Nucleotides in the Eye: Focus on Functional Aspects and Therapeutic Perspectives

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ABSTRACT

The presence and activity of nucleotides and dinucleotides in the physiology of most, if not all, organisms, from bacteria to humans, have been recognized by the scientific community, and the eye is no exception. Nucleotides in the dynamic fluids interact with many ocular structures, such as the tears and aqueous humor. Moreover, high concentrations of nucleotides in these secretions may reflect disease states such as dry eye and glaucoma. Apart from the nucleotide concentration in these fluids, P2 purinergic receptors have been described on the ocular surface (cornea and conjunctiva), anterior pole (ciliary body, trabecular meshwork), and posterior pole (retina). P2X and P2Y purinergic receptors are essential in maintaining the homeostasis of ocular processes, such as tear secretion, aqueous humor production, or retinal modulation. When they are functioning properly, they allow the eye to do its job (to see), but in some cases, a lack or an excess of nucleotides or a malfunction in the corresponding purinergic receptors leads to disease. This Perspective is focused on the nucleotides and dinucleotides and the P2 purinergic receptors in the eye and how they contribute to normal and disease states. We also emphasize the action of nucleotides and their receptors and antagonists as potential therapeutic agents.

Introduction

The role of purine nucleotides and nucleosides as extracellular messengers was first proposed in the 1970s (Burnstock, 1972). Since then, the rapidly accelerating progress in this field has helped determine the involvement of these compounds in key biochemical and physiologic processes in different tissues. Their therapeutic potential in a wide range of diseases is also attracting increasing interest (Burnstock, 2012).

Extracellular nucleotides act by stimulating P2 purinergic receptors on the cell surface. This group of receptors is divided into two families, the ionotropic P2X receptors and the metabotropic P2Y receptors that are coupled to G proteins (von Kugelgen and Harden, 2011). P2X receptors are ligand-gated ion channels that allow calcium influx from the extracellular space after nucleotide binding. Seven mammalian purinergic receptor subunits, denoted P2X1 through P2X7, and several spliced forms of these subunits have been identified. Cloning of P2X receptors revealed that all subunits have a large extracellular loop, two transmembrane domains, and intracellular-located amino and carboxyl termini of variable lengths. Three subunits are necessary for forming a native functional channel, and these channels are organized as homotrimers or heterotrimers (Coddou et al., 2011).

P2Y receptors, as G protein-coupled receptors, contain seven hydrophobic transmembrane domains connected by three extracellular loops and three intracellular loops (Jacobson and Boeynaems, 2010). The P2Y family is composed of eight members encoded by distinct genes that can be subdivided into two groups based on their coupling to specific G proteins. The

ABBREVIATIONS: Ap3A, diadenosine triphosphate; Ap4A, diadenosine tetraphosphate; Ap5A, diadenosine pentaphosphate; ATPγS, adenosine-5’-O-(3-thiotriphosphosphate); BBG, Brilliant Blue G; BzATP, 2’,3’-O-(4-benzoylbenzoyl)adenosine-5’-triphosphate; ERK, extracellular-signal-regulated kinases; IL, interleukin; IN5356, diuridine tetraphosphate; INS37217, P(1’)-(uridine 5’)-P(4’)-(2’-deoxyxycytidine 5’)-tetraphosphate; IOP, intraocular pressure; IP3, diinosine tetraphosphate; MRS2179, 2’-deoxy-5’-methyl adenosine 3’-5’-diphosphate; MRS2578, 1,4-di(3-(3-isothiocyanatophenyl)thioureidol)butane; PVR, proliferative vitreoretinopathy; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; RPE, retinal pigment epithelium; SRS, subretinal space; υP2U, diuridine tetraphosphate; Y27632, (R)-(−)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide; YO-PRO-1, 4’-[3-methyl-1,3-benzoxazol-2’(3H)-ylidene]methyl]-1-[(3-methylammonio)propyl]quinolinium diiodide.
P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors couple to Gq to activate phospholipase C β; the P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors associate with Gi to inhibit adenyl cyclase. The P2Y₁₁ receptor has the unique property of coupling through both Gq and Gi (Jacobson et al., 2012).

The P2X receptors are more structurally restrictive than P2Y receptors with regard to agonist selectivity. They respond principally to ATP and their analogs 3’-O-(4-benzoyl) benzoyl ATP, α,β-methylene ATP, β,γ-methylene ATP, and 2-methylthio ATP as active ligands, while P2Y receptors, other than ATP and its analogs, are also activated by nucleotides such as ADP, UTP, or UDP. In particular, the ligand preferences [shown in brackets] of the eight human P2Y receptors are as follows: P2Y₁ [ADP], P2Y₂ [UTP = ATP], P2Y₄ [UTP], P2Y₆ [UDP], P2Y₁₁ [ATP, NAD⁺], P2Y₁₂ [ADP], P2Y₁₃ [ADP], and P2Y₁₄ [UDP, UDP-glucose, and other nucleotide sugars] (Zimmermann et al., 2012; Burnstock, 2013).

Dinucleoside polyphosphates, known as dinucleotides, also act as agonists for P2X and P2Y receptors. They are composed of two nucleoside moieties linked by their ribose 5’-ends to a variable number of phosphates (2–7), the diadenosine polyphosphates (containing two adenosine moieties) being the most abundant and widely studied of these compounds (Guzman-Aranguez et al., 2007).

