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Pharmacological characterization of human recombinant melatonin mt_1 and MT_2 receptors

*,[1]Christopher Browning, [1]Isabel Beresford, [2]Neil Fraser & [1]Heather Giles

[1]Receptor Pharmacology Glaxo Wellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts, SG1 2NY and [2]Molecular Pharmacology, Glaxo Wellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts, SG1 2NY

1 We have pharmacologically characterized recombinant human mt_1 and MT_2 receptors, stably expressed in Chinese hamster ovary cells (CHO-mt_1 and CHO-MT_2), by measurement of [^{3}H]-melatonin binding and forskolin-stimulated cyclic AMP (cAMP) production.

2 [^{3}H]-melatonin bound to mt_1 and MT_2 receptors with pK_D values of 9.89 and 9.56 and B_{max} values of 1.20 and 0.82 pmol mg^{-1} protein, respectively. Whilst most melatonin receptor agonists had similar affinities for mt_1 and MT_2 receptors, a number of putative antagonists had substantially higher affinities for MT_2 receptors, including luzindole (11 fold), GR128107 (23 fold) and 4-P-PDOT (61 fold).

3 In both CHO-mt_1 and CHO-MT_2 cells, melatonin inhibited forskolin-stimulated accumulation of cyclic AMP in a concentration-dependent manner (pIC_{50} 9.53 and 9.74, respectively) causing 83 and 64% inhibition of cyclic AMP production at 100 nM, respectively. The potencies of a range of melatonin receptor agonists were determined. At MT_2 receptors, melatonin, 2-iodomelatonin and 6-chloromelatonin were essentially equipotent, whilst at the mt_1 receptor these agonists gave the rank order of potency of 2-iodomelatonin > melatonin > 6-chloromelatonin.

4 In both CHO-mt_1 and CHO-MT_2 cells, melatonin-induced inhibition of forskolin-stimulated cyclic AMP production was antagonized in a concentration-dependent manner by the melatonin receptor antagonist luzindole, with pA_2 values of 5.75 and 7.64, respectively. Melatonin-mediated responses were abolished by pre-treatment of cells with pertussis toxin, consistent with activation of G_i/G_o G-proteins.

5 This is the first report of the use of [^{3}H]-melatonin for the characterization of recombinant mt_1 and MT_2 receptors. Our results demonstrate that these receptor subtypes have distinct pharmacological profiles.

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Keywords: Melatonin; mt_1 receptor; MT_2 receptor; [^{3}H]-melatonin; 2-[^{125}I]-iodomelatonin; receptor classification; subtypes; cyclic AMP; luzindole

Abbreviations: cAMP, cyclic adenosine 3′:5′ monophosphate; CHO, Chinese hamster ovary; 5-HT, 5-hydroxytryptamine, MT, melatonin; 8-M-PDOT, 8-methoxy-2-propionamidotetraline; 4-P-PDOT, 4-phenyl-2-propionamidotetraline

Introduction

Melatonin is the principle hormone of the pineal gland and is believed to have a central role in the regulation of the mammalian circadian system and reproductive function in seasonally breeding animals (for a review see Arendt, 1995). To date, two human melatonin receptors have been cloned, termed mt_1 and MT_2, and have been shown to be seven transmembrane G-protein coupled receptors (Reppert *et al*., 1994; 1995). When expressed in immortalized mammalian cell lines, both mt_1 and MT_2 receptors bind melatonin with high affinity and couple to the inhibition of adenylate cyclase (Reppert *et al*., 1994; 1995).

Radioligand binding studies using 2-[^{125}I]-iodomelatonin have identified high affinity binding sites in rat and human suprachiasmatic nucleus, sheep pars tuberalis and chicken retina (for a review see Morgan *et al*., 1994). *In situ* hybridization studies have shown the presence of mt_1 mRNA in the human suprachiasmatic nucleus (Weaver & Reppert, 1996), whilst MT_2 mRNA is found in human retina and brain (Reppert *et al*., 1995). Subtype selective agonists and antagonists would greatly assist the elucidation of the relative contributions of melatonin receptor subtypes to the physiological actions of melatonin, such as its effects in the suprachiasmatic nucleus to regulate the circadian system. The expression of mt_1 and MT_2 receptors as isolated, single populations of receptors allows the investigation of melatonin receptor pharmacology and the search for subtype selective ligands which cannot be achieved in physiological systems where mixed populations of melatonin receptors may exist.

Thus, we have stably expressed mt_1 and MT_2 receptors in Chinese hamster ovary (CHO) cells and characterized the pharmacology of these receptors using both radioligand binding assays and measurement of the inhibition of forskolin-stimulated cyclic adenosine 3′:5′ monophosphate (cyclic AMP). Previous melatonin receptor binding studies have generally employed 2-[^{125}I]-iodomelatonin as a radioligand. However, high receptor expression levels have enabled us to develop a radioligand binding assay utilising [^{3}H]-melatonin. This radioligand has advantages over 2-[^{125}I]-iodomelatonin, as it is safer, more economical to use, and is chemically identical to the endogenous hormone. We have used a number of melatonin receptor agonists, including the indolene GR196429 (Beresford *et al*, 1998a) and the napthalenic compound S-20098 (Yous *et al*., 1992) to characterize CHO-mt_1 and CHO-MT_2 receptors. In addition,

*Author for correspondence at: National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA.

a number of putative melatonin receptor partial agonists and antagonists with selectivity for the MT_2 receptor were evaluated, including GR128107, 5-MCA-NAT, 8-M-PDOT and 4-P-PDOT (Dubocovich *et al*., 1997). The most selective of these compounds, the amidotetraline 4-P-PDOT, was compared to the previously characterized melatonin receptor antagonist luzindole (Dubocovich, 1988) for its ability to antagonize melatonin mediated inhibition of forskolin-stimulated cyclic AMP production. Preliminary accounts of this work have already been presented (Browning *et al*., 1997; 1998).

The nomenclature and classification of melatonin receptors used here was recently approved by the Nomenclature Committee of the International Union of Pharmacology (Dubocovich *et al*., 1998a). The denomination 'mt_1' corresponds to that of the recombinant receptor previously termed Mel_{1a}. MT_2 refers to native functional receptors with pharmacological characteristics similar to that of the recombinant receptor mt_2, previously termed Mel_{1B}. MT_3 corresponds to the pharmacologically defined melatonin receptor subtype, with unknown gene sequence, previously referred to as ML_2.

