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Dual coupling of MT_1 and MT_2 melatonin receptors to cyclic AMP and phosphoinositide signal transduction cascades and their regulation following melatonin exposure

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Abstract

In this investigation, we wanted to determine whether MT_1 or MT_2 melatonin receptors are capable of coupling to the phosphoinositide (PI) signal transduction cascade. In addition, we wanted to assess the effects of chronic melatonin exposure on MT_1 and MT_2 melatonin receptor-mediated stimulation of PI hydrolysis. We also assessed the effects of chronic melatonin exposure on other parameters of the MT_2 melatonin receptor function including total specific 2-[^{125}I]-iodomelatonin binding, the affinity of melatonin for the receptor, and melatonin (1 nM)-mediated inhibition of cyclic 3′,5′-adenosine monophosphate (cAMP) accumulation. Investigation of the PI signal transduction cascade activated by either the MT_1 or MT_2 melatonin receptor expressed in Chinese hamster ovary (CHO) cells showed that melatonin (1 pM to 1 mM) was able to stimulate the formation of PIs to ~40–60% over basal [EC_{50}: MT_1 = 29 nM (2–300 nM) and MT_2 = 1.1 nM (0.32–3.5 nM), $N = 5$]. This response was mediated via receptors based upon the findings that melatonin did not stimulate the formation of PIs in CHO cells devoid of receptor and that antagonism of MT_2 melatonin receptors by 4P-PDOT (AH 024; 4-phenyl-2-propionamidotetralin) attenuated melatonin-mediated stimulation of PI hydrolysis in CHO cells expressing the MT_2 melatonin receptor. The consequence of chronic melatonin exposure on MT_1 and MT_2 receptor function was also examined. Pretreatment of either MT_1- or MT_2-CHO cells with melatonin (1 μM for 5 hr) resulted in: (a) a complete loss of melatonin-mediated stimulation of PI hydrolysis, and (b) an attenuation of melatonin (1 nM)-mediated inhibition of forskolin-induced cAMP accumulation by ~20–40%. The desensitization of the PI hydrolysis signal transduction cascades coupled to either MT_1 or MT_2 melatonin receptors following chronic melatonin exposure was not due to depleted phospholipid pools, to elevated basal levels, or to decreases in receptor affinity and density. This dual coupling of melatonin receptors to different signal transduction cascades may contribute to the diversity of melatonin receptor function *in vivo*.
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Keywords: Melatonin; MT_1 melatonin receptor; MT_2 melatonin receptor; Phosphoinositide hydrolysis; Cyclic AMP

1. Introduction

Melatonin is the primary hormone of the vertebrate pineal gland and is a small and highly lipophilic molecule that can traverse membranes easily [1,2] and accumulate up to 30 times its concentration within the blood [1,3], thus affecting both cytosolic and nuclear components of the cell. Therefore, melatonin may mediate its effects through receptor-dependent and -independent mechanisms.

Two high-affinity human melatonin receptors have been cloned and, by IUPHAR classification, denoted as either the MT_1 (formerly known as Mel_{1a} [4]) or MT_2 (formerly known as Mel_{1b} [5]) subtypes. These receptors belong to the family of G-protein-coupled receptors. Using both pharmacological and molecular biological approaches, a role for MT_1 melatonin receptors in inhibiting neuronal firing in the mouse SCN [6], and in vasoconstriction of the rat artery [7,8] has been demonstrated, and reviewed [9]. Additionally, MT_2 melatonin receptors may also play a role in retinal physiology [10], in the entrainment of circadian rhythms [11], and, in contrast to the role of MT_1 melatonin receptors, may cause dilation of the rat caudal artery [7], as reviewed [9]. Activation of either the MT_1 or MT_2 receptor by melatonin (0.1 pM to 1 nM)

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Abbreviations: cAMP, cyclic 3′,5′-adenosine monophosphate; CHO, Chinese hamster ovary; DMEM, Dulbecco's Modified Eagle's Medium; PI, phosphoinositide; PLC, phospholipase C; SCN, suprachiasmatic nucleus of the hypothalamus.

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results in the inhibition of forskolin-induced cAMP formation [4,5,12–14] with a subsequent decrease in activated protein kinase A [12,15] and a decrease in the phosphorylation of cAMP response element binding (CREB) protein [15,16]. Higher concentrations of melatonin (1 nM to 1 μM) have been shown to stimulate PI hydrolysis in Syrian hamster RPMI melanoma cells [17] and in chick brain tissues slices [18]. In another study, activation of MT_1 melatonin receptors expressed in HEK 293 cells or in cultured pars tuberalis cells by melatonin (1 μM) increased intracellular calcium levels [19]. This increase in intracellular calcium levels is due most probably to an activation of the PI hydrolysis signal transduction cascade [19].

