Adenosine Tetrabphosphate, Ap₄, a Physiological Regulator of Intraocular Pressure in Normotensive Rabbit Eyes

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ABSTRACT

Adenosine 5′-tetraphosphate, Ap₄, is a natural nucleotide present in many biological systems. This nucleotide has been found as a constituent of the nucleotide pool present in the aqueous humor of New Zealand rabbits. HPLC analysis confirmed its identity and calculated its concentration levels to be 197 ± 21 nM. When applied topically to the rabbit eyes, this mononucleotide produced a reduction in the intraocular pressure, which was dose-dependent. The pD₂ value calculated from the dose-response curve was 7.28 ± 0.47, which is equivalent to 52.48 nM. The time course of such intraocular pressure reduction presented a maximal decrease of IOP to 75.1 ± 2.3% compared with the vehicle control value (100%), and the effect lasted for more than 2 h. Cross-desensitization studies demonstrated that Ap₄ effect was mediated via a P2X receptor in this system. P2 receptor antagonists suramin, pyridoxal phosphate 6-azophenyl-2,4′-disulfonic acid (PPADS), and reactive blue 2 (RB-2) showed that only the latter was able to revert the effect of Ap₄. Antagonists of adrenoceptors and cholinceptors were able to partially reverse the effect of this nucleotide; this might indicate a connection with the neural mechanisms that control the intraocular pressure.

Adenosine 5′-tetraphosphate, Ap₄, is a natural nucleotide present in neurosecretory vesicles together with other nucleotides and classical transmitters (Gualix et al., 1996). Although it has been described in many biological systems, it is the least investigated for its physiological/pharmacological actions compared with other nucleotides. Compounds such as ATP, UTP, or diadenosine polyphosphates Ap₅A have been investigated for their physiological effects and their receptor function (Hourani et al., 1998; Pintor et al., 2000a), but Ap₄ has not been studied as much attention.

Ap₄ was identified first from horse muscle (Lieberman, 1955) and also in other tissues such as rabbit muscle and platelets, yeast spores, and rat liver (Small and Cooper, 1966; Lobaton et al., 1975; Van Dyke et al., 1996). This nucleotide is present and releasable from neurochromaffin cells (Winkler and Carmichael, 1982; Gualix et al., 1996), and its synthesis is carried out by a transphosphorylation process involving ATP (Gualix et al., 1996). When stored in the vesicles, it can be released to the extracellular space together with other transmitters. Once at the extracellular space, this nucleotide can activate both ionotropic P2X and metabotropic P2Y receptors. In this sense, the effect of Ap₄ on the vascular system is to reduce blood pressure by means of the activation of a P2Y receptor; however, in stress situations, such as in an hemorrhage, it produces vasoconstriction by the activation of smooth muscle P2X receptors (Lee et al., 1995b; Lewis et al., 2000). Very recently, Ap₄ has been identified in human myocardial tissue. The application of this nucleotide in isolated rat heart can produce vasodilatation (Westhoff et al., 2003).

It is only recently that the nucleotides are considered as active compounds in the eye (Pintor, 1999). Processes in which extracellular nucleotides can modify ocular physiology include tear secretion, intraocular pressure, and retinal fluid reabsorption (Yerxa 2001; Pintor et al., 2002). Mononucleotides, such as ATP and its synthetic analogs, can modulate intraocular pressure (IOP). There is a clear difference in the way they can modify IOP, because those nucleotides with a P2Y agonistic profile tend to increase IOP, whereas P2X agonists reduce it (Pintor and Peral, 2001). Recently, it has been demonstrated that diadenosine polyphosphates can modify IOP. Diadenosine tetraphosphate (Ap₅A), induces a...
Materials and Methods

Animals. Twenty-four normotensive 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 cycles (12/12-h light/dark). Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the statement of the Association for Research in Vision and Ophthalmology on the Use of Animals in Ophthalmic and Vision Research.

Aqueous Humor Collection and HPLC Procedures. Aqueous humor was collected from anesthetized rabbits by means of an intracamer al injection. These experiments were performed at the same time, 3:00 PM, when IOP has recovered completely from the night period. Briefly, New Zealand White rabbits were anesthetized with 1.5 mg/kg propofol (Abbott Laboratories, Madrid, Spain). Aqueous humor (500 μl) was removed with a syringe connected to a 30-gauge needle in the scero-corneal limbus. Samples were stored at −35°C before treatment through SEP-Pak Accell QMA cartridges (Waters, Milford, MA). Samples were chromatographed through these cartridges, and the elution of these compounds was obtained by applying 500 μl of a mixture containing 0.1 N HCl, 0.2 N KCl. Eluates were neutralized with 10 N KOH before HPLC analysis (Rotllán et al., 1991).

