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Ocular hypotensive effects of melatonin receptor agonists in the rabbit: further evidence for an MT_3 receptor

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1 Melatonin is involved in the control of intraocular pressure during the night and day photoperiod. We have investigated the receptor that regulates intraocular pressure in New Zealand white rabbits by means of agonists and antagonists of melatonin receptors.

2 Melatonin and its analogues: 2-Phe-melatonin, 6-Cl-melatonin, 2-I-melatonin, 5- methoxycarbonylamino-*N*-acetyltryptamine (5-MCA-NAT) and *N*-acetyltryptamine all produced a reduction in intraocular pressure. Dose-response analysis for these compounds gave pD_2 values of 9.3 ± 0.24 for melatonin; 9.0 ± 0.09 for 6-Cl-melatonin; 9.0 ± 0.84 for 2-I-melatonin; 8.9 ± 0.07 for 5-MCA-NAT; 8.7 ± 0.18 for 2-Phe-melatonin and 9.4 ± 0.30 for *N*-acetyltryptamine (all $n = 8$).

3 At a dose of 0.5 nmol (in 10 μl) melatonin and the selective melatonin MT_3 agonist 5-MCA-NAT, induced greater reductions of intraocular pressure ($22.8 \pm 2.3\%$ and $32.5 \pm 1.4\%$, respectively) than the other compounds.

4 The melatonin-receptor antagonists, prazosin, DH-97 and 4-P-PDOT, reversed the effect of 5-MCA-NAT in a dose-dependent manner, with pA_2 values of 13.5 ± 0.17 for prazosin, 10.6 ± 0.16 for DH-97 and 9.4 ± 0.20 for 4-P-PDOT ($n = 8$).

5 Cholinoceptor antagonists (hexamethonium and atropine) and α_2- and β_2-adrenoceptor antagonists (yohimbine and ICI 118,551) partially reversed the effects produced by melatonin and 5-MCA-NAT, suggesting the possible involvement of cholinergic and noradrenergic systems in the hypotensive actions mediated by melatonin agonists. The α_1-adrenoceptor antagonist, corynanthine, had no significant effect.

6 The strong hypotensive effect of the MT_3 agonist, 5-MCA-NAT, suggests that this compound may be a useful agent for treating those pathologies where intraocular pressure is abnormally elevated.
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Keywords: Glaucoma; intraocular pressure; 5-methoxycarbonylamino-*N*-acetyltryptamine (5-MCA-NAT); melatonin; ocular hypertension

Abbreviations: 6-Cl-melatonin, 6-chloromelatonin; 2-I-melatonin, 2-iodomelatonin; 5-MCA-NAT, 5-methoxycarbonylamino-*N*-acetyltryptamine; 2-Phe-melatonin, 2-phenylmelatonin; ANOVA, analysis of variance; DMSO, dimethylsulphoxide; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride; IOP, intraocular pressure; s.e., standard error

Introduction

Melatonin is a neurohormone that mediates lightness–darkness signals to synchronize cellular physiology with the photoperiod (Bartness & Goldman, 1989). Melatonin also affects processes such as cell proliferation and differentiation (Benitez-King *et al*., 1990) and sleep promotion (Cutler *et al*., 1997), and it can also act as free radical scavenger (for a review see Reiter *et al*., 2000).

Specific functions of melatonin are mediated through cell membrane MT_1, MT_2 and MT_3 melatonin receptors (Dubocovich, 1995). MT_1 and MT_2 melatonin receptors are negatively coupled to adenylate cyclase while the MT_3 seems to be coupled to phospholipase C (Mullins *et al*., 1997). Melatonin MT_1 and MT_2 receptors have been cloned in several species, nevertheless, little is known about the structure of the melatonin MT_3 receptor, however, the melatonin MT_3 receptor has been identified as the enzyme quinone reductase 2 (QR_2) in some mammalian species (Nosjean *et al*., 2000).

Melatonin, whose presence and receptors have been found in the eye, has been investigated in detail in the retina, where it can modify visual physiology (Vanecek, 1998). It can affect dopamine-release during light-adaptation in amacrine cells. Receptors for melatonin have been identified in retinal ganglion cells (Blazynski & Dubocovich, 1991; Djamgoz & Wagner, 1992; Laitinen & Saavedra, 1990).