In the eye, the identification of nucleotides and dinucleotides in ocular secretions such as tears and aqueous humor (Mitchell et al., 1998; Pintor et al., 2002a,b) and the expression of P2 receptors in different eye locations (see Table 1) suggests the involvement of these compounds in the physiology of the eye. In fact, during the last decade, the role of several nucleotides and their receptors in ocular processes, including tear secretion, corneal wound healing, regulation of intraocular pressure (IOP), or retinal detachment, has been established (Pintor et al., 2003a; Crooke et al., 2008).

Moreover, the expanding knowledge of the contribution of nucleotides to ocular functions reveals that these compounds can provide new opportunities for therapeutic interventions in ocular diseases (Fig. 1).

In this article, we review the main biologic roles of nucleotides in the eye and the applications of these compounds as therapeutic agents for the treatment of eye disease.

**Nucleotides at the Ocular Surface**

**Identification of Nucleotides in Tears and Changes in Pathologic Conditions.** The tear film covering the ocular surface consists of two distinct layers: an outer lipid layer produced by meibomian glands and an inner layer containing a mixture of mucins, glycoproteins, and different aqueous components such as electrolytes and glucose, together with other proteins (lysozyme and lactoferin, among others). The aqueous phase is mainly derived from the main and accessory lacrimal glands, but the conjunctiva can also contribute to secretion of fluid into the tear film. Finally, the mucin component is provided by the conjunctival goblet cells as well as corneal and conjunctival epithelial cells (Murube, 2012).

Mono- and dinucleotides have been detected in rabbit and human tears using high-performance liquid chromatography (Pintor et al., 2002a,b). Particularly, diadenosine polyphosphates, diadenosine tetraphosphate (Ap₂A), and diadenosine pentaphosphate (Ap₃A) were found at micromolar concentrations in rabbit tears. Human tears also contain these dinucleotides together with diadenosine triphosphate (Ap₃A) and Ap₄A, the latter being the most abundant diadenosine polyphosphate in human tears. Interestingly, the concentrations of diadenosine polyphosphates in rabbits were one order of magnitude higher than that found in humans. Despite this difference, the Ap₃A/Ap₄A ratio in rabbits and humans remained stable, at 2.97 and 2.91, respectively. (Pintor et al., 2002a,b).

Nucleotides in tears can be released into the extracellular medium by cell rupture or neural exocytosis, and it also has been suggested that the release of nucleotides may be a consequence of mechanical shear stress on the corneal epithelium (Srinivas et al., 2002). In humans, there is evidence pointing to a mechanical release of nucleotides, in particular Ap₂A and Ap₄A, as a consequence of the lid effect on the corneal surface. Normal individuals instructed to blink at different frequencies release more nucleotides the more often they blink (Peral et al., 2006) (Fig. 2).

An increase in blinking frequency is a typical sign of eye dryness, which occurs to compensate tear instability (Tsutobu et al., 1996). Therefore, the concentrations of these molecules should be elevated in cases of dry eye, and indeed they are. In symptomatic non-Sjögren dry eye patients with normal tear production, Ap₂A levels were increased by 5-fold and Ap₄A levels by 1.5-fold, and almost by 100- and 345-fold for Ap₃A and Ap₄A, respectively, in symptomatic non-Sjögren dry eye patients with low tear production (Peral et al., 2006). Interestingly, differences were found between male and female tear samples. Samples from symptomatic women apparently had higher levels of diadenosine polyphosphates than those of men. This divergence gives rise to the possibility that there may be a hormonal factor involved, which may deserve further investigation.

Additionally, Ap₂A concentration increased 42-fold and Ap₃A concentration 595-fold in Sjögren dry eye patients, compared with normal individuals (Carracedo et al., 2010). The association between the increase of diadenosine polyphosphates in tears and dry eye pathology has been proposed as a potential molecular biomarker for this disease (Pintor, 2007).

**Effects on Tear Secretion and Tear Composition.** The physiologic surface of both mammalian animals and humans may be modified by nucleotides in tears. One of the physiologic processes regulated by extracellular nucleotides on the ocular surface is tear production.

Topical instillation of the mononucleotides, UTP and ATP, in an equipotent way, increased tear secretion in rabbits around 4-fold as determined by Schirmer tests (Murakami et al., 2000), whereas UDP and ADP did not modify tear secretion (Pintor et al., 2002b). Based on this response to nucleotides, the effect seems to be mediated by P2Y₂ receptors. This conclusion is reinforced by the promotion of chloride secretion and net fluid flux in the serosal-to-mucosal direction induced by P2Y₂ receptor activation in conjunctival epithelium (Li et al., 2001b). Dinucleotides can also stimulate tear fluid secretion. Diadenosine polyphosphate Ap₂A markedly increased tear production to around 60% higher than normal in New Zealand White rabbits. Ap₃A and Ap₄A also significantly increased tear production by about 20% (Pintor et al., 2002b). Likewise, the application of diuridine tetraphosphate (Up₄U, INS365), a selective P2Y₂ agonist, induced a 1.5-fold transient increase in tear fluid secretion in a rat model (Fujihara et al., 2001).

Nucleotide application not only alters tear volume (water and electrolytes) but also affects tear composition, modifying...
its protein content. Activation of P2X7 receptors in rat lacrimal gland by the nucleotide (benzoylbenzoyl)adenosine 5’-triphosphate (BzATP) increased intracellular calcium and stimulated protein secretion (Hodges et al., 2009). Furthermore, the expression of specific tear proteins such as secreted mucins can also be modified by nucleotides. UTP and ATP stimulated mucin release from isolated rabbit and human conjunctival tissues through P2Y2 activation (Jumblatt and Jumblatt, 1998). Similarly, the mononucleotides ATP and UTP and the dinucleotide Up2U increased mucin secretion from conjunctival goblet cells in living animals (Fujihara et al., 2001; Murakami et al., 2003).

