Presence of Diadenosine Polyphosphates in the Aqueous Humor: Their Effect on Intraocular Pressure

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ABSTRACT

Adenine dinucleotides are present in many biological systems and may serve as physiological regulators of processes such as neurotransmitter release, vascular tone or corneal hydration. The presence of diadenosine polyphosphates was investigated in New Zealand White rabbit aqueous humor. Diadenosine tetraphosphate (Ap4A) and diadenosine pentaphosphate (Ap5A) were identified and quantified in the aqueous humor with concentrations of 0.34 ± 0.1 and 0.08 ± 0.01 μM, respectively. The effects of topical corneal application of diadenosine pyrophosphate (Ap2A), diadenosine triphosphate (Ap3A), Ap4A, and Ap5A on intraocular pressure in rabbits were also studied. Ap2A, Ap3A, and Ap5A increased intraocular pressure with threshold doses of approximately 0.1 to 1.0 μg · 10 μl⁻¹. Ap4A decreased intraocular pressure with an IC50 value of 0.12 μg · 10 μl⁻¹ (or 0.13 nmol). Cross-desensitization studies suggested the activation of a P2X receptor for the hypotensive effect of Ap4A and a P2Y receptor in the case of Ap5A. The ATP receptor antagonists (all 100 μg · 10 μl⁻¹), pyridoxal phosphate-6-azophenyl-2’,4’-disulfonic acid (PPADS), suramin, and reactive blue 2 (RB-2) alone had no effect on intraocular pressure but attenuated responses to diadenosine polyphosphates by approximately 80%. It is concluded that Ap2A, Ap3A, and Ap5A increase intraocular pressure, and Ap4A decreases intraocular pressure via mechanisms that involve P2 receptors, and that Ap5A present in aqueous humor may serve to regulate intraocular pressure. Furthermore, we suggest that topical application of Ap2A to the cornea has therapeutic potential for lowering intraocular pressure, a major risk factor for glaucoma.

Diadenosine polyphosphates (abbreviated to ApnA, where n = 2–7) are natural compounds. In particular diadenosine triphosphate (Ap3A), diadenosine tetraphosphate (Ap4A), diadenosine pentaphosphate (Ap5A), Ap6A, and Ap7A are found in exocytotic vesicles, such as those in nerve terminals, adrenal medullary chromaffin cells, and platelets (Floodgaard and Klenow, 1982; Rodriguez del Castillo et al., 1988; Pintor et al., 1992a,b,c, 1997; Schlüter et al., 1994; Pintor and Miras-Portugal, 1995; Jankowski et al., 1999), and diadenosine pyrophosphate (Ap2A) has recently been identified in secretory granules of cardiac myocytes (Luo et al., 1999). They have diverse actions in peripheral and central tissues because of their roles as extracellular signal molecules (Hoyle, 1990; Pintor et al., 1996; Kisselev et al., 1998; Hoyle et al., 2001).

Ap2A and Ap3A have been found in rabbit tears (Pintor et al., 2002a,b), and both of these dinucleotides together with Ap4A have been isolated from human tears (Pintor et al., 2002a,b). In rabbits, topical corneal application of Ap2A or Ap3A, but not Ap4A or Ap5A, evokes tear secretion (Pintor et al., 2002a,b), suggesting that these two dinucleotides play a role in the regulation of corneal hydration and cleaning.

The mechanisms that control and regulate intraocular pressure are not fully understood, but it results from the dynamic equilibrium between the production and drainage (or resorption) of aqueous humor. In general terms, sympathetic activity results in a reduction in intraocular pressure, and parasympathetic activity results in an increase. It seems that the main point of control of intraocular pressure is the outflow of aqueous humor, rather than its production (Burke and Potter, 1986; Judge and Flitcroft, 2000; Jumblatt, 2000). Intraocular pressure may also be regulated by effectors of circadian rhythms, and we have recently shown that melatonin and the MT3 receptor ligand, 5-methoxy-3β-acetyltryptamine-N-acetyltryptamine (5-MCA-NAT or GR 135531), can cause profound decreases in pressure (Pintor et al., 2001).