Another location where melatonin receptors are present is the ciliary body, as described by autoradiographic studies of rabbit eyes (Osborne & Chidlow, 1994). More recently, Wiechmann and co-workers have described the presence of a Mel_{1b} (MT_2) melatonin receptor in *Xenopus laevis* non-pigmented ciliary epithelial cells (Wiechmann & Wirsig-Wiechmann, 2001). The presence of these receptors here suggests that they modulate physiological processes such as

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the formation of aqueous humour, and are therefore involved in the control of intraocular pressure.

Intraocular pressure changes diurnally, increasing during the day and decreasing during the night. That this effect is mediated by melatonin has been demonstrated in different experimental models (Samples *et al*., 1988; Liu & Dacus, 1991). Topical application of melatonin or 5-methoxycarbonylamino-*N*-acetyltryptamine (5-MCA-NAT), a selective MT_3 melatonin receptor agonist, to the cornea of New Zealand white rabbits produces a clear reduction in intraocular pressure, suggesting the involvement of this receptor (Pintor *et al*., 2001). The effect of 5-MCA-NAT is more sustained than that of melatonin (Pintor *et al*., 2001). To date, no other melatonin analogues have been tested for their ability to reduce intraocular pressure. Furthermore, prazosin, familiarly known as an α_1-adrenoceptor antagonist, acts as an MT_3 receptor antagonist (Dubocovich, 1995), and as yet has not been tested against the ocular hypotension produced by 5-MCA-NAT.

The aim of the present experimental work was to characterize the putative MT_3 receptor that controls intraocular pressure in New Zealand white rabbit eyes, by means of melatonin agonists and antagonists. Previous studies performed in our laboratory have shown that luzindole blocks the hypotensive effects of melatonin and 5-MCA-NAT (Pintor *et al*., 2001). We further extended this study by testing the MT_2 receptor antagonists DH-97 and 4-P-PDOT (Lucchelli *et al*., 1997; Paredes *et al*., 1999; Paul *et al*., 1999) and prazosin. Furthermore, in order to see whether or not the actions of melatonin and 5-MCA-NAT were mediated by local cholinergic or adrenergic nerves that control intraocular pressure, the nicotinic and muscarinic cholinoceptor antagonists, hexamethonium and atropine, and adrenoceptor antagonists, yohimbine and ICI 118,551, were tested. In a previous work, we demonstrated the ability of these compounds to interfere with cholinoceptor and adrenoceptors which modulate intraocular pressure in rabbits (Pintor & Peral, 2000).

Methods

Animals

Twenty-four male New Zealand white rabbits (2.5–3.0 kg) were kept in individual cages with free access to food and water. They were submitted to controlled 12 h/12 h light/dark cycles. All the protocols here described adhere to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research and also is in accordance with the European Communities Council Directive (86/609/EEC).

Intraocular pressure measurements

Intraocular pressure was measured by means of a Tonopen XL contact tonometer supplied by Mentor (U.S.A.). Since the application of the tonometer may produce discomfort in the rabbits, corneas were anaesthetized by applying 10 μl of 1 : 10 (v v^{-1}) oxibuprocaine/tetracaine (4 and 1 mg respectively). Experiments were performed using a blinded design: no visible indication was given to the experimenter as to the applied solution (agent or saline). The different compounds were applied unilaterally to the cornea at fixed volumes of 10 μl. The contralateral eye received the same volume of sterile 0.9% w v^{-1} saline (vehicle). Two intraocular pressure measurements were taken before any compound was instilled.

Pharmacological protocols

Melatonin agonists were assayed across a range of doses from 10 amol to 1 nmol in order to generate dose–response curves. For these experiments, intraocular pressure was measured during the maximal effect of the agent. Also, single doses of 0.5 nmol (10 μl) were assayed in order to study the time-course of the effect, up to 6 h or more if necessary. On any one day, only a single dose was tested on a single animal, which was rested at least 2 days between doses. Melatonin agonists were prepared at 10–100 fold higher concentrations in dimethylsulphoxide (DMSO) to be later diluted in saline solution to reach the desired final concentration.

