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Melatonin receptors trigger cAMP production and inhibit chloride movements in non-pigmented ciliary epithelial cells

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Running title: Melatonin reduces the ciliary release of chloride via cAMP

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Abbreviations: MQAE, N-(6-methoxyquinolyl) acetoethyl ester; 4-P-P-DOT, 4-Phenyl-2-propionamidotetralin; prazosin; bis-I, Bisindolylmaleimide; Forsk, forskolin; Ksv, Stern-Volmer constant; MTn, melatonin receptor n.

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Abstract

Melatonin and its analogue 5-MCA-NAT are active compounds reducing intraocular pressure (IOP). This action is mediated through MT$_2$ and the putative MT$_3$ melatonin receptor producing a transient reduction of IOP which last for a few hours and has not yet been characterized. The use of melatonin and its analogue are causing a decrease in chloride efflux from rabbit non-pigmented epithelial cells (NPE), possibly explaining the decrease in IOP. Melatonin and 5-MCA-NAT inhibited rabbit NPE chloride release in a concentration-dependent manner, while the pD$_2$ values were between 4.5±1.2 and 4.4±1.0, respectively. Melatonin hypotensive action was enhanced by the presence of MT$_2$ antagonists such as DH97 and 4-P-P-DOT, and by the non-selective melatonin receptor antagonist luzindole. Prazosin 1.5 µM partially reverses the melatonin action by acting as a selective MT$_3$ antagonist. However, at 15 nM it acts as an alpha-adrenergic receptor antagonist, enhancing the melatonin effect.

Regarding the intracellular pathways triggered by melatonin receptors, neither PLC/PKC pathway nor the canonical reduction of intracellular cAMP were responsible for melatonin or 5-MCA-NAT actions. On the contrary, the application of these substances produced a concentration-dependent increase of cAMP, with pD$_2$ values of 4.6±0.2 and 4.9±0.7, for melatonin and 5-MCA-NAT respectively. To sum up then, melatonin reduces the release of chloride concomitantly to cAMP generation. The reduction of Cl$^-$ secretion accounts for a decrease in the water outflow and therefore a decrease in aqueous humour production. This could be one of the main mechanisms responsible for the reduction of IOP after application of melatonin and 5-MCA-NAT.
Introduction

Melatonin is a relevant hormone controlling various physiological actions, many of which are related to the photoperiod (Pandi-Perumal et al., 2006). It is generally accepted that blood melatonin levels increase overnight as a consequence of its production and release from the pineal gland (Caprioli and Sears, 1984; Alarma-Estrany and Pintor, 2007; Stehle et al., 2011). This gland is not exclusively responsible for melatonin production. In the orbital cavity and in the eye, for instance, some areas such as the retina (Cardinali and Rosner, 1971a; Cardinali and Rosner, 1971b; Alarma-Estrany and Pintor, 2007), ciliary body (Martin et al., 1992; Alarma-Estrany and Pintor, 2007) and hardierian glands (Djeridane et al., 1998; Alarma-Estrany and Pintor, 2007), have the ability to synthesise and release melatonin. As occurs in other tissues, the eye melatonin exerts many of its actions by means of membrane receptors termed melatonin receptors, divided into MT₁, MT₂ and the putative MT₃ melatonin receptors (Alarma-Estrany and Pintor, 2007; Dubocovich et al., 2010).

One significant physiological process in the eye undergoing circadian control is the regulation of the intraocular pressure (IOP). IOP is the result of the balance between the production of the aqueous humour by the ciliary body (Civan and Macknight, 2004; Do and Civan, 2004) and its drainage by the trabecular meshwork and uvesoscleral pathway (An and Ji, 2011; Pattabiraman et al., 2012). In this sense, the circadian fluctuation of IOP has been widely studied (Rowland et al., 1981; Liu et al., 2011), as well as its melatonin levels relationship (Samples et al., 1988). Interestingly, this circadian pattern can be modified by applying exogenously melatonin or any of its analogues (Pintor et al., 2001; Serle et al., 2004). In this sense, the topical application of melatonin on the ocular surface produces a transient reduction in IOP which is enhanced by melatonin analogues such as 5 methylcarboxyamino N-acetyl tryptamine (5-MCA-NAT) or N-Butanoyl 2-(9-methoxy-6H-iso-indolo[2,1-a]indol-11-yl)-ethan-amine (IIK7). Melatonin and these two analogues acting through MT₃ and MT₂
melatonin receptors, can modify IOP by acting on the ciliary body (Pintor et al., 2001; Pintor et al., 2003; Alarma-Estrany et al., 2007).