In the present experimental work, we describe the presence of diadenosine polyphosphates in the aqueous humor and the differential effects of a series of diadenosine polyphosphates, Ap$_2$A, Ap$_3$A, Ap$_4$A, and Ap$_5$A, which includes those known to be present in tears, on intraocular pressure in the rabbit. Our results suggest that one of them, Ap$_4$A, has the potential to be used as a therapeutic agent in conditions where intraocular pressure is elevated.

**Materials and Methods**

**Animals.** New Zealand White rabbits (males, 2–3 kg) were used. The animals were kept in individual cages with free access to food and water, under controlled 12-h light/dark cycles. Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

**Aqueous Humor Collection and Sample Preparation.** New Zealand White rabbits were anesthetized with 1.5 mg kg$^{-1}$ propofol (Abbott Laboratories, Madrid, Spain). Aqueous humor was removed with a syringe connected to a 30-gauge needle in the sclero-corneal limbus area. Samples were stored at –35°C before treatment. The treatment consisted of their chromatography through a SEP-PAK Acell QMA cartridges (Waters, Milford, MA) and elution of the retained nucleotides and dinucleotides by means of a mixture of 0.1 N HCl, 0.2 N KCl. Eluates were neutralized with 10 N KOH before HPLC injection (Rotllán et al., 1991).

**HPLC Procedures.** The HPLC system consisted of a Waters 1515 isocratic HPLC pump, a 2487 dual absorbance detector and a Reodyne injector, all managed by the software Breeze from Waters. The column was a Novapak C-18 (15 cm length, 0.4 cm diameter) from Waters.

The system was equilibrated overnight with the following mobile phase: 10 mM KH$_2$PO$_4$, 2 mM tetrabutyl ammonium, 17% acetoni-trile, pH 7.5. Detection was monitored at 260 nm wavelength. All the peaks identified as putative diadenosine polyphosphates were taken for phosphodiesterase treatment. Phosphodiesterase (EC 3.1.15.1) from Crotalus durissus (Sigma-Aldrich, St. Louis, MO) at a concentration of 0.3 U/ml was incubated for 10 min with the corresponding putative dinucleotide and the digestion products were analyzed by HPLC.

**Intraocular Pressure Measurements.** Intraocular pressure was measured by means of a Tono-Pen XL contact tonometer (Mentor Massachusetts Inc., Norwell, MA). This device has been shown to be the tonometer of choice for measuring intraocular pressures within the range of 3 to 30 mm Hg in rabbits (Abrams et al., 1996). All measurements fell within this diapason: the mean baseline value within the range of 3 to 30 mm Hg in rabbits (Abrams et al., 1996). Variance and post hoc Tukey’s tests, using α = 0.05.

**Results**

**Presence of Diadenosine Polyphosphates in the Aqueous Humor.** The analysis of nucleotide and dinucleotide content in rabbit aqueous humor indicated the presence of several peaks, two of which were tentatively identified as Ap$_2$A and Ap$_3$A, when compared to commercial standards (Fig. 1). To confirm the nature of the putative dinucleotides, samples were enriched with commercial dinucleotides that coeluted together with the peaks present in the samples (results not shown). To fully confirm the existence of Ap$_2$A and Ap$_3$A in the aqueous humor, peaks were collected and submitted to phosphodiesterase treatment. This enzyme cleaves the dinucleotide giving AMP plus another nucleotide that depends on the length of the phosphate chain in the original dinucleotide. When diadenosine tetraphosphate was submitted to this treatment, it was possible to observe the presence of AMP and ATP (Fig. 2, upper traces); Ap$_2$A digestion with phosphodiesterase gave AMP and adenosine 5'-tetraphosphate (Ap$_3$A) as products (Fig. 2, lower traces). This treatment therefore confirmed that the two dinucleotide after the two baseline measurements and 30 min before application of saline or a dinucleotide. Because maximum responses to the dinucleotides occurred within 2 h of application in the presence of P2 antagonists, measurements were made over a period of 3 h. Control experiments were performed in which 10 μl of sterile saline (0.9% w/v) was applied instead of a dinucleotide or in the opposite eye from the dinucleotide.