Melatonin antagonists were added, in volumes of 10 μl, at doses ranging from 1 amol to 0.1 μmol, 30 min before the application of 5-MCA-NAT (0.5 nmol, 10 μl). Measurements were taken 2 h after the application of this agonist, as described by Pintor *et al*., 2001. The $-$log dose of antagonist that inhibited the response to 5-MCA-NAT by 50%, i.e. the pA_2, was calculated.

Experiments with antagonists of cholinoceptors and adrenoceptors

The cholinoceptors antagonists, atropine and hexamethonium were applied together, as were the α_2- and β_2-adrenoceptor antagonists yohimbine and ICI 118,551, all at a dose of 100 μg μl^{-1} (10 μl). Both α_2- and β_2-adrenoceptors have been implicated in the control of IOP (Toris *et al*., 1995a, b). In a separate series of experiments, the α_1-adrenoceptor antagonist, corynanthine (1 nmol), was applied. Melatonin and 5-MCA- NAT (0.5 nmol, 10 μl each), were subsequently applied 30 min later and measurements were taken after a further hour.

Materials

Melatonin, hexamethonium, corynanthine and atropine were purchased from Sigma (St. Louis, U.S.A.). 2-Phe-melatonin, 6-Cl-melatonin, 2-I-melatonin, 5-MCA-NAT, N-acetyltryptamine, prazosin, DH-97 and 4-P-PDOT, yohimbine and (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride (ICI 118,551) were from Tocris (Bristol, U.K.). Oxibuprocaine/tetracaine anaesthetic was from CUSI labs (Spain). Other reagents were analytical grade from Merk (Darmstadt, Germany).

Statistical analysis

All data are presented as the mean ± s.e.mean. Significant differences were determined by two-tailed Student's *t*-tests. The plotting and fitting of dose–response curves was carried out with the computer programme Microcal Origin v.5.0 (Microcal Software, U.S.A.).

Results

Effect of melatonin receptor agonists on rabbit intraocular pressure

The resting IOP was 23.2±2.1 mm Hg. Melatonin and all of its analogues, 2-Phe-melatonin, 6-Cl-melatonin, 2-I-melatonin, 5-MCA-NAT and N-acetyltryptamine produced a reduction in intraocular pressure. Among these compounds 5-MCA-NAT was the most effective, reducing intraocular pressure maximally by almost 45% (Figure 1, Table 1). The other analogues had smaller maximal effects, causing a reduction in intraocular pressure between 10 and 22% (Figure 1, Table 1). The pD_2 values ranged from 9.4 to 8.7, but none was statistically significantly different from any other (ANOVA, Table 1). At a single dose of 50 μM (10 μl), followed for 6–9 h, melatonin and 5-MCA-NAT were the most effective, reducing intraocular pressure by 22.8±2.3% and 32.5±1.4% respectively (Figure 2). 2-Phe-melatonin and 6-Cl-melatonin reduced intraocular pressure by 17.7±2.1% and 15.5±1.7% (Figure 3A), and 2-I-melatonin and *N*-acetyltryptamine reduced intraocular pressure by 13.2±1.8% and 5.4±1.3%, respectively (Figure 3B) ($n=8$).

Concerning the pattern of the time courses, for all the compounds except 5-MCA-NAT, intraocular pressure returned to its normal level within 5 h of the instillation (Figures 2 and 3). 5-MCA-NAT evoked a sustained hypotensive effect which lasted for more than 8 h (Figure 2).

Effect of melatonin receptor antagonists

Prazosin evoked an increase in IOP at 30 min, but this rapidly declined and IOP was not significantly different from resting levels by 1 h (Figure 4A). Neither DH-97 nor 4-P-PDOT had a significant effect on IOP. Prazosin, DH-97 and 4-P-PDOT all caused a dose-dependent inhibition of the hypotensive effect evoked by 0.5 nmol 5-MCA-NAT (Figure 4B). The pA_2 values for these three antagonists were 13.5±0.17 for prazosin, 10.6±0.16 for DH-97 and 9.4±0.20 for 4-P-PDOT ($n=8$).

