Evidence for Multiple P2Y Receptors in Trabecular Meshwork Cells

Craig E. Crosson, Phillip W. Yates, Aruna N. Bhat, Yuriy V. Mukhin, and Shahid Husain

Departments of Ophthalmology (C.E.C., P.W.Y., A.N.B., S.H.), MUSC-Hewitt Laboratory of the Ola B. Williams Glaucoma Center, and Medicine (Y.V.M.), Medical University of South Carolina, Charleston, South Carolina

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ABSTRACT

The purpose of this study was to determine whether functional purinergic P2 receptors are present in trabecular meshwork cells. The human trabecular cell line HTM-3 and cultured bovine trabecular cells were used to assess the effects of P2 agonists on intracellular Ca^{2+} levels, extracellular signal-regulated kinase (ERK1/2) activation, and P2Y receptor expression. ATP, UTP, ADP, and 2-methyl-thio-adenosine triphosphate (2-MeS-ATP) each produced a concentration-dependent increase in intracellular Ca^{2+} in bovine trabecular cells and the HTM-3 cell line. The addition of UDP did not produce any detectable rise in intracellular Ca^{2+}. Pretreatment with the P2Y_1 receptor antagonist 2'-deoxy-N_9-methyladenosine-3',5'-diphosphate (MRS-2179) blocked the ADP- and 2-MeS-ATP-induced rise in intracellular Ca^{2+}. However, the ATP- or UTP-induced rise in intracellular Ca^{2+} was not inhibited by MRS-2179 pretreatment. The addition of ADP, 2-MeS-ATP, ATP, or UTP were also found to activate the ERK1/2 signaling pathway. This activation of ERK1/2 was blocked by pretreatment with the mitogen-activated protein kinase kinase inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene (U-0126) or the protein kinase C inhibitor chelerythrine chloride, but not by MRS-2179. Analysis of mRNA from HTM-3 cells by reverse transcription-polymerase chain reaction revealed the expression of P2Y_1, P2Y_4, and P2Y_11 receptor subtypes. These data demonstrate that multiple P2Y receptors are present in trabecular cells. Our results are consistent with the idea that the mobilization of intracellular Ca^{2+} results from the activation of P2Y_1 and P2Y_4 receptors, whereas the activation of the ERK1/2 pathway results from the activation of P2Y_11 receptors alone. However, a role for the P2Y_11 receptors in mobilization of Ca^{2+}, or activation of the ERK1/2 pathway, cannot be discounted.

The existence of two families of purinergic receptors, P2X and P2Y, that are activated by adenine or uracil nucleotides was proposed by Abbracchio and Burnstock (1994). To date, eight mammalian P2Y receptor subtypes and seven P2X receptor subtypes have been cloned and characterized pharmacologically. In general, mammalian P2Y receptors (i.e., P2Y_1,2,4,6,11) are coupled to the G_q/11 family of G proteins and P2X receptors function as ligand-gated cation channels (North, 2002). The presence of adenine nucleotides in the humor of the eye has been known for some time (Greiner et al., 1991). Recent studies have also provided evidence that the activation of ocular P2 receptors can modulate intraocular pressure (IOP) (Pintor et al., 2003). However, little is known about the expression and associated signaling events of P2 purinergic receptor subtypes in anterior segment tissues of the eye. The trabecular meshwork is a specialized region in the anterior chamber of the eye composed of connective tissue beams lined with smooth-muscle-like trabecular meshwork cells (Wiederhold et al., 2000). This meshwork forms the primary pathway for drainage of aqueous humor from the anterior chamber. Cells of the trabecular meshwork are thought to influence IOP through their phagocytic actions (Tripathi and Tripathi, 2002; Abbracchio et al., 2003). The P2X family of receptors are coupled receptors but have been found to primarily associate with G_q/o signaling systems (Communi et al., 2001; Zhang et al., 2002; Abbracchio et al., 2003). The P2X family of receptors in the meshwork cells is likely involved in the phagocytosis of cellular debris, which is thought to modulate IOP.
1984), morphological changes altering inter trabecular space (Wiedenholt et al., 2000), and influencing the extracellular matrix turnover (Yue, 1996; Shearer and Crosson, 2001). Consequently, pharmacological agents that target trabecular meshwork cells have the potential to regulate outflow resistance and IOP.