From a therapeutic point of view, the implications of melatonin and analogues on IOP control are relevant. IOP is elevated in primary open angle glaucoma, affecting more than 65 million patients all over the world (Quigley and Broman, 2006). In most of the cases, the treatment for the reduction of the abnormally elevated IOP is by means of adrenergic compounds, carbonic anhydrase inhibitors, prostaglandins or parasympathomimetics (Webers et al., 2008; Lee and Goldberg, 2011; Carta et al., 2012). Since melatonin reduces IOP in experimental models it would be of interest to see whether this effect is also feasible in humans. In this sense, some ophthalmologists have started to use melatonin to reduce IOP in patients undergoing cataract surgery, indicating the relevance of this molecule as regulator of IOP (Ismail and Mowafi, 2009), suggesting its possible use as a treatment of ocular hypertension.

The actions of melatonin in the reduction of IOP are taking place mainly on the ciliary body as commented above. Interestingly, the actions melatonin and analogues can exert on this part of the eye, are not only the short-term IOP reduction (Pintor et al., 2001; Pintor et al., 2003) but they also produce a long-term effect. This second aspect of melatonin action is due to the modification in the expression of key genes encoding for proteins relevant in the control of aqueous humour production such as adrenergic receptors (Crooke et al., 2011) and carbonic anhydrases (Crooke et al., 2012). These proteins indirectly regulate water efflux, therefore controlling aqueous humour production and subsequently IOP.

On the contrary, little is known about the mechanisms of IOP rapid reduction by melatonin or 5-MCA-NAT. It is supposed that melatonin and analogues may also modify the aqueous humour water production, but to date there is no evidence of the possible mechanism involved.
One of the ions driving the water movement from the ciliary body to the posterior chamber to form aqueous humour is chloride (Civan and Macknight, 2004; Do and Civan, 2004). Since there may be a connection between melatonin receptor activation, chloride movement and aqueous humour production, the present experimental work studies the ability of melatonin and 5-MCA-NAT to modify the chloride efflux in ciliary body non-pigmented epithelial cells.

Methods

Material and Methods

Cell culture

Non-pigmented epithelial cells, NPE cells, an immortalized cell line of rabbit ciliary non-pigmented epithelium, were kindly supplied by Dr. Coca-Prados. Cells were grown in high glucose DMEM (Gibco-Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS) (Sigma, St. Louis, USA) and 0.05 mg/ml Gentamicin (Gibco), at 37°C in humidified atmosphere 5% CO₂-95% air.

Chloride efflux studies

Chloride efflux was measured using 6-(6-methoxyquinolyl) acetoethyl ester (MQAE) (Invitrogen) as chloride indicator (Lee et al., 1984). Briefly, cells were seeded in 48-wells plates (Iwaki, Tokyo, Japan) at a density of 10⁴ cells/well, and grown to confluence. 20 hours before the experiment, the cells were incubated in DMEM containing MQAE 1mM (West and Molloy, 1996). After incubation, the cells were washed three times in chloride-containing buffer, and incubated in this buffer, with or without different antagonists, during 10 min (at 37°C) to induce chloride channel activation. This buffer consisted of: 2.4 mM K₂HPO₄, 0.6 mM KH₂PO₄, 1 mM
CuSO₄, 1 mM MgSO₄, 10 mM Hepes, 10 mM D-glucose and 130 mM NaCl. (Panreac, Barcelona, Spain). After this new incubation, the buffer was replaced by a chloride-free buffer with or without the corresponding agonist or antagonist. In this buffer, NaCl was replaced by an equimolar concentration of NaNO₃ (Panreac). Plates were then read on Fluoroskan FL fluorescence plate reader (Thermo Labsystems Inc. Waltham, MA) following the methodology described by Huete et al (2010).

Melatonin receptor antagonists luzindole (non-specific antagonist melatonin receptor), 4-P-P-DOT, DH-97 (MT₂ receptor antagonists) (Tocris, Bristol, UK) (100µM) and prazosin (alpha-1 and MT₃ receptor antagonist) (Santa Cruz Biotechnology) (15nM, 150nM, 1.5µM), specific alpha-1 antagonist corynanthine (Santa Cruz Biotechnology) (100µM), protein kinase C inhibitors staurosporine (100nM) and Bisindolylmaleimide (bis-I)(1 µM), phospholipase C inhibitor U73122 (3 µM) and protein phosphatase 1/2A okadaic acid (100nM) (Tocris), were added in chloride-containing buffer and maintained in a chloride-free buffer. Forskolin (Forsk)(40 µM) and IBMX (50 µM) were added only in a chloride-free buffer.

Data are expressed as mean ± S.E.M. of relative fluorescence units (RFU) normalized to the initial time 

\[ F_t - F_0 \]

where \( F_t \) is the fluorescence at time \( t \), and \( F_0 \) is the initial fluorescence.

**Calculation of the Stern-Volmer constant**

To calculate Stern-Volmer constant (\( K_{sv} \)) in NPE cells, we used the double ionophore technique. As described by West and Molloy (1996), isosmotic buffers with different concentrations of chloride were used to create a range of chloride concentrations. The whole range of concentrations was assayed simultaneously in the same plate to avoid MQAE leakage problems during monitoring of chloride concentrations in the given sample.
To equilibrate intracellular and extracellular buffer chloride concentration, 10μM tributyltin (Sigma) and 5μM nigericin (Sigma) were added to the buffer. Applying changes in fluorescence in cells under these different chloride buffers, a Stern-Volmer plot was obtained, responding to the equation:

\[ \frac{F_0}{F} = 1 + K_{sv} [Cl^-] \]

Where \( F_0 \) are the MQAE fluorescence in absence, \( F \) in presence of chloride and \( K_{sv} \) (Stern-Volmer constant) is the slope of linear plot representing the efficiency of collisional quenching.