Methods

Generation of CHO-mt_1 and CHO-MT_2 cells

Clones representing the sequence of the human mt_1 receptor were amplified using degenerate primers based on the sequence of the *Xenopus laevis* melatonin receptor (Ebisawa *et al*., 1994). These clones defined the genomic sequence flanking the intron within the coding sequence of the receptor and enabled the coding exons to be amplified from genomic DNA independently and reassembled in frame using an engineered site. Cloning was in pBluescript (Stratagene). The human MT_2 sequence was cloned in a similar manner using the sequence described by Reppert and co-workers (1995). The nucleotide sequences obtained encoded for the same amino acid sequences as described by Reppert *et al*. (1994; 1995). The sequences encoding the receptor were cloned into the mammalian expression vector pcDNA3 (Invitrogen) and introduced into CHO cells by conventional calcium phosphate precipitation techniques, which were then placed under G418 selection (1 mg ml^{-1}). Several G418 resistant mt_1 and MT_2 cell lines were selected for [^{3}H]-melatonin saturation binding studies and measurement of melatonin-mediated inhibition of forskolin-stimulated cyclic AMP production. The cell-lines which gave the highest levels of [^{3}H]-melatonin binding and greatest melatonin-induced inhibition of cyclic AMP accumulation were chosen for further [^{3}H]-melatonin radioligand binding and cyclic AMP studies, respectively.

Preparation of cell membranes

CHO cells stably expressing human mt_1 or MT_2 receptors were maintained in DMEM-F12 medium, supplemented with 10% (v v^{-1}) foetal calf serum, 2 mM L-glutamine, 1 mg ml^{-1} G418 and 400 μg ml^{-1} hygromycin. Cells were grown at 37°C in 5% CO_2 in air. Membranes were prepared by harvesting CHO cells using Hanks balanced salt solution, containing EDTA (5 mM). The suspension was homogenized and centrifuged at $4500 \times g$ for 35 min. The pellet was re-homogenized, resuspended in (mM): Tris-HCl buffer 50, containing $MgCl_2$ 2, EDTA 1, 0.1% ascorbic acid, pH 7.4, and aliquots ($\sim$2 mg protein ml^{-1}) stored at -80°C until use.

Radioligand binding assays

The assay was performed in 96-well plates in a final assay volume of 500 μl. Drugs and [^{3}H]-melatonin (0.3 nM) or 2-[^{125}I]-iodomelatonin (50 pM) were incubated with membranes (50 μg protein ml^{-1}) for 120 min at 37°C in a buffer comprising (mM): Tris-HCl 50, $MgCl_2$ 2, EDTA 1, 0.1% ascorbic acid, pH 7.4. At the concentration of protein used, less than 10% of added radioligand was bound to membrane (data not shown). Non-specific binding was defined with melatonin (1 μM). For association studies, membranes (50 μg ml^{-1}) were added to [^{3}H]-melatonin (0.3 nM) and incubated for increasing periods of time (2–180 min). For dissociation studies, membranes were incubated with [^{3}H]-melatonin (0.3 nM) for 2 h prior to the addition of cold melatonin (1 μM) to initiate dissociation, and then incubated for increasing periods of time (0–240 min). Bound radioactivity was separated by rapid filtration through GF/B filter paper using a Brandel or Wallac cell harvester and filters were washed with 4×1 ml Tris-HCl (50 mM; pH 7.4). Radioactivity was measured by liquid scintillation spectrometry.

cyclic AMP assays

Cells were cultured as described above. The assay was performed in 96-well plates in a final assay volume of 200 μl. Confluent CHO-mt_1 or CHO-MT_2 cells were incubated at 37°C with DMEM-F12, containing 300 μM isobutylmethylxanthine (IBMX) to inhibit phosphodiesterase activity. Following 60 min incubation, agonist (0.01 pM–100 μM) was added. Sixty minutes later, forskolin (30 μM) was added and cells were incubated for a further 15 min. For antagonist experiments, melatonin was co-incubated with luzindole (0.1–100 μM) for 60 min prior to addition of forskolin. The reaction was terminated by removal of media and addition of ice-cold ethanol (100 μl) for 30 min at 4°C. Ethanol samples were evaporated to dryness and cyclic AMP concentrations determined by [^{125}I]-cyclic AMP scintillation proximity assay (Amersham).

Data analysis

Saturation binding experiments were analysed by fitting the sum of a hyperbola and a straight line to the total binding data to generate pK_D and B_{max} values. Competition binding data were analysed by fitting with a four-parameter logistic equation. pIC_{50} values were converted to pK_i values using the equation of Cheng & Prusoff (1973). Association kinetic data were analysed by fitting total binding data to the equation $B = C + B_{max}.(1 - \exp^{-K*t})$, where B = binding at time t, C = non-specific binding and k = observed association rate constant. Dissociation kinetic data were analysed by fitting total binding data to the equation $B = C + B_{max}.\exp^{-K*t}$, where k = dissociation rate constant.

Cyclic AMP data were fitted with a four parameter logistic equation to determine pIC_{50} values and Hill coefficients. Drug responses were expressed as percentage inhibition of forskolin-stimulated cyclic AMP. The potency ratio was defined as the ratio of the EC_{50} of the drug relative to that of melatonin determined in the same experiment. The pA_2 values for luzindole were determined using a modified form of the Schild equation (Black *et al*., 1985; Lew & Angus, 1995).

All data are expressed as arithmetic mean $\pm$ s.e.mean. Statistical comparisons were made using two-tailed Student's *t*-test. For luzindole studies, the upper and lower asymptotes

and Hill slopes of the melatonin concentration-effect curves were compared using one-way analysis of variance (ANOVA) followed by Dunnett's test for significance. Where data are presented as the mean of separate experiments, curves were generated from the mean of the individual curve fitting parameter estimates.

Drugs

GR196429, S20098, 5-MCA-NAT (GR135531), GR128107 and luzindole were synthesized by Medicinal Chemistry, Glaxo Wellcome. Melatonin, 6-hydroxymelatonin, 6-chloromelatonin, N-acetyl-5-HT, 5-HT, forskolin and IBMX were supplied by Sigma. 2-iodomelatonin, 4-phenyl-2-propionamidotetraline (4-P-PDOT) and 8-methoxy-2-propionamidotetraline (8-M-PDOT) were purchased from RBI. [^{3}H]-melatonin (85 Cimmol^{-1}) and 2-[^{125}I]-iodomelatonin ($\sim$2000 Cimmol-1) were supplied by Amersham.

