Short-Term Exposure to Melatonin Differentially Affects the Functional Sensitivity and Trafficking of the hMT$_1$ and hMT$_2$ Melatonin Receptors

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ABSTRACT

The hormone melatonin mediates a variety of physiological functions in mammals through activation of pharmacologically distinct MT$_1$ and MT$_2$ G protein-coupled melatonin receptors. We therefore sought to investigate how the receptors were regulated in response to short melatonin exposure. Using 2-[125I]iodomelatonin binding, cAMP functional assays, and confocal microscopy, we demonstrated robust differences in specific 2-[125I]iodomelatonin binding, receptor desensitization, and cellular trafficking of hMT$_1$ and hMT$_2$ melatonin receptors expressed in Chinese hamster ovary (CHO) cells after short (10-min) exposure to melatonin. Exposure to melatonin decreased specific 2-[125I]iodomelatonin binding to CHO-MT$_2$ cells (70.3 ± 7.6%, $n = 3$) compared with vehicle controls. The robust decreases in specific binding to the hMT$_2$ melatonin receptors correlated both with the observed functional desensitization of melatonin to inhibit forskolin-stimulated cAMP formation in CHO-MT$_2$ cells pretreated with 10 nM melatonin (EC$_{50}$ of 159.8 ± 17.8 nM, $n = 3$, $p < 0.05$) versus vehicle (EC$_{50}$ of 6.0 ± 1.2 nM, $n = 3$), and with the arrestin-dependent internalization of the receptor. In contrast, short exposure of CHO-MT$_2$ cells to melatonin induced a small decrease in specific 2-[125I]iodomelatonin binding (34.2 ± 13.0%, $n = 5$) without either desensitization or receptor internalization. We conclude that differential regulation of the hMT$_1$ and hMT$_2$ melatonin receptors by the hormone melatonin could underlie temporally regulated signal transduction events mediated by the hormone in vivo.

In mammals, the hormone melatonin regulates a myriad of physiological functions, including visual, circadian, cardiovascular, and neuroendocrine, through activation of high-affinity membrane receptors belonging to the G protein-coupled receptor (GPCR) superfamily (Brzezinski, 1997; Masana and Dubocovich, 2001). These physiological functions are mediated through activation of at least two molecularly and pharmacologically distinct melatonin receptors, the MT$_1$ and MT$_2$ (Dubocovich et al., 2000). Activation of the MT$_1$ receptor mediates inhibition of neuronal firing in the mouse suprachiasmatic nucleus (SCN) (Liu et al., 1997) and vasoconstriction in rat vascular smooth muscle (Doolen et al., 1998; Masana et al., 2002). Activation of the MT$_2$ receptor mediates phase shifts of both the circadian rhythm of wheel running activity in C57/HeN mice (Dubocovich et al., 1998) and the peak of the circadian rhythm of neuronal firing rate in the rat SCN (Hunt et al., 2001), inhibition of dopamine release in the rabbit retina (Dubocovich et al., 1997), and vasodilatation in rat vascular smooth muscle (Doolen et al., 1998; Masana et al., 2002).

Activation of both recombinant and endogenous MT$_1$ and MT$_2$ melatonin receptors by the hormone melatonin induces a variety of signal transduction cascades (Masana and Dubocovich, 2001). Melatonin inhibits forskolin-stimulated cAMP production through a pertussis toxin-sensitive G protein (Carlson et al., 1989; Witt-Enderby and Dubocovich, 1996) and stimulates protein kinase C (PKC)-dependent processes in recombinant systems and native tissues through activation of either receptor (Godson and Reppert, 1997; Hunt et al., 2001). Melatonin potentiates prostaglandin E$_{2}$-induced phospholipase C stimulation and arachidonate release through a pertussis toxin-sensitive G protein and PKC-dependent pathway via activation of the MT$_1$ melatonin receptor (Godson and Reppert, 1997). In the SCN, activation of the

ABBR EVIATIONS: GPCR, G protein-coupled receptor; SCN, suprachiasmatic nucleus; CHO, Chinese hamster ovary; PKC, protein kinase C; HEK, human embryonic kidney; 4P-PDOT, 4-phenyl-2-propionamidotetraline; GFP, green fluorescent protein; PBS, phosphate-buffered saline; ANOVA, analysis of variance.
MT₂ receptor phase shifts the circadian rhythm of the peak of neuronal firing through a PKC-dependent mechanism (Hunt et al., 2001). In contrast, activation of the MT₁ and MT₂ melatonin receptors exhibits different coupling mechanisms to the cGMP-signaling pathway. Activation of the MT₂ melatonin receptor expressed in human embryonic kidney (HEK-293) cells decreases cGMP levels in a dose-dependent manner via the soluble guanylyl cyclase pathway; however, melatonin did not affect cGMP levels in HEK-293 cells expressing the MT₁ melatonin receptor (Petit et al., 1999).