Concentration-response curves
To determine concentration-response curves, different concentrations of melatonin (Sigma) and 5-MCA-NAT (Tocris) were tested according to the previous methodology. Concentrations tested varied in ranges from 1 nM to 150μM. The \( F_{max} \) and the slope of the straight segment of each dose curve were converted into % of fluorescence (\( F_t - F_0 \)) versus control (taken as 100%) and plotted. Data are plotted as % of mean fluorescence (vs. control) in RFU ± s.e.m., versus logarithm of agonist concentration.

Cyclic AMP Studies
Cyclic AMP accumulation was measured using cAMP Enzyme Immune Assay EIA KIT (Cayman Chemical Company, Ann Harbor, USA). Cells were grown to confluence in 6-wells plates (Iwaki). Then, medium was replaced with fresh medium containing different concentration of agents. Antagonist were pre-incubated during 15 minutes and after 10 minutes of incubation with agonists, the medium was removed and immediately each well was incubated for 20 minutes containing 275 μl HCl 0.1M. The cells were then scraped and centrifuged at 1000xg
during 10 minutes, and the supernatant was assayed as indicated in the protocol of cAMP Cayman EIA Kit. The results were expressed as pmol/ml to avoid errors due to the re-suspension and protein quantification in small volumes. All wells were examined before the assay and cells were counted, to ensure homogeneity of the study. Results were shown as a mean ± s.e.m.

**Statistical analysis**

GraphPad Prism (GraphPad software Inc. San Diego, USA) was used to obtain the linear regression (for the straight lines), non-linear regression curves and calculation of slope (for the straight lines and straight segments of the curves), pD$_2$ and IC$_{50}$ values. Statistical significance was calculated by ANOVA (Bonferroni post-tests) and Student’s $t$-test, when needed. Value of $p$ < 0.05 was taken as significant.

**RESULTS**

**Effect of melatonin and 5-MCA-NAT on ciliary body epithelial cells**

MQAE is a high sensitive chloride fluorescence probe. This fluorescence is quenched in presence of chloride, so that changes in fluorescence are inversely proportional to changes in chloride concentration. Using protocol specified in methods, it was possible to verify the linear relationship between dye fluorescence and intracellular chloride concentrations in this cell type (Figure 1A). Using this data it was also possible to calculate the Stern-Volmer constant ($K_{sv}$), whose value was 12.18 ± 0.89 M$^{-1}$ (n=6), in rabbit NPE cells. This constant permits us to calculate the intracellular Cl$^-$ concentration which was 74.37±3.8 mM (n=6) in rabbit non-pigmented ciliary epithelial cells when external Cl$^-$ concentration was 130 mM.
Using this fluorescence probe, we were able to measure changes in the intracellular chloride concentrations after challenging the cells with melatonin and analogues. In particular, melatonin and 5-MCA-NAT were able to modify intracellular chloride. Normalized plots of fluorescence vs. time always presented sigmoid patterns. From these plots, three different parameters were calculated: On the one hand, the maximal fluorescent signal, $F_{\text{max}}$, which corresponded to the minimal intracellular chloride concentration $([\text{Cl}^-])$. On the other hand $t_{50}$, which corresponded to the time necessary to produce 50% of $F_{\text{max}}$ that is an indication of how fast the release of chloride was. Finally, the slope of the curve straight segment representing the velocity ($V$) of the chloride efflux. We have used these parameters to evaluate the treatments versus the untreated cells which were taken as controls. These control cells showed the normal release of chloride of this cell type in chloride-free buffer. As previously indicated, non-treated cells depicted a sigmoid behaviour that was consistent to a chloride efflux from inside the cells to the extracellular milieu (Figure 1B). From this curve it was possible to calculate $F_{\text{max}}$, $t_{50}$ and $V$ values presented in table 1.

Melatonin and 5-MCA-NAT (100 $\mu$M), clearly and significantly changed the Cl$^-$ efflux as can be seen in Figure 1B. There were differences in the $F_{\text{max}}$, $t_{0.5}$ and $V$, when comparing melatonin and 5-MCA-NAT with control (Figure 2, table 1). Interestingly, both melatonin and 5-MCA-NAT showed a strong inhibition when compared to control. Indeed, melatonin completely inhibited chloride release for about 602 s (10.0 min) and 5-MCA-NAT for roughly 860 s (14.3 min). After that, and in the presence of these two compounds, the slope of their respective curves was not as steep as the control and moreover, they did not reach the $F_{\text{max}}$ the control did (Figure 1B).
Concentration-response curves for melatonin and 5-MCA-NAT

In order to fully study the effect of melatonin and 5-MCA-NAT on chloride fluxes, cells were challenged with graded concentrations of both compounds following the protocol described in methods. We focused on how these two compounds were able to diminish cell fluorescence (Fmax) and its concentration dependency. In this sense, and as can be seen in Figure 2A, both compounds depicted concentration-response curves which were almost identical. From both curves it was possible to obtain pD2 value of 4.5 ± 1.2 for melatonin and 4.4 ± 1.0 for 5-MCA-NAT (n=5). These values corresponded to EC50 values of 31.6 μM and 39.8 μM for melatonin and 5-MCA-NAT respectively.

