



SPECIAL REPORT

A₁-, A_{2A}- and A₃-subtype adenosine receptors modulate intraocular pressure in the mouse

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Despite the potential importance of the mouse in studying the pharmacology of aqueous dynamics, measurement of intraocular pressure (IOP) in its very small eye has been problematic. Utilizing a novel servo-null electrophysiologic approach recently applied to the mouse, we have identified a diversity of adenosine-receptor mechanisms in modulating IOP in this species. We report the first evidence that A₃ receptors increase IOP in any species, and verify in the mouse reports with larger mammals that A₁ receptors lower and A_{2A} receptors increase IOP.

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Introduction The mouse provides a potentially powerful vehicle for studying the pharmacology of aqueous humour dynamics, particularly in view of the increasing availability of knockout animals. Further clarification of this pharmacologic area would be helpful in generating novel drugs in treating glaucoma, a major world-wide cause of blindness usually associated with elevated intraocular pressure (IOP). The mouse is especially suitable for this initiative since it and the human display similar functional responses to drugs reducing inflow and enhancing outflow of aqueous humour (Avila *et al.*, 2001). However, progress has been limited by the technical challenge of measuring IOP in the small eye of the mouse, whose anterior chamber contains only 2–4 μ l of aqueous humour (Lea *et al.*, 2000; Avila *et al.*, 2001). The novel application of an electrophysiologic, non-manometric approach has addressed this challenge, permitting reliable monitoring of mouse IOP (Avila *et al.*, 2001).

As the initial extension of this technique to ocular pharmacology in the mouse, we sought a potential role for adenosine receptors in modulating IOP. A₁ and A₂ receptors have been associated with reduction and increase of IOP, respectively, in rabbits (Crosson, 1995; Crosson & Gray, 1996) and monkeys (Tian *et al.*, 1997), an effect ascribed in monkeys entirely to actions on aqueous humour outflow (Tian *et al.*, 1997). In contrast, A₃ agonists activate Cl⁻ channels of the nonpigmented ciliary epithelial cells which face the eye's aqueous humour *in vivo* (Carré *et al.*, 1997; 2000; Mitchell *et al.*, 1999). This effect is expected to increase inflow and consequently IOP, but until now A₃ receptor mechanisms potentially related to IOP control have been studied only *in vitro*, not in living mammals.

Methods *Animals and anaesthesia* Black Swiss outbred mice of mixed sex, 7–9 weeks old and ~30 gm in weight, were obtained from Taconic, Inc. (Germantown, NY,

U.S.A.), maintained under 12-h light–dark illumination cycle and allowed unrestricted access to food and water. All procedures conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Mice were anaesthetized with intraperitoneal ketamine (250 mg kg⁻¹) supplemented by topical proparacaine HCl 0.5% (Allergan, Hormigueros, Puerto Rico) for the IOP measurements, which were conducted at 6–11 h into the light phase.

Measurement of IOP IOP was measured with the Servo-Null Micropipette System (SNMS) previously detailed in detail (Avila *et al.*, 2001). The SNMS has been validated by comparing imposed and measured pressures in the mouse eye, by comparing repeated measurements in the same eye and between right and left eyes, and by monitoring the responses to anisosmotic solutions and drugs known to modulate inflow and outflow of aqueous humour in other species.

Drugs Drugs were applied topically in 10 μ l droplets with an Eppendorf pipette at the stated concentrations. Agents were initially dissolved in DMSO. The final droplet solution was isosmotic saline solution (310 mOsm) containing 1–2% DMSO and 0.003% benzalkonium chloride (Sigma, commonly used to enhance ocular penetration of topical drugs). We have previously found that the DMSO-benzalkonium solution itself has no effect on mouse IOP (Avila *et al.*, 2001). We have also verified in three sets of experiments that topical administration alters IOP through local ocular, and not systemic, actions: (1) In our initial study, 1% pilocarpine HCl (Alcon, Fort Worth, TX, U.S.A.) triggered oculotensive responses (Avila *et al.*, 2001) and miosis in the treated eye but no miosis contralaterally. (2) We now report that 10 μ l droplets of 1% mydriacyl (Alcon Humacao, Puerto Rico) produced mydriasis 45 min later in the treated ($\Delta = +1.18 \pm 0.07$ mm, $n = 8$, $P < 0.001$), but not in the contralateral, eye ($\Delta = -0.12 \pm 0.05$ mm). (3) Topical application of 100 μ M adenosine to one eye also had no effect on IOP in the contralateral eye ($n = 3$), but topical adenosine

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application to the monitored eye produced the largest IOP change of all agents tested (Results, Table 1, Figures 1 and 2).