Results

Kinetics and saturation of [^{3}H]-melatonin binding to mt_1 and MT_2 receptors

At 37°C the specific binding of [^{3}H]-melatonin (0.4 nM) to mt_1 receptors reached equilibrium within 15–30 min (Figure 1A; $t_{1/2}$ 3.9$\pm$1.2 min; observed association rate constant (k_{obs}) 3.89e^8 M^{-1}.min^{-1}; $n=3$) and remained stable for more than 360 min. Binding of [^{3}H]-melatonin (0.4 nM) to MT_2 receptors occurred more slowly, taking 60–90 min to reach equilibrium (Figure 1B; $t_{1/2}$ 14$\pm$2 min; k_{obs} 9.18e^7 M^{-1}.min^{-1}; $n=3$). For both receptors, an incubation time of 120 min was used routinely. Specific [^{3}H]-melatonin binding (0.4 nM) to mt_1 receptors dissociated rapidly to non-specific binding levels (Figure 1C), with a dissociation rate constant (k_{-1}) of 0.056$\pm$0.003 min^{-1} and half-time of 12.5$\pm$0.8 min ($n=3$). In contrast, binding of [^{3}H]-melatonin (0.4 nM) to MT_2 receptors dissociated more slowly (Figure 1D), with a dissociation rate constant (k_{-1}) of 0.011$\pm$0.002 min^{-1} and half-time of 64$\pm$8 min ($n=3$). Whilst only 3.6$\pm$0.6% ($n=3$) of [^{3}H]-melatonin remained specifically bound to mt_1 receptors after 240 min, 22$\pm$2% ($n=3$) of the specific [^{3}H]-melatonin remained bound to MT_2 receptors, which was significantly different from non-specific binding ($P<0.05$). From the kinetic data, pK_D values of 9.78$\pm$0.16 and 9.92$\pm$0.03 ($n=3$) for mt_1 and MT_2 receptors, respectively, were determined.

Saturation binding studies indicated that [^{3}H]-melatonin bound in a specific, concentration-dependent and saturable manner to both mt_1 and MT_2 receptors (Figure 2A,B), with pK_D values of 9.89$\pm$0.13 and 9.56$\pm$0.03, respectively ($n=3$). B_{max} values were 1.20$\pm$0.10 and 0.82$\pm$0.06 pmol mg^{-1} protein, respectively. Hill coefficients were 1.02$\pm$0.13 and 1.06$\pm$0.06, respectively, suggesting interactions with single populations of binding sites. In the cell lines selected for cyclic AMP assays, [^{3}H]-melatonin saturation binding studies yielded B_{max} values of 0.39$\pm$0.07 and 0.23$\pm$0.06 pmoles mg^{-1} protein, and pK_D values of

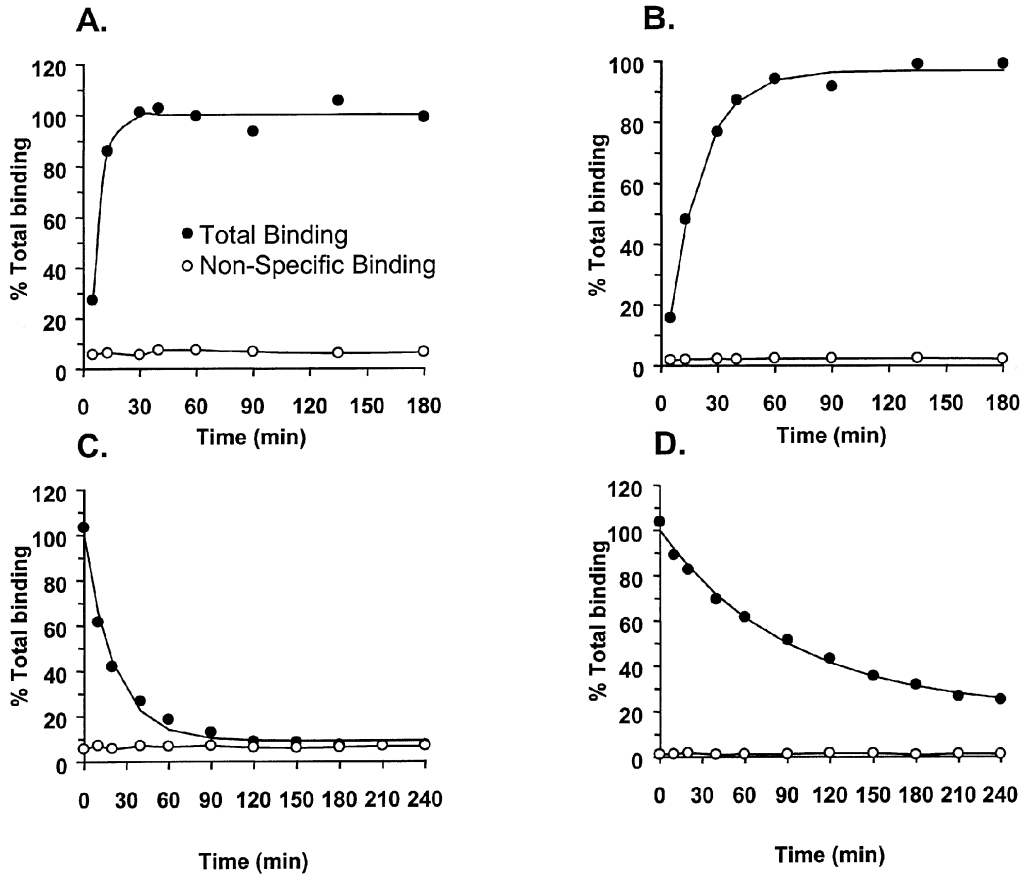

Figure 1 Time-courses of association (A, B) and dissociation (C, D) of [^{3}H]-melatonin binding to the human recombinant mt_1 (A, C) and MT_2 (B, D) receptors. Data are the mean of duplicate points and representative of three similar experiments. For association experiments, upper asymptote (mean$\pm$s.e.mean) = 0.37$\pm$0.05 pmol mg^{-1} (mt_1) and 0.39$\pm$0.01 pmol mg^{-1} (MT_2). For dissociation experiments, binding at time t_0 (mean$\pm$s.e.mean) = 0.38$\pm$0.03 pmol mg^{-1} (mt_1) and 0.40$\pm$0.03 pmol mg^{-1} (MT_2).

9.43 ± 0.06 and 9.04 ± 0.18 at mt_1 and MT_2 receptors respectively ($n=3$).

Comparison of [^{3}H]-melatonin and 2-[^{125}I]-iodomelatonin binding to mt_1 receptors

There was excellent agreement between the abilities of a range of melatonin analogues to compete for [^{3}H]-melatonin and 2-[^{125}I]-iodomelatonin binding to mt_1 receptors (Table 1). Linear regression analysis yielded a correlation coefficient of 0.99 and a slope of 1.01 ± 0.05 ($n=8$).