When, instead of studying the relationship between concentration and cell fluorescence, we analysed concentration vs. changes in the slope (velocity) for melatonin, 5-MCA-NAT, sigmoidal curves were obtained, providing interesting data. As observed in Figure 2A, melatonin presented a pD2 value of 4.7 ± 0.2, while 5-MCA-NAT provided a pD2 value of 5.0 ± 0.1, corresponding to EC50 values of 19.9 μM and 10 μM for melatonin and 5-MCA-NAT respectively (n=5). Interestingly, the Hill slopes for both compounds were different, their values being 0.6 ± 0.3 for melatonin and 1.8 ± 0.2 for 5-MCA-NAT (Figure 2B) (n=5).

Studies with antagonists

Although the presence of melatonin receptors, mainly MT2 and MT3 have already been described in the ciliary body non-pigmented epithelial cells, we have tried to investigate which receptor is involved in the changes in the intracellular chloride concentrations. In this sense, the MT2 antagonist 4-P-P-DOT was unable to modify the effect of melatonin. Interestingly another MT2 antagonist, DH97 and the non-selective melatonin receptor antagonist luzindole, enhanced the effect triggered by melatonin and 5-MCA-NAT. Melatonin effect in the presence of DH97 reduced Cl⁻ efflux from 47.2 ± 5.3 % to 21.1±1.4 % and luzindole to 19.8 ±1.1 % (n=6).
5-MCA-NAT effect changed from 43.4±3.4 % (alone) to 33.3±1.3 % when 4-P-P-DOT was present, to 17.9±2.3 % in the presence of DH97 and to 17.5±2.1% when luzindole was present (n=6) (Figure 3). In the same sense, prazosin (MT$_3$ antagonist receptor) 15nM, surprisingly enhanced the effect of 5-MCA-NAT to 29.5±4.3 %. Nevertheless, the values were very close to those of 5-MCA-NAT alone (44.8±3.5 %) when we used prazosin 150nM, and partially reverted 5-MCA-NAT effect (78.3±16.5 %) when it was used at 1.5µM concentration (n=6) (Figure 4A). Similar results were obtained using melatonin (n=6) (Figure 4B), however no statistical significance differences were reached between melatonin and melatonin + prazosin 15 nM. This lack of statistical significance difference is probably due to a relatively high S.E.M regarding “n” used, and not to a difference in the behavior of both substances.

**Adrenoreceptors Alpha 1 implications in chloride regulation**

The results obtained with prazosin at a low concentration, produced an enhancement of 5-MCA-NAT hypotensive effect while at high concentration it produced the partial inhibition of 5-MCA-NAT effect suggesting the involvement of an adrenoreceptor alpha-1 in the regulation of chloride secretion.

As is shown in Figure 5A and 5B (n=6), corynanthine (alpha-1 antagonist) was able to enhance the effect of 5-MCA-NAT on chloride efflux from 43.36±5.53% to 28.18±3.46%, confirming the role of alpha-1 receptors in the regulation of chloride flux. Corynanthine alone has no effect on the chloride secretion (data not shown).

**Second messengers triggered by melatonin and 5-MCA-NAT**

It has been claimed that MT$_3$ melatonin receptors are coupled to phospholipase C/protein kinase C (PLC/PKC) pathway (Huang et al., 2001). In order to see whether the effect of melatonin and 5-MCA-NAT was triggering the mentioned intracellular pathway, different
blocking agents of this route were tested in their ability to modify the Cl⁻ effluxes triggered by melatonin and 5-MCA-NAT.

As is shown in Figure 6, none of the compounds tested to inhibit the PLC-PKC pathway was able to produce a change in the fluorescence signal either alone or in the presence of melatonin or 5-MCA-NAT.

**Involvement of cAMP pathway**

We decided to investigate the canonical cAMP pathway which has been described to be negatively coupled to both MT₁ and MT₂ receptors. When adenylate cyclase activity was increased by means of a forskolin and IBMX mixture (see methods), we could notice a reduction in Cl⁻ efflux as observed in Figure 7A. Since the chloride efflux triggered by adenylate cyclase activation resembled the behaviour of melatonin and 5-MCA-NAT, we studied the ability of these two substances to increase cAMP concentrations. The results showed that melatonin 100μM was able to increase intracellular cAMP levels of 58.05 ± 4.22 to 84.59 ± 6.78 pmol/ml (n=6). In the case of 5-MCA-NAT, 100μM results were similar, obtaining an intracellular cAMP concentration of 90.55 ± 5.53 pmol/ml (n=6) in the presence of the melatonin analogue.