The HPLC system consisted of a Waters 1515 Isocratic HPLC pump, a 2487 dual wavelength absorbance detector, and a Reodyne injector, all controlled by the software Breeze from Waters. The column used was a NovaPak C18 (15 cm in length, 0.4 cm in diameter) also from Waters. The mobile phase consisted of 10 mM potassium phosphate, 2 mM tetrabutyl ammonium, 17% acetonitrile. Detection was monitored at 260-nm wavelength.

To confirm the chemical nature of the putative peak identified as Ap4, enzymatic digestion with alkaline phosphatase (EC 3.1.3.1) 0.3 U/ml (molecular biology grade) was used. Incubation with this enzyme and the putative nucleotide was carried out for 10 min, and the digested products were analyzed by HPLC.

Animals used for aqueous humor studies were not used for IOP experiments after 2 weeks of recovery.

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). Because IOP changes from the night to day, all the experiments were performed at the same time, 3:00 PM. At this time, IOP remains more stable and permits an objective comparison with vehicle treatment. All measurements fell within this diapason: the mean baseline measurement and 30 min before application of saline or a nucleotide.

Results

Presence and Quantification of Ap4 in the Aqueous Humor. The chromatographic analysis of the samples obtained from rabbit’s aqueous humor revealed the presence of various peaks that were identified as mono and dinucleotides. In particular, a peak occurred between the corresponding ATP and diadenosine tetraphosphate, Ap4A peaks. This peak was tentatively identified as Ap4 with a nucleotide standard chromatographed that contained the commercial Ap4 (Fig. 1). Because the identification by the retention time and comparison with a standard is not enough to verify the presence of this nucleotide in the aqueous humor of the rabbit, the peak was collected and submitted to alkaline phosphatase treatment as described under Materials and Methods. The products obtained after the enzyme digestion, with the presence of ATP, ADP, and AMP, and the concomitant reduction in the peak of the putative Ap4, indicated that the nucleotide investigated was Ap4 (Fig. 2). The concentration obtained for this compound in the aqueous humor was 197 ± 21 nM (n = 8), which is smaller than the one obtained for other mononucleotides (Pintor et al., 2002).

Effect of Ap4 on Intraocular Pressure. Because Ap4 is present in the rabbit aqueous humor, we wanted to know whether there was any change in the IOP values when this nucleotide was topically applied. Graded doses of Ap4 ranging from 10−2 to 10−11 M were instilled as described previously, to construct a dose-response curve. As it is shown in Fig. 3, Ap4 was able to reduce intraocular pressure in rabbits in a concentration dependent manner. IOP was reduced to 75.1 ± 2.3% compared with control the values (100%). The analysis of the curve permitted the calculation of the pD2 value, which was 7.28 ± 0.47, equivalent to an EC50 of 52.48 nM (n = 8).

The time course of Ap4-induced IOP changes was examined. A single dose of 100 μM Ap4 produced a reduction of
IOP, from 17.1 ± 0.6 to 12.5 ± 1.5 mm Hg, which was maximal 2 h after the application of the nucleotide (Fig. 4). The effect was sustained for about 2 to 3 h and returned to normal values within 6 h after (*n* = 6). To compare the effect of the nucleotide with other substances that are known to reduce IOP, the commercial hypotensive compound Xalatan (latanoprost) was topically applied to the rabbit eyes. Ten microliters of 0.005% Xalatan, produced a marked reduction in the normotensive rabbit’s IOP, from 16.9 ± 0.8 to 11.3 ± 0.1 mm Hg, as observed in Fig. 4.

**Cross-Desensitization and Studies with P2 Antagonists.** To investigate the P2 purinergic receptor involved in the effect of Ap₄, cross-desensitization studies were performed by means of ATP-γ-S and β,γ-meATP, hypertensive and hypotensive nucleotides, in this model as described previously (Pintor and Peral, 2001).

Homologous cross-desensitization of Ap₄ at a maximal dose of 100 μM, allowed the measurement of the first hypotensive effect but not the one due to the second dose of this nucleotide (*n* = 6; Fig. 5). A similar behavior was observed when the P2X agonist β,γ-meATP was applied before Ap₄. When the P2Y agonist ATP-γ-S was applied, it was possible to measure the hypotensive effect of Ap₄, despite the huge hypertensive effect displayed by ATP-γ-S (*n* = 6; Fig. 5). These experiments suggest the activation of a P2X receptor that is involved in a reduction of IOP as occurs with β,γ-me ATP and diadenosine tetraphosphate (Pintor and Peral, 2001; Pintor et al., 2003).

Three different P2 antagonists were tested in their ability to reverse the hypotensive effect of Ap₄: suramin, RB-2, and PPADS. As shown in Fig. 6, only RB-2 was able to significantly reverse the effect of Ap₄. Neither PPADS nor suramin could modify the reduction in IOP produced by Ap₄ in a significant way at the single dose tested (*n* = 6).