**Drugs Used.** Diadenosine pyrophosphate sodium salt, diadenosine triphosphate ammonium salt, diadenosine tetraphosphate ammonium salt, and diadenosine pentaphosphate sodium salt, β,γ-methylATP, suramin, and RB-2 were all obtained from Sigma-Aldrich. ATP$^8$S was purchased from Roche Diagnostics (Mannheim, Germany). Pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid, was obtained from Sigma/RBI (Natick, MA). All the dinucleotides were dissolved in sterile saline (0.9%), to produce stocks with concentrations in the range from 1 ng · ml$^{-1}$ to 1 mg · ml$^{-1}$. Suramin, RB-2, and PPADS were diluted to produce a stock of 1 mg · ml$^{-1}$.

**Analysis of Data.** Numerical values are given as mean ± S.E.M. Means of two groups were compared using Student’s t test (unpaired, two-tailed, unless stated) with a 5% fiducial point of significance. Means of three or more groups were compared using analysis of variance and post Tukey’s tests, using α = 0.05.

![Fig. 1. Presence of diadenosine polyphosphates in rabbit aqueous humor.](image-url)
peaks present in the aqueous humor corresponded to Ap 4A and Ap 5A. The concentrations of these dinucleotides were then calculated by comparison with samples of commercial external standards. Ap 4A and Ap 5A were present in the aqueous humor at concentrations of 0.34 ± 0.01 and 0.08 ± 0.01 M, respectively (n = 8).

Mononucleotides were also present and identified in aqueous humor samples. When identified by their retention times and comparison with external standards, the most relevant adenine mononucleotides were AMP, ADP, and ATP, which presented concentration values of 10.47 ± 0.34, 1.93 ± 0.42, and 1.07 ± 0.21 μM, respectively.

Effect of Diadenosine Polyphosphates on Intraocular Pressure. Ap 2A and Ap 3A both caused a dose-dependent increase in intraocular pressure (Figs. 3 and 4). The threshold dose was between 0.1 and 1.0 μg · 10 μl⁻¹, and a maximum response at a dose of 10 to 100 μg · 10 μl⁻¹ (Fig. 5). The highest dose caused a fall in pressure of 29.6 ± 2.2% (n = 8). Because there was a clear maximum it was possible to calculate an IC₅₀, which was 0.12 ± 0.03 μg · 10 μl⁻¹, with 95% confidence limits of 0.03 and 0.62 μg · 10 μl⁻¹. This is equivalent to an absolute concentration of 13 μmol · l⁻¹ (3.2, 67.2) μmol · l⁻¹ (mean with 95% confidence limits).

All four dinucleotides produced responses with a latency of at least 30 min, with a maximum effect observable at 1 or 2 h after application. Responses to Ap 2A and Ap 3A were relatively short-lived, and baseline values of intraocular pressure were restored by 3 h after application (Fig. 6A). Responses to both Ap 4A and Ap 5A were more sustained, taking 5 h to return to control levels (Fig. 6B). Saline alone had no significant effect on intraocular pressure (IOP), whether instilled extremely variable, with a coefficient of variability greater than the value of the mean (data not shown). At doses of 10, 100, and 300 μg · 10 μl⁻¹, increases in pressure were clear, but lacked dose dependence (Fig. 4).

Ap 4A evoked a decrease in intraocular pressure, with a threshold between 0.1 and 1.0 ng · 10 μl⁻¹, and a maximum response at a dose of 10 to 100 ng · 10 μl⁻¹ (Fig. 5). The highest dose caused a fall in pressure of 29.6 ± 2.2% (n = 8). Because there was a clear maximum it was possible to calculate an IC₅₀, which was 0.12 ± 0.03 μg · 10 μl⁻¹, with 95% confidence limits of 0.03 and 0.62 μg · 10 μl⁻¹. This is equivalent to an absolute concentration of 13 μmol · l⁻¹ (3.2, 67.2) μmol · l⁻¹ (mean with 95% confidence limits).