In view of the simultaneous effect of nucleotides via P2Y2 receptors on fluid/mucin secretion, P2Y2 receptor agonists have been tested in different dry eye animal models for restoring moisture and rehydration of the ocular surface. In fact, the dinucleotide diuridine tetraphosphate (INS365, Diquafosol) has been under development as a new treatment of dry eye (Nichols et al., 2004) and was recently launched in Japan by Santen Pharmaceutical after completion of clinical trials.

Interestingly, as mentioned above, non-Sjögren and Sjögren dry eye patients showed higher Ap4A and Ap5A dinucleotide levels than normal subjects (Peral et al., 2006; Carracedo et al., 2010). These increased levels may represent a compensatory attempt by the ocular surface epithelium to preserve a wet-surface phenotype by increasing tear fluid and mucin secretion, although this hypothesis remains to be confirmed.

Finally, it has been observed that nucleotides can modify the levels of not only secreted mucins, but also other tear proteins. In particular, changes in lysozyme levels, the most abundant protein in tears, have been detected after topical application of nucleotides (Peral et al., 2008). UTP, Ap4A, and Up2U increased lysozyme concentrations by 67, 93, and 119%, respectively, compared with lysozyme basal levels. Because this enzyme is one of the first defense mechanisms against bacterial infection, increasing the levels of this natural bacteriologic agent with these nucleotides could provide additional protection against pathogen invasion.

Effects on Wound Healing and Cell Migration. The cornea is a multilayered tissue of the eye characterized by its transparency, avascularity, and its ability to refract light. Its most superficial layer is the corneal epithelium, which is susceptible to damage by several factors. After injury, normal epithelial regeneration is initiated by the wound healing process (Dupps and Wilson, 2006).

Nucleotides have been shown to stimulate events associated with epithelial wound repair. The mononucleotides ATP and UTP and the dinucleotide Ap4A induce cell migration and stimulate corneal re-epithelialization, both in vitro and in vivo (Pintor et al., 2004a; Mediero et al., 2008). In particular, UTP increased the rate of healing by 168% and Ap4A by 130% in experiments in New Zealand White rabbits (Pintor et al., 2004a). Pharmacologic studies using different P2 receptor antagonists showed that the wound healing effect triggered by these nucleotides was mediated by P2Y2 receptor activation (Pintor et al., 2004a; Mediero et al., 2006).

Experiments performed in vitro with immortalized rabbit corneal epithelial cells indicate that diadenosine polyphosphates can produce several effects on corneal epithelium, which differ depending on phosphate chain bridging between the two adenosines. Ap4A accelerated the rate of re-epithelialization, whereas Ap3A or Ap5A did the opposite, delaying the wound-healing process (Mediero et al., 2006).

An increased re-epithelialization rate has been observed with nucleotides such as Ap4A, UTP, and ATP. This agonistic profile coincides with the observations of Lazarowski et al. (1995) showing Ap4A and UTP to be the best agonists on the cloned P2Y2 receptor.

Concerning the second messenger systems triggered by the P2Y2 receptor to accelerate the rate of cell migration during wound healing, two intracellular pathways are implicated: extracellular-signal-regulated kinases (ERK) and Rho-associated protein kinase (ROCK). ROCK-I is linked to the actin cytoskeleton, modifying cellular contractility. The involvement of this pathway has been demonstrated with compounds such as Y27632 [(R)(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide] or (+)-blebbistatin, which inhibit ROCK-1 and myosin light chain kinase (Mediero et al., 2008). When these compounds were used, the ability of epithelial cells to migrate after Ap4A treatment disappeared. This notion has been recently confirmed by specific silencing of the P2Y2 receptor using small interfering RNA (Crooke et al., 2009; Boucher et al., 2010).

The P2X7 receptor may also mediate later phases of wound repair in the cornea. In vivo studies with P2X7 knockout mice (P2X7−/−) revealed that P2X7 is necessary for the timely healing of abrasions and the normal structural organization of the corneal stroma (Mayo et al., 2008).

Effects on Cell Proliferation. After the damaged area has been covered with a cell monolayer, the final step in corneal wound healing is to initiate the mitotic process for restoring normal corneal epithelial thickness. In vitro experiments have shown that after treatment with Ap4A or Ap3A, corneal epithelial cell proliferation increased similarly with both dinucleotides at 24 and 36 hours (Mediero et al., 2010; http://www.journalofemmetropia.org/2171-4703/jemmetropia.2010.1.81.87.php). Thus, pretreatment with several inhibitors demonstrated that Ap4A is linked to the phospholipase C/protein kinase C/RhoA/ROCK-1 and Erk1/2 pathways, and Ap3A modulates proliferation by activating ERK1/2 and p38 mitogen-activated protein kinase cascades (Mediero et al., 2010). Pharmacologic experiments have demonstrated the sequential involvement of P2Y2 and P2Y6 receptors in the proliferative stage of wound healing. It is interesting that corneal epithelial cell proliferation during the wound healing process was found to be initiated by P2Y2 receptor activation via the ERK1/2 pathway (Muscella et al., 2004). Wound healing could not be completed without the participation of a P2Y6 receptor to maintain the mitotic process. In the case of the P2Y6 receptor, the natural activator would be Ap3A, which recruits the p38 mitogen-activated protein kinase pathway to lead the whole proliferation process (Mediero et al., 2010).