Different concentrations of melatonin and 5-MCA-NAT were tested to characterize the dose-dependent behaviour of these substances. As shown in Figure 7B, graded concentrations of melatonin and 5-MCA-NAT evoked the accumulation of concomitant amounts of cAMP in rabbit NPE cells. The concentration-response curve provided a pD₂ value of 4.6 ± 0.2 for melatonin and 4.9 ± 0.7 for 5-MCA-NAT, which were equivalent to EC₅₀ values of 22.0 μM and 19.4 μM (n=6) for melatonin and 5-MCA-NAT respectively.

To investigate the involvement of the MT₃ receptor in this signalling pathway we blocked this receptor using prazosin 1.5 μM (n=6), measuring the accumulation of intracellular cAMP. The results were presented in Figure 8. Data showed a partial and significant reversion of
intracellular cAMP from 92.34±6.36 (5-MCA-NAT) to 74.36±4.32 pmol/ml (5-MCA-NAT+prazosin).

Discussion

The present manuscript describes the effect of melatonin and its analogue, 5-MCA-NAT acting on melatonin receptors of ciliary body non-pigmented epithelial cells. The main action of melatonin and its analogue is the modulation of intracellular chloride concentrations, which is important since chloride rules water movement and therefore is the key ion driving the production of the aqueous humour (Civan and Macknight, 2004). The aqueous humour is responsible for the correct eye shape and acts as a nutritional fluid for avascular structures such as the lens or the cornea (Civan, 1998). Under certain circumstances, a lack of drainage of the aqueous humour produces an elevation of intraocular pressure (IOP) possibly responsible for the pathology termed glaucoma.

There are different reports indicating that melatonin application reduces or increases IOP (Caprioli and Sears, 1984). We claim that melatonin and analogues reduce IOP in New Zealand white rabbits, to glaucomatous monkeys (Serle et al., 2004) and even human beings (Ismail and Mowafi, 2009).

Aqueous humour formation relies on the ability of the ciliary body cells (PE and NPE) to mobilize chloride ions from the stromal part of the ciliary body to the posterior chamber of the eye (Do and Civan, 2004). The results presented in this manuscript suggest that melatonin and 5-MCA-NAT may reduce IOP since they decrease the efflux of chloride from the cytoplasm of NPE towards the extracellular space. The effect of both melatonin and 5-MCA-NAT inhibiting this ion movement was strong during 10 and 14 min for melatonin and 5-MCA-NAT (100µM) respectively. After this interval of inhibition the rate of efflux increases, even though slope and Fmax, were always below the control values. This fact indicates that the inhibitory effect does
not only affect initial chloride efflux but inhibition was also present when the equilibrium was reached (see Figure 1). The substantial inhibition of the chloride release and the presumable inhibition of the aqueous humour formation seem to be higher than the IOP reduction observed “in vivo” (Pintor et al., 2003). In this sense, it is important to notice that the “in vitro” model we are using, although mimicking the conditions present in the ciliary body, has certain limitations. One of the main “in vitro” restrictions is that we are measuring the secretory layer NPE cells, producers of the aqueous humour, and not the drainage system, possibly the reason for such differences. Another limitation to take into consideration is that the measurements we have performed involve only Cl⁻ efflux processes. This implies that there is a Cl⁻ efflux following a concentration gradient (as occurs in the “in vivo” model) and, due to the lack of this ion in the extracellular buffer, mechanisms transporting this ion from the extracellular space to NPE cells cytoplasm are not fully activated. Consequently, the data obtained are dependent solely on the state of chloride channels and transporters. Also, we should emphasize the absence of the pigment epithelium (PE), which takes chloride from stromal and transfers it to NPE layer cells. The importance of PE and its function has been extensively described in the literature (McLaughlin et al., 1998; Do and To, 2000; Do et al., 2004a; Ni et al., 2006). However, the simultaneous study of both layers complicates precise conclusions concerning the contribution of each structure involved.