Melatonin is released during the hours of darkness. The duration of high melatonin synthesis and, therefore, blood levels during the night depend upon the length of the light/dark cycle [1]. Therefore, melatonin receptors are exposed to high levels of melatonin for 8–16 hr each day. Prolonged exposure to melatonin may result in the regulation of specific melatonin receptor subtypes. To date, it has been shown that a desensitization of MT_1 melatonin receptors linked to cAMP-dependent pathways occurs following melatonin exposure [14,15,20]. In addition, it was shown recently in primary or recombinant cell cultures that a desensitization of human MT_2 melatonin receptors coupled to cAMP-dependent pathways occurs following chronic melatonin exposure [14]. Even though no desensitization of melatonin receptors has been measured *in vivo* using functional analysis, decreases in melatonin receptor affinity and density have been reported during the hours of darkness [21–23]. Thus, the high levels of melatonin during this time may contribute to this reduced affinity and density of melatonin receptors.

In this study, we wanted to determine whether specific human melatonin receptor subtypes, MT_1 and MT_2, couple to the cascade involved with PI hydrolysis. Also, we wanted to examine whether chronic agonist exposure results in a desensitization of MT_1 and MT_2 melatonin receptors coupled to this pathway. Our approach was to use transfected cellular models to explore these events because only an antagonist selective for the MT_2 melatonin receptor is available, and it was essential to control for the receptor-independent effects of melatonin in the cell lines (especially MT_1-CHO).

2. Materials and methods

2.1. Cell culture and pretreatment conditions

To determine the effects of melatonin treatment on MT_1 and MT_2 melatonin receptor function, three cell lines were developed: CHO cells transfected with the PSV2-neo plasmid (Clontech; neo-CHO) and either the human MT_1 melatonin receptor cDNA [4] (MT_1-CHO) or the human MT_2 melatonin receptor cDNA [5] (MT_2-CHO). All three cell lines were maintained in F-12 medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (GIBCO-BRL) and 1% penicillin/streptomycin (GIBCO-BRL). The development and characterization of these cell lines have been described elsewhere [13,24]. Cells were incubated at 37° in a 5% CO_2 atmosphere. The initial passage number was noted for each cell line, and cells were passed twice weekly and utilized for experiments up to passage number forty. At that time, new cells were grown from a frozen stock. Following 2 days of growth, cells (neo-CHO, MT_1-CHO, MT_2-CHO) were treated as described in Section 3. Melatonin (Sigma Chemical Co.), solubilized in ethanol and then diluted in F12 or DMEM, was added to achieve a final concentration of 1 μM. Control treatments consisted of the addition of the appropriate amounts of ethanol vehicle alone. All treatments were carried out for 5 hr at 37° in a 5% CO_2 atmosphere.

2.2. Radioligand binding assays

To determine whether chronic melatonin pretreatment regulated melatonin receptor affinity and/or total 2-[^{125}I]-iodomelatonin binding, both saturation analysis and competition of melatonin for 2-[^{125}I]-iodomelatonin binding assays were performed.

2.2.1. Saturation analysis

Briefly, cells (neo-CHO, MT_1-CHO, and MT_2-CHO) were grown to subconfluent densities on plates. Cells were replenished with F-12 medium and subjected to vehicle or melatonin. Following pretreatment, cells were washed in buffer (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 0.62 mM KH_2PO_4, pH 7.4), removed in LIFT buffer (10 mM KH_2PO_4/$KHPO_4$, 1 mM EDTA, pH 7.4), and centrifuged (365 *g* for 5 min at 4°). The cells were resuspended in Tris–HCl (50 mM, pH 7.4) and added to tubes containing 0–1 nM 2-[^{125}I]-iodomelatonin (NEN/DuPont) in the absence (total binding) or presence (non-specific binding) of 1 μM melatonin. Cells were incubated for 1 hr at room temperature, harvested by filtration onto glass fiber filters, and counted for 5 min in a gamma counter as described [13]. Protein concentrations were determined by the method of Bradford using Bio-Rad Protein Assay reagents (Bio-Rad Inc.). Curves were generated by non-linear regression analysis least squares fit using Graphpad Prism software (Graphpad Inc.), and K_D and B_{max} values were calculated.

2.2.2. Competition binding

To determine whether melatonin pretreatment affected the affinity of melatonin for the receptor, cells (MT_1-CHO and MT_2-CHO) were grown to subconfluent densities on plates and then were subjected to the treatment conditions described for the total binding assays. Following treatment, cells were washed in PBS incomplete, removed from the plate in LIFT buffer, and centrifuged (500 *g* for 10 min at 25°). The cells were resuspended in Tris (50 mM, pH 7.4)

and added to tubes containing 2-[^{125}I]-iodomelatonin (80 pM for MT_1-CHO cells or 100 pM for MT_2-CHO cells) in the absence or presence of appropriate concentrations of melatonin (1 fM to 10 μM). Cells were incubated for 1 hr at room temperature (equilibrium conditions already established and reported [13]), harvested by filtration onto glass fiber filters, and counted as described previously [13]. Competition curves were constructed, and K_i values were determined by non-linear regression analysis least squares fit using GraphPad Prism software.

