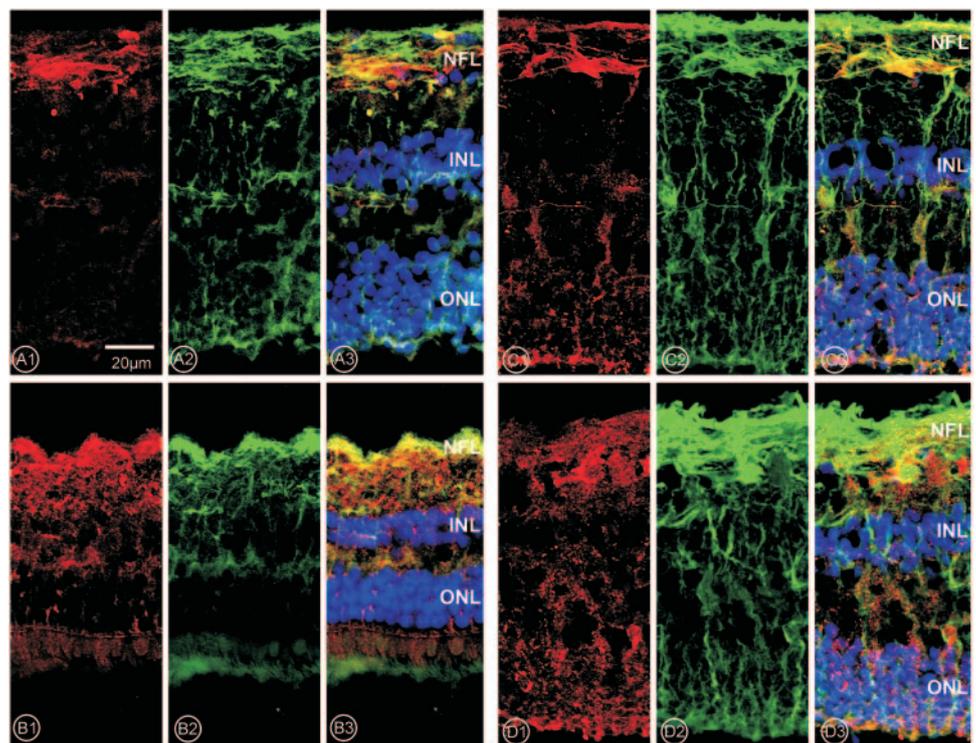


FIGURE 4. Immunohistochemical evidence for P2Y receptors in the human retina. Retinal slices from organ donors were stained with antibodies against the P2Y₁ (A1), P2Y₂ (B1), P2Y₄ (C1), and P2Y₆ (D1) receptors (red). (A2, B2, C2, D2) Double-staining against vimentin (green). (A3, B3, C3, D3) Double-stained areas (i.e., cells that express the P2Y receptor and the filament protein) in the merged images (yellow). Cell nuclei were stained with Hoechst 33258 (blue). NFL, nerve fiber layer; INL, inner nuclear layer; ONL, outer nuclear layer.

express these receptors as well. Both phenomena would result in the existence of structures positive for P2Y receptors but devoid of vimentin (i.e., red-stained structures in the merged images; Fig. 4). Moreover, some of the double-stained structures in the nerve fiber layer probably belong to astrocytes, which also express vimentin.

DISCUSSION

In the present study, human Müller cells expressed at least four different P2Y receptor subtypes. This confirms and extends previous data demonstrating Ca^{2+} responses in human Müller cells evoked by several P2 agonists.²³ The existence of different functional P2Y receptor subtypes has also been confirmed in pharmacologic studies on isolated salamander Müller cells³¹ and on Müller cells in the intact rat retina.²¹ However, because of the lack of highly specific agonists and/or antagonists for most of the P2Y subtypes, the unequivocal identification of all subtypes expressed in Müller cells was not possible. Because of the pharmacologic profile, it was concluded that rat Müller cells express P2Y₁ receptors.²¹ Responses evoked by UTP in approximately one third of the cells indicate that P2Y₂ and/or P2Y₄ receptors may also exist. Additional P2 agonists were used,³¹ demonstrating salamander Müller cells to express P2Y₁, P2Y₂, P2Y₆, P2Y₁₁, and possibly P2Y₄ and P2Y₁₃ receptors. In the retina of the rabbit and the rhesus macaque the P2Y₂ receptor was found by in situ hybridization; however, an unambiguous cellular localization in Müller cells has not been possible.⁴⁶ Comparative studies on different mammalian species in the past few years have shown that there may be significant species differences regarding the expression of certain receptors or membrane channels.^{16,38,47} Therefore, we decided to investigate the expression of P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors in human Müller cells. These subtypes have recently been described in the rat retina and Müller cells.^{29,30}