Although there are some limitations in the model we are using, it is important to emphasize that an analysis of the concentration-curves demonstrates that the effects depicted by melatonin and 5-MCA-NAT were highly similar but not identical (especially in Fmax), as we can see in Figure 1. This matches the results obtained for both substances on IOP in New Zealand white rabbits, where the effect of melatonin as an hypotensive agent is less robust than that of 5-MCA-NAT (Pintor et al., 2003).
An interesting and unexpected observation was the effect of the classical melatonin antagonists. The MT<sub>2</sub> antagonist DH97 and the MT<sub>1</sub>/MT<sub>2</sub> non-selective antagonist luzindole, potentiated the inhibitory effect of both melatonin and 5-MCA-NAT on chloride movement. This may suggest that melatonin and 5-MCA-NAT are acting through a different MT<sub>1</sub>/MT<sub>2</sub> or luzindole sensitive receptor. Moreover, when MT<sub>1</sub>/MT<sub>2</sub> or luzindole sensitive receptors were blocked, the effect of this putative receptor was enhanced, suggesting a different signalling pathway from the canonical described for melatonin receptors. This indirectly implies that the action of MT<sub>1</sub>/MT<sub>2</sub> may have an opposite effect, increasing the efflux of Cl<sup>-</sup>. Since MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors are negatively coupled to adenylate cyclase and there is a concomitant reduction in the concentrations of cAMP, the blockade of these two receptors might involve PLC/PKC pathway as happens in some models (Bowler et al., 1996; Godson and Reppert, 1997; Dortch-Carnes and Tosini, 2013). Interestingly, new studies are appearing indicating that new second messenger systems can be coupled to melatonin receptors. For instance, melatonin receptors produce a reduction in SNP-released nitric oxide and cGMP levels in human non-pigmented ciliary epithelial cells (Dortch-Carnes and Tosini, 2013). These authors indicate that, at least in part, melatonin and analogue effects can use this pathway in human NPE cells and that this second messenger system might be responsible for the melatoninergetic compound hypotensive effect. These results are perfectly compatible with those presented here and with previous ones in which we suggested that MT<sub>2</sub> melatonin receptor agonists reduce IOP (Alarma-Estrany et al., 2008).

Coming back to the second messengers being activated by melatonin and 5-MCA-NAT in our model, we were unable to detect any involvement of the PLC/PKC pathway. Some authors claimed that 5-MCA-NAT is not a selective MT<sub>3</sub> receptor agonist but it could activate MT<sub>1</sub> or MT<sub>2</sub> melatonin receptors (Vincent et al., 2010). If this is so, we should expect reductions
in the cytosolic concentration of cAMP, since, as already mentioned above, MT₁ or MT₂ melatonin receptors are negatively coupled to adenylate cyclase (Vanecek, 1998). The study on the ability of melatonin and 5-MCA-NAT to inhibit cAMP formation pointed in the opposite direction as it was impossible to see a reduction in cAMP production. On the contrary, we could observe that melatonin and its analogue increased cAMP concentrations in a concentration-dependent manner. This is not a common mechanism of signal amplification but it has been described in some models (Raviola, 1974; Beraldo and Garcia, 2005; Schuster et al., 2005). Indeed it modified chloride efflux, and this effect was mimicked when we applied forskolin and IBMX. The relationship between the increase of cAMP and the decrease of IOP is widely studied in the eye. In rabbits and monkeys, it is known that this decrease in IOP is associated to a significant decrease in AH secretion. Interestingly, it has been reported that 5-MCA-NAT is able to produce important increases in cAMP in chick retinas by a mechanism that may involve an MT₃ (Mel1c) binding site (Sampaio Lde, 2009).

When the involvement of the putative MT₃ melatonin receptor was studied, the use of the only available antagonist prazosin was tested. Low concentrations of this antagonist produced an unexpected potentiation of the action triggered by 5-MCA-NAT. Nevertheless, at higher concentrations (1.5 μM), prazosin did its antagonistic effect blocking melatonin and 5-MCA-NAT actions, indicating that at low micromolar concentrations it acts as a MT₃ receptor antagonist as previously described elsewhere (Dubocovich et al., 2003; Pintor et al., 2003; Alarma-Estrany et al., 2011).

Concerning the results obtained with prazosin at nanomolar concentrations, the observed contradictory effect potentiating 5-MCA-NAT action can be explained as an effect performed on alpha-1 adrenoceptors. This point was confirmed when the same effect was obtained with the selective alpha-1 adrenoceptor antagonist corynanthine, as was also described regarding the modulation of IOP in rabbits by other authors (Chidlow et al., 2001).
In the same way, we tested the action of prazosin, in micromolar range, on the cAMP accumulation induced by 5-MCA-NAT. Prazosin was able to partially revert the increased intracellular cAMP. This fact relates the cAMP increase with the MT₃ melatonin receptor.