Desensitization of GPCRs is the waning of receptor responsiveness in the presence of persistent agonist challenge. Both MT₁ and MT₂ recombinant melatonin receptors expressed in mammalian cells desensitize after prolonged exposure to high concentrations of melatonin as shown by the attenuation of cAMP formation and phosphoinositide hydrolysis (MacKenzie et al., 2002). Desensitization of endogenous melatonin receptors has also been reported in ovine pars tuberalis cells using cAMP functional assays (Hazlerigg et al., 1993). Regulation of melatonin binding sites by endogenous melatonin is ambiguous. In the rat SCN and pars tuberalis, there is an inverse relationship between melatonin receptor density and serum melatonin levels (Gauer et al., 1993; Tenn and Niles, 1993), whereas in the hamster SCN no such correlation was observed (Recio et al., 1996). Together, these studies suggest that melatonin can negatively regulate its own receptors after long-term exposure (>5 h) to either endogenous or exogenous melatonin. With the exception of one study (MacKenzie et al., 2002), however, these regulatory effects could not be attributed to either the MT₁ or MT₂ melatonin receptors.

Melatonin regulation of the MT₁ and MT₂ melatonin receptors by short-term exposure to melatonin has not been reported. Therefore, the goal of the present study was to assess the effects of short melatonin exposure on the functional sensitivity and cellular trafficking of the MT₁ and MT₂ melatonin receptors using cAMP accumulation assays, confocal microscopy, and radioligand binding. We demonstrated that the hMT₂ melatonin receptor stably expressed in CHO cells desensitized and internalized through an arrestin-dependent mechanism after short exposure to melatonin, but these effects were not observed in CHO cells expressing the hMT₁ melatonin receptor.

Materials and Methods

Materials. cDNA containing the complete coding region of the hMT₁ (human Mel₁α cloned into pcDNA) and hMT₂ melatonin receptor (human Mel₁β cloned into pcDNA-3) were provided by Dr. S. M. Reppert (Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA) (Reppert et al., 1994, 1995). Effectene transfection and plasmid DNA purification kits were obtained from QIAGEN (Valencia, CA). Cell culture products were obtained from Invitrogen (Carlsbad, CA). [3H]Iodomelatonin (specific activity 2000 Ci/mmol) and [3H]adenosine 3′,5′-cyclic monophosphate (specific activity 23 Ci/mmol) were purchased from Amersham Biosciences, Inc. (Piscataway, NJ). Melatonin and other general reagents were obtained from Sigma-Aldrich (St. Louis, MO). Luzindole (2-benzyl-N-acetyltryptamine) and 4-phenyl-2-propionamidotetra line (4P-PDOT) were obtained from Tocris Cookson (Ballwin, MO) and 2-iodomelatonin from Sigma/RBI (Natick, MA). Rat β-arrestin 1 (pCMV5) and β-arrestin 2 (pCR3.1) were provided by Dr. Richard J. Miller (Northwestern University, Chicago, IL).

FLAG and GFP Epitope Tagging. The FLAG peptide (DYKDDDK) coding sequence was fused to the coding region of the hMT₁ and hMT₂ melatonin receptors by subcloning the corresponding receptor cDNA into the vector pFLAG-CMV-2 (Sigma-Aldrich). These constructs were used for expression of N-terminal FLAG-tagged hMT₁ or hMT₂ melatonin receptor fusion proteins in CHO cells (CHO-MT₁ and CHO-MT₂). The GFP coding sequence was fused to the carboxy terminus of FLAG-tagged hMT₁ and FLAG-tagged hMT₂ melatonin receptors by subcloning the corresponding receptor cDNA into the vector pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA). These constructs were used for expression of N-terminal FLAG-tagged/C-terminal GFP-tagged hMT₁ or hMT₂ melatonin receptor fusion proteins in CHO cells (CHO-MT₁-GFP and CHO-MT₂-GFP). The successful construction of carboxy terminus GFP-tagged and/or amino terminus FLAG-tagged hMT₁ and hMT₂ receptors was confirmed by DNA sequencing (Northwestern University Biotechnology Facility, Chicago, IL).