Anterior Chamber Nucleotides

Nucleotides in Aqueous Humor and Their Effect on Intraocular Pressure. Aqueous humor, produced by ciliary processes, is a transparent fluid that provides nutritional support to avascular intraocular structures, mainly the cornea and the lens. Aqueous humor is drained through the trabecular meshwork, Schlemm’s canal, and episcleral veins (To et al., 2002).
<table>
<thead>
<tr>
<th>Ocular surface</th>
<th>Species</th>
<th>Function</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Conjunctival epithelium</strong></td>
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<tr>
<td><strong>P2X7</strong></td>
<td>Human</td>
<td>Membrane permeabilization associated with iatrogenic pathology</td>
<td>Dutot et al., 2008</td>
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<tr>
<td><strong>P2Y2</strong></td>
<td>Rabbit, macaque, and human</td>
<td>Stimulation of tear secretion</td>
<td>Jumblatt and Jumblatt, 1998; Cowlen et al., 2003</td>
</tr>
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</table>

| Lacrimal gland | | | |
| **P2X1, P2X2, P2X4, P2X6, P2X7** | Rat | Not determined | Hodges et al., 2011 |
| **P2X1, P2Y1, P2Y11, P2Y13** | Rat | Induction of intracellular calcium concentration and protein secretion | Ohtomo et al., 2011 |

| Anterior and posterior chamber | | | |
| **Ciliary processes** | | | |
| **P2X2** | Cow | Not determined | Pintor and Peral, 2001 |
| **P2X7** | Cow | Membrane permeabilization | Li et al., 2011 |
| **P2Y1** | Rabbit | Not determined | Farahbakhsh and Cilluffo, 2002 |
| **P2Y2** | Rabbit, cow, monkey, rat, and human | Increase intraocular pressure | Shahidullah and Wilson, 1997; Cullinane et al., 2001a,b; Farahbakhsh and Cilluffo, 2002; Cowlen et al., 2003; Pintor et al., 2004b; Martin-Gil et al., 2012 |
| **P2Y4** | Rat | Not determined | Pintor et al., 2004b |
| **P2Y6** | Rabbit, rat | Not determined | Pintor et al., 2004b; Markovskaya et al., 2008b |

| Trabecular meshwork | | | |
| **P2X2** | Rabbit and cow | Decrease IOP (increase aqueous humor outflow) | Pintor and Peral, 2001 |
| **P2X7** | Human | Membrane permeabilization, ATP release | Li et al., 2012 |
| **P2Y1** | Cow, human, pig, and rat | Decrease IOP (increase aqueous humor outflow). Cell swelling | Crosset et al., 2004; Pintor et al., 2004b; Soto et al., 2005; Chow et al., 2007 |
| **P2Y4** | Cow and pig | Not determined | Soto et al., 2005; Chow et al., 2007 |
| **P2Y11** | Human | Not determined | Crosset et al., 2004 |

| Retina | | | |
| **Photoreceptors** | | | |
| **P2X2** | Rat | Not determined | Greenwood et al., 1997 |
| **P2X7** | Mouse, rat, and marmoset | Modulation of synaptic transmission | Franke et al., 2005; Puthussery et al., 2006; Notomi et al., 2011 |
| **P2Y1, P2Y2, P2Y4, P2Y6, P2Y8** | Rat, rabbit, and macaque | Not determined | Cowlen et al., 2003; Fries et al., 2004a; Pintor et al., 2004b |

| Bipolar cells | | | |
| **P2X3, P2X4, P2X5, P2X7** | Rat | Not determined | Wheeler-Schilling et al., 2000 |
| **P2X7** | Mouse and rat | Modulation of synaptic transmission | Vessey and Fletcher, 2012 |
| **P2Y1, P2Y2, P2Y4, P2Y6** | Rat | Not determined | Fries et al., 2004b |
| **P2Y4** | Mouse and rat | Modulation of synaptic transmission | Ward et al., 2008 |
| **Horizontal cells** | | | |
| **P2X7** | Mouse and rat | Modulation of synaptic transmission | Vessey and Fletcher, 2012 |

| Amacrine cells | | | |
| **P2X1, P2X3, P2X4, P2X5** | Mouse and rat | Not determined | Wheeler-Schilling et al., 2001; Yazulla and Studholme, 2004; Puthussery and Fletcher, 2007; Shigematsu et al., 2007 |
| **P2X2** | Mouse | Inhibition of Ach release from OFF cholinergic amacrine | Kaneda et al., 2004; Puthussery et al., 2006; Kaneda et al., 2008, 2010 |
| **P2X7** | Mouse | Modulation of synaptic transmission | Vessey and Fletcher, 2012 |

(continued)
TABLE 1—Continued

<table>
<thead>
<tr>
<th>P2 Receptor Subtypes</th>
<th>Species</th>
<th>Function</th>
<th>References</th>
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<tbody>
<tr>
<td>Ganglion cells</td>
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<tr>
<td>P2X2, P2X3, P2X4, P2X5</td>
<td></td>
<td>Not determined</td>
<td>Greenwood et al., 1997; Brandle et al., 1998; Wheeler-Schilling et al., 2001; Puthussery et al., 2006</td>
</tr>
<tr>
<td>P2X7</td>
<td>Mouse</td>
<td>Increase intracellular calcium concentrations</td>
<td>Brandle et al., 1998; Franke et al., 2005; Zhang et al., 2006; Reigada et al., 2008; Mitchell et al., 2009; Hu et al., 2010; Vessey and Fletcher, 2012</td>
</tr>
<tr>
<td>P2Y1, P2Y2, P2Y4, P2Y6</td>
<td>Rabbit, rat, and macaque</td>
<td>Not determined</td>
<td>Cowlen et al., 2003; Fries et al., 2004a</td>
</tr>
</tbody>
</table>