In these studies, we sought to determine whether trabecular meshwork cells express receptors for adenine and uracil nucleotides, and begin to assess the signal transduction pathways coupled to these receptors. Our results show that trabecular meshwork cells express P2Y1, P2Y4, and P2Y11 purinergic receptor subtypes. The activation of these receptors by P2 agonists leads to mobilization of intracellular Ca\textsuperscript{2+} and activation of the extracellular signal-regulated kinase (ERK)1/2 pathway.

**Materials and Methods**

**Materials.** Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT) and DMEM was purchased from Invitrogen (Carlsbad, CA). ATP, 2-methyl-thio-triphosphate (2-MeS-ATP), ADP, UTP, UDP, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), MRS-2179, suramin, 8-sulfophenylthephpine (8-SPT), chelerythrine chloride, and U-0126 were purchased from Sigma-Aldrich (St. Louis, MO). Fluoro 3-AM was purchased from Molecular Probes (Eugene, OR).

**Cell Culture.** Primary bovine trabecular cell cultures were established from trabecular meshwork explants by techniques described previously (Shearer and Crosson, 2001). Briefly, small strips of trabecular meshwork tissue were dissected from one or two eyes and homogenized by means of a Teflon hand-held homogenizer in DMEM containing 15% fetal bovine serum (FBS). The homogenized tissue was plated onto a 60-mm collagen-I-coated (Biocoat, Fort Washington, PA) cell culture plate and allowed to grow for 2 weeks in DMEM containing 15% FBS. The resultant cells were harvested and plated onto polypropylene cell culture plates in DMEM containing 10% FBS (Pang et al., 1994). These cells were allowed to grow to approximately 80% confluence.

**Determination of Intracellular Calcium.** Intracellular free Ca\textsuperscript{2+} was determined using a fluorometric imaging plate reader system (Molecular Devices Corp., Sunnyvale, CA). Cells for intracellular Ca\textsuperscript{2+} measurements were subcultured into 96-well clear-bottom black microplates (Costar; Cambridge, MA). On the day of each experiment, cells were incubated with 4 μM fluo 3-AM (excitation at 488 nm, emission at 540 nm; Molecular Probes) in HEPES buffer (pH 7.4) containing 2.5 mM probenecid for 1 h at 37°C. Cells were then washed four times, placed in the fluorometric imaging plate reader, and each well of the microplate was monitored at 1.5-s intervals over 6 min. Six wells were averaged for each individual value and experiments were repeated at least three times. In selected experiments, the contribution of extracellular Ca\textsuperscript{2+} to these responses was investigated by omitting Ca\textsuperscript{2+} from the incubation buffer and adding 2 mM EDTA. In cross-desensitization studies, agonist treatments were separated by 2 min. For antagonist studies, cells were treated for 10 min with individual antagonists before the addition of the P2 agonists.

**ERK Assay.** Cells were maintained in serum-free medium for 16 h before the addition of any agent. Cells were then treated with P2 agonists for 10 min. In experiments evaluating the P2 antagonist U-0126 or chelerythrine chloride, cells were pretreated for 30 min with individual agents before the addition of the agonist. At the end of the incubation periods, cells were rinsed with ice-cold phosphate-buffered saline and lysed by the addition of 0.5 ml of lysis buffer (50 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl\textsubscript{2}, 1 mM Na\textsubscript{2}VO\textsubscript{4}, 1 mM dithiothreitol, and 1 μg/ml of a protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN). To determine the level of ERK1/2 activation (phosphorylation), equivalent amounts of protein were loaded. Levels of 2% SDS, glyceraldehyde 3-phosphate dehydrogenase, and proteins were separated according to molecular weight using standard SDS-polyacrylamide gel electrophoresis protocols and transferred to a nitrocellulose membrane. Total ERK levels (phosphorylated and nonphosphorylated forms) were determined by immunoblot techniques using polyclonal anti-phospho-ERK antibodies (New England Biolabs, Beverly, MA). Bands were visualized by the addition of anti-rabbit horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Biosciences Inc., Piscataway, NJ). Blots were then stripped by incubation in "stripping buffer" (62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS) for 30 min at 50°C. The level of phosphorylated (activated) ERK1/2 was then determined by immunoblot analysis with polyclonal anti-phospho-ERK antibodies (New England Biolabs) and visualized by the addition of anti-rabbit horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents. Band densities were quantified by means of a Versa Doe imaging system (Bio-Rad, Hercules, CA), and the level of phosphorylated ERK1/2 isoforms was normalized for differences in loading, using band intensities from immunoblots of total ERK protein.