The physiological responses of isolated human Müller cells are consistent with the existence of different types of P2Y receptors. Increases in $[Ca^{2+}]_i$, indirectly recorded by the increase of BK currents, were observed during application of the P2Y₁-specific agonists ADP β S, MeSADP, and MeSATP, and of the P2Y₆-specific agonist UDP. However, discrimination between P2Y₂- and P2Y₄-mediated responses was not possible. Both subtypes are activated by UTP, whereas ATP activates the P2Y₂ but not the human P2Y₄ receptor.² This pharmacologic profile is not helpful when investigating cells that express both receptors and/or additional subtypes that are activated by ATP. Moreover, the used P2 agonists may activate other P2Y subtypes that were not included in the present study. Therefore, we did not attempt to perform a more detailed physiological study. Instead, the goal was to complete the pharmacologic receptor characterization by means of RT-PCR and immunohistochemistry.

The mRNA of all four receptor subtypes was found in our RT-PCR experiments. The percentage of cells positive for P2Y₁, P2Y₄, and P2Y₆ subtypes varied between 64% and 82% in those experiments in which a large part of the cytoplasm or even the whole cell content was used. The negative result in the remaining cells may be due to methodical problems, mainly the degradation of mRNA before reverse transcription or a failure in harvesting the respective mRNA. Because we wanted to use as many samples as possible for P2Y receptor-specific PCR, we avoided including a positive control (i.e., amplification of a house-keeping gene). Therefore, it cannot be decided whether the negative cells did not express the respective receptor subtype (which is unlikely because physiological responses were present in all cells) or whether the mRNA was lost during the procedure. Nevertheless, the significantly lower

incidence of P2Y₂-positive cells can hardly be explained by technical reasons. Thus, it appears that only a subpopulation of Müller cells contains this subtype.

Immunoreactivity for all investigated P2Y receptors was found in vimentin-positive cells in immunohistochemical experiments on retinas from organ donors. The results confirmed that Müller cells in the human retina express P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors. A quantitative analysis of the immunohistochemical data was not possible.

P2 receptors seem to be involved in the pathomechanism(s) of reactive gliosis,⁴⁸ as shown in cultured astrocytes^{49,50} and in astrocytes within the brain^{51,52} and within the optic nerve.⁵³ In Müller cells, the expression of functional P2Y receptors was found to be changed under pathologic conditions.^{27,28} Whereas these studies were performed on rabbit and rat Müller cells, no significant differences regarding Ca^{2+} responses evoked by P2 agonists were found between human Müller cells from healthy organ donors and those from patients with PVR.²³ This finding was in contrast to the downregulation of inwardly rectifying K^+ currents⁵⁴ and upregulation of P2X₇-mediated currents⁵⁵ in Müller cells from diseased human retinas. In the present RT-PCR study, we were able to investigate only Müller cells from patients with PVR. The investigation of human material is always limited by the clinical requirements. The only source we found to be usable for a single-cell RT-PCR experiment was retinal tissue removed during vitreoretinal surgery. Müller cells can also be isolated from the retina of organ donors; however, we did not succeed in single-cell RT-PCR when using such cells in preliminary experiments, probably because these eyes were supplied several hours after death and the mRNA was already degraded. Rather, retinal tissue from organ donors was used for immunohistochemistry. Because immunoreactivity for all four P2Y receptors was found in Müller cells of those retinas, based on double-labeling with vimentin, there is no evidence for any dramatic changes in the expression of these receptors during PVR. This is in agreement with the fact that virtually all human Müller cells from healthy and diseased retinas responded to an activation by P2Y agonists (Ref. 23 and present study). Thus, there seems to be no alteration of the incidence of human cells expressing functional P2Y receptors, an observation that contrasts findings in experimental PVR in rabbits²⁷ and in Borna disease virus-infected rats.²⁸

There is increasing evidence supporting the notion that the expression of a certain mRNA does not necessarily imply the existence of a functional receptor. For example, the mRNA for several P2X receptor subunits was found in rat Müller cells¹⁵; however, P2X receptor-mediated currents were never demonstrated in these cells. There are similar data regarding *N*-methyl-D-aspartate (NMDA) receptor mRNA in rat Müller cells (Pannicke et al., unpublished data, 1999). Therefore, RT-PCR studies should be supported by physiological experiments, whereas identification of mRNA by PCR may help to analyze pharmacologic data. Recently, the physiological study of rat retina²¹ was confirmed by finding P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptor mRNA in rat Müller (and other retinal) cells.³⁰ In the current study, we provide evidence based on different techniques that human Müller cells may express the same receptor subtypes as found in rat. This finding should be a prerequisite for future studies on the specific roles of the various P2Y receptor subtypes in retinal physiology.

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