Cell Culture, Transfections, and Stable Melatonin Receptor Expression in CHO Cells. CHO cell cultures were grown as monolayers in F12 media supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C. CHO cells stably expressing the hMT₁-FLAG or hMT₂-FLAG receptors were generated by transfecting pcDNA1-hMT₁ or pcDNA3-hMT₂ with pSV-NEO plasmids using Effectene (QIAGEN). Cells expressing the melatonin receptor were selected for their resistance to the antibiotic G418 at 300 μg/ml (Invitrogen) and the ability of these G418-resistant clones to specifically bind 2-[125I]Iodomelatonin (2000 Ci/mmol; Amersham Biosciences, Inc.) in whole cell lysates. The CHO-MT₁ and CHO-MT₂ cell lines used in this study originated from a single cell selected using the limited dilution protocol. For transient expression of the hMT₁-FLAG-GFP or hMT₂-FLAG-GFP receptors, CHO cells at 40 to 50% confluency were transiently transfected with the corresponding constructs using Effectene (QIAGEN).

Melatonin and Drug Treatments. CHO-MT₁ and CHO-MT₂ cells were cultured 2.5 days to 80% to 90% confluence. The cells were then incubated in serum-free media for 5 h and washed once with phosphate-buffered saline (PBS). Cells were then treated with vehicle, melatonin, luzindole, or 4P-PDOT in serum-free media at 37°C as appropriate. In some studies, this was followed by short or melatonin treatment for 10 min in the absence or presence of the drugs as appropriate.

Membrane Preparation and 2-[125I]Iodomelatonin Binding Studies. Cells were washed twice with PBS on ice for 5 min, lifted in potassium phosphate buffer (10 mM, pH 7.4) containing 0.25 M sucrose and 1 mM EDTA and then pelleted by centrifugation (13,800g). Pellets were resuspended in 50 mM Tris-HCl (pH 7.4, 10 mM MgCl₂) and pelleted by centrifugation (13,800g). Membrane pellets were stored at −80°C until used. 2-[125I]Iodomelatonin binding was determined in cell membranes (10–25 and 15–40 μg protein assay for MT₁ and MT₂, respectively) as described previously (Witt-Endeber and Dubocovich, 1996). Briefly, binding reactions were started by adding cell membranes to tubes containing binding buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂) for 2-[125I]Iodomelatonin (102.6 ± 6.0 pM, n = 5, unless otherwise indicated), and appropriate concentrations of vehicle or competing agents in a total assay volume of 0.26 ml. After incubating for 1 h at 25°C, reactions were terminated by rapid vacuum filtration through glass fiber filters (Schleicher & Schuell 30; Schleicher & Schuell, Keene, NH) soaked in 0.5% polyethyleneimine solution. Non-specific binding was determined in the presence of 1 μM melatonin. Homologous competition assays were performed by competition of 2-iodomelatonin (0.2 pM–1 μM) for 2-[125I]Iodomelatonin (33.3 ± 6.7 pM, n = 3) binding to cell membranes. Saturation analyses were performed with 2-[125I]Iodomelatonin (1–4000 pM) and nonspecific binding to cell membranes defined with 1 μM melatonin.

cAMP Assay. CHO-MT₁ or CHO-MT₂ cells were seeded in 12-well plates and grown for 24 h. Cells were then serum starved for 5 h and treated with 100 nM (hMT₁) or 10 nM (hMT₂) melatonin or vehicle...
(F12 media) for 10 min. Cells were washed twice with PBS for 5 min at room temperature and then incubated with 30 μM rolipram for 50 min at 37°C. Cells were stimulated with 20 μM forskolin for 10 min in the absence or presence of melatonin (0.1 nM–1 μM). The amount of cAMP in the cells was determined by radioreceptor binding (~65,000 cpm of [3H]cAMP/tube) using cAMP as standard and the purified regulatory subunit of protein kinase A as binding protein. After incubation at 0°C for 2 h, the reaction was terminated by vacuum filtration using glass fiber filters (Schleicher & Schuell) soaked in 0.5% polyethyleneimine (v/v). Filters were washed with ice-cold 50 mM Tris-HCl and the radioactivity counted by liquid scintillation.