9-chloro-2-(2-furyl)-5-([phenylacetyl]amino)[1,2,4]-triazolo-[1,5-c]quinazo-line (ZM241385) was purchased from Tocris (Ballwin, MO, U.S.A.) and 3,5-diethyl 2-methyl-6-phenyl-4-(trans-2-phenylvinyl)-1,4(R,S)-dihydro-pyridine-3,5-dicarboxylate (MRS 1097) was a gift from Drs Kenneth A. Jacobson and Bruce L Liang. The following agents were obtained from RBI Sigma (St. Louis, MO, U.S.A.): adenosine, N⁶-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropyl-xanthine (DCPCX), 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS-21680), N⁶-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide (IB-MECA), 2-chloro-IB-MECA (Cl-IB-MECA), 3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenyl-ethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191), and 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS 1523).

Data analysis Means ± s.e.mean were calculated from recording periods of usually 3–5 min. Numbers of experiments or of eyes are indicated by 'N'. For those few drug concentrations tested in duplicate, the uncertainty was estimated as half the difference between the two values. The probability P of the null hypothesis was estimated with the paired, 2-tailed Student's *t*-test.

Results *A₃ receptors* As indicated in the Introduction, the primary aim was to test whether AR agonists and antagonists modulate IOP in the mouse and to focus on the putative role of *A₃* ARs. For the latter purpose, MRS 1191 is particularly favourable as a highly selective *A₃* antagonist of both human and murine ARs (Jacobson *et al.*, 1997).

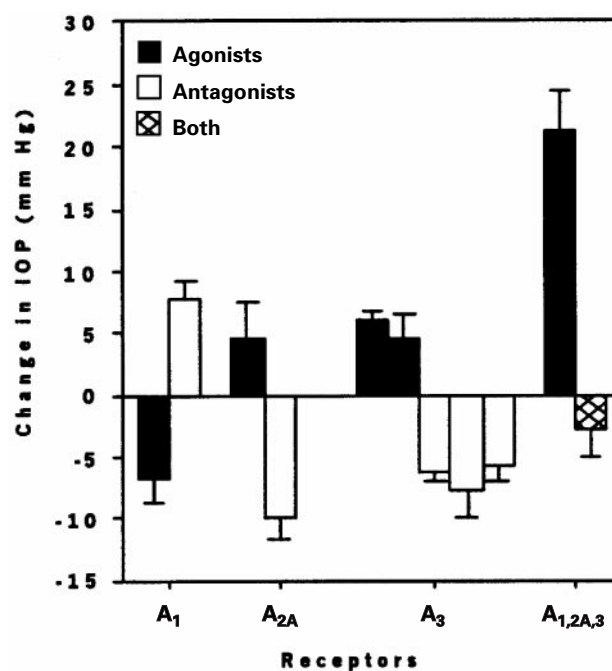


Figure 1 Means ± s.e.mean of the responses to minimally effective concentrations of AR agonists and antagonists: 100 nM CPA, 100 nM DCPCX, 60 μM CGS 21680, 100 nM ZM241385, 200 nM Cl-IB-MECA, 140 nM IB-MECA, 25 μM MRS 1191, 11 μM MRS 1097, and 400 μM MRS 1523. At the extreme right of the bar graph, data are presented for 100 μM adenosine with and without pretreatment with the *A₃* AR antagonist MRS 1191.

Table 1 IOP effects of adenosine agonists and antagonists

Class	Name	Conc	N	Δ(IOP) from baseline in mmHg	P (vs baseline)
<i>A₁</i> agonist	CPA	100 nM	3	-6.8 ± 1.8	<0.05
		1 mM	3	-1.0 ± 2.3	>0.7
<i>A₁</i> antagonist	DCPCX	100 nM	7	+7.8 ± 1.4	<0.005
		100 μM	2	+10.5 ± 4.4	-
<i>A_{2A}</i> agonist	CGS 21680	3 μM	3	+2.8 ± 1.8	>0.2
		60 μM	3	+21.2	-
<i>A_{2A}</i> antagonist	ZM 241385	100 nM	9	-9.7 ± 1.6	<0.001
		5 μM	4	-6.6 ± 1.6	<0.05
		25.5 μM	2	-10.5 ± 2.7	-
<i>A₃</i> agonist	IB MECA	140 nM	7	+6.0 ± 0.9	<0.001
		200 nM	9	+5.7 ± 2.3	=0.01
<i>A₃</i> antagonist	MRS 1191	2.5 μM	4	-1.4 ± 1.1	>0.2
		25 μM	23	-6.3 ± 0.7	<0.001
		2.5 mM	4	-7.9 ± 1.2	<0.01
	MRS 1097	11 μM	8	-5.7 ± 1.2	<0.005
		20 μM	2	+0.5	-
	MRS 1523	200 μM	2	-6.4 ± 5.1	<0.01
		400 μM	11	-7.7 ± 2.4	<0.025
10 mM		3	-7.2 ± 2.6	>0.1	
Nonselective agonist	Adenosine	2 μM	3	-0.5 ± 0.5	=0.5
		100 μM	5	+21.2 ± 3.2	<0.01
<i>A₃</i> antagonist/ + <i>A₁</i> agonist	MRS1191/ + CPA	25 μM/ + 100 nM	8	-6.0 ± 1.7/ -6.0 ± 1.6	<0.01 <0.01
		<i>A₃</i> antagonist/ + nonselective	MRS1191/ + Adenosine	25 μM/ + 100 μM	6
<i>A₃</i> antagonist/ + <i>A₃</i> agonist	MRS1191/ + Cl-IB-MECA			25 μM + 200 nM	4