Pharmacological characterization of [^{3}H]-melatonin binding to mt_1 and MT_2 receptors

The abilities of fourteen melatonin analogues to compete for [^{3}H]-melatonin binding to mt_1 and MT_2 receptors were determined (Table 1; Figure 3A,B). All Hill coefficients were not significantly different from unity, with the exception of 2-iodomelatonin at mt_1 receptors (1.24 ± 0.07, $P<0.05$). Most melatonin receptor agonists bound with similar affinities to both receptor subtypes (linear regression analysis yielded a correlation coefficient of 0.87 and a slope of 0.71 ± 0.12). Greatest selectivity was exhibited by 2-iodomelatonin, which had 5 fold higher affinity for mt_1 receptors, and 6-chloromelatonin, which had 5 fold higher affinity for MT_2 receptors. A number of putative partial and silent receptor antagonists had substantially higher affinities for MT_2 than mt_1 receptors. Greatest selectivity was demonstrated by 4-P-PDOT, which had a 61 fold higher affinity for MT_2 compared to mt_1 receptors.

Inhibition of forskolin-stimulated cyclic AMP in CHO-mt_1 and CHO-MT_2 cells by melatonin

Melatonin produced a potent, monophasic and concentration-dependent inhibition of forskolin-stimulated cyclic AMP in both CHO-mt_1 and CHO-MT_2 cells, with pEC_{50} values of

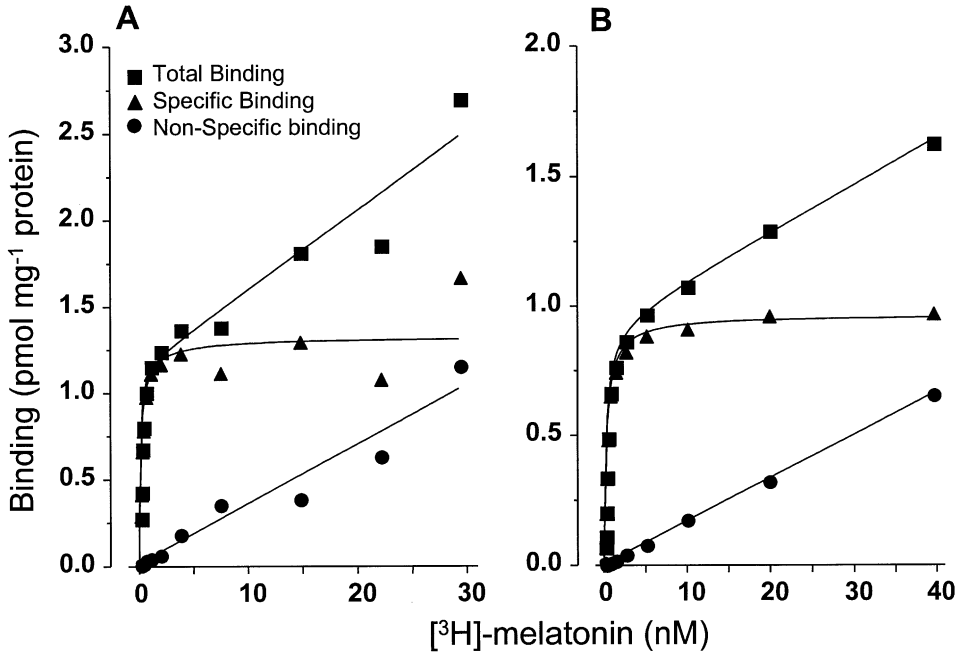

Figure 2 [^{3}H]-melatonin saturation binding to human recombinant mt_1 (A) and MT_2 (MT_2) receptors. Data are mean of duplicate points and representative of three separate experiments.

Table 1 Affinities of melatonin analogues to compete for [^{3}H]-melatonin and 2-[^{125}I]-iodomelatonin binding to human recombinant mt_1 and MT_2 receptors

Compound	mt_1 pK_i 2-[^{125}]-iodomelatonin	mt_1 pK_i [^{3}H]-melatonin	MT_2 pK_i [^{3}H]-melatonin	mt_1K_i/MT_2K_i
2-Iodomelatonin	10.57 ± 0.09	10.55 ± 0.11	9.87 ± 0.10	0.2
S20098	9.82 ± 0.09	10.09 ± 0.09	10.23 ± 0.09	1.4
Melatonin	9.67 ± 0.06	9.93 ± 0.06	9.65 ± 0.05	0.5
GR196429	9.39 ± 0.11	9.69 ± 0.09	9.66 ± 0.07	0.9
6-Hydroxymelatonin	8.94 ± 0.07	9.23 ± 0.05	8.79 ± 0.05	0.4
6-Chloromelatonin	8.90 ± 0.07	9.10 ± 0.05	9.77 ± 0.05	4.8
8-M-PDOT	ND	8.23 ± 0.07	8.95 ± 0.02	5.2
5-methoxyluzindole	ND	7.97 ± 0.03	9.20 ± 0.05	16.9
GR128107	ND	7.71 ± 0.08	9.07 ± 0.01	22.8
N-acetyl-5-HT	7.13 ± 0.35	7.08 ± 0.04	7.00 ± 0.14	0.8
Luzindole	6.21 ± 0.20	6.61 ± 0.04	7.63 ± 0.08	10.7
4-P-PDOT	ND	6.59 ± 0.04	8.37 ± 0.19	60.7
5-MCA-NAT	ND	5.85 ± 0.05	5.98 ± 0.05	1.4
5-HT	ND	<5.6	<5.6	

Values are mean $pK_i \pm$ s.e.mean from 3–12 separate experiments. All slope values were not significantly different from unity, with the exception of 2-iodomelatonin at mt_1 receptors (1.24 ± 0.07, $P<0.05$). ND = not determined.

9.53±0.16 and 9.74±0.05 and maximum inhibitions of 83±4 and 64±3%, respectively (Table 2; Figure 4A,B). Melatonin had no effect on basal levels of cyclic AMP in control or pertussis toxin treated cells, or on forskolin-stimulated cyclic AMP levels in mock transfected CHO cells (data not shown). Overnight incubation with pertussis toxin (100 ng ml^{-1}) completely abolished responses to melatonin in both CHO-mt_1 and CHO-MT_2 cells (Figure 4A,B). In CHO-mt_1 cells, pertussis toxin incubation revealed a small stimulatory response to melatonin in the presence of forskolin, with a pEC_{50} value of 8.37±0.17 and maximum response of 26±3% (0.1 μM) of forskolin-stimulated cyclic AMP (Figure 4A). This stimulatory response of melatonin was not observed in pertussis toxin-treated CHO-MT_2 cells (Figure 4B).