**Reverse Transcription-Polymerase Chain Reaction.** Total RNA was isolated from HTM-3 cells using a TRIzol reagent RNA isolation kit (Invitrogen) according to the manufacturer’s instructions. Two micrograms of total RNA were reversely transcribed for cDNA synthesis using SuperScript RNase H-Reverse Transcriptase and oligo(dT)-12-18 primer (Invitrogen). Amplifications of targeted purinergic receptor cDNA were performed with specific primers that were designed based on GenBank nucleotide sequences. The PCR was allowed to proceed in a final volume of 20 μl in a programmable Master Cycler Gradient Thermocycle (Eppendorf, Mansfield, TX) with the following settings: 5 min at 95°C for initial denaturation followed by repeated cycles of denaturation at 95°C for 3 min, primer annealing for 1 min at 55°C, and extension at 72°C for 1 min 30 s. After the initial cycle, further extension was allowed to proceed for another 10 min at 72°C. The PCR products were resolved on a 1.0% ethidium bromide-stained agarose gel and then visualized under ultraviolet light transillumination. PCR product sizes were estimated from the migration of a DNA size marker run concurrently (1 kilobase plus DNA Ladder; Invitrogen). For each sample, PCR was performed on RNA that had not been reversely transcribed to confirm that no genomic DNA was present in the samples. The positive reaction products were sequenced to confirm cDNA identity. Primers for each receptor were as follows: P2Y\textsubscript{2} (forward primer) TGTTGTT- GTACCCCTCCAATGGCC (reverse primer) ATCCGTAACAGCGCA-GAATCAGCA; P2Y\textsubscript{4} (forward primer) GAGCATCTGAGCTTGAGAG (reverse primer) AGTGCATCAGACACAGCCAG; P2Y\textsubscript{6} (forward primer) GAGTACATCGACAGACACGAC; P2Y\textsubscript{11} (forward primer) CCACCTGGCATTGTCAGACCC (reverse primer) GAGTACATCGACAGACACGAC; P2Y\textsubscript{13} (forward primer) CTCTTCTATGCCAACC (reverse primer) CCATCCTGGCGGCA-GAATCAGCA; P2Y\textsubscript{27} (forward primer) CAATCCTGGCGGCA-GAATCAGCA; P2Y\textsubscript{29} (forward primer) CPGTCT-CCTCTTCTATGCCAACC (reverse primer) CCATCCTGGCGGCA-GAATCAGCA; P2Y\textsubscript{31} (forward primer) CAATCCTGGCGGCA-GAATCAGCA; and P2Y\textsubscript{14} (forward primer) ACAAGCCTATACGCTTTGT-GTTG (reverse primer) ACTCAGGCGTATAGAGTAG.

**Statistical Analysis.** Data are presented as the mean ± S.E. and were analyzed using analysis of variance followed by Duncan’s multiple range test for detecting differences, with P < 0.05 considered as significant. The dose-response curves were analyzed by nonlinear regression analysis (GraphPad Software Inc., San Diego, CA).