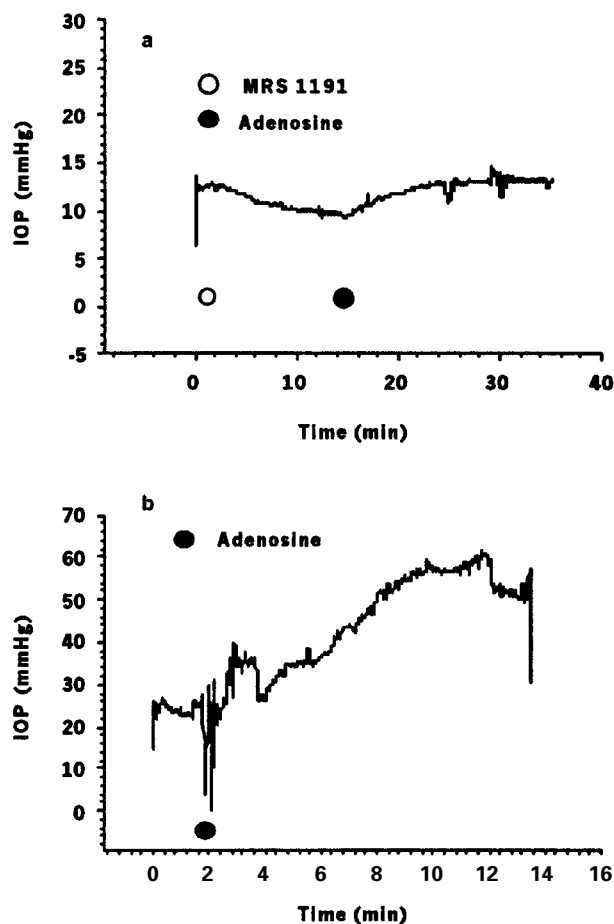


Figure 2 Representative IOP tracings assessing effects of MRS 1191 and adenosine on mouse IOP. (a) MRS 1191 ($25 \mu\text{M}$) lowered baseline IOP by $2.62 \pm 0.04 \text{ mmHg}$, following which the response to $100 \mu\text{M}$ adenosine ($+2.52 \pm 0.03 \text{ mmHg}$) was substantially attenuated; see, e.g., Figure 2b). (b) In the absence of the A_3 antagonist, $100 \mu\text{M}$ adenosine consistently produced several-fold larger increases in IOP, in this eye raising IOP by $31.9 \pm 0.1 \text{ mmHg}$.

Consistent with our earlier data (Avila *et al.*, 2001), baseline IOP was $17.2 \pm 0.8 \text{ mmHg}$ ($n=134$). MRS 1191 ($25 \mu\text{M}$ in the topical droplet) lowered IOP by $6.3 \pm 0.7 \text{ mmHg}$ ($n=23$, $P<0.001$, Figure 1). A representative trace appears in Figure 2a. From measurements (Table 1) obtained at $2.5 \mu\text{M}$, $25 \mu\text{M}$ and 2.5 mM , we calculated a half-maximal inhibitory concentration (ID_{50}) for MRS 1191 in the droplet of $16 \pm 4 \mu\text{M}$. Another dihydropyridine A_3 antagonist MRS 1097 (Jacobson *et al.*, 1997) similarly lowered IOP by $5.7 \pm 1.2 \text{ mmHg}$ at $11 \mu\text{M}$ ($n=8$, $P<0.005$, Figure 1, Table 1). A structurally dissimilar third A_3 AR antagonist MRS 1523 (Li *et al.*, 1999) also reduced baseline IOP at higher concentrations (Figure 1, Table 1). These data indicated that antagonism of A_3 ARs lowers baseline mouse IOP.