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**Fig. 1.** Presence of adenosine tetraphosphate in rabbit aqueous humor. Samples analyzed as described under Materials and Methods presented a peak (upper trace) identified tentatively as Ap₄, compared with a commercial standard (lower trace, 1000 pmol each). Inset, blowup of the putative adenosine tetraphosphate peak. Aqueous humor was taken always at the same time, 3:00 PM, to avoid the possible effect of circadian rhythms.

**Fig. 2.** Alkaline phosphatase treatment and analysis of putative adenosine tetraphosphate. Ap₄ was treated with alkaline phosphatase (molecular biology grade) as described under Materials and Methods. The treatment of the putative Ap₄ (top trace) completely changed the chromatographic profile with peaks corresponding to AMP and ADP occurring as digestion products (second trace). The bottom trace represents a standard of 1000 pmol AMP, ADP, and ATP.

**Fig. 3.** Dose-response analysis of adenosine tetraphosphate on rabbit intraocular pressure. Effect of instillation of graded doses of Ap₄ into the rabbit eye on IOP (solid squares) and the effect of vehicle control (open squares). Each point was obtained 3 h after the application of the nucleotide (maximal effect of Ap₄). Initial IOP values were 17 ± 0.39 mm Hg, whereas the maximal effect reduced IOP to 12.6 ± 1.3 mm Hg. All the IOP experiments were started at 3:00 PM to avoid the possible effect of circadian rhythms. Points represented the mean ± S.E.M. (*n* = 8).
Intraocular pressure is a process controlled by the nervous system (Bergmanson, 1982). To see whether the effect of Ap4 is coupled to the nervous system, antagonists of the cholinergic and noradrenergic systems were tested.

The application of the cholinergic antagonists hexamethonium and atropine alone produced a clear increase in the IOP (Fig. 7). When the two cholinergic antagonists were applied 30 min before the instillation of Ap4, the hypertensive effect of hexamethonium and atropine disappeared, and it was possible to observe a reduction in IOP (due to Ap4), which was not statistically different from the effect of Ap4 alone (n = 6; Fig. 7).

The application of the adrenergic antagonists yohimbine and ICI-118.551 produced also an increase in IOP (Fig. 7). When Ap4 was applied after the previous instillation of these antagonists, there was a measurable reduction in IOP, nevertheless there were significant differences between this value and the one by Ap4 alone (n = 6; Fig. 7).

**Discussion**

The nucleotide Ap4 is a naturally occurring nucleotide present in rabbit aqueous humor as demonstrated by high-performance liquid chromatography. Comparison with a commercial standard, digestion with alkaline phosphatase,
and analysis of the digestion products permitted to characterize and identify this compound as a component of the aqueous humor.

Nucleotides and nucleosides have been described in the aqueous humor of different animal models. In this sense, Maul and Sears (1979) described the release of ATP after stimulation of the trigeminal nerve of the rabbit eye as an extracellular source of nucleotides. In the same way, Mitchell et al. (1998) described the release of ATP from bovine ocular ciliary epithelial cells into the aqueous humor. Also, Greiner et al. (1991) showed the presence of phosphate metabolites in ocular humors as well as the presence of the nucleoside adenosine in rabbits (Crosson and Petrovich, 1999). All these works are principally focused on ATP, which has been generally considered as the natural agonist of P2 receptors together with ADP, UTP, and UDP. Nevertheless, other nucleotides such as diadenosine polyphosphates have been described in the rabbit aqueous humor: Ap4A and diadenosine pentaphosphate (Ap5A) are present at concentrations in the low micromolar range, and they can exert differential action on IOP (Pintor et al., 2003). In a recent study, a calculation of the concentrations of mononucleotides in the aqueous humor demonstrated that the values obtained for ATP or ADP were about 1 order of magnitude higher than the concentration value obtained for Ap4 in the present study (Pintor et al., 2003). A lower concentration in the aqueous humor is not indicative that Ap4 is less relevant than other mononucleotides. For example, in terms of extracellular stability, Ap4 lasts longer than ATP, as demonstrated by Gomez-Villafuertes et al. (2000). In this way, the rate of hydrolysis of Ap4 in synaptic terminals is 1.89% per 2 min, whereas ATP hydrolyzes 24.64% for the same period of time. Also, the time course for ATP on IOP in these rabbits is different from the one obtained for Ap4 (data not shown). ATP effect presents a sharp reduction in IOP, which rapidly returns to normal values continuing toward an hypertensive effects 2 h after the application of the nucleotide (Peral et al., 2001; Pintor et al., 2000b). This biphasic effect is probably due to the gradual degradation of ATP to adenosine, which has been described as an hypertensive compound when acting through adenosine A2 receptors (Crosson and Gray, 1996). There is, therefore, a difference between the effect of ATP and Ap4 that suggests that Ap4 remains stable as an active molecule longer than ATP and therefore may act through purinergic receptors.