It has been demonstrated that the chloride efflux from NPE cells is one of the most, if not the most important, ion controlling aqueous humour secretion as previously commented (Jacob and Civan, 1996; Forrester, 2002). However, the nature of the proteins ruling the movement of Cl⁻ (JC, 1989; Paulmichl et al., 1992; Chen et al., 1999; Do et al., 2006) and the transmitters regulating chloride secretion and secondarily IOP is not clear. For example, traditionally it has been accepted that the blockade of β-adrenergic receptors is a pharmacological approach to decrease IOP (Freddo, 1987). β-adrenergic receptors are positively coupled to adenylate cyclase but surprisingly, cAMP activates some chloride channels. The explanation seems to be that the effects of cAMP itself and β-adrenergic receptors may reflect a complex regulation evolving different mechanisms (McLaughlin et al., 2001) and presumably several channels and transporters as suggested by Do and Civan (2004). Recently, it has been demonstrated that the increase in cAMP levels does not only activate PKA but also may have a direct effect on ion channels. Fleishauer and co-workers demonstrated that cAMP action is conducted via a direct action on chloride channels and not by means of PKA in the ciliary body (Fleischhauer et al., 2001). Also experiments performed using patch clamp techniques have demonstrated that cAMP can activate chloride channels such as the maxi-Cl⁻, although its physiological role “in situ” has not been fully elucidated (Mitchell and Civan, 1997; Do et al., 2004b). Small changes in cAMP cytosolic concentrations may produce vast changes in the chloride efflux. In this sense, Huang and co-workers (Huang et al., 2001) demonstrated that small changes in cAMP levels can cause profound variations in the lung chloride efflux. In addition, transepithelial chloride secretion in the bovine and porcine ciliary body is severely inhibited by cAMP (Do et al., 2004a;
Ni et al., 2006). These results may explain and reinforce the results obtained with melatonin and 5-MCA-NAT described in the present manuscript, also matching the results obtained by Sampaio (Sampaio Lde, 2009).

The results obtained in this study, suggest that a prazosin sensitive melatonin receptor, different from the MT₁ or MT₂, is responsible for the decrease in Cl⁻ outflow. Both melatonin and 5-MCA-NAT would bind to the three melatonin receptor subtypes, probably with different affinity. MT₁/MT₂ receptors produce an opposite effect on intracellular cAMP levels, but not so intense as the non-cloned melatonin receptor (putative MT₃ receptor) would do. This could explain the observed effect when the inhibition of MT₁ and MT₂ melatonin receptors is performed. Differences in the affinity of the agonists for each receptor subtype, may explain the observed changes in cAMP at non-saturating concentrations of these two agonists. When melatonin acts, it does so mainly through MT₁/MT₂ receptors while 5-MCA-NAT by MT₂ and preferentially by MT₃. Also, there is more and more evidence pointing to melatonin receptor heterodimerization to explain these differences. In this sense, Ayub and co-workers have described that MT₂ melatonin receptors can form heterodimers with MT₁ receptors, and that the formation of these heterodimers modifies the affinity for the agonists and antagonists (Ayoub et al., 2004). Moreover, there are cases where heterodimerization produces significant changes in ligand binding, signaling, or trafficking, and this may also explain the activation of AC by melatonin and 5-MCA-NAT (Gazi et al., 2002). More work in this area is necessary to fully understand the real mechanism underlying the melatonin receptors involved in the production of the aqueous humor in our model.

In summary, we can conclude that melatonin and 5-MCA-NAT acting through MT₃ melatonin receptors can participate in the modulation of certain chloride channels, by a process
that involves cAMP increase. This might be the potential mechanism to reduce and modulate net aqueous humour secretion explaining why melatonin and some of its analogues can reduce IOP in experimental animals (Pintor et al., 2001; Pintor et al., 2003).
Authorship contributions

Participated in research design: Pintor

Conducted experiments: Huete-Toral

Performed data analysis: Martínez-Águila

Wrote or contributed to the writing of the manuscript: Crooke
References


Footnotes

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Legends for Figures

Figure 1: Effect of melatonin and 5-MCA-NAT on chloride efflux.

A- Stern-Volmer linear regression fit in NPEsv cells. We can see that there is a linear relationship between intracellular chloride and fluorescence intensity. Data represents mean ± s.e.m. of normalized fluorescence (Ft-F0) in varying ratios of intracellular chloride (n=6).

B- Traces represent normalized fluorescence at time 0 (Ft-F0) of control and treated cells with melatonin (open circles) and 5-MCA-NAT (filled triangles) both at 100 μM, versus time. There were extremely significant differences between treated cells and control in Fmax, t50 and v (slope of straight segment) in all the cases with a significance of $p<0.001$ (n=6). Values represent the mean ± s.e.m.

Figure 2: Concentration-response curves of melatonin and 5-MCA-NAT analyzing Fmax and changes in the slope.

A.- Concentration-response curves for melatonin (open circles) and 5-MCA-NAT (filled triangles) assayed at concentrations ranging from $10^{-9}$ M to $10^{-4}$ M. No significant differences have been found between melatonin and 5-MCA-NAT. Values are the mean ± s.e.m. (n=5).

B.- Concentration-response curves for melatonin (open circles) and 5-MCA-NAT (filled triangles) assayed at concentrations ranging from $10^{-9}$ M to $10^{-4}$ M. Points on the graph represents the variation of the slope (V) in the straight segment of the fluorescence curve at the mentioned concentrations. Non-linear regression asymmetric (five parameter) was plotted. R square for melatonin and 5-MCA-NAT was respectively 0.96 and 0.98. Values are the mean ± s.e.m (n=5).

Figure 3: Effect of MT2 and non-selective MT receptor antagonists on the melatonin and 5-MCA-NAT secretory responses.