2.3. Second messenger assays

2.3.1. cAMP determination

These assays were performed as described previously with a slight modification [13]. To determine the effect of melatonin treatment on the ability of melatonin (1 nM) to inhibit forskolin-induced cAMP accumulation, cells (neo-CHO, MT_1-CHO, and MT_2-CHO) were grown overnight in a 24-well plate (0.5 mL cells/well) in an incubator at 37° in a 5% CO_2 atmosphere. The following day, cells were labeled with 2 μCi/mL of [^{3}H]adenine in F-12 medium containing vehicle or melatonin. Following the treatment, cells were washed five times with 1 mL PBS to remove free [^{3}H]adenine from the medium and any residual drug from the cells. cAMP formation was stimulated by the addition of F-12 medium containing: 30 μM rolipram alone (basal), 30 μM rolipram and 100 μM forskolin (maximal accumulation), or 30 μM rolipram, 100 M forskolin, and 900 pM to 1 nM melatonin. In some experiments, vehicle-treated cells were exposed to 1 μM 4P-PDOT (AH 024; 4-phenyl-2-propionamidotetralin; Tocris) alone or in combination with melatonin. The plates were incubated for 10 min at 37°. Reactions were stopped by aspiration of medium from each well and the addition of 1 mL of ice-cold trichloroacetic acid (5%) for 16 hr. [^{3}H]cAMP was separated from [^{3}H]ATP using Dowex (AG50W-X4; Bio-Rad) and alumina column chromatography as described previously [13]. Data were expressed as a percentage of the forskolin (maximal) response within each treatment group or within a cell line.

2.3.2. PI hydrolysis

To determine the effect of melatonin treatment on melatonin-mediated PI release, cells (neo-CHO, MT_1-CHO, and MT_2-CHO) were grown to a subconfluent density in 6-well plates and labeled with 2 μCi/mL of *myo*-[2-^{3}H]inositol in DMEM for 60 hr in an incubator at 37° with 5% CO_2. DMEM, instead of F12 medium, was used for these experiments because no incorporation of *myo*-[2-^{3}H]inositol into the lipid bilayer of the CHO cells occurred using the F12 medium. During the last 5 hr of the incubation with *myo*-[2-^{3}H]-inositol, cells were exposed to either vehicle or melatonin. Immediately following the pretreatment period, the medium was aspirated from all wells, and the cells were washed extensively to remove any residual melatonin or vehicle from the wells. Basal levels of [^{3}H]PIs were recorded: one at time zero (i.e. as soon as the incubation with melatonin began) and another following a 1-hr incubation with vehicle alone to account for any fluctuations in basal PIs during the course of the exposure. The wells used for basal PI determinations received 0.5 mL of freshly oxygenated Krebs (100 mM NaCl) solution (pH 7.4) containing 10 mM LiCl, whereas all of the other wells received 0.45 mL of freshly oxygenated Krebs, 10 mM LiCl plus 0.05 mL melatonin (1 pM to 1 mM). In some experiments, vehicle-treated cells were exposed to 0.05 mL 4P-PDOT (1 μM final concentration) alone or in combination with melatonin (1 nM, 30 nM, 1 μM, 30 μM). All reactions, except for basal time zero wells which were stopped immediately, were incubated for 1 hr at 37°. After the treatment period, all reactions were stopped upon the immediate addition of 0.75 mL of ice-cold methanol. The contents of each well were scraped into the methanol and placed in a 15-mL polypropylene centrifuge tube. Each well was rinsed a second time with ice-cold methanol and combined in the tube and frozen at −80° overnight. The next day, chloroform (1 mL) and deionized water (0.5 mL) were added to each tube for extraction. The tubes were mixed for 1 min on a vortex and centrifuged at 230 *g* for 20 min at 25°. Next, 1.5 mL of the aqueous layer was applied to columns containing 1 mL AG1-X8 resin (formate form) prewashed with 12 mL of deionized water. The phosphate esters were eluted by the stepwise addition of solutions with increasing concentrations of formate as described previously [18]. A 0.2-mL aliquot of the organic layer was placed in a scintillation vial and evaporated under a fume hood at room temperature. This was used as an estimate of the incorporation of total *myo*-[2-^{3}H]inositol label into the lipid bilayer. Ten milliliters of scintillation fluid was added to vials containing eluate from the columns or the dried organic layer. The radioactivity was quantified by liquid scintillation counting. All data were expressed as percent basal (after 1 hr) release, and curves were generated by non-linear regression analysis least squares fit using Graphpad Prism software. Potency (EC_{50}) values were calculated from the curves.

2.4. Statistical analysis

All data were analyzed by a two-tailed, unpaired *t*-test or by a one-way ANOVA followed by the Newman–Keul's post-hoc test (Graphpad Prism software). Significance was defined as $P < 0.05$.