Astrocytes and Müller cells

| P2X7 | Human | Increase DNA synthesis | Pannicke et al., 2000; Bringmann et al., 2001 |
| P2Y1 | Guinea pig | Increase DNA synthesis | Moll et al., 2002; Milenkovic et al., 2003 |
| P2Y4 | P2Y6 | Elicitation of Ca2+ responses | Li et al., 2001a; Fries et al., 2004b, 2005 |
| P2Y7 | Mouse and rat | Elicitation of Ca2+ responses | Fries et al., 2005 |
| P2Y1, P2Y2, P2Y4, P2Y6 | Human | Homeostasis of the extracellular space volume | Uckermann et al., 2006; Iandiev et al., 2007; Wurm et al., 2008, 2010 |

Retinal pericytes

| P2X7 | Rabbit and rat | Depolarization and contraction | Kawamura et al., 2003; Sugiyama et al., 2005, 2006 |
| P2Y4 | Rat | Regulation of calcium-activated chloride channel | Kawamura et al., 2003; Sugiyama et al., 2005 |

Retinal pigment epithelium

| P2X7 | Human | Not determined | Reigada et al., 2005 |
| P2Y2 | Human, cow, rat, and human | Increase of fluid flow from apical to basolateral membrane. | Peterson et al., 1997; Sullivan et al., 1997; Maminishkis et al., 2002; Meyer et al., 2002; Relvas et al., 2009 |
| P2Y4 | Human | Increase IL-8 secretion | Relvas et al., 2009 |
| P2Y4, P2Y7 | Human | Not determined | Reigada et al., 2005 |
| P2X7 | Human | Not determined | Dutot et al., 2008; Yang et al., 2011 |

Measurable amounts of nucleotides and dinucleotides, such as ATP or Ap4A, have been detected in aqueous humor (Mitchell et al., 1998; Pintor et al., 2003b). Purinergic receptors P2X and P2Y are present in the main structures bathed by the aqueous humor (the ciliary process and trabecular meshwork) (Table 1), suggesting that these compounds contribute to physiologic functions in these areas, such as the control of IOP. The mechanisms that control and regulate IOP are not fully understood, but they result from the dynamic equilibrium between the production and drainage of aqueous humor. Disruption of this equilibrium results in raised IOP, which is an important risk factor in the pathogenesis of glaucoma, a multifactorial optic neuropathy characterized by damage to the optic nerve head and irreversible field of vision loss (Friedman et al., 2004). For this reason, most glaucoma drug treatments involve lowering IOP, either by decreasing aqueous humor production. Thus, the nucleotide UDP (adenosine-5′-O-(3-thiotriphosphate)) (Martin-Gil et al., 2012). Pharmacologic studies using antagonists have suggested that the P2Y2 receptor is involved in the hypertensive effect induced by these nucleotides. In fact, silencing the P2Y2 receptor by small interfering RNA produced a robust decrease in IOP of 48% ± 5% compared with control (Martin-Gil et al., 2012). Therefore, the use of small interfering RNA against the P2Y2 receptor may be an interesting therapeutic approach in the reduction of IOP in the treatment of the abnormally raised IOP observed in many glaucoma patients.

It is worth pointing out that there are also nucleotides that, when topically applied, produce a hypotensive effect. Among the nucleotides with a hypotensive effect on IOP, there are compounds capable of facilitating the aqueous humor outflow. For instance, α,β-me ATP and β,γ-me ATP produce a clear and marked decrease in IOP (14.2% ± 2.2% and 33.2% ± 1.3% decrease, respectively) (Peral et al., 2009). This effect seems to be mediated by P2X2 receptors present in the cholinergic terminals that innervate the ciliary muscle. The P2X2 receptor triggers the release of acetylcholine, leading to the widening of the iridocorneal angle with the corresponding increased aqueous humor drainage and reduced IOP (Peral et al., 2009).

Other nucleotides can also reduce IOP, improving aqueous humor outflow by the direct activation of metabotropic P2Y receptors on the trabecular meshwork. Thus, Ap4A induces a hypotensive effect by increasing trabecular outflow through activation of P2Y1 receptors (Soto et al., 2005).

When instilled topically, nucleotides can also stimulate purinergic receptors in the ciliary processes responsible for aqueous humor production. Thus, the nucleotide UDP...
stimulates P2Y6 receptors of the blood vessels of the ciliary processes, inducing vasoconstriction and significantly reducing aqueous humor production (Markovskaya et al., 2008a) with a concomitant decrease in IOP (around 20%).

Very recently, the topical application of diinosine polyphosphates has been seen to have profound effects on IOP in normotensive rabbits. Diinosine pentaphosphate produced an increase in IOP, while diinosine triphosphate and Ip4I (diinosine tetraphosphate) produced a decrease, the latter being the most effective in reducing IOP, with an EC50 of 0.63 μM (Guzman-Aranguez et al., 2012). The same study suggested that diinosine tetraphosphate behaved as both an agonist and an antagonist. This was the first time that an agonistic profile for Ip4I was described (Pintor et al., 1997). Studies performed with selective and nonselective P2 receptor antagonists demonstrated that it was possible to reverse the hypotensive effect of Ip4I. The selective P2Y1 antagonist MRS2179 (2'-deoxy-N6-methyladenosine 3',5'-diphosphate) and the selective P2Y6 antagonist MRS2578 (1,4-di[3-(3-isothiocyanatophenyl)thiourea]butane) significantly blocked the hypotensive effect induced by Ip4I on IOP, indicating that Ip4I effect is mediated partially by P2Y1 and P2Y6 receptors. Moreover, the use of other nonselective antagonists also supported this finding (Guzman-Aranguez et al., 2012).