Confocal Microscopy. CHO cells in culture at 40 to 50% confluence were transiently transfected with the hMT1-FLAG-GFP or hMT2-FLAG-GFP melatonin receptors and/or cotransfected with rat β-arrestin 1 and 2 using the Effectene transfection kit (QiAGEN). After 24 h, cells were lifted with trypsin and seeded on poly-D-lysine-treated coverslips for another 24 h. Coverslips were then mounted with a 40% glycerol/PBS solution and visualized on an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Data Analysis and Statistics. Percent decrease in specific 2-[125I]iodomelatonin binding relative to control were calculated by dividing the average specific binding (fmol/mg per milligram of protein) defined by a particular melatonin concentration by the average specific binding (fmol/mg per milligram of protein) of the vehicle-treated control. IC50 values from homologous competition experiments were obtained as the concentration that reduced a robust and statistically significant decrease (70.3 ± 7.6% of control) in specific 2-[125I]iodomelatonin (103.3 ± 0.3 nM, n = 3) binding beginning at a concentration as low as 10 nM (vehicle-treated: 52.2 ± 10.4 fmol/mg protein, n = 6, versus melatonin-treated: 14.4 ± 3.6 fmol/mg protein, n = 3) compared with vehicle-treated controls (Fig. 1B). Specific 2-[125I]iodomelatonin (33.3 ± 6.7 μM, n = 3) binding defined with 2-iodomelatonin was also decreased when CHO-MT2 cells were pretreated with 100 nM melatonin (Fig. 2B) (vehicle-treated: 36.6 ± 7.22 fmol/mg protein, n = 3, versus melatonin-treated: 8.3 ± 1.7 fmol/mg protein, n = 3, 77% reduction).

Melatonin (1 nM–1 μM) pretreatment of CHO-MT2 cells induced a robust and statistically significant decrease (70.3 ± 7.6% of control) in specific 2-[125I]iodomelatonin (103.3 ± 0.3 nM, n = 3) binding beginning at a concentration as low as 10 nM (vehicle-treated: 52.2 ± 10.4 fmol/mg protein, n = 6, versus melatonin-treated: 14.4 ± 3.6 fmol/mg protein, n = 3) compared with vehicle-treated controls (Fig. 1B). Specific 2-[125I]iodomelatonin (33.3 ± 6.7 μM, n = 3) binding defined with 2-iodomelatonin was also decreased when CHO-MT2 cells were pretreated with 100 nM melatonin (Fig. 2B) (vehicle-treated: 36.6 ± 7.22 fmol/mg protein, n = 3, versus melatonin-treated: 8.3 ± 1.7 fmol/mg protein, n = 3, 77% reduction).

Short Exposure to Melatonin Did Not Affect the Affinity of the hMT1 and hMT2 Melatonin Receptors for 2-[125I]iodomelatonin. The effect of melatonin pretreatment on the affinity (Kd) of 2-[125I]iodomelatonin for the CHO-MT1 and CHO-MT2 melatonin receptors was assessed in homologous competition experiments using 2-iodomelatonin (0.2 μM–1 μM) (Fig. 2). Competition curves were analyzed by nonlinear regression and best fitted to one site. In vehicle-treated cells, 2-iodomelatonin (0.2 μM–1 μM) competed for 2-[125I]iodomelatonin (33.3 ± 6.7 μM, n = 3) binding to the hMT1, and hMT2 melatonin receptors with an affinity (Kd) of 262 ± 0.03 μM (n = 3) and 331 ± 0.07 μM (n = 3), respectively (Fig. 2, A–D). Melatonin pretreatment did not

**TABLE 1**

Affinity and potency of melatonin at WT and epitope-tagged hMT1, and hMT2, melatonin receptors