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treated it was possible to see a small but not statistically significant reduction in the \( \beta,\gamma\text{-meATP} \) effect. The application of \( \text{ATP}_\gamma\text{S} \) (100 \( \mu \text{g} \cdot 10 \text{ \( \mu \text{l} \)^{-1}} \)) abolished completely the effect of \( \text{Ap}_4\text{A} \), suggesting the activation of the same \( P_2 \) receptor (\( n = 8 \); Fig. 7B).

To demonstrate the involvement of \( P_2 \) receptors on the effects produced by \( \text{Ap}_4\text{A} \) and \( \text{Ap}_5\text{A} \), three \( P_2 \) antagonists were used: suramin, RB-2 and PPADS. Application of suramin, RB-2, or PPADS (100 \( \mu \text{g} \cdot 10 \text{ \( \mu \text{l} \)^{-1}} \)) had no significant effect on intraocular pressure; 150 min after application of the antagonists the intraocular pressure had hardly changed from the control level of 16.8 \( \pm \) 0.5 mm Hg (\( n = 10 \)) to 17.2 \( \pm \) 1.1 mm Hg (no significant difference, paired \( t \) test).

Of the three antagonists (all tested at 100 \( \mu \text{g} \cdot 10 \text{ \( \mu \text{l} \)^{-1}} \)) only RB-2 was unable to reverse the hypotensive effect of \( \text{Ap}_4\text{A} \), while both suramin and PPADS could completely abolish \( \text{Ap}_4\text{A} \) response (\( n = 8 \); Fig. 8A). In the case of \( \text{Ap}_5\text{A} \), only PPADS antagonized the hypertensive effect of this dinucleotide. Neither suramin nor RB-2 were able to revert the effect of \( \text{Ap}_5\text{A} \) (\( n = 8 \); Fig. 8B). Following application of PPADS, the responses to all four dinucleotides (100 \( \mu \text{g} \cdot 10 \text{ \( \mu \text{l} \)^{-1}} \)) were severely and significantly attenuated (Fig. 9).

**Discussion**

These results show, for the first time, the presence of diadenosine polyphosphates in the aqueous humor of that New Zealand White rabbits. Chromatographic analysis of these dinucleotides demonstrated that \( \text{Ap}_4\text{A} \) and \( \text{Ap}_5\text{A} \) are present in the aqueous humor in the low micromolar range. Adenine mononucleotides were also identified in the aqueous humor, and they may also participate in the regulation of the IOP as previously indicated (Pintor and Peral, 2001).

Our results also show that topical application of diadenosine polyphosphates to the cornea can have profound effects on intraocular pressure in the rabbit. \( \text{Ap}_4\text{A} \), \( \text{Ap}_5\text{A} \), and \( \text{Ap}_A \) all produced increases in pressure, while \( \text{Ap}_A \) produced a decrease. Cross-desensitization studies and antagonism with suramin, PPADS, and RB-2 indicate that the polyphosphates were acting via \( P_2 \) receptors, although the subtypes involved have not been fully characterized.