3. Results

3.1. Effect of melatonin pretreatment on the induction of PI hydrolysis in MT_1- and MT_2-CHO cells

In vehicle-treated MT_1- and MT_2-CHO cells, melatonin (1 pM–1 mM) increased PI release by ∼45% over basal levels. However, following melatonin treatment, no melatonin-mediated increases in PI hydrolysis over basal

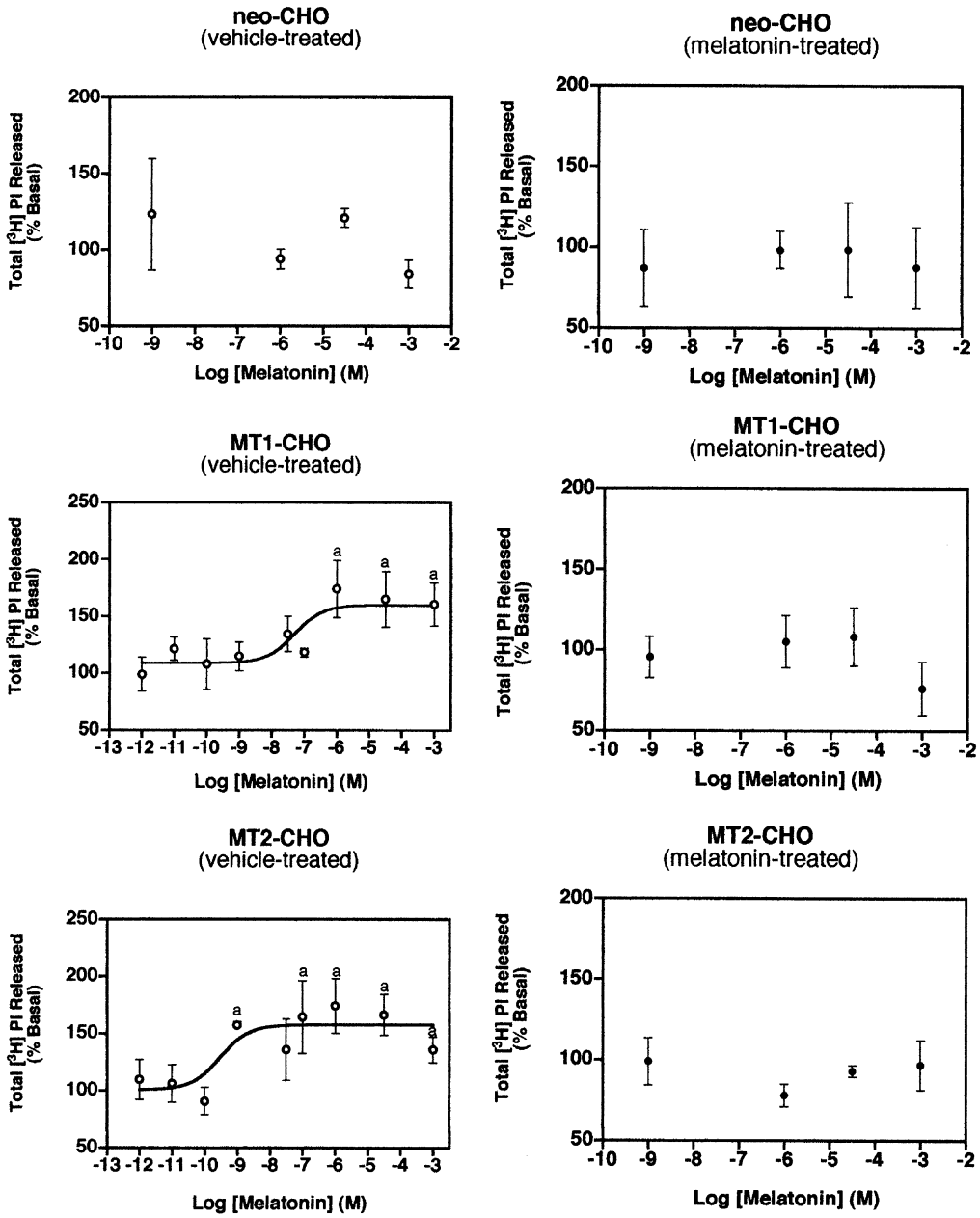

Fig. 1. Effect of melatonin pretreatment on melatonin-mediated stimulation of PI hydrolysis in MT_1- and MT_2-CHO cells. Shown is the percent PI released in response to increasing concentrations of melatonin in MT_1- and MT_2-CHO cells subjected to vehicle alone or melatonin (1 μM for 5 hr). Data represent the means ± SEM of 3–12 experiments. Key: (a) $P < 0.05$ when compared to basal PI (after 1 hr) levels.

levels occurred in either the MT_1- or MT_2-CHO cells. This lack of response was not due to elevated levels of basal PI hydrolysis in melatonin-treated cells as these levels were similar between groups (Fig. 1, Table 1). In both vehicle- and melatonin-treated neo-CHO cells, no melatonin-mediated stimulation of PI hydrolysis over basal occurred (Fig. 1).

3.2. Effect of melatonin pretreatment on melatonin-mediated inhibition of forskolin-induced cAMP accumulation in MT_1- and MT_2-CHO cells

In both MT_1- and MT_2-CHO cells, melatonin (1 nM) inhibited forskolin-induced cAMP accumulation (Fig. 2). However, following treatment with melatonin for 5 hr followed by extensive washing, there was a total loss of the inhibitory effect of melatonin at both the MT_1 and MT_2 melatonin receptors compared with untreated cells. Melatonin did not inhibit forskolin-induced cAMP accumulation in neo-CHO cells (Fig. 2).