We tested whether the preferred murine A_3 antagonist MRS 1191 also attenuated the responses to non-selective and A_3 -selective AR antagonists. Adenosine had no effect at $2 \mu\text{M}$ concentration, but increased IOP over baseline by $21.2 \pm 3.2 \text{ mmHg}$ ($n=5$, $P<0.01$) at $100 \mu\text{M}$ (Figures 1 and 2b, Table 1). Increasing the adenosine concentration 20 fold to 2 mM had no further effect (Table 1). The concentration in the aqueous humour following topical application of $100 \mu\text{M}$

adenosine was likely high enough (Discussion) to stimulate A_1 , A_{2A} and A_3 , but not A_{2B} receptors (Fredholm *et al.*, 1994).

Following pretreatment with $25 \mu\text{M}$ MRS 1191, $100 \mu\text{M}$ adenosine increased IOP by only $5.9 \pm 2.2 \text{ mmHg}$ ($n=6$, $P<0.05$, Figures 1 and 2a, Table 1); thus, the adenosine-triggered elevation in IOP was $\sim 70\%$ -attenuated by pretreatment with the A_3 AR antagonist (compare Figures 2a and 2b). The combined effect of MRS 1191 and adenosine was to lower IOP slightly (with respect to the initial baseline level) by $1.8 \pm 2.1 \text{ mmHg}$ (Figures 1 and 2a, Table 1).

Opposite to the reduction in IOP produced by A_3 AR antagonists, A_3 agonists increased pressure. IB-MECA (Jacobson *et al.*, 1993) increased IOP by $6.0 \pm 0.9 \text{ mmHg}$ ($n=7$, $P<0.001$, Figure 1, Table 1) at 140 nM . The more polar A_3 AR agonist Cl-IB-MECA (Jacobson *et al.*, 1995) raised IOP above baseline by $5.7 \pm 2.3 \text{ mmHg}$ at 200 nM ($n=9$, Figure 1, $P=0.01$) and by $4.6 \pm 1.8 \text{ mmHg}$ at 2 mM ($n=3$), and this response was abolished by pretreatment with the A_3 AR antagonist MRS 1191 ($25 \mu\text{M}$, $n=5$, Table 1).

The foregoing results obtained with selective A_3 antagonists and agonists and with adenosine constitute the first evidence that A_3 ARs modulate mammalian OP *in vivo*. We also tested whether mouse IOP is modulated by A_1 and A_2 ARs, as reported for rabbit (Crosson, 1995; Crosson & Gray, 1996) and monkey (Tian *et al.*, 1997).

A₁ and A₂ receptors The A_1 -agonist CPA (Fredholm *et al.*, 1994; Klotz *et al.*, 1998) reduced IOP by $6.8 \pm 1.8 \text{ mmHg}$ (100 nM , $n=4$, $P<0.05$, Figure 1, Table 1) and the A_1 -antagonist DCPCX (Haleen *et al.*, 1987) increased IOP by $7.8 \pm 1.4 \text{ mmHg}$ (100 nM , $n=6$, $P<0.005$, Figure 1, Table 1). At a substantially higher concentration (1 mM), CPA exerted no significant effect (Table 1). The A_{2A} -agonist CGS-21680 (Jacobson *et al.*, 1995) increased IOP at $60 \mu\text{M}$ ($n=3$, Table 1) but also produced corneal oedema and was associated with unstable records, complicating analysis of the IOP measurements. However, a role of A_{2A} ARs was clearly detected by applying the A_{2A} -antagonist ZM 241385 (Ongini *et al.*, 1999) (100 nM , $n=11$), which triggered a transient increase in IOP ($8.4 \pm 2.5 \text{ mmHg}$), followed by a sustained reduction of $9.7 \pm 1.6 \text{ mmHg}$ ($P<0.001$, Figure 1, Table 1). Similar sustained reductions were noted at higher concentrations of this drug (Table 1).