<table>
<thead>
<tr>
<th>Receptor Construct</th>
<th>2-[125I]iodomelatonin Binding (Kd)</th>
<th>Forskolin-Stimulated cAMP Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (nM)</td>
<td>Efficacy</td>
</tr>
<tr>
<td>WT, MT1</td>
<td>0.15 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>MT1* FLAG</td>
<td>0.24 ± 0.02</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>MT1* FLAG/GFP</td>
<td>0.18 ± 0.01</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>WT, MT2</td>
<td>0.36 ± 0.06</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>MT2* FLAG</td>
<td>0.45 ± 0.09</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>MT2* FLAG/GFP</td>
<td>0.33 ± 0.07</td>
<td>5.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>2.5 ± 0.9</td>
<td>2.5 ± 0.9</td>
</tr>
</tbody>
</table>

WT, wild-type.
affect the affinity ($K_D$) of 2-iodomelatonin for competition with 2-$^{[125]I}$iodomelatonin binding to either the hMT1 (202 ± 0.02 pM, $n = 3$) or hMT2 (360 ± 0.09 pM, $n = 3$) compared with results obtained in vehicle-treated cells (Fig. 2, A–D).

Luzindole and 4P-PDOT Differentially Affected Specific 2-$^{[125]I}$iodomelatonin Binding to CHO-MT1 and CHO-MT2 Cells. The indole ligand luzindole shows 15 to 25 times higher affinity for the MT2 than the MT1 melatonin receptor, whereas the amidotetraline 4P-PDOT is a selective MT2 receptor ligand (Dubocovich et al., 1997, 1998). Short pretreatment (10 min) of CHO-MT1 cells with luzindole (10 nM–10 μM) or 4P-PDOT (1 nM–10 μM) did not affect specific 2-$^{[125]I}$iodomelatonin binding (Fig. 3, A and C). Pretreatment of CHO-MT2 cells with luzindole (1 nM–10 μM) significantly...
decreased specific 2-[125I]iodomelatonin binding only at concentrations higher than 10 μM (Fig. 3B). In contrast, short pretreatment of CHO-MT2 cells with 4P-PDOT (1 nM–1 μM) induced a concentration-dependent inhibition of 2-[125I]iodomelatonin binding, which was significant at concentrations of 10 nM and higher (Fig. 3D). 4P-PDOT also inhibited in a concentration-dependent manner forskolin-stimulated cAMP formation in CHO-MT2 cells (pEC50 of 7.5 ± 0.1 nM, efficacy 51.7 ± 7.7%, n = 3, for 1 μM).

Luzindole and 4P-PDOT Blocked the Melatonin-Mediated Decreases in Specific 2-[125I]Iodomelatonin Binding to the hMT2 Melatonin Receptor. To assess whether the melatonin-induced decreases in specific 2-[125I]iodomelatonin binding to the hMT2 melatonin receptor were receptor-mediated, we used the competitive melatonin receptor ligands luzindole and 4P-PDOT. Pretreatment of CHO-MT2 cells with luzindole (100 and 1000 nM) alone did not affect specific 2-[125I]iodomelatonin binding to the hMT2 melatonin receptor (Fig. 4A). However, there was a significant interaction (p < 0.05) between luzindole and melatonin. In the presence of luzindole, the melatonin-mediated (10 nM) decrease in specific 2-[125I]iodomelatonin binding was completely antagonized (Fig. 4A). In contrast, pretreatment of CHO-MT2 cells with 30 nM 4P-PDOT alone reduced specific 2-[125I]iodomelatonin binding (Fig. 4B). There was, however, a significant interaction (p < 0.001) between 4P-PDOT and melatonin. Furthermore, pretreatment with 30 nM 4P-PDOT antagonized the decrease in specific 2-[125I]iodomelatonin binding reduced by pretreatment with melatonin (Fig. 4B).