Pharmacological characterization of functional responses in CHO-mt_1 and CHO-MT_2 cells

The inhibitory effect of melatonin was mimicked by a range of indolic and non-indolic melatonin analogues (Figure 5A,B; Table 2). At the mt_1 receptor, the rank order of potency was 2-iodomelatonin > melatonin = S20098 > 6-chloromelatonin = GR196429 > 6-hydroxymelatonin > N-acetyl-5-HT, whilst at the MT_2 receptor the rank order was S20098 = 2-iodomelatonin > melatonin = 6-chloromelatonin > GR196429 > 6-hydroxymelatonin > N-acetyl-5-HT. At both mt_1 and MT_2 receptors all compounds tested were full agonists with respect to melatonin, with the exception of 6-hydroxymelatonin at the MT_2 receptor, which was a partial agonist with an intrinsic activity of 0.82±0.05 ($P<0.05$ vs melatonin, $n=4$). 2-iodomelatonin showed the greatest degree of mt_1 selectivity relative to melatonin, being 30 fold more potent than melatonin to inhibit forskolin-stimulated cyclic AMP in CHO-mt_1 cells, whilst only 2 fold more potent than melatonin in CHO-MT_2 cells. 6-chloromelatonin showed the greatest degree of MT_2 receptor selectivity relative to melatonin. In CHO-mt_1 cells, 6-chloromelatonin was 25 fold less potent than melatonin, whilst in CHO-MT_2 cells it was equipotent with melatonin. All other agonists had similar potency ratios, with respect to melatonin, at both mt_1 and MT_2 receptors.

Antagonist studies in CHO-mt_1 and CHO-MT_2 cells

The putative melatonin receptor antagonist luzindole (0.1–100 μM) produced a concentration-dependent rightward shift

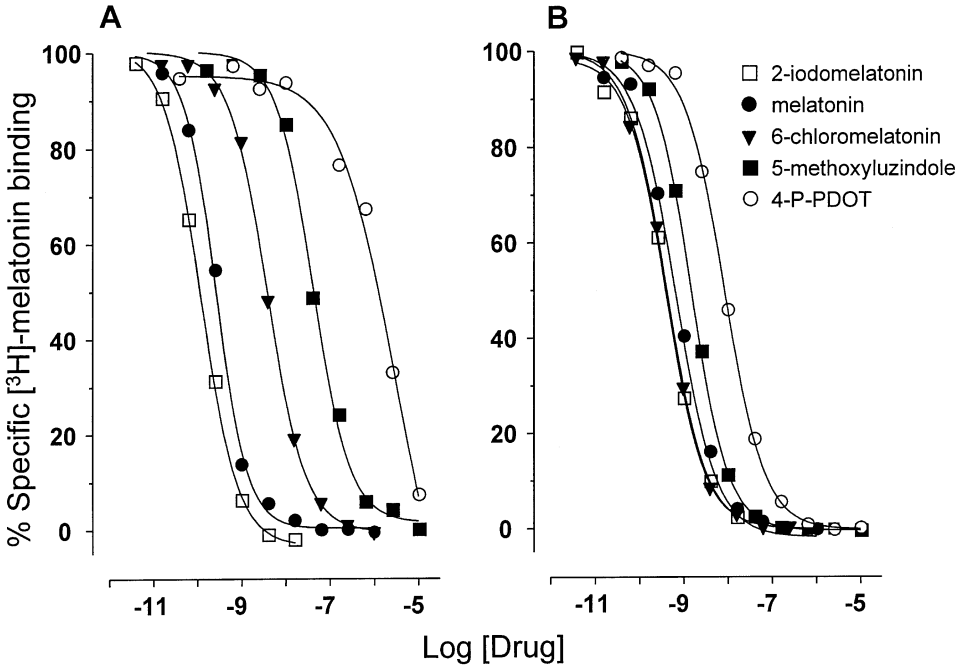

Figure 3 Competition for [^{3}H]-melatonin binding at human recombinant mt_1 (A) and MT_2 (B) receptors. Data are mean of duplicate points and representative of at least three separate experiments. Binding in the absence of competitor (mean±s.e.mean) = 0.42±0.02 pmol mg^{-1} (mt_1) and 0.35±0.02 pmol mg^{-1} (MT_2).

Table 2 Potencies of melatonin receptor agonists to inhibit forskolin stimulated cyclic AMP accumulation in CHO-mt_1 and CHO-MT_2 cells

	mt_1			MT_2			
Compound	pEC_{50}	E_{max}	*PR*	pEC_{50}	*PR*	E_{max}	*PR mt_1/PR MT_2*
2-Iodomelatonin	11.0±0.1	82±3	0.03	10.1±0.2	0.50	76±3	0.07
Melatonin	9.5±0.2	81±4	1.00	9.7±0.1	1.00	65±3	1.0
S20098	9.4±0.2	82±4	1.42	10.3±0.1	0.30	72±2	4.7
6-Chloromelatonin	8.1±0.1	85±4	24.6	9.8±0.2	1.02	60±9	24.0
GR196429	8.0±0.1	87±2	36	9.1±0.1	4.61	68±3	7.79
6-Hydroxymelatonin	7.2±0.1	76±3	205	7.4±0.1	231	50±5*	0.88
N-Acetyl-5-HT	5.7±0.1	84±5	7150	6.6±0.1	1170	65±2	6.1

Values are mean pEC50±s.e.mean of 3–11 separate experiments. PR, potency ratio with respect to melatonin; E_{max}, maximum response to agonist expressed as percentage inhibition of forskolin stimulated cyclic AMP; *significantly different from melatonin ($P<0.05$, $n=4$).