Antagonists for melatonin receptors at the concentrations described in our methods, were tested in their ability to block melatonin and 5-MCA-Nat effects. All data are expressed as % of the normal (control) chloride intracellular concentration at Fmax. Antagonists did not reverse melatonin and 5-MCA-NAT effect, and on the contrary, they were able to increase melatonin and 5-MCA-NAT effect (***$p<0.001$; n=6). Values are the mean ± s.e.m.

Figure 4: Effect of prazosin antagonism on the secretory response triggered by melatonin and 5-MCA-NAT.
A.- Effect of prazosin at 15 nM, 150 nM and 1.5 µM on chloride efflux elicited by 5-MCA-NAT 100µM. Prazosin at the lowest concentration enhanced the action of 5-MCA-NAT, however at the highest concentration partially antagonized the effect of 5-MCA-NAT ($p<0.05; n=6$).

B.- Effect of prazosin at different concentration (see A), on chloride efflux elicited by melatonin 100µM. The results were very close to those obtained with 5-MCA-NAT ($p<0.05; n=6$).

Figure 5: Action of corynanthine (alpha-1 antagonist receptor) on the secretory effect of 5-MCA-NAT.

A.- Normalized fluorescence versus time in NPE$_{sv}$ cells using 5-MCA-NAT 100µM, and corynanthine 100µM (n=6).

B.- Normalized fluorescence in $F_{\text{max}}$ at different treatments. Corynanthine clearly enhances the action of 5-MCA-NAT alone ($p<0.05; n=6$). Corynanthine alone did not differ from controls.

Figure 6: Effect of PKC-PLC inhibitors on the secretory response triggered by melatonin and 5-MCA-NAT.

The responses of melatonin or 5-MCA-NAT in the presence of a variety of PLC/PKC pathway (see methods) were completely unaffected when treated with these agents (n=8). Values are the mean ± s.e.m.

Figure 7: Effect of melatonin and 5-MCA-NAT cAMP production on forskolin + IBMX on chloride efflux

A.- Effect of forskolin + IBMX on chloride efflux. The plot represents normalized fluorescence at time 0 ($F_t-F_0$) of control and Forsk-IBMX treated cells. There was a statistically significant reduction in the rate of increase of fluorescence in Forsk-IBMX group compared to control, and therefore a decrease in chloride efflux ($p<0.01; n=15$).

B.- Concentration-response curves for melatonin (open circles) and 5-MCA-NAT (filled triangles) assayed at concentrations ranging from $10^{-6}$ M to $10^{-3}$ M, on the generation of cAMP. Values represent the mean ± s.e.m (n=6).

Figure 8: Effect of prazosin on cAMP production promoted by 5-MCA-NAT

Addition of prazosin 1.5 µM with 5-MCA-NAT 100 µM as described in methods. Prazosin was able to partially revert the cAMP accumulation produced by 5-MCA-NAT ($p<0.05$, n=6).
Table 1.- Effect of melatonin and 5-MCA-NAT on chloride efflux

<table>
<thead>
<tr>
<th></th>
<th>Fluorescence max $F_{\text{max}}$ (RFU)</th>
<th>$T_{50}$ (sec)</th>
<th>Slope (RFU/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.283 ± 0.088</td>
<td>2748.08</td>
<td>6.66x10^{-4} ± 3.80x10^{-6}</td>
</tr>
<tr>
<td>Melatonin</td>
<td>3.151±0.02515</td>
<td>5242.95</td>
<td>3.23x10^{-4} ± 1.34x10^{-5}</td>
</tr>
<tr>
<td>5-MCA-NAT</td>
<td>1.756±0.1054</td>
<td>4010.27</td>
<td>2.70x10^{-4} ± 3.94x10^{-6}</td>
</tr>
</tbody>
</table>

Data of $F_{\text{max}}$, $T_{50}$ and slope are expressed as mean ± s.e.m. $F_{\text{max}}$ was estimated by sigmoidal allosteric curve regression ($R^2$ square for control, melatonin and 5-MCA-NAT are respectively 0.94, 0.93 and 0.86). Highly significant differences ($p<0.001$) were found in $F_{\text{max}}$, $T_{50}$ and slope between treated cells and controls (***). However, there was no significant difference between treatments of melatonin and 5-MCA-NAT except $F_{\text{max}}$ ($p<0.05$) (##) (n=6).
Figure 1
Figure 2

A

%Normalized fluorescence (Ft-F0) RFU vs control

log [Agonist] M

B

Slope of normalized fluorescence (Ft-F0) RFU

log [Agonist] M
Figure 3
A

B

Figure 4
Figure 5

A

Normalized fluorescence ($F/F_0$) RFU vs time (sec)

- control
- 5-MCA-NAT
- 5-MCA-NAT + corynanthine

B

% Normalized fluorescence ($F/F_0$) RFU vs control

- control
- 5-MCA-NAT
- 5-MCA-NAT + corynanthine

Figure 5
Figure 7

A

![Graph A: Normalized fluorescence (Ft-F0) RFU over time (sec)]

B

![Graph B: [AMPc] pmol/ml vs. log [Compound] M]

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