**Results**

**Effect of P2 Agonists on Ca\textsuperscript{2+} Mobilization.** Exposure of HTM-3 or bovine trabecular cells to ATP, UTP, or ADP (10°6 mol/l) produced a rapid increase in intracellular free Ca\textsuperscript{2+} concentration, peaking in 20 to 30 s (Fig. 1). The rise in
in intracellular Ca\(^{2+}\) was followed by a return to basal level in 60 to 80 s. At equivalent doses ATP and UTP produced similar increases in intracellular free Ca\(^{2+}\); however, the rise in Ca\(^{2+}\) measured after ADP addition was consistently less than that observed for ATP or UTP. In cells incubated in Ca\(^{2+}\)-free buffer for 10 min, a rapid increase in intracellular free Ca\(^{2+}\) and subsequent decline in response to P2 agonists, was measured (data not shown). Figure 2 shows the concentration-response curve for peak rise in intracellular Ca\(^{2+}\) increase after the addition of various P2 agonists to BTM cells. The EC\(_{50}\) and response maxima for P2Y agonists in bovine trabecular cells and the HTM-3 cell line are listed in Table 1. Except for UDP, all agonists produced a dose-related increase in intracellular Ca\(^{2+}\); however, maximum response to ADP and 2-MeS-ATP was 35 to 45% lower than that observed for ATP and UTP.

To further characterize the P2 agonist-induced response in trabecular cells, the effects of P2Y\(_{1}\)-receptor antagonist MRS-2179 and the nonselective P2 antagonists suramin and PPADS were evaluated. The increases in intracellular-free Ca\(^{2+}\) induced by 2-MeS-ATP and -ADP were blocked by the presence of MRS-2179 (10 \(\mu\)M) (Fig. 3). However, the ATP- and UTP-induced increase in Ca\(^{2+}\) mobilization was not altered by the presence of MRS-2179. Pretreatment of cells with nonselective P2 antagonists suramin and PPADS (10 \(\mu\)M) each significantly inhibited the ADP- and 2-MeS-ATP-induced rise in intracellular Ca\(^{2+}\) by 60 to 80%, but it did not significantly alter the responses to ATP or UTP. Pretreatment with the adenosine receptor antagonist 8-SPT (10 \(\mu\)M) did not significantly alter the rise in intracellular Ca\(^{2+}\) induced by any of the P2 agonists (data not shown).

To evaluate cross-desensitization between ATP and UTP, agonists were administered sequentially within a 2-min interval. The addition of ATP (10 \(\mu\)M) did not alter the subsequent addition of UTP. However, the addition of UTP (10 \(\mu\)M) reduced the response to ATP by 26% \((P < 0.05)\).

**Effects of P2 Agonists on ERK1/2 Activation.** As shown in Fig. 4, the addition of 10\(^{-7}\) mol/l ATP, UTP, ADP, and 2-MeS-ATP to bovine trabecular meshwork cells produced a significant increase in ERK1/2 phosphorylation. No increase in ERK1/2 activation was observed after the addition of UDP. In HTM-3 cells the addition of ATP, UTP, ADP, and 2-MeS-ATP increased ERK1/2 activation by 233, 228, 247, and 190%, respectively. In both culture systems, the increase in ERK1/2 activation induced by each P2 agonist was not altered by pretreatment with MRS-2179, PPADS, or suramin (data not shown).

To investigate the upstream signaling events associated with P2 agonist-induced stimulation of ERK1/2, cells were pretreated with the MEK inhibitor U-0126 or the PKC inhibitor chelerythrine chloride. As shown in Fig. 5, pretreatment of BTM cells with U-0126 (1.0 mol/l) blocked the ERK activation induced by ATP or UTP (10\(^{-7}\) mol/l). Pretreatment with the PKC inhibitor chelerythrine chloride (20 \(\mu\)M), also completely blocked the ATP- and UTP-induced ERK1/2 activation in these cells. In HTM-3 cells, pretreatment with U-0126 or chelerythrine also completely blocked the ERK1/2 activation induced by ATP or UTP (10\(^{-7}\) mol/l).