Short Exposure to Melatonin Desensitized the hMT2 Receptor and not the hMT1 Receptor. The functional sensitivity of the hMT1 and hMT2 melatonin receptors after short-term exposure to melatonin was assessed by determining the potency of melatonin to inhibit forskolin-stimulated cAMP formation in vehicle- and melatonin-treated CHO-MT1 and CHO-MT2 cells. In vehicle-treated CHO-hMT1 (Fig. 5A) and CHO-hMT2 (Fig. 5B) cells, melatonin (0.1–1000 nM) inhibited forskolin-stimulated cAMP formation in a dose-dependent manner. Forskolin stimulation was similar in vehicle (81.4 ± 12.0 pmol/well cAMP, n = 5) and melatonin-pretreated (82.8 ± 21.6 pmol/well cAMP, n = 5) CHO-MT1 cells. Pretreatment of CHO-MT1 cells for 10 min with 100 nM melatonin did not affect the potency of melatonin to inhibit forskolin-stimulated cAMP formation compared with vehicle control (Fig. 5A). In contrast, although melatonin pretreatment of CHO-MT2 cells did not affect forskolin-stimulated cAMP formation (59.4 ± 14.8 pmol/well cAMP, n = 3) compared with vehicle control (80.0 ± 17.2 pmol/well cAMP, n = 3), the potency of melatonin was significantly decreased in CHO-MT2 cells pretreated with 10 nM melatonin for 10 min (EC50 of 159.8 ± 17.8 nM, n = 3, p < 0.05) compared with vehicle control (EC50 of 6.0 ± 1.2 nM, n = 3) (Fig. 5B).

Short Exposure to Melatonin Internalized the hMT2 but Not the hMT1 Melatonin Receptors When Arrestins Were Overexpressed. To investigate the cellular localization of the hMT1 and hMT2 melatonin receptors after pretreatment with melatonin, CHO cells were transiently transfected with hMT1 or hMT2 melatonin receptor cDNA.
constructs containing an N-terminal FLAG epitope and a C-terminal GFP (CHO-MT1-GFP or CHO-MT2-GFP). Expression of the GFP sequence on the melatonin receptor C terminus did not affect the binding affinity of 2-[125I]iodomelatonin (100.2 ± 1.3 pM, n = 5) binding expressed as femtomoles per milligram of protein. Data represent mean ± S.E.M. of two to three independent experiments performed in duplicate. Data were analyzed using two-way ANOVA with Bonferroni’s post test (**, p < 0.01 compared with vehicle control). MLT, melatonin; V, vehicle.

Fig. 4. Luzindole and 4P-PDOT antagonized the decrease in 2-[125I]iodomelatonin binding to the hMT2 receptors induced by short pretreatment with melatonin. CHO-MT2 (A and B) cells in culture were serum starved for 5 h and then pretreated in situ for 10 min at 37°C with either vehicle or 10 nM melatonin in the absence or presence of luzindole (100 nM, 1000 nM (A)) or 4P-PDOT (30 nM (B)). Luzindole or 4P-PDOT was added 10 min before acute pretreatment with melatonin. Cells were harvested and membranes were prepared as described under Materials and Methods. Specific binding was defined with 1 μM melatonin. The ordinate represents specific 2-[125I]iodomelatonin (100.2 ± 1.3 pM, n = 5) binding expressed as femtomoles per milligram of protein. Data represent mean ± S.E.M. of two to three independent experiments performed in duplicate. Data were analyzed using two-way ANOVA with Bonferroni’s post test (**, p < 0.01 compared with vehicle control). MLT, melatonin; V, vehicle.

Fig. 5. Short exposure to melatonin functionally desensitized the hMT2 melatonin receptor. CHO-MT1 (A) and CHO-MT2 (B) cells were serum starved 5 h and treated with either vehicle (○) or melatonin (■), 100 nM MT1, and 10 nM MT2. The ordinate (A and B) represents the percentage of forskolin-stimulated cAMP (picomoles). Curves represent the percentage of forskolin-stimulated cAMP (picomoles) inhibited by increasing concentrations of melatonin (0.1–1000 nM). In Fig. 5A, melatonin potently inhibited forskolin-stimulated cAMP formation in both vehicle- (EC50 of 1.7 ± 0.4 nM, n = 5) and melatonin-treated (EC50 of 3.1 ± 1.4 nM, n = 5) CHO-MT1 cells. In Fig. 5B, melatonin potently inhibited forskolin-stimulated cAMP formation in vehicle- (EC50 of 6.0 ± 1.2 nM, n = 3), but not melatonin-treated (EC50 of 159.8 ± 17.8 nM, n = 3, *, p < 0.05) CHO-MT2 cells. Data represent mean ± S.E.M. of three to five independent experiments performed in duplicate. MLT, melatonin.