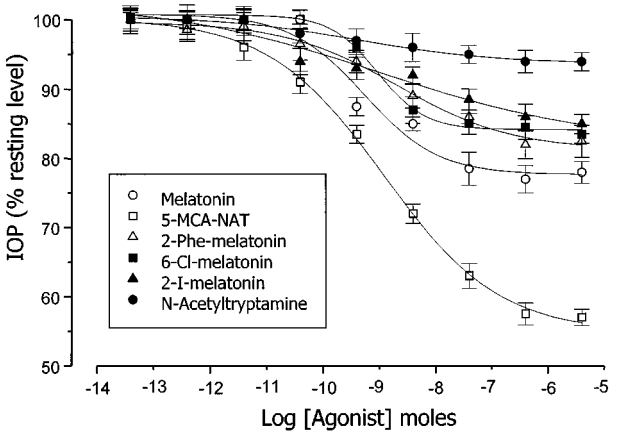

Figure 1 Dose–response curves for melatonin and analogues on intraocular pressure in New Zealand white rabbits. Melatonin, 5-MCA-NAT, 2-Phe-melatonin, 2-I-melatonin, 6-Cl-melatonin and *N*-acetyltryptamine were tested at doses between 10^{-14} mol to 10^{-5} mol. 100% represents the intraocular pressure before application of any drug and was equivalent to 23.2±2.1 mm Hg. Values represent the mean±s.e.mean of eight independent experiments.

Table 1 pD_2 values and maximum reduction in intraocular pressure for melatonin and melatonin analogues

	pD_2	%
Melatonin	9.3±0.24	22.0±1.6 (4)
N-Acetyltryptamine	9.4±0.30	6.0±1.3 (8)
5-MCA-NAT	8.9±0.07	42.5±1.6 (8)
6-Chloromelatonin	9.0±0.09	16.5±1.3 (8)
2-Iodomelatonin	9.0±0.84	15.0±1.4 (8)
2-Phenylmelatonin	8.7±0.18	18.0±2.0 (8)

The pD_2 value was taken as the $-\log(EC_{50})$. Reduction in intraocular pressure was expressed relative to the resting level.

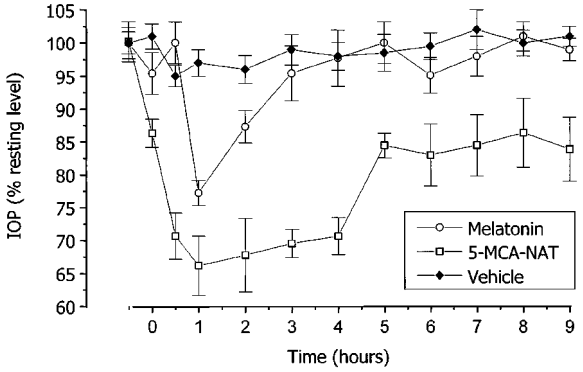

Figure 2 Effects of melatonin and 5-MCA-NAT on rabbit intraocular pressure. Time course of melatonin and 5-MCA-NAT (0.5 nmol) followed for 9 h. 100% represents the intraocular pressure before application of any drug (i.e. at t_0) and was equivalent to 23.2±2.1 mm Hg. Values represent the mean±s.e.mean of eight independent experiments.

Effects of cholinoceptor and adrenoceptor antagonism on melatonin and 5-MCA-NAT

Corynanthine (1 nmol), an α_1-adrenoceptor antagonist, evoked a small, sustained decrease in IOP (Figure 5) and pupillary constriction (data not shown). Responses to 5-MCA-NAT were not significantly affected by corynanthine (Figure 5).

Application of the combined muscarinic and nicotinic cholinoceptor antagonists evoked an increase in intraocular pressure of 25.4±4.2% ($n=8$). Application of the combined α_2- and β_2-adrenoceptor antagonists evoked an increase in intraocular pressure of 16.9±2.7% ($n=8$).