\( \text{Ap}_4\text{A} \) was a potent agonist and produced a decrease in intraocular pressure at concentrations 3 orders of magnitude below those at which \( \text{Ap}_A \), \( \text{Ap}_A \), or \( \text{Ap}_A \) produced an increase. The dose-response curve for \( \text{Ap}_4\text{A} \) did not appear to inflect at the highest concentrations tested points at which activation of the excitatory receptor might be expected. At the lowest concentrations tested, none of \( \text{Ap}_A \), \( \text{Ap}_A \), and \( \text{Ap}_A \) produced a decrease in intraocular pressure, which implies that in addition to there being two separate populations of receptors, one mediating an increase and the other a decrease in intraocular pressure, this latter receptor is specific for \( \text{Ap}_A \). It is possible that \( \text{Ap}_A \) also activates the excitatory receptor, but in the mixed population the effects of activation of the receptor that mediates a decrease in pressure predominated. In this sense, cross-desensitization studies suggest that \( \text{Ap}_A \) acts through the same receptor as \( \beta,\gamma\text{-meATP} \) (a P2X receptor), while \( \text{Ap}_A \) acts to the same as \( \text{ATP}_\gamma\text{S} \) (a P2Y receptor) (Pintor and Peral, 2001). It has not been possible to identify the molecular mechanisms that link these two receptors and the physiological processes that control IOP.
Ap2A and Ap5A are also present in rabbit tears (Pintor et al., 2002b), and both stimulate tear secretion in rabbits, as does Ap6A, while Ap3A and Ap9A do not (Pintor et al., 2002b). Their presence in tears and aqueous humor and their effects on both tear secretion and intraocular pressure suggests that they are serving physiological roles in maintaining corneal hydration and eye pressure. The endogenous tear concentration of Ap4A is close to 3 μM (Pintor et al., 2002b), and this is within its range of activity for reducing intraocular pressure when topically applied as a single 10-μl dose. The IC50 concentration was equivalent to 13 μM. It is important to point out that the concentrations of diadenosine polyphosphates determined in the aqueous humor may reflect a more physiological concentration than those that occur when the dinucleotides are applied topically. Diadenosine polyphosphates may be partially hydrolyzed when passing through the cornea before reaching the aqueous humor, therefore the real IC50 values for these compounds in the eye anterior chamber should be lower than that apparently obtained.

It is unusual to find a tissue or organ in which members of the homologous series of diadenosine polyphosphates, with a chain length from two to five, have opposing actions. However, there are examples of receptors that are selective or specific for one or members of this group of compounds. For example, receptors that are activated by ApA but not other diadenosine nucleosides have been described in recombinant rat P2X2 receptors, expressed in Xenopus laevis oocytes. This receptor is activated by ApA, but not Ap2A, ApA, or Ap9A (Pintor et al., 1996; Wildman et al., 1999). The MM39 cell line, derived from human tracheal gland epithelium, possesses a native receptor that is also sensitive to ApA but not Ap2A or Ap5A (Saleh et al., 1999). The P2 receptor on rat mast cells (the P2Z receptor, which opens a membrane pore) is activated by ApA, but not other dinucleotides. This is because ApA can bear four negative charges, one per phosphate group, whereas smaller dinucleotides cannot, and larger dinucleotides do not because they chelate divalent cations (Tatham et al., 1988). In contrast receptors that are activated by Ap2A, ApA, and ApA but not ApA have not been identified with any clarity.

P2X receptors in the guinea pig vas deferens and urinary bladder are insensitive to ApA, and ApA is only a weak agonist (Hoyle et al., 1995). Similarly at P2X receptors in the rat mesenteric arterial bed ApA, ApA, and ApA are all agonists, while ApA and ApA are not (Ralevic et al., 1995). In contrast, Ap2A and ApA are both good agonists of P2Y receptors in the guinea pig taenia coli and rat mesenteric arterial endothelial cells (Hoyle et al., 1995; Ralevic et al., 1995; Hourani et al., 1998), and in ECV304 cells, a cell line derived from human umbilical endothelial cells, there is a P2Y receptor that is sensitive to ApA but not Ap2A, Ap3A, or Ap6A (Conant et al., 1998).

In conclusion, diadenosine polyphosphates are present in the rabbit aqueous humor, moreover, intraocular pressure can be regulated by topical corneal application of adenine dinucleotides. ApA potently decreases intraocular pressure; Ap2A, Ap3A, and Ap5A increase intraocular pressure. The receptors involved remain to be characterized, but the one activated by ApA bears similarities to some previously described P2X receptors. The fact that these adenine dinucleotides modulate intraocular pressure leads to the suggestion that they may be useful compounds in the development of therapeutic agents. In particular, ApA, or a derivative, may be useful in the treatment of ocular disorders such as forms of
of glaucoma, in which a reduction of intraocular pressure would be beneficial.

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