**Expression of P2Y Receptor Subtype mRNA in BTM and HTM-3 Cells.** To investigate the expression of P2Y-receptor subtypes, mRNA from human cell line (HTM-3) was analyzed by RT-PCR. As shown in Fig. 6, mRNA for P2Y\(_{1}\), P2Y\(_{4}\), and P2Y\(_{11}\) receptors was detected in HTM-3 cells. However, no message for P2Y\(_{2}\) and P2Y\(_{6}\) receptors could be detected in these cells.
Adenine nucleotides have been identified in the aqueous humor of animals (Greiner et al., 1991). However, our understanding of the role P2 receptors play in regulating anterior segment function has been limited. Although P2X receptors have been identified in the mammalian retina (Jabs et al., 2000; Wheeler-Schilling et al., 2000, 2001), the expression of P2X receptors in anterior segment tissues have not been reported. In contrast, molecular and functional studies have provided evidence that P2Y receptors are expressed in a number of anterior segment tissues including corneal and ciliary epithelium, lens, and conjunctiva (Merriman-Smith et al., 1998; Collison and Duncan, 2001; Cullinane et al., 2001; Farahbakhsh and Cilluffo, 2002; Cowlen et al., 2003). In this study, we investigated whether trabecular meshwork cells express functional P2 receptors.

The addition of a P2 agonist to human or bovine trabecular cells produced a rapid rise in intracellular Ca\(^{2+}\). This increase in intracellular Ca\(^{2+}\) did not seem to result from the activation of ionotropic P2X receptors, because it was not blocked by the incubation of cells in Ca\(^{2+}\)-free media. Although adenosine receptors have been identified on trabecular meshwork cells (Shearer and Crosson, 2002), the inability of the adenosine antagonist 8-SPT to alter the response to adenine and uracil nucleotides demonstrates that the activation of adenosine receptors did not contribute to responses observed in these studies. Together, these data support the idea that the responses induced by P2 agonists in trabecular cells are mediated by P2Y receptors.

The difference in response maxima (Table 1) and the selective blockade of P2 agonists responses by MRS-2179 (Fig. 3), indicate that the activation of at least two P2Y-receptor subtypes can mobilize intracellular Ca\(^{2+}\) in trabecular cells. The moderately selective P2Y1 agonists ADP and 2-MeS-ATP exhibited response maxima that were 35 to 45% lower than values determined for ATP and UTP. The EC\(_{50}\) values measured for ADP and 2-MeS-ATP are consistent with the EC\(_{50}\) values for P2Y1 receptors measured in other mammalian tissues (Simon et al., 1995; Nicholas et al., 1996; Pacaud et al., 1996; Ralevic and Burnstock, 1996; Palmer et al., 1998). In addition, the ADP- or 2-MeS-ATP-induced increase in intracellular Ca\(^{2+}\) was blocked by the pretreatment of P2Y1 antagonist MRS-2179. These data support the idea that both ADP- and 2-MeS-ATP stimulate the release of intracellular Ca\(^{2+}\) in trabecular cells through activation of the P2Y1 receptor. The expression of P2Y1 receptors in the HTM-3 cell line was confirmed by RT-PCR. The precise signal transduc-

### Discussion

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### Table 1

Dose-response parameters for P2 agonist-induced mobilization of intracellular Ca\(^{2+}\) in trabecular meshwork cells

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Bovine Trabecular Cells</th>
<th>HTM-3 Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC(_{50})</td>
<td>Response Maximum</td>
</tr>
<tr>
<td>ATP</td>
<td>(0.58)</td>
<td>428</td>
</tr>
<tr>
<td>UTP</td>
<td>(0.37)</td>
<td>419</td>
</tr>
<tr>
<td>ADP</td>
<td>(0.35)</td>
<td>269</td>
</tr>
<tr>
<td>2-MeS-ATP</td>
<td>(0.070)</td>
<td>225</td>
</tr>
<tr>
<td>UDP</td>
<td>ND</td>
<td>ND</td>
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Parameters for UDP were not determined (ND) because this agonist did not produce any significant elevation in intracellular Ca\(^{2+}\) at the concentrations tested.