Fig. 6, A and E, shows confocal images of CHO-MT1-GFP or CHO-MT2-GFP cells pretreated with vehicle. The hMT1 melatonin receptor localized to the cell plasma membrane, whereas the hMT2 seemed more diffusely distributed within the perinuclear regions. Short exposure with 100 nM melatonin for 10 min did not affect the membrane localization of hMT1 melatonin receptor (Fig. 6A) compared with vehicle-treated control (Fig. 6A). In contrast, pretreatment with 10 nM melatonin seemed to increase the perinuclear fluorescence of the hMT2 melatonin receptor (Fig. 6B) compared with vehicle-treated control (Fig. 6A). Overexpression of arrestin 1 and 2 did not affect the cellular localization of the hMT1 melatonin receptors treated with vehicle or melatonin (Fig. 6, C and D). However, overexpression of arrestin 1 and 2 led to the formation of more defined intracellular puncta after acute melatonin (10 nM) treatment of the hMT2 melatonin receptor (Fig. 6H) compared with vehicle-treated control (Fig. 6G).
coupling of receptor and G protein (Ferguson, 2001). Subsequent binding of arrestins to phosphorylated receptors targets GPCRs for endocytosis (Ferguson, 2001). Internalization as a mechanism of receptor desensitization has been shown for endogenous G$_i$/G$_o$-coupled somatostatin receptors in NG108-15 cells (Beaumont et al., 1998). Using a similar melatonin treatment paradigm that functionally desensitized the hMT$_2$ melatonin receptor we observed that overexpression of arrestin 1 and 2 led to a marked enhancement of melatonin-mediated internalization of the hMT$_2$ melatonin receptor. Thus, the robust decrease in specific 2-$^{125}$Iiodomelatonin binding after short melatonin exposure of hMT$_2$ melatonin receptors seems consistent with rapid receptor desensitization followed by internalization.

The hMT$_1$ melatonin receptors did not desensitize or internalize after short melatonin exposure even when coexpressed with arrestins 1 and 2. In contrast, Roy et al. (2001) found a rapid arrestin-dependent internalization of the MT$_1$ melatonin receptor in GT1-7 neurons after acute exposure to melatonin. GT1-7 cells express endogenous MT$_1$ melatonin receptors and thus the presence of endogenous signaling partners, which may be absent in CHO cells, may have facilitated MT$_1$ melatonin receptor internalization. In addition, there is a high proportion of constitutively active MT$_1$ melatonin receptors expressed in non-neuronal mammalian cells (Dubocovich and Masana, 1998; Roka et al., 1999). Constitutively active GPCRs are thought to be constitutively phosphorylated and desensitized; however, constitutively active mutant AT$_{1A}$ receptors do not show elevated basal phosphorylation (Thomas et al., 2000) and α1b adrenergic receptors do not internalize due to an inability to activate arrestins (Mhouty-Kodja et al., 1999). Therefore, constitutively active hMT$_1$ melatonin receptors expressed in CHO cells may exist in a receptor conformation that does not exhibit elevated basal phosphorylation and is therefore unable to interact with arrestins to mediate internalization. Additionally, melatonin-mediated receptor internalization could be species specific because our study uses the human melatonin receptor, whereas Roy et al. (2001) used the mouse melatonin receptor. Species-specific patterns of internalization have been observed for the opioid receptors because the human κ-opioid receptor internalizes, whereas the rat κ-opioid does not (Li et al., 1999).

The small decrease in 2-$^{125}$Iiodomelatonin binding to the hMT$_1$ melatonin receptor after acute melatonin exposure (−30%) is likely due to residual melatonin on the receptor given that the receptor did not desensitize or internalize after short melatonin exposure and has slightly higher affinity for melatonin than the hMT$_2$ melatonin receptor. Tight melatonin binding to the super high-affinity state of the hMT$_1$ melatonin receptor was suggested by Witt-Enderby and Dubocovich (1996) where 1 µM melatonin pretreatment of CHO cells stably expressing hMT$_1$ melatonin receptors for 1 h reduced binding of 2-$^{125}$Iiodomelatonin to the super high-affinity site in whole cell lysates. However, we propose that the decrease in specific 2-$^{125}$Iiodomelatonin binding after melatonin pretreatment of CHO-MT$_1$ cells represents residual melatonin bound to the high-affinity site of the hMT$_1$ melatonin receptor. In the present study, competition of 2-iodomelatonin for the radioligand 2-$^{125}$Iiodomelatonin to crude washed membranes from either vehicle- and melatonin-treated CHO-MT$_1$ cells resulted in monophasic curves
with identical affinity for a single site. Similarly, 2-[125I]iodomelatonin binds to a single high-affinity site in crude washed CHO or HEK cell membranes expressing the hMT1 receptor (Roka et al., 1999; Browning et al., 2000). Melatonin binding to the high-affinity state is supported by the fact that 100 nM melatonin pretreatment did not significantly affect specific binding to the MT1 melatonin receptor when defined with 1 μM 2-iodomelatonin, whereas a significant decrease in specific binding was observed when defined using 1 μM melatonin. This difference is due to 2-iodomelatonin being able to compete for 2-[125I]iodomelatonin with 10 times higher affinity compared with melatonin (Browning et al., 2000).