**Nucleotides in the Retina**

The main function of the retina is to transform the photic input into electrical messages that will be sent to the lateral geniculate nucleus that projects to the visual cortex. It contains various types of neurons, Müller cells (radial glia), astrocytes, and microglial cells.

The most representative nucleotide in the mammalian retina is ATP, which is released from Müller cells and broken down into adenosine by enzymatic degradation resulting in autocrine activation of the P1 receptors in these glial cells (Newman, 2003). ATP may also activate P2 receptors of neighboring retinal neurons, such as photoreceptors, amacrine cells, and ganglion cells (Greenwood et al., 1997; Puthussery et al., 2006; Kaneda et al., 2008). The co-release of ATP together with acetylcholine from cholinergic amacrine cells has also been reported (Neal and Cunningham, 1994), and ATP can also be released from retinal pigmented epithelial cells.
(RPE cells) and may activate P2 receptors on photoreceptor membranes (Mitchell and Reigada, 2008).

Histologic, biochemical, and pharmacologic data support the presence of different P2 receptors in most types of neurons and glial cells in the mammalian retina (Table 1). Their role in retinal function is not well determined, although there is evidence that the activation of some P2 receptors, such as P2X7 (Vessey and Fletcher, 2012), P2X2 (Kaneda et al., 2010), and P2Y4 (Ward et al., 2008), is involved in the modulation of outer and inner retinal processing. Recent studies have suggested that altered purinergic signaling mediated by P2 receptors may be an important player in pathophysiological processes of the retina. Therefore, P2 receptors could be interesting targets for the development of future treatments for retinal diseases.

### Neuronal Apoptosis
Inherited retinal diseases cause increased extracellular ATP levels that may lead to photoreceptor apoptosis. Selective photoreceptor apoptosis mediated by P2X7 receptors (Notomi et al., 2011) occurred after intravitreal injection of ATP in murine retina (Puthussery and Fletcher, 2009; Notomi et al., 2011). In contrast, intracellular injections of Brilliant Blue G, a selective P2X7 receptor antagonist, reduced ATP-induced photoreceptor apoptosis (Notomi et al., 2011). Interestingly, P2X7 receptor-mediated photoreceptor apoptosis was reported in BALB/Crd mice, a retinitis pigmentosa model (Franke et al., 2005), as upregulated expression of P2X7 receptor, and peak photoreceptor apoptosis occurred at the same time between P20 and P40. Injections of a purinergic receptor antagonist also decreased photoreceptor apoptosis in rd1 mice (Puthussery and Fletcher, 2009).

In vitro studies have demonstrated that ATP triggered ganglion cell apoptosis through P2X7 receptor activation (Zhang et al., 2006). Moreover, hypoxia and acute increase of hydrostatic pressure induced retinal ganglion cell death due to the increase of extracellular ATP, which activates P2X7 receptors (Resta et al., 2007; Sugiyma et al., 2010). Acute pressure-induced ganglion cell apoptosis also was reduced by blocking P2X7 receptors with Brilliant Blue G or oxidized ATP or degrading extracellular ATP with apyrase (Resta et al., 2007).

### Müller Cell Volume Regulation
P2 receptors are also involved in Müller cell volume regulation. ATP is released after glutamate receptor activation from these glial cells and is catabolized to ADP, which activates P2Y1 receptors, triggering adenosine release (Uckermann et al., 2006; Wurm et al., 2010). The stimulation of adenosine A1 receptors then triggers the opening of potassium and chloride channels in the plasma membrane of Müller cells and inhibits their osmotic swelling (Iandiev et al., 2007; Wurm et al., 2010). Moreover, hypotonic exposure of retinal slices from P2Y1 receptor-deficient mice induced Müller cell swelling (Wurm et al., 2010).

Exogenous UDP and UTP reduced Müller cell volume in murine retinal slices (Wurm et al., 2010), probably via the activation of P2Y4 and P2Y6 receptors. Given that, Müller cells exhibited impaired cell volume regulation in ischemic (Uckermann et al., 2006) and diabetic rat retina (Wurm et al., 2008). Autocrine P2Y1 signaling contributes to the homeostasis of the extracellular space volume, preventing the osmotic swelling of Müller cells. Therefore, this receptor may be interesting as a therapeutic target for treating retinal diseases accompanied by ischemic or hypoxic processes.

### Müller Cell Proliferation
The mitogenic effect of ATP in cultured guinea pig Müller cells has been demonstrated (Moll et al., 2002; Milenkovic et al., 2003). The activation of P2Y receptors led to membrane conductance alterations through stimulation of large-conductance calcium-activated potassium channels and phosphorylation of ERK1/2, inducing stimulation of DNA synthesis in guinea pig Müller cells (Moll et al., 2002; Milenkovic et al., 2003). In contrast, the mitogenic action of ATP on DNA synthesis rate was mediated by P2X7 receptors in cultured human Müller cells (Bringmann et al., 2001).