3.3. Effect of melatonin pretreatment on the affinity and density of MT_1 or MT_2 melatonin receptors

3.3.1. Receptor density

Because melatonin at a concentration of 1 μM can activate both the cAMP-dependent [13] and PI hydrolysis

Table 1
Pharmacological and functional characterization of human MT_1 or MT_2 melatonin receptors expressed in CHO cells

	MT_1		MT_2	
	Vehicle-treated	Melatonin-treated	Vehicle-treated	Melatonin-treated
Affinity				
K_i (range of SEM)	95 pM (46–200 pM)	370 pM (230–600 pM)	270 pM (150–510 pM)	1300 pM (680–2400 pM)
K_D (range of SEM)	332 pM (310–356 pM)	325 pM (303–348 pM)	289 pM (251–331 pM)	286 pM (249–329 pM)
Density				
B_{max} (fmol/mg protein)[a]	781 ± 29	2302 ± 526	300 ± 90	658 ± 171
PI hydrolysis				
EC_{50} (range of SEM)	29 nM (2–300 nM)	No potency	1.1 nM (0.32–3.5 nM)	No potency
Efficacy (% max PI stimulation over basal)[b]	45 ± 8	No efficacy	46 ± 12	No efficacy
Basal 0 hr (% PI released)[b]	3.4 ± 0.88	3.4 ± 0.51	3.4 ± 0.38	3.9 ± 0.69
Basal 1 hr (% PI released)[b]	3.1 ± 0.34	4.4 ± 1.5	4.4 ± 0.74	5.2 ± 1.1
Forskolin-induced cAMP levels (pM/well)[c]	166 ± 21	180 ± 15	150 ± 23	141 ± 16

[a] Values are means ± SEM, $N = 3$.
[b] Values are means ± SEM, $N = 3$–12.
[c] Values are means ± SEM, $N = 4$–8.

pathways maximally, the effects of this same concentration of melatonin on MT_1 or MT_2 melatonin receptor affinity, function, and regulation were explored. Following vehicle or melatonin pretreatment, no statistically significant change in 2-[^{125}I]-iodomelatonin binding occurred in MT_1- or MT_2-CHO cells even though there was a trend towards an increase in the B_{max}. No specific 2-[^{125}I]-iodomelatonin binding was detected in neo-CHO cells (Fig. 3).