As shown in Figure 6A, the combined cholinoceptor antagonists and the combined adrenoceptor antagonists markedly inhibited the hypotensive effects of 5-MCA-NAT. The cholinoceptor antagonists alone had a small hypertensive effect, and in their presence both melatonin and 5-MCA-NAT caused small but significant decreases in intraocular pressure: 12.5±3.8%, $n=8$, $P<0.01$ and 11.3±3.7%, $n=8$, $P<0.02$, respectively. In the presence of the noradrenergic antagonists the reductions in intraocular pressure when compared to the effect of noradrenergic antagonists alone

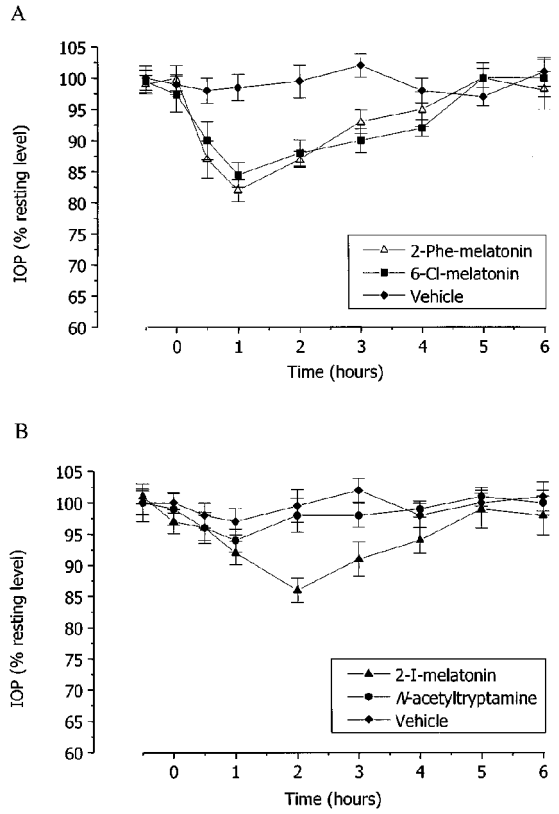

Figure 3 Effects of melatonin analogues on rabbit intraocular pressure. (A) Time course of 2-Phe-melatonin and 6-Cl-melatonin (0.5 nmol) followed for 6 h. (B) Time course of 2-I-melatonin and *N*-acetyltryptamine (0.5 nmol), followed for 6 h. In both plots, 100% represents the intraocular pressure before application of any drug (i.e. at t_0) and was equivalent to 23.2±2.1 mm Hg. Values represent the mean±s.e.mean of eight independent experiments.

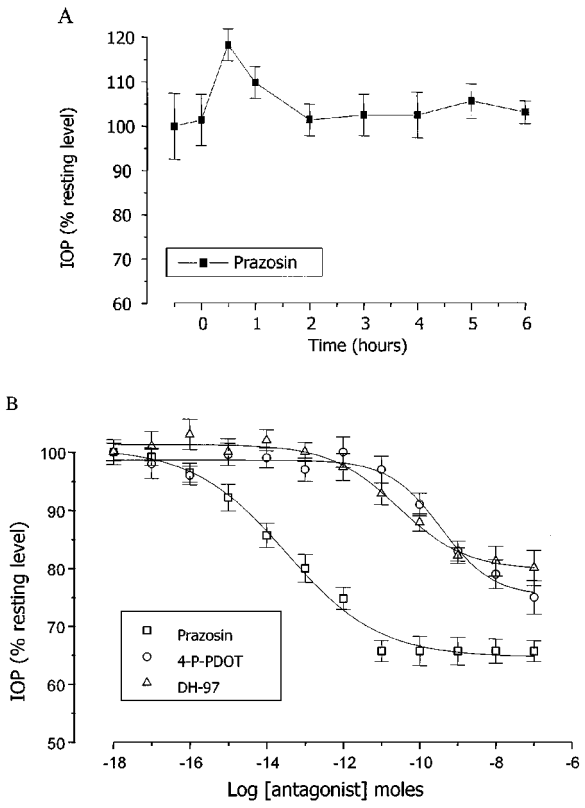

Figure 4 Effects of melatonin receptor antagonists on the action of 5-MCA-NAT. (A) Prazosin alone caused a transient increase in intraocular pressure. (B) Graded doses of prazosin, DH-97 and 4-P-PDOT were applied as described in Methods. The maximal reduction in intraocular pressure due to 5-MCA-NAT was 35±2% (n=8). Values represent the mean±s.e.mean of eight independent experiments.

were: 12.1±2.8%, n=8, P<0.02; 22.0±1.9%, n=8, P<0.001 for melatonin and 5-MCA-NAT, respectively.