Müller cell proliferation occurs in the retina of patients with proliferative vitreoretinopathy (PVR), causing the formation of epiretinal membranes (glial scars) which can result in
retinal detachment and finally blindness (Bringmann and Wiedemann, 2009). Müller cells from patients with PVR exhibited upregulated currents through P2X7 receptors compared with normal donors, which led to increased activity of calcium-activated large-conductance potassium channels (Bringmann et al., 2001). Hence, the increased density of P2X7 receptor currents may contribute to the activation of some mitogen pathways involved in stimulation of Müller cell proliferation.

It would be interesting to identify effective P2 receptor agonists for reducing the proliferation of Müller cells and to investigate their therapeutic application in the prevention of retinal detachment in PVR.

**Regulation the Physiology of Retinal Pericytes.** ATP acts as a vasoactive molecule regulating the function of the pericytes located in rat (Kawamura et al., 2003) and rabbit (Sugiyama et al., 2006) retinal microvessels. ATP modifies ionic currents, increases Ca$^{2+}$ levels, and causes contraction of pericytes (Kawamura et al., 2003). ATP effects were mediated by P2X7 receptor activation, which initially caused a rise in the steady-state and transient inward current of pericytes, narrowing the microvascular lumen (Kawamura et al., 2003); thus, ATP acts as a vasoconstrictor through the P2X7 receptor and decreases retinal blood velocity. UTP-induced P2Y$_4$ receptor activation also regulates calcium-activated chloride channel activity and increases the transient inward current but not the steady-state inward current (Kawamura et al., 2003). Consequently, P2Y$_4$ receptor stimulation can also induce retinal pericyte contractions.

Diabetic retinal microvessels exhibited more pericyte vulnerability to the lethal effect of P2X7 receptor activation (Kawamura et al., 2003; Sugiyama et al., 2006). Low BzATP concentrations (≤100 μM), nonlethal for normal retina, triggered pericyte apoptosis through the P2X7 receptor in diabetic retinal microvasculature (Sugiyama et al., 2004). This diabetes-induced increase in sensitivity suggests that extracellular nucleotide concentrations that are not lethal in the normal retina may cause pores to open and, consequently, microvascular cells to die in the diabetic retina. Thus, diabetes appears to facilitate the channel-to-pore transition that occurs during activation of P2X7 receptors, although the mechanism by which diabetes induces this effect remains unclear. Interestingly, activation of P2Y$_4$ receptors inhibited P2X7 pore formation and microvascular cell death (Sugiyama et al., 2005). These findings suggest that ATP vasotoxicity may be improved by P2Y$_4$-mediated inhibition of the lethal effects of P2X7 on the diabetic retina.

**RPE Nucleotides**

The RPE is a monolayer of cells located between the outer segments of photoreceptors and the vessels of the choriocapillaris. The RPE plays an important role in the homeostasis of the retina by forming the outer blood-retinal barrier regulating the transport of ions, water, and metabolic end products from the subretinal space (SRS) to the choroid (Strauss, 2005; Simo et al., 2010). Moreover, RPE cells also phagocytose the shed photoreceptor outer segments (Strauss, 2005; Simo et al., 2010), which is an essential function in the maintenance of photoreceptor excitability. Additionally, RPE cells produce cytokines, which are necessary for retinal development and survival (Wenkel and Streilein, 2000; Bian et al., 2011). RPE cells can release ATP into the SRS and act as an autocrine or paracrine signal by stimulating P2 receptors (Mitchell, 2001; Eldred et al., 2003; Reigada et al., 2005). The effects of ATP, ADP, and UTP are mediated through different P2 receptors in RPE cells (Table 1). Moreover, ATP and UTP can trigger the release of ATP by RPE cells, thus acting as signal amplifiers (Mitchell, 2001; Reigada et al., 2005).

Ectonucleotidase expression has been shown in human ARPE-19 cells (Reigada et al., 2005; Mitchell and Reigada, 2008). These enzymes are involved in ATP degradation to ADP, the effects of which are mediated by PY$_1$ and P2Y$_{12}$ receptors (Reigada et al., 2005). Furthermore, the apical membrane localization of nucleotidases in ARPE-19 cells suggests a role in the balance of nucleotides in SRS that would affect purinergic signaling of RPE cells.

**Retinal Detachment.** Deregulation of the transepithelial transport of nutrients, ions, and water contributes to fluid accumulation in the SRS, reducing adhesion between the retina and the RPE and producing the retinal detachment that is characteristic of a wide variety of ocular diseases with loss of vision (Macherer, 1984; Cox et al., 1988; Kent et al., 2000).

Interestingly, previous in vitro studies have shown P2Y$_2$ receptor activation with ATP or UTP triggered upregulation of the membrane fluid flow in the apical-to-basolateral direction in bovine (Peterson et al., 1997) and rat RPE monolayers (Maminishkis et al., 2002). Moreover, intravitreal injection of P2Y$_2$ receptor agonist INS37217 (a synthetic dinucleotide, deoxycytidine 5’-tetraphospho 5’-uridine) enhanced fluid reabsorption from experimental subretinal blebs and retinal reattachment in rabbit (Meyer et al., 2002) and rat (Maminishkis et al., 2002) retinal detachment models. Surprisingly, UTP intravitreal injection neither increased fluid reabsorption nor retinal reattachment in a retinal detachment rat model (Maminishkis et al., 2002). These authors suggested that UTP may be degraded in vivo by ectonucleotidases located in retinal outer segments that were not present in cultured RPE cells.