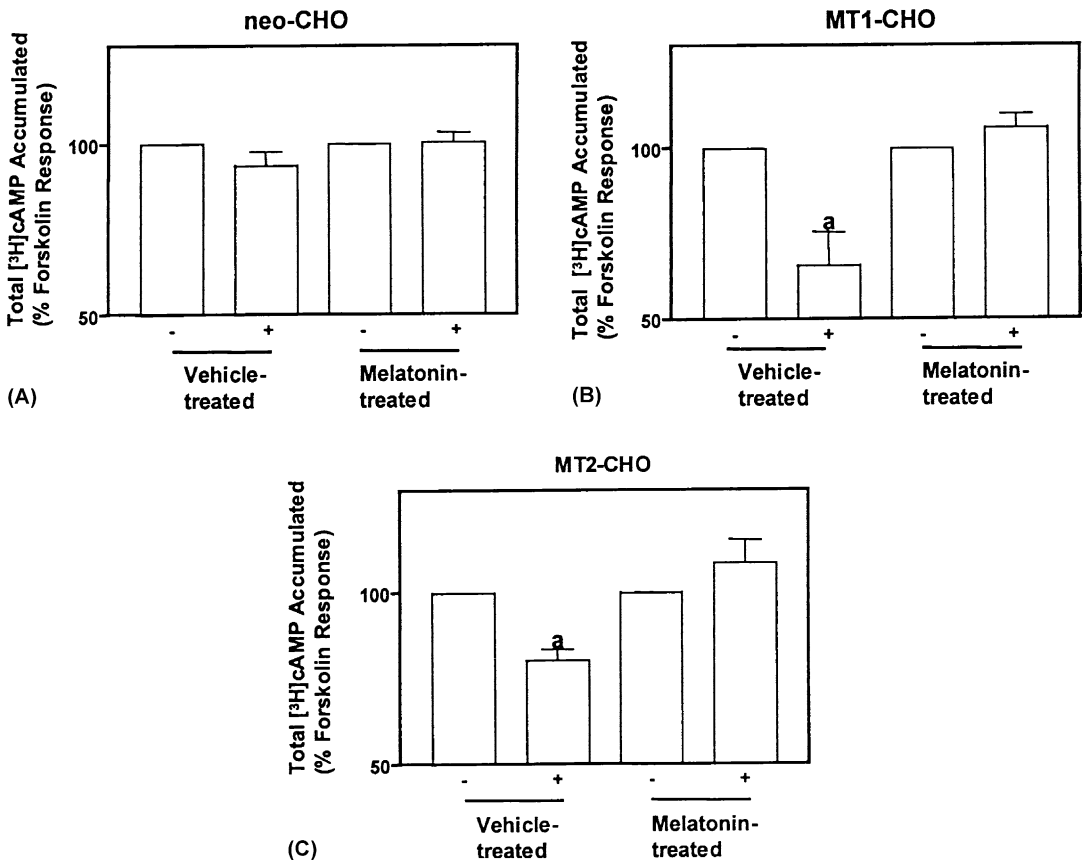

Fig. 2. Effect of melatonin pretreatment on melatonin-mediated inhibition of cAMP accumulation in MT_1- and MT_2-CHO cells. Shown above is the ability of (+) melatonin (1 nM) to inhibit forskolin-induced [^{3}H]cAMP accumulation compared with forskolin stimulation in the absence of melatonin (−) in cells subjected to vehicle alone or melatonin (1 μM for 5 hr). Data represent the means ± SEM of 4–8 individual experiments performed in duplicate. Key: (a) $P < 0.05$ when compared with vehicle-treated (−) cells.

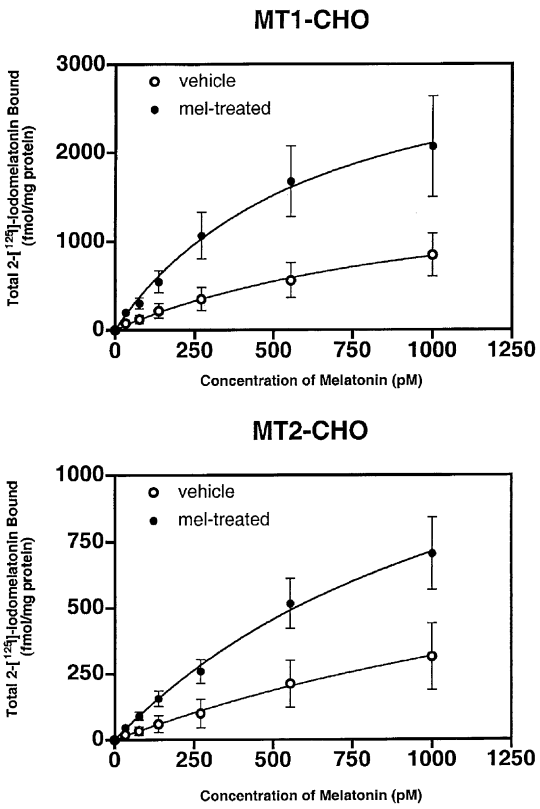

Fig. 3. Effect of melatonin pretreatment on the affinity and density of 2-[^{125}I]-iodomelatonin to MT_1-CHO and MT_2-CHO cells. Shown above are saturation isotherms of 2-[^{125}I]-iodomelatonin (0–1 nM) to MT_1- and MT_2-CHO cells subjected to vehicle alone or melatonin (1 μM for 5 hr). Each point represents 3 individual experiments performed in duplicate.

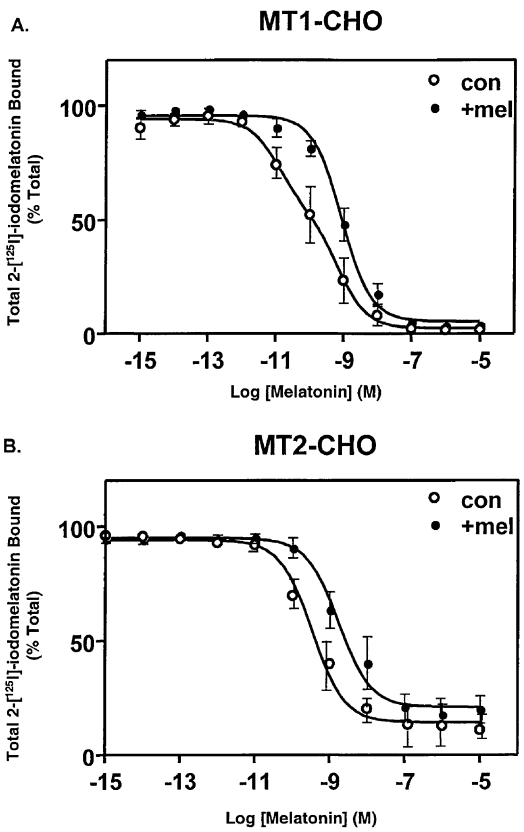

Fig. 4. Effect of melatonin pretreatment on the affinity of melatonin for the MT_1 and MT_2 melatonin receptors. Shown above is the competition of melatonin for 2-[^{125}I]-iodomelatonin (80–100 pM) binding to MT_1- and MT_2-CHO cells subjected to vehicle alone or melatonin (1 μM for 5 hr). Data represent the means ± SEM of 4–7 individual experiments performed in duplicate.

3.3.2. Affinity of melatonin for the MT_1 or MT_2 melatonin receptor

Even though there was a general trend towards a decrease in the affinity of melatonin for each of the receptors following melatonin exposure as revealed through competition analysis (Fig. 4), no change in the affinity (K_D) of 2-[^{125}I]-iodomelatonin occurred under the same conditions using saturation analysis (Table 1). Competition of melatonin for 2-[^{125}I]-iodomelatonin binding to neo-CHO cells was not performed because they do not express melatonin receptors revealed by the total binding experiments (Fig. 3).