When the time course for 5-MCA-NAT was determined in the presence of the adrenoceptor antagonists, although the magnitude of the inhibitory response was reduced, the duration of the hypertensive effect was not similarly affected (Figure 6B).

Discussion

The results show that topical application of melatonin and its analogues, 2-Phe-melatonin, 6-Cl-melatonin, 2-I-melatonin, 5-MCA-NAT and its precursor N-acetyltryptamine, to the cornea evokes dose-dependent reductions in intraocular pressure in New Zealand white rabbits. Among the compounds tested, 5-MCA-NAT had the greatest maximum effect. However, when potencies were compared, based on their pD_2 values, there were no significant differences.

5-MCA-NAT has been claimed to be a selective MT_3 receptor ligand (Paul *et al.*, 1999). The first evidence for a physiological role of the putative MT_3 receptor by means of melatonin and 5-MCA-NAT was reported by our group when describing the hypotensive effect on intraocular pressure by these two compounds in New Zealand white rabbits (Pintor *et al.*, 2001). A controversy arose recently,

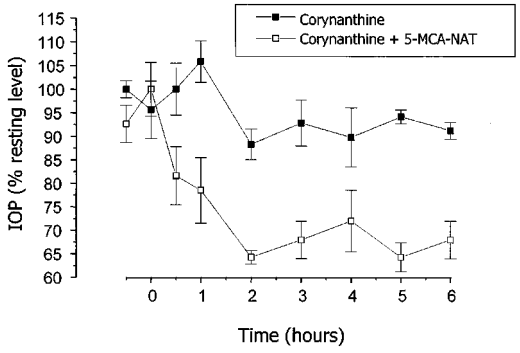

Figure 5 Effects of corynanthine on responses to 5-MCA-NAT. Corynanthine alone caused a small reduction in IOP but did not significantly inhibit ocular hypotensive effects of 5-MCA-NAT. Points show mean±s.e.mean of eight independent experiments.

when the MT_3 receptor was identified as QR_2, thus shedding new light on the possibilities of melatonin analogues being modulators of this enzyme (Nosjean *et al.*, 2000). Determination of the tissue distribution of the MT_3/QR_2, by means of 2-[^{125}I]-MCA-NAT showed the correlation between the binding and the enzymatic activity. There are, nevertheless,

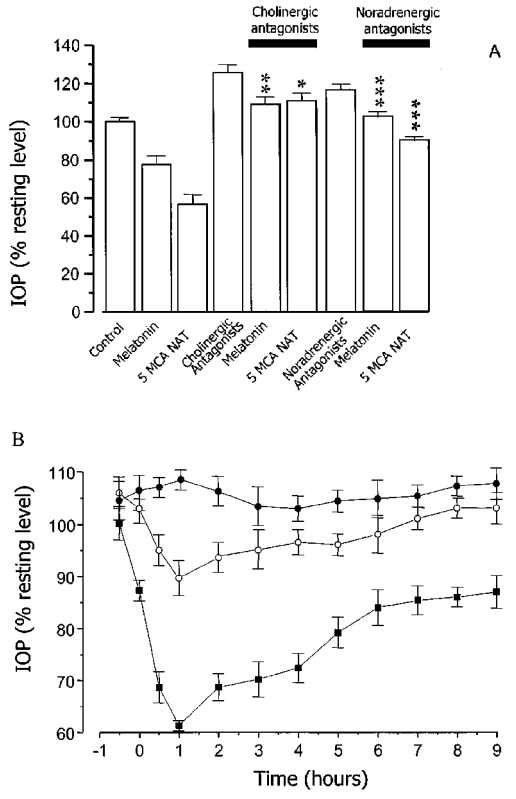

Figure 6 Effect of cholinoceptor and adrenoceptor antagonists on the effects mediated by melatonin and 5-MCA-NAT. (A) The effect of the cholinoceptor antagonists (hexamethonium and atropine) and α_2 and β_2-adrenoceptor antagonists (yohimbine and ICI 118,551) were assayed both alone and on responses to 0.5 nmol of melatonin and 5-MCA-NAT. (B) Time-course for the effect of 5-MCA-NAT (0.5 nmol), alone ■, in the presence of noradrenergic antagonists ○ and cholinergic antagonists ●. All the values are the mean ± s.e.mean of eight independent experiments. $^*P<0.01$, $^{**}P<0.05$, $^{***}P<0.005$ *vs* the corresponding controls, Student's *t*-test.

some discrepancies which appear in mammals such as dogs and monkeys. In these animal models, MT_3 binding and QR_2 activity do not fully correlate, this being particularly different in *Macaca fasciculatus* (Nosjean *et al*., 2001). Taken together, this may indicate the presence of more than one protein showing the same pharmacological profile but different biochemical and physiological properties.