In view of these results, the P2Y$_2$ receptor may be a good therapeutic target for resolving retinal detachment, although it would be necessary evaluate the hydrolytic resistance of P2Y$_2$ receptor agonists.

**RPE Apoptosis.** ATP is released into SRS under multiple pathologic conditions, such as chemical ischemia, induced oxidative stress, osmotic stress, growth factors, cell swelling, or glutamate (Eldred et al., 2003; Reigada et al., 2005, 2006; Dutot et al., 2008), inducing apoptosis through P2X7 receptor activation. However, little is known about the apoptotic role of ATP in RPE cells. P2X7 receptor expression has been shown in cultured human ARPE-19 cells (Dutot et al., 2008) and human RPE cells isolated from donor eyes (Yang et al., 2011). P2X7 activation is caused by exposing human ARPE-19 cells to ATP and BzATP, triggering pore formation and YO-PRO-1 (4-[[3-methyl-1,3-benzoxazol-2(3H)-ylidene)methyl]-1-[3-(trimethylammonio)propyl]quinolinium diiodide) dye uptake in these cells (Dutot et al., 2008). Based on these results, Dutot and coworkers proposed that the P2X7 receptor could induce RPE apoptosis. A recent paper supports this hypothesis, as increases in Ca$^{2+}$ levels are concomitant with apoptotic markers such as caspase-3 activation and nuclear condensation in native RPE cells treated with ATP or BzATP, and these events are impeded by preincubation with oxidized

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ATP, an irreversible inhibitor of the P2X7 receptor (Yang et al., 2011).

Oxidative stress is also an important contributing factor to RPE apoptosis during the development of retinal diseases such as age-related macular degeneration or diabetic retinopathy (Cai et al., 2000; Hao et al., 2012; Sreeekumar et al., 2012). Interestingly, overproduction of reactive oxygen species (ROS) and increased P2X7-mediated permeabilization of YO-PRO-1 were reported in cultured human RPE cells treated with tert-butyl hydroperoxide (Dutot et al., 2008), a chemical oxidant. Further studies are required to determine the implication of ROS overproduction in RPE apoptosis induced by P2X7 receptor activation and the breakdown of the outer blood-retina barrier in age-related macular degeneration or diabetic retinopathy. Furthermore, it would be very interesting to investigate whether treatment with P2X7 receptor antagonists can reduce RPE apoptosis and improve the damaged visual function and homeostasis of the retina in these diseases.

**Immunе Responses.** Monocyte-derived proinflammatory cytokines such as tumor necrosis factor α, interleukin-1β (IL-1β), or IL-18, among others, can be released to the SRS and act as danger signals, stimulating inflammatory cytokine production in human RPE cells (Bian et al., 2011). Interestingly, activation of IL-8 secretion through an ERK1/2-dependent pathway was demonstrated in stimulated human ARPE-19 cells with ATPγS, UTP and UDP (Relvas et al., 2009). In addition, these nucleotides and tumor necrosis factor α have a synergic effect on induced IL-8 secretion by human ARPE-19 cells (Relvas et al., 2009). Relvas et al. (2009) proposed that ATPγS and UTP effects on IL-8 production may be mediated through the P2Y2 receptor. UDP action can be mediated by P2Y6 or P2Y4 receptors, but considering that P2Y4 receptors are not expressed in ARPE-19 cells, it is clear that the increase of IL-8 secretion is mediated through the P2Y4 receptor.

Proinflammatory cytokines induce ROS overproduction in human RPE apoptosis (Yang et al., 2007). Moreover, increases in 
\[\text{Ca}^{2+}\] levels, ROS, and caspase activation have been involved in activated monocyte-induced mouse (Yang et al., 2011) and human (Elner et al., 2003) RPE cell apoptosis. In addition, IL-8 attracts and activates neutrophils, and this also involves the breakdown of the outer blood-retina barrier and monocyte infiltration into the SRS, the main hallmarks of immune response in a variety of retinal pathologic processes (Penfold et al., 2001). Moreover, abnormal IL-8 production and immune cell infiltration into SRS occur in these eye diseases, so it would be very interesting to investigate whether the therapeutic application of P2Y4 and P2Y6 receptor antagonists can contribute to the repair of RPE breakdowns during retinal inflammation.

**Perspectives and Conclusions**

Nucleotides and dinucleotides participate in several relevant physiologic processes in the eye, and they have also been shown to be involved in disease. From a pharmacologic point of view, these molecules may help in restoring the normal conditions of eye physiology.

The possibilities, therefore, of using nucleotides and dinucleotides agonists or antagonists in the treatment of ocular disease are wide ranging. It is important to bear in mind that due to the widespread distribution of P2 receptors in ocular structures, sometimes we may need agonists and other times antagonists to achieve the desired effect. Moreover, there are few studies on the presence of ectonucleotidases in the eye, which may limit the effect of some nucleotides. Finally, some ocular structures, such as the retina, need special delivery systems because the application of purinergic compounds is not easy.

All these, and probably many others, are the challenges facing purinergic investigators in the search for effective medicines for the treatment of eye diseases. To date, the dinucleotide UppU (diuridine tetraphosphate), marketed under the name Diquas (Inspire Pharmaceuticals, Raleigh, NC), is the only nucleotide-based medicine to treat an eye disease (i.e., dry eye). This should encourage researchers to continue in the search for new compounds and strategies based on nucleotides or dinucleotides for the treatment of ocular diseases.

**Authorship Contributions**

_Wrote or contributed to the writing of the manuscript: Guzman-Aranguex, Santanox, Martin-Gil, Fonseca, Pintor._

**References**


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