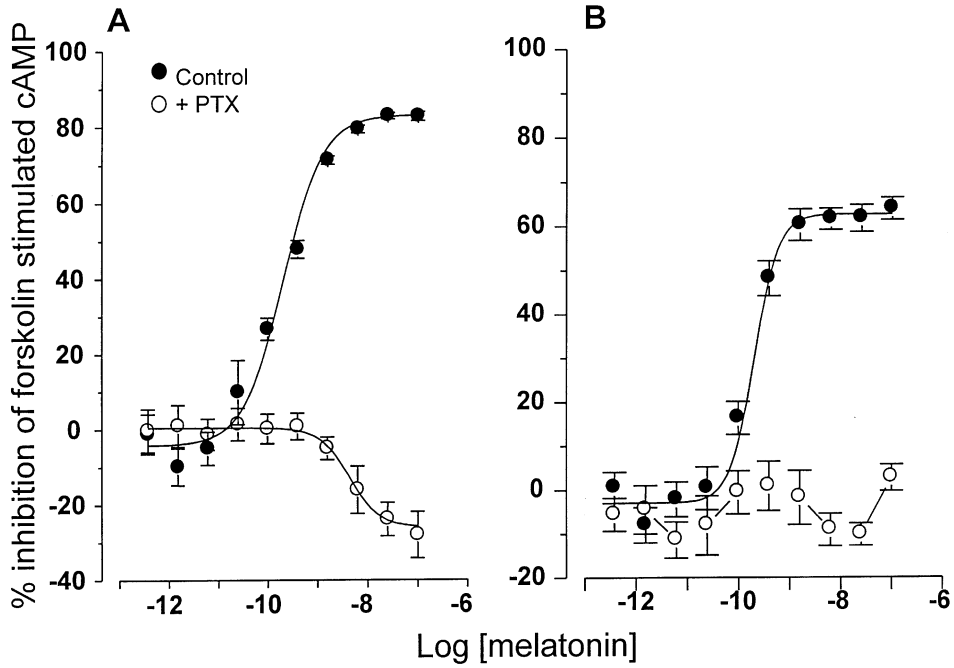

Figure 4 The effect of pertussis toxin (100 ng ml^{-1}) on melatonin-induced inhibition of forskolin-stimulated cyclic AMP production in CHO-mt_1 (A) and CHO-MT_2 (B) cells. Data are mean ± s.e.mean of three separate experiments. Mean basal cyclic AMP levels were 0.30 pmol $well^{-1}$ (range 0.18–0.35); forskolin-stimulated cyclic AMP levels were 27 pmol $well^{-1}$ (range 14–40). Similar values were obtained in subsequent experiments.

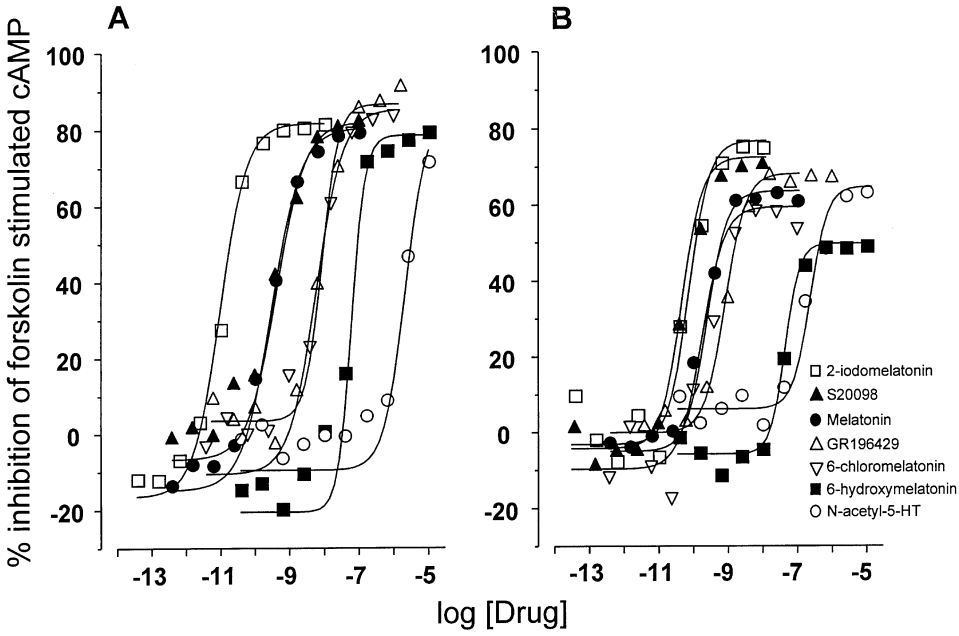

Figure 5 Agonist concentration-effect curves for inhibition of forskolin-stimulated cyclic AMP in CHO-mt_1 (A) and CHO-MT_2 cells. The data are mean of 3–11 separate experiments.

of the melatonin concentration-effect curves in both CHO-mt_1 and CHO-MT_2 cells (Figure 6A,B). Non-linear regression analyses yielded pA_2 values of 5.75 ± 0.10 and 7.64 ± 0.11 for mt_1 and MT_2 respectively ($n = 3–4$). Schild slope parameters for luzindole were 1.00 ± 0.10 and 0.94 ± 0.07 for mt_1 and MT_2 respectively, which were not significantly different to unity ($P > 0.05$). In CHO-mt_1 cells luzindole had no significant effect ($P > 0.05$) on Hill slopes or maximum responses to melatonin (data not shown). However, luzindole caused a concentration-dependent enhancement of the lower asymptote of the melatonin concentration-effect curve of 95 ± 20% ($P < 0.05$) at 30 μM, and 134 ± 36% ($P < 0.01$) at 100 μM, of forskolin-stimulated cyclic AMP (Figure 6A). Luzindole alone produced a small and variable enhancement of forskolin-stimulated cyclic AMP, which was not of the same magnitude as that seen in the presence of melatonin (Figure 6A and 7A). In contrast, in CHO-MT_2 cells, luzindole was without effect on forskolin-stimulated cyclic AMP, and had no effect on the maximum response to melatonin (Figure 6B and 7B). However, luzindole reduced the Hill slopes for the melatonin concentration-effect curves in a concentration-dependent manner (e.g. $n_H = 1.65 \pm 0.33$ and 0.89 ± 0.19, control and 10 μM luzindole

respectively; $P<0.05$). For these reasons, pA_2 rather than pK_B values have been quoted.

In CHO-mt_1 cells the amidotetraline 4-P-PDOT (10 μM) had no effect on forskolin-stimulated cyclic AMP levels, either alone (Figure 7A), or in the presence of melatonin (data not shown). In contrast, in CHO-MT_2 cells, 4-P-PDOT was an agonist, producing a concentration-dependent inhibition of forskolin stimulated cyclic AMP, with a pEC_{50} value of 8.72 ± 0.29 and intrinsic activity of 0.86 ± 0.15 ($n=3$; Figure 7B). This degree of agonism precluded the determination of a pA_2 value for 4-P-PDOT at MT_2 receptors.

Discussion

This study has described the pharmacology of human recombinant melatonin mt_1 and MT_2 receptors stably expressed in CHO cells, using a number of indolic and non-indolic analogues of melatonin. Human recombinant mt_1 and MT_2 receptors were expressed in identical cell lines using the same expression vectors and achieved similar receptor expression levels. This has allowed the pharmacology of each receptor to be compared in systems with identical cellular backgrounds.