Discussion This manuscript provides the first information concerning modulation of mouse IOP by the three adenosine receptors (A_1 , A_{2A} and A_3) postulated to regulate human IOP. In principle, caution is necessary in analyzing the data in the mouse eye since we do not know the concentrations of agonists and antagonists in the small volume of aqueous humour. MRS 1191 is known to be a highly selective A_3 antagonist for human and rat ARs (Jacobson *et al.*, 1997). We can calculate an absolute, unrealistic upper bound to the true aqueous humour concentration of MRS 1191 resulting from topical administration by assuming that the cornea presents no permeability barrier whatsoever. The topically applied drug would have been thereby distributed uniformly and thus diluted throughout the applied droplet, tear and anterior chamber. Based on this assumption, the concentration in the aqueous humour would not have exceeded 40% of that in the applied droplet. Thus, topical application of

25 μM MRS 1191 could not have led to concentrations higher than 10 μM in the aqueous humour. Even at this unrealistically high level, only 25% of murine A_1 and <10% of murine A_{2A} receptors would have been occupied (Jacobson *et al.*, 1997). We conclude that the direct effect of MRS 1191 to lower baseline IOP and its marked action to attenuate the subsequent response to adenosine are mediated by A_3 receptors, consistent with results of *in vitro* studies (Carré *et al.*, 1997; 2000; Mitchell *et al.*, 1999).

In extending analysis of the data presented in Table 1, we have compared the minimally effective concentrations in the topical droplets with published functional estimates of binding, largely to human receptors. From this, drug penetration ratios (PR) of aqueous-to-drop concentration can be estimated. For example, the A_3 -selective agonist IB-MECA significantly raised IOP at a drop concentration of 140 nM (Figure 1, Table 1) and the K_d in mouse cerebellar membranes is 1.39 ± 0.04 nM (Jacobson *et al.*, 1993), so that the penetration ratio was $\sim 1:100$. By this approximate measure, almost all adenosine agonists and antagonists displayed penetration ratios of 1:200–250. The exceptions were CI-IB-MECA (PR ~ 600) and MRS 1523 (PR ~ 770), presumably reflecting slower penetrance of these agents. Thus, a conservatively high estimate of the drug concentration in the aqueous humour can be calculated as $\sim 1\%$ of the applied droplet concentration. Interestingly, this observation conforms to the commonly-applied assumptions of $\sim 1\%$ penetration of topically applied drug to rabbits (Honjo *et al.*, 2001) and primates (Asseff *et al.*, 1973). This rough rule of thumb apparently holds for the mouse eye, as well as for eyes with thicker corneas from other species.

Taking the likely aqueous humour concentrations to be no higher than 1% the droplet concentrations, the antagonists MRS 1097 and 1191, and the agonists IB-MECA and CI-IB-MECA must have exerted their IOP effects by binding specifically to A_3 ARs, whereas MRS 1523 (Li *et al.*, 1999) may possibly have antagonized A_{2A} as well as A_3 receptors. Correspondingly, the A_1 agonist CPA lowered IOP at a calculated equivalent aqueous concentration of ~ 1 nM, at which <1% of the A_{2A} and A_3 receptors were likely occupied (Fredholm *et al.*, 1994; Klotz *et al.*, 1998). Conversely, the A_1 antagonist DCPCX likely raised IOP by selective action at A_1 ARs. When the droplet concentration was markedly raised to 1 mM, ensuring occupancy of A_1 , A_{2A}

and A_{2B} adenosine receptors, CPA had no significant effect on IOP, suggesting that the negative oculotensive effect of the A_1 receptors was offset by the opposing effects of the A_3 and possibly A_{2A} receptors. The role of A_{2A} receptors in elevating mouse IOP was separately substantiated by the observation that the selective A_{2A} antagonist ZM 241385 triggered a sustained reduction in IOP at 100 nM droplet concentration. At an equivalent aqueous concentration of 1 nM, ZM 241385 was occupying 2% of the A_{2B} and <1% of the other receptors.

It is intriguing that A_1 and A_3 ARs exert opposing effects on IOP although both receptors trigger reductions in intracellular cyclic-AMP (Fredholm *et al.*, 1994). This suggests that A_1 and A_3 agonists reduce IOP through other signalling pathways and/or that they act on different cells. Indeed, the major oculotensive effects of A_1 and A_3 agonists may be at the trabecular meshwork and/or inner wall of Schlemm's canal (Tian *et al.*, 1997) and nonpigmented ciliary epithelial cells (Carré *et al.*, 1997; 2000; Mitchell *et al.*, 1999), respectively.

In summary, the current data provide the first evidence that A_3 -selective adenosine agents and antagonists modulate IOP in the mammalian eye, extending *in vitro* observations implicating A_3 receptors in tissues controlling aqueous humour physiology. The pharmacologic responses *in vivo* conform to expectations based on A_3 AR responses by ciliary epithelial cells *in vitro* suggesting that A_3 antagonists would lower IOP. The results also agree with findings in other mammalian eyes implicating A_1 and A_{2A} receptors in control of IOP. The work further supports the potential utility of the mouse in studying the physiology and pharmacology of aqueous dynamics.

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