Ap4 presents an interesting effect of reducing intraocular pressure in normotensive rabbits. Other adenosine nucleotides have shown a similar behavior. ATP and the analogs β,γ-meATP and α,β-meATP can produce a reduction in the IOP. The reduction in IOP is similar in magnitude to the one produced by Ap4 (Pintor and Peral, 2001). Moreover, it is probable that in the regulation of IOP by these nucleotides and Ap4, they are stimulating the same P2X receptor. This conclusion partially supported on the cross-desensitization studies when using β,γ-meATP and the lack of effect to a second dose of Ap4. Also because the effect of the hypertensive nucleotide ATP-γ-S (a P2Y agonist in this model) is severely reduced when an application of Ap4 is used. The blockade by means of the P2 antagonist RB-2 suggests that the action of β,γ-meATP and Ap4 are performed through the same P2X receptor. It is interesting to compare the differential effect of the P2 antagonists tested. It would be expected to have a reduction in the effect when suramin is applied, because it is a general P2 receptor antagonist. It needs to be taken into account that all the antagonists have been tested at a single dose and that this does not need to the most effective for all the antagonists. Another possibility is to have differential antagonist permeability. The cornea may be selective to the transport of some of the P2 antagonists but not to others.

Comparison of the pharmacology for the effect of Ap4 on IOP with other tissues demand a more detailed pharmacological study; nevertheless, some conclusions can be made. For example, Ap4 has been demonstrated to activate P2X receptors in areas such as smooth muscle and the central nervous system (Lee et al., 1995b; Gomez-Villafuertes et al., 2000). In rat midbrain synaptic terminals, this nucleotide stimulates a heteromeric P2X receptor that present features of a P2X2/P2X3 purinergic receptor (Gomez-Villafuertes et al., 2000). This receptor is pharmacologically different from the one described in ocular cells/tissues because in the rat synaptic terminals the nucleotide ATP-γ-S is full agonist, whereas in the rabbit eye is not. This observation suggests the existence of a P2X receptor in the rabbit eye that presents a subunit composition that does not match with the one described in the rat brain.

Other locations where Ap4 stimulates P2X receptors are vas deferens, vascular smooth muscle, and mesenteric artery (Bailey and Hourani, 1995b; Lee et al., 1995a; Lewis et al., 2000). In these models, adenosine 5′-tetraphosphate produces vasoconstriction, with this nucleotide being especially active on P2X1 receptors (Lewis et al., 2000). It is premature to elucidate which subunits form the P2X receptor involved in the reduction of intraocular pressure, according to the preliminary pharmacological studies presented in this work. It is necessary to start with studies at the molecular level with isolated cells from the trabecular meshwork and ciliary processes to fully understand the location and characteristics of this receptor.

An interesting finding is that the levels found in the aqueous humor for Ap4 are in the range for promoting IOP decrease, as illustrated in the dose-response experiments presented here. A concentration of 197 nM is near to the EC50 value. This suggests that under certain physiological circumstances, the humor levels of Ap4 can oscillate and therefore they may modulate intraocular pressure. We still do not know what factors influence the possible variations in the Ap4 concentrations, but if Ap4 is released from ocular nerve terminals, its concentrations may be regulated, as happens with other neurotransmitters in the eye, such as acetylcholine or epinephrine.

It is also possible that Ap4 is generated from the hydrolytical cleavage of the dinucleotide Ap4A. This process, carried out by means of an asymmetrical phosphodiesterase, is relevant extracellularly and could be an alternative to neural release (Zimmermann, 1996; Mateo et al., 1997). Nevertheless this cannot be the only mechanism for Ap4 production because the concentrations measured of Ap4A in the aqueous humor were only 80 nM, insufficient to justify the presence of this mononucleotide as a consequence of Ap4A cleavage if we consider that 100% of Ap4A is hydrolyzed (Pintor et al., 2003). So far, we do not know which one of these processes is the most likely one or whether there are alternative sites for its release such as the ciliary epithelial cells, which can
release adenine nucleotides as reported previously (Mitchell et al., 1998).

Concerning the possible side effects the application of Ap4 can produce, we focused rabbit’s corneal transparency, corneal thickness, and changes in the corneal endothelium (possible polymegathism and changes in cell hexagonality). During the time of the present research, no changes in the parameters measured were observed (data not shown).

In summary, Ap4 is a mononucleotide present and physiologically active in the rabbit eye. It can modulate intraocular pressure through a mechanism that involves the activation of a P2X receptor. This compound may be used for the treatment of those pathologies involved in an abnormal increase in the intraocular pressure.

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