The mechanism through which melatonin differentially affects desensitization and internalization of the hMT1 and hMT2 melatonin receptors is not well understood. In COS-7 and HEK-293 cells, activation of the D2, but not the D3, dopamine receptor results in internalization after receptor phosphorylation and translocation of arrestins to the membrane (Kim et al., 2001). Similarly, the β2-adrenergic receptor undergoes agonist-mediated internalization, whereas the β1-adrenergic receptor does not internalize due to its low affinity for β-arrestins (Shina et al., 2001). The δ-opioid receptor activates arrestin more efficiently than the μ-opioid receptor, resulting in faster desensitization kinetics (Lowe et al., 2002). Thus, differences in affinity and/or activation of arrestins between the hMT1 and hMT2 melatonin receptors may underlie their different patterns of desensitization and internalization. Recently, MacKenzie et al. (2002) reported desensitization of both hMT1 and hMT2 melatonin receptors after exposure to 1 μM melatonin for 5 h. The observations in the present study were made after acute (10 min) exposure of hMT1 and hMT2 melatonin receptors to melatonin. Therefore, desensitization and internalization of the hMT1 melatonin receptor may require longer periods of exposure. It is also possible that the GFP on the hMT2 melatonin receptor C terminus may have compromised contact between the putative PDZ domain on the receptor and potential trafficking partners (Stricker et al., 1997).

Luzindole and 4P-PDOT, two well-characterized competitive melatonin receptor ligands, were used to determine whether the decreases in specific 2-[125I]iodomelatonin binding to CHO-hMT2 melatonin receptors was receptor-mediated. Luzindole is an MT2 melatonin receptor antagonist in both recombinant systems (Browning et al., 2000) and in native tissue (Dubocovich et al., 1997; Hunt et al., 2001). Pretreatment of CHO-MT2 cells with luzindole did not affect specific 2-[125I]iodomelatonin binding determined in cell membranes, except at concentrations higher than 10 μM. However, pretreatment with luzindole followed by coinubcation with melatonin blocked the melatonin-mediated decrease in specific 2-[125I]iodomelatonin binding to the hMT2 melatonin receptor induced by short exposure, suggesting a receptor-mediated event. In contrast, 4P-PDOT is a competitive MT2 melatonin receptor antagonist in native tissue (Dubocovich et al., 1998; Hunt et al., 2001) but also shows partial agonist/antagonist efficacy in recombinant systems (Nonno et al., 1999; Browning et al., 2000; MacKenzie et al., 2002) and partial agonist efficacy on leukocyte rolling in the rat microcirculation (Lotufo et al., 2001). Similarly, the endogenous MT1 and MT2 melatonin receptors to melatonin. There are differences between MT1 and MT2 melatonin receptor-mediated physiological responses, including exacerbating vasoconstriction (Doolen et al., 1998; Masana et al., 2002) and impairing phase shifts of circadian rhythms (Dubocovich et al., 1998; Hunt et al., 2001).

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References


Dubocovich ML, Masana MI, Iacob S, and Sauri DM (1997) Melatonin receptor antagonists that differentiate between the human Mel1α and Mel1β, recombinant subtypes are used to assess the pharmacological profile of the rabbit retina Mel1α, presynaptic heteroreceptor. Naunyn-Schmiedebergs Arch Pharmac 355:365–373.


Hazlerigg DG, Gonzalez-Brito A, Lawson W, Hastings MH, and Morgan PJ (1993) Prolonged exposure to melatonin leads to time-dependent sensitization of adenyl-

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