3.4. Effect of the selective MT_2 melatonin receptor antagonist 4P-PDOT on melatonin-induced stimulation of PI hydrolysis or on melatonin-mediated inhibition of forskolin-induced cAMP in MT_2-CHO cells

In vehicle-treated MT_2-CHO cells, 4P-PDOT (1 μM) added in combination with melatonin (1 nM, 30 nM, 1 μM, 30 μM) attenuated melatonin-mediated stimulation of PI hydrolysis ($P < 0.05$). 4P-PDOT alone was without effect on eliciting any PI response in MT_2-CHO cells (Fig. 5A). In addition, 4P-PDOT attenuated melatonin (1 nM)-mediated inhibition of forskolin-induced cAMP accumulation in MT_2-CHO cells. Also, similar to the PI assays, 4P-PDOT alone had no effect on forskolin-induced cAMP accumulation in these cells (Fig. 5B).

4. Discussion

The results from this study have demonstrated that melatonin is able to stimulate PI hydrolysis in CHO cells expressing either the human MT_1 or MT_2 melatonin receptor. In addition, the results from this study showed that treatment of MT_1-CHO or MT_2-CHO cells with 1 μM melatonin for 5 hr resulted in a complete loss of melatonin-stimulated PI hydrolysis and in an attenuation of melatonin-mediated decreases in cAMP accumulation.

The complexity of melatonin's function within the body is becoming quite apparent. Perhaps contributing to this diversity is the presence of multiple receptor subtypes located throughout the body, which may be able to couple to multiple signal transduction cascades. In this study, we show that both the MT_1 and MT_2 melatonin receptors are able to couple to cAMP-dependent and PLC-dependent pathways. Dual coupling of MT_1 melatonin receptors

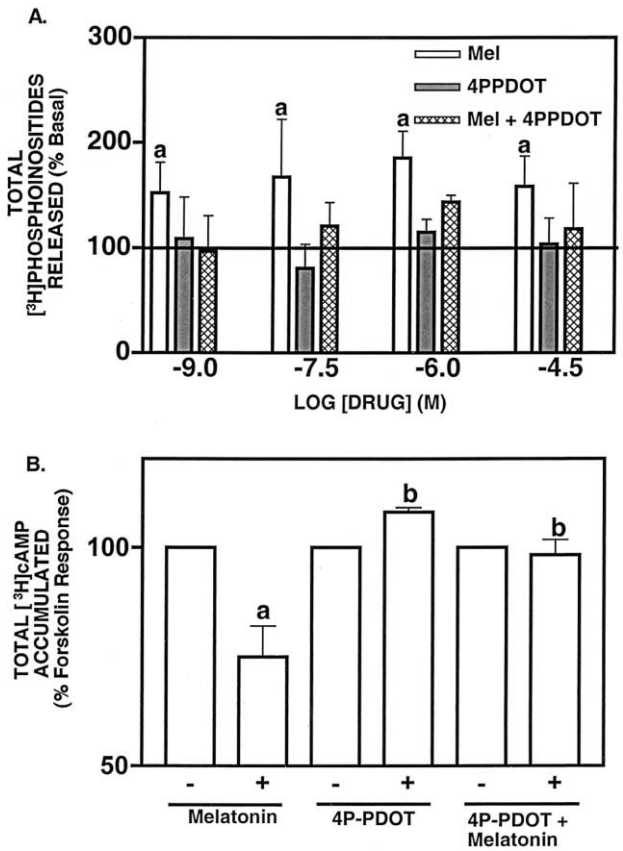

Fig. 5. Effect of 4P-PDOT on melatonin-mediated stimulation of PI hydrolysis or on melatonin-mediated inhibition of forskolin-induced cAMP accumulation on MT_2-CHO cells. Shown above are the effects of 4P-PDOT (1 M) on melatonin-mediated stimulation of PI hydrolysis (A) or inhibition of forskolin-induced cAMP accumulation (B). Data represent the means ± SEM of 4–10 individual experiments performed in duplicate. In panel A, (a) $P < 0.05$ when compared with basal (1 hr) cells. In panel B, (a) $P < 0.05$ when compared with cells exposed to forskolin alone, and (b) $P < 0.05$ when compared with cells exposed to melatonin alone.

expressed in HEK 293 cells to these two signal transduction cascades has been observed [19]. Also, melatonin has been shown to decrease cAMP levels [25] and to increase PI hydrolysis [18] in the same brain region of different species. However, which melatonin receptor subtype is responsible for eliciting these responses in the SCN was not determined. The ability of melatonin to activate or potentiate multiple signal transduction cascades has also been demonstrated [26–30].