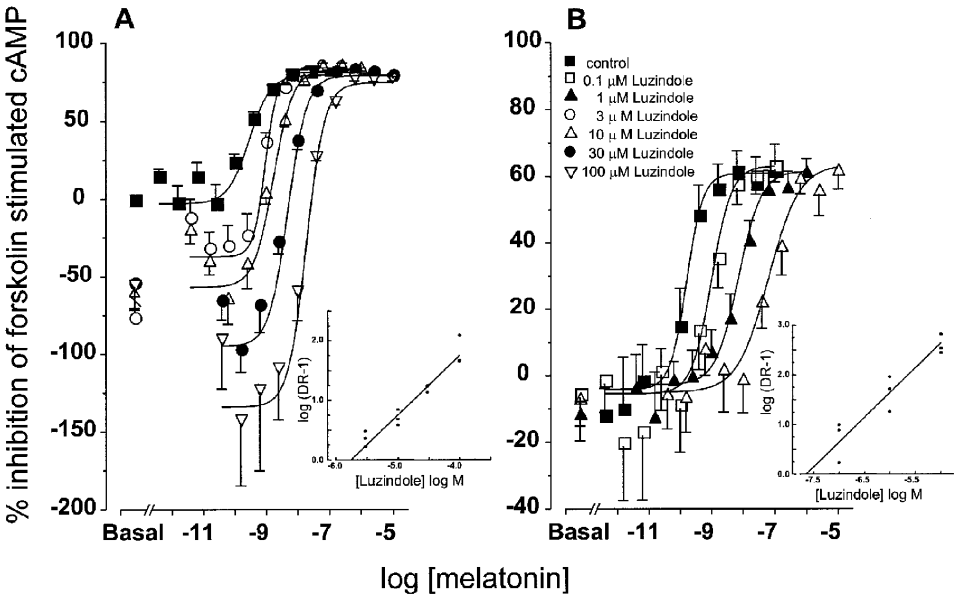

Figure 6 Antagonism by luzindole of melatonin-induced inhibition of forskolin-stimulated cyclic AMP production in CHO-mt_1 (A) and CHO-MT_2 (B) cells. Data are mean$\pm$s.e.mean of 3–4 separate experiments. Inset: Schild plots depicting antagonism of melatonin responses by luzindole. The line through the data was plotted using the parameters obtained from the analysis described in the Methods.

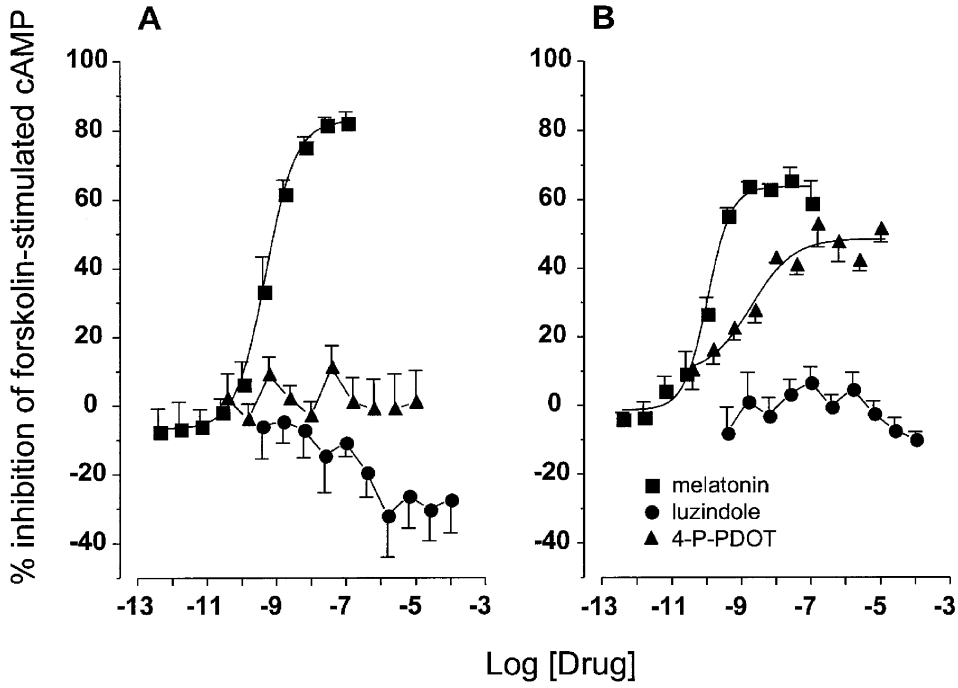

Figure 7 The effect of melatonin, luzindole and 4-P-PDOT on forskolin-stimulated cyclic AMP production in CHO-mt_1 (A) and CHO-MT_2 (B) cells. Data are mean$\pm$s.e.mean of 3–4 separate experiments.

The affinities of melatonin analogues at the mt_1 receptor measured using [^{3}H]-melatonin are in good agreement with affinities obtained using 2-[^{125}I]-iodomelatonin. This is the first report of the use of [^{3}H]-melatonin for the pharmacological characterization of melatonin receptor subtypes and validates the use of this radioligand as an alternative to 2-[^{125}I]-iodomelatonin. Similarly, Kennaway *et al*. (1994) showed comparable pharmacological profiles for the binding of these two radioligands to chicken brain. In addition to its safety benefits, [^{3}H]-melatonin is chemically identical to the endogenous hormone and is therefore the ligand of choice for the generation of structure-activity relationships. Indeed, Kennaway *et al*. (1994) concluded that 2-[^{125}I]-iodomelatonin may not be an appropriate ligand for melatonin binding studies because of its disproportionately slow dissociation rate.

Studies of association binding kinetics at mt_1 receptors showed that [^{3}H]-melatonin associated rapidly and reached equilibrium after 30 min. At the MT_2 receptor, [^{3}H]-melatonin associated more slowly and reached equilibrium after approximately 60 min. Interestingly, [^{3}H]-melatonin dissociated from the MT_2 receptor over a much longer period of time, and failed to reach non-specific binding levels, with 22% of specifically bound radioligand still present after 240 min. Our data suggests that, over the time frame of the experiment, a component of the [^{3}H]-melatonin binding to the MT_2 receptor may be irreversible. This result may be explained using a model of multiple receptor states. Long term exposure of receptor with radioligand may 'lock' a proportion of occupied receptor into a super-high affinity state which may appear irreversible over the time frame of the experiment. This behaviour of slowly dissociating radioligands has been reported for a number of 7-transmembrane, G-protein coupled receptors and has been shown to be sensitive to the presence of guanine nucleotides, suggesting the presence of high affinity, agonist occupied, G-protein linked receptors (Cohen *et al*., 1996). Interestingly, whilst the pK_D of 9.78 for [^{3}H]-melatonin at the mt_1 receptor determined from kinetic studies was in good agreement with the value of 9.89 estimated from equilibrium saturation binding, at the MT_2 receptor the pK_D of 9.92 determined from kinetic studies was nearly half a log unit higher than the pK_D of 9.56 estimated from equilibrium saturation binding. The irreversible component of the binding of [^{3}H]-melatonin to the MT_2 receptor may explain this discrepancy.

The affinities of a range of melatonin analogues at mt_1 and MT_2 receptors are in broad agreement with those obtained by other groups (Dubocovich *et al*., 1997; Reppert *et al*., 1995). Most ligands were either non-selective or showed some degree of MT_2 selectivity. Ligands which exhibited the greatest degree of selectivity were 2-iodomelatonin, having 5 fold higher affinity at the mt_1 receptor, and the amidotetraline derivative, 4-P-PDOT, with 60 fold higher affinity for the MT_2 receptor. Similarly, Dubocovich and co-workers (1997) have reported that 4-P-PDOT had 300 fold higher affinity at the MT_2 receptor.