Prazosin is a widely used α_1-adrenoceptor antagonist, although it has been claimed to be a melatonin MT_3 receptor antagonist when used at nanomolar concentrations (Dubocovich, 1995). Prazosin has been used as a melatonin receptor antagonist in the guinea-pig colon (Lucchelli *et al*., 1997), in mouse morphine tolerance (Raghavendra & Kulkarni, 1999), on melatonin acetylcholine-induced release (Paredes *et al*., 1999) and in rat nociception (Yu *et al*., 2000). The fact that corynanthine did not modify responses to 5-MCA-NAT indicates that 5-MCA-NAT is not an agonist of α_1-adrenoceptors, and that in the effects blocked by prazosin are mediated *via* a melatonin receptor. In the present study, of all the melatonin antagonists tested, prazosin was approximately 1000 times more potent at blocking the responses induced by 5-MCA-NAT than was DH-97 or 4-P-PDOT. It is also similarly much more potent than luzindole (Pintor *et al*., 2001). Thus, the melatonin receptor that mediates the reduction in intraocular pressure appears to be an MT_3 receptor.

The maximum reduction in IOP evoked by 5-MCA-NAT (approximately 45%) is comparable to that evoked by flesinoxan, a hybrid 5-HT_{1A} receptor agonist/α_1-adrenoceptor antagonist (Chidlow *et al*., 2001). This is slightly greater than that of approximately 25% evoked by the 5-HT_{1A} receptor agonist, 8-hydroxy-dipropyl-aminotetralin (Chu *et al*., 1999).

Cholinoceptor and adrenoceptor antagonists can alter intraocular pressure values both in the presence or in the absence of added compounds (Pintor & Peral, 2000; Sugiyama *et al*., 2001). The pre-incubation with atropine and hexamethonium or yohimbine and ICI 118,551 showed that the effect of melatonin is related to the innervation that controls either the formation or drainage of aqueous humour. It is not clear at present how melatonin and 5-MCA-NAT produce a reduction in intraocular pressure, but the implication is that it evokes release of acetylcholine and noradrenaline, presumably from parasympathetic and sympathetic nerve terminals in the eye. In several other systems, interactions between melatonin on cholinergic nerves have been described. For example, it has been reported that melatonin can increase acetylcholine release by the activation of cholinergic neurons in the nucleus accumbens (Paredes *et al*., 1999). Melatonin can also modify vasopressin levels by a mechanism which is atropine-sensitive (Bojanowska & Forsling, 1997). Melatonin and 5-MCA-NAT may act through a similar mechanism, however, it is unlikely to be the only mechanism of action since it was possible to see only a partial block of the hypotensive effect of 5-MCA-NAT by the adrenoceptor antagonists.

Melatonin has been claimed to be the endogenous compound responsible for the reduction in intraocular pressure during the night (Samples *et al*., 1988; Liu & Dacus, 1991). The levels of this neurohormone have been quantified in various animal models. Concentrations of 2.0 nM in human aqueous humour (Martin *et al*., 1992) and 1 nM in pigmented rabbit aqueous humour (Yu *et al*., 1990), indicate that this compound may act as the physiological effector regulating intraocular pressure during the circadian period (Liu & Dacus, 1991). The lack of effect of the melatonin receptor antagonists on resting levels of IOP indicates that during the day there is no endogenous melatonin controlling the IOP. This is consistent with the observation that luzindole has no effect on IOP at doses shown to antagonise exogenous melatonin (Pintor *et al*., 2001).

We have demonstrated that instillation of the selective MT_3 agonist 5-MCA-NAT is able to reduce intraocular pressure acting *via* specific receptors. Considering the strong reduction of intraocular pressure, 5-MCA-NAT may be useful for treating those pathologies where intraocular pressure is abnormally elevated.

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