### Fig. 3

Effect of the P2Y₁ antagonist MRS-2179 on the P2 agonist-induced increase in intracellular Ca\(^{2+}\). Bovine trabecular meshwork cells were pretreated with MRS-2179 (10 \(\mu\)M) for 10 min before the addition of P2 agonists. Asterisks denote significant difference (\(P < 0.05\)) between agonist stimulation alone and agonist stimulation after pretreatment with MRS-2179 (\(n = 4\)).

### Fig. 4

Effects of P2 agonists on ERK1/2 activation. Serum-deprived bovine trabecular cells were incubated for 10 min in the presence or absence (control) of individual P2 agonists (0.1 \(\mu\)M). A, summary data from five experiments. Values are the means ± S.E. of densitometry measurements from immunoblots of cell lysates. Asterisks denote significant difference (\(P < 0.05\)) from control levels. B, representative immunoblots of phospho-ERK and total ERK from bovine trabecular cell lysates.
of any measurable increase in intracellular Ca$^{2+}$ from P2Y4 receptor activation. However, recent studies have shown that UTP can mobilize intracellular Ca$^{2+}$, activating calcium-sensitive PKC, and eventually activating ERK1/2 in these cells. Unlike the Ca$^{2+}$-mobilization studies, no significant difference in response magnitude was measured in these experiments. The activation of ERK1/2 by UTP in these studies indicates that uracil-sensitive P2Y$_4$ receptors are linked to ERK activation. Additionally, the inability of MRS-2179 to inhibit ERK activation induced by ADP or 2-MeS-ATP argues that the P2Y$_1$ receptor is not linked to this pathway. However, we cannot exclude the possibility that ERK activation observed after the addition of nucleotides also results from the activation of P2Y$_{11}$ receptors.

Original studies considered the trabecular meshwork a passive filter for aqueous humor, with changes in resistance to aqueous flow occurring indirectly through ciliary muscle contraction. Recent studies have demonstrated that trabecular cells are contractile in nature and are capable of modifying the extracellular matrix within the region, supporting the idea that these cells participate in the regulation of trabecular function and aqueous outflow resistance. Recent studies have demonstrated that trabecular P2Y receptors may play an important role in regulation of trabecular cells, and human HTM-3 cell line, and the calculated EC$_{50}$ value for UTP is similar to that reported for P2Y$_{12}$ receptors in other cell types. However, recent studies have shown that UTP administration, and the absence of any detectable mRNA in bovine or human trabecular meshwork (Shearer and Crosson, 2002). Hence, the activation of the ERK1/2 signaling pathway seems to play a central role in the regulation of trabecular function (Shearer and Crosson, 2002). The addition of ATP, UTP, ADP, or 2-MeS-ATP each activated ERK1/2 in HTM-3 and bovine trabecular cells. The ATP- and UTP-induced activation of ERK1/2 was blocked by pretreatment with the MEK inhibitor U-0126 or the PKC antagonist chelerythrine chloride. These results are consistent with the idea that activation of P2Y receptors leads to mobilization of intracellular Ca$^{2+}$, activating calcium-sensitive PKC, and eventually activating ERK1/2 in these cells. Unlike the Ca$^{2+}$-sensitive agents targeting trabecular P2Y receptors may prove to be efficacious agents for the treatment of glaucoma.

In summary, these data demonstrate that multiple P2Y receptors are present in human and bovine trabecular meshwork cells. Our results are consistent with the idea that the activation of P2Y$_1$, P2Y$_4$, and P2Y$_{11}$ receptors leads to the mobilization of intracellular Ca$^{2+}$. The stimulation of the ERK1/2 signaling pathway seems to result from the activation of P2Y$_4$ receptors via a PKC-dependent system. However, a role for
the P2Y<sub>11</sub> receptor in the activation of this pathway cannot be excluded.

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References


Address correspondence to: Dr. Craig E. Crosson, Storm Eye Institute, 167 Ashley Ave., Charleston, SC 29425. E-mail: ccrosson@musc.edu