Functional studies demonstrated that melatonin receptor activation resulted in the inhibition of forskolin-stimulated cyclic AMP production. The abrogation of the response to melatonin following pre-treatment with pertussis toxin indicates that this response is mediated *via* G_i/G_o G-proteins, confirming previous reports using both recombinant human receptor systems (Reppert *et al*., 1994; 1995) and sheep pars tuberalis (Morgan *et al*., 1990). In CHO-mt_1 cells, pertussis toxin treatment revealed a small enhancement by melatonin of forskolin-stimulated cyclic AMP, suggesting that the mt_1 receptor can promiscuously couple to G-proteins other than G_i/G_o, presumably G_s. Promiscuous coupling of melatonin receptors has been previously reported for cloned *Xenopus* receptors expressed in human embryonic kidney 293 cells (Yung *et al*., 1995) and endogenous receptors in sheep pars tuberalis (Morgan *et al*., 1990; 1995). Interestingly, the stimulatory response to melatonin following pertussis toxin treatment was not observed in CHO-MT_2 cells, indicating a difference in the second-messenger coupling characteristics of these receptors.

In both CHO-mt_1 and CHO-MT_2 cells, luzindole antagonised melatonin responses in a concentration-dependent manner, with approximately 80 fold higher affinity at the MT_2 receptor. This is the first direct comparison of the affinity of luzindole for mt_1 and MT_2 receptors measured in a functional assay and confirms previous reports that luzindole has selectivity for MT_2 receptors (Dubocovich *et al*., 1997). However, the pA_2 value of 5.75 for luzindole at the mt_1 receptor is somewhat lower than the values of 7.2 and 7.3 determined by Beresford and co-workers (1998b) and Witt-Enderby & Dubocovich (1996) respectively. It is not clear the extent to which the pA_2 value for luzindole at the mt_1 receptor determined in this study is compromized by its complex action in this system. In CHO-mt_1, but not in CHO-MT_2 cells, luzindole alone produced a small increase in forskolin-stimulated cyclic AMP, suggesting that luzindole may act as an inverse agonist at the mt_1 receptor. In support of this hypothesis, Dubocovich & Masana (1998) have shown that the affinity of luzindole for the mt_1 receptor increased in the presence of GTP, a property which is displayed by inverse agonists. Interestingly, in the presence of low concentrations of melatonin, luzindole enhanced forskolin-stimulated cyclic AMP, indicating that the inverse agonist response can be augmented in the presence of melatonin. Alternatively, as our experiments with pertussis toxin suggest that the mt_1 receptor can promiscuously couple to non G_i/G_o G-proteins, luzindole may potentiate the signalling of melatonin through this alternative G-protein pathway. Further studies are clearly required to elucidate the exact nature of the effects of luzindole and its interaction with melatonin at the mt_1 receptor.

In CHO-mt_1 cells, the putative MT_2 receptor selective antagonist 4-P-PDOT, at concentrations up to 10 μM, had no effect on forskolin-stimulated cyclic AMP levels alone, and did not affect melatonin concentration-effect curves. In contrast, in CHO MT_2 cells, 4-P-PDOT was a partial agonist with respect to melatonin, producing a concentration-dependent inhibition of forskolin-stimulated cyclic AMP accumulation with a pEC_{50} of 8.7 and intrinsic activity of 0.87. The intrinsic activity of 4-P-PDOT at the MT_2 receptor precluded the determination of a pA_2 value for this compound. Whilst this result supports the claim that 4-P-PDOT is a MT_2 receptor selective compound (Dubocovich *et al*., 1997), 4-P-PDOT did not behave as a neutral antagonist in our assay. In agreement, Nonno and co-workers (1999) recently reported that 4-P-PDOT was a partial agonist (intrinsic activity of 0.37) at the MT_2 receptor in a [^{35}S]-GTPγS binding assay, a functional measure of receptor activation. In contrast, 4-P-PDOT has previously been shown to be a silent, simple competitive antagonist with high affinity at the presynaptic MT_2 receptor mediating the inhibition of electrically-evoked dopamine release in rabbit retina (Dubocovich *et al*., 1997). The lack of agonism in the rabbit retina may be explained by the lower level of receptor expression in this tissue (8.5 fmol mg^{-1} protein; Dubocovich, 1995), compared with 0.2 pmol mg^{-1} protein in our CHO-MT_2 cells and 0.5 pmol mg^{-1} in the NIH3T3 cells used by Nonno *et al*. (1999). It is well established that a reduction in receptor number may reduce the sensitivity of a tissue to low efficacy

agonists (Furchgott, 1966). Therefore, the rabbit retina may not reveal the agonist activity of low efficacy compounds. Interestingly, it has recently been demonstrated that 4-P-PDOT is a silent antagonist of melatonin responses in an animal model of circadian rhythms (Dubocovich *et al*., 1998b).

While antagonist affinity estimates provide a theoretically robust method of classifying and characterizing G-protein coupled receptors, functional data from agonist experiments can also be used to characterize receptors (Leff, 1987). Estimates of agonist potency alone are of little value in receptor classification, as agonist potency is dependent on both receptor density and transduction efficiency (Kenakin & Beek, 1980). However, the potency of an agonist, when expressed relative to the potency of a reference agonist, provides a robust parameter for receptor classification, since this value will be tissue-independent for full agonists. Therefore, agonists that yield different potency ratios with respect to melatonin can be used to discriminate between mt_1 and MT_2 receptors. 2-iodomelatonin was 30 fold more potent than melatonin in CHO-mt_1 cells, but had a similar potency to melatonin in CHO-MT_2 cells. Conversely, 6-chloromelatonin was 25 fold less potent than melatonin in CHO-mt_1 cells, whilst being equipotent with melatonin in CHO-MT_2 cells. At MT_2 receptors, melatonin, 2-iodomelatonin and 6-chloromelatonin are essentially equipotent, whilst at the mt_1 receptor these agonists have the rank order of potency of 2-iodomelatonin > melatonin > 6-chloromelatonin. Thus, in addition to antagonist data, these three agonists may be of use in classifying melatonin receptors.

In conclusion, we have expressed human recombinant melatonin receptors in CHO cells using identical expression systems and have characterized these receptors using [^{3}H]-melatonin binding assays and measurement of forskolin stimulated cyclic AMP accumulation. Luzindole is an antagonist at melatonin receptors, with higher affinity at MT_2 receptors. In addition to the use of antagonists, the rank order of potency of melatonin, 2-iodomelatonin, and 6-chloromelatonin can be used to classify melatonin receptors.

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