The physiological consequences of specific melatonin receptor subtype coupling to distinct signal transduction cascades are unknown. However, activation of specific signal transduction cascades in tissues has led to responses similar to that achieved by melatonin alone. For example, activation of PKC in cultured neurons from the SCN has been shown to phase-shift their firing rates similar to the effects of melatonin [31]. These data suggest the involvement of PKC and possibly PLC-dependent pathways in modulating physiological responses like circadian entrainment.

To date, no one has studied the effects of a chronic agonist exposure on melatonin receptor-mediated stimulation of PI hydrolysis. This is important to study because endogenous melatonin receptors are exposed to melatonin for prolonged periods of time (between 8 and 16 hr each night), depending upon the length of the light/dark cycle [32]. Thus, our findings that MT_1 or MT_2 melatonin receptors coupled to cAMP-dependent and PLC-dependent pathways desensitize following chronic melatonin exposure are significant. Whether or not the loss of melatonin receptor function is caused by a loss of receptor affinity and density remains unclear due to interexperimental variability. However, based upon the results obtained from competition analysis, total binding analysis (data not shown), and saturation analysis, no change in receptor affinity or density occurs in a statistically significant frequency. In a previous study, it was shown that exposure of MT_1-CHO cells to the same concentration of melatonin (1 μM) but for a shorter period of time (1 hr) resulted in no change in receptor density or function [13]. Thus, the time-course of desensitization must occur between 1 and 5 hr. Also, consistent with a previous study [15] is the finding that a trend towards an increase in 2-[^{125}I]-iodomelatonin binding occurs in melatonin-exposed MT_1-CHO cells. In this previous study [15], it was concluded that the increase in 2-[^{125}I]-iodomelatonin binding in agonist-exposed MT_1-CHO cells was, perhaps, due to changes in the coupling states of the receptor [i.e. an increase in the heterotrimeric state ($G_{\alpha\beta\gamma}$) and a decrease in the dissociated state (G_α) of the G-protein] [15]. However, we do not believe that this phenomenon is occurring in these cells, because, in contrast to this previous paper [15], no significant increase in forskolin-induced cAMP accumulation occurred following melatonin exposure in the present study.

The inability of melatonin to activate either one of these pathways in melatonin-treated MT_1- or MT_2-CHO cells was not due to a depletion of the ATP or phospholipid pools. Examination of the [^{3}H]ATP and [^{3}H]phospholipid pools by chromatographic analysis in both treatment groups showed no significant difference in the levels of [^{3}H]ATP or [^{3}H]phospholipids (data not shown). We also believe that the decrease in function of the MT_1 or MT_2 melatonin receptors was not due to residual melatonin remaining on the receptor due to tight coupling as previously reported [13] because vehicle-treated cells exposed to 1 μM melatonin for 2 min and then washed in parallel with the other plates exhibited no decrease in 2-[^{125}I]-iodomelatonin binding (data not shown). In addition, under exact wash conditions, no residual melatonin remained on cells exposed to even greater concentrations of melatonin (10–100 μM) as already reported [14]. If residual melatonin remained in the medium during any of these assays, then we would have expected a decrease in 2-[^{125}I]-iodomelatonin binding and a decrease in forskolin-induced cAMP accumulation following melatonin pretreatment. Finally, the attenuation of melatonin response in both

MT_1- and MT_2-CHO cells following melatonin exposure was not due to increases in the basal levels of PI within the melatonin-exposed cells when compared with vehicle-treated cells.

Desensitization of the MT_1 melatonin receptor following melatonin pretreatment has been demonstrated in both cultured pars tuberalis cells [12] and in previous studies using CHO cell lines [15] or NIH3T3 cell lines [14] transfected with the human MT_1 melatonin receptor. Additionally, desensitization of human MT_2 melatonin receptors expressed in NIH3T3 cells occurred following exposure to melatonin. In this previous study, the loss of potency of melatonin at either the MT_1 or MT_2 receptor following agonist exposure may be due to decreases in receptor density [14]. No decrease in 2-[^{125}I]-iodomelatonin binding occurred in CHO cells expressing the MT_2 melatonin receptor following melatonin pretreatment. Perhaps other mechanisms of desensitization (e.g. phosphorylation) underlie the phenomenon observed in this study. Phosphorylation, as a mechanism underlying the desensitization of other G-protein-coupled receptors, has been demonstrated for m2 muscarininc receptors [33], for A_3-adenosine receptors [34], κ opioid receptors [35], and μ opioid receptors [36] to name a few. The consequences of such phosphorylation events on distinct mechanisms of receptor desensitization have been reviewed [37]. Based upon these past studies, it would be interesting to determine whether phosphorylation events underlie melatonin receptor desensitization. Also, it would be interesting to determine the role of PLC-mediated processes in melatonin receptor function *in vivo*. The findings that (a) acute exposure of MT_1- and MT_2-CHO cells to melatonin can increase PI hydrolysis, and (b) that melatonin pretreatment desensitizes this pathway are important because the ability of melatonin receptors to couple to PI hydrolysis may have serious physiological implications perhaps in circadian entrainment. This study has given us novel insight into underlying MT_1 and MT_2 melatonin receptor function and may explain how one hormone is capable of producing such diverse physiological effects.

Acknowledgments

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