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Pharmacological characterization of the human melatonin Mel_{1a} receptor following stable transfection into NIH3T3 cells

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1 Mouse fibroblasts (NIH3T3) transfected with the full-length coding region of the Mel_{1a} melatonin receptor stably expressed the receptor, coupled to a pertussis toxin-sensitive G-protein(s) and exhibiting high affinity and adequate pharmacological profile.

2 The receptor protein had the tendency of a strong coupling to the G-protein and therefore low-affinity state was induced by uncoupling the receptor from its G-protein in presence of high concentrations of NaCl (500–700 mM) and/or GTPγS (100 μM). Thereafter, the affinity of a series of melatonin analogues was determined to both, high- and low-affinity receptor states, thus providing a basis for the prediction of their efficacy, according to the ternary complex model.

3 The cells were subsequently used to study the agonist-induced G-protein activation, determined by calculating the rate of GDP-GTP exchange measured in presence of ^{35}S-labelled GTPγS. The natural ligand melatonin induced a significant increase in the GDP-GTP exchange rate, the presence of GDP and NaCl being necessary to observe this effect.

4 The full agonists 2-phenylmelatonin, 2-bromomelatonin and 6-chloromelatonin equally induced an increase of the GDP-GTP exchange. 5-Hydroxy-*N*-acetyltryptamine activated the GTP-GDP exchange to a much lesser extent (53%) than melatonin, thus behaving as a partial agonist. As predicted by the model, the melatonin antagonist (*N*-[(2-phenyl-1*H*-indol-3-yl)ethyl]cyclobutanecarboxamide) was without effect on basal G protein activation. Coincubation of this compound with melatonin induced a dose-dependent rightward shift in the melatonin concentration-effect curve, thus exhibiting the behaviour of a competitive and surmountable antagonist.

5 Using the equation proposed by Venter (1997) we were able to determine that there were no 'spare' receptors in the system. Therefore, the approach proposed in the present work can be successfully used for the determination of 'drug action' at the level of the human Mel_{1a} melatonin receptor and evaluation of the efficacy of new selective melatonin analogues.

Keywords: Human melatonin receptor; melatonin analogues; efficacy; G_i-coupled receptors; [^{35}S]-GTPγS binding

Introduction

Melatonin, the principal hormone secreted by the human pineal gland, has a number of physiologically relevant actions associated with the regulation of circadian-related phenomena (Dollins *et al*., 1994; Hagan & Oakley, 1995; Lewy *et al*., 1995). Melatonin actions are interceded through high affinity binding sites belonging to the superfamily of the seven-transmembrane domain receptors that couple to heterotrimeric G-proteins (Reppert *et al*., 1994; 1995). Currently, two subtypes of human melatonin receptors have been identified: Mel_{1a} (preferentially expressed in the brain) (Reppert *et al*., 1994) and Mel_{1b} (preferentially expressed in the retina) (Reppert *et al*., 1995). Expression of the human melatonin receptor in CHO and COS cells have also demonstrated receptor coupling to a pertussis toxin-sensitive G protein (Reppert *et al*., 1994; 1995; Witt-Enderby & Dubocovich, 1996).

Agonist occupation of the melatonin receptor leads to inhibition of stimulated adenilyl cyclase and cyclic AMP (Morgan *et al*., 1994), an event mediated by pertussis toxin-sensitive G protein(s). The ability of forskolin to stimulate the intracellular cyclic AMP accumulation and the capacity of melatonin and its agonists to inhibit the effects of forskolin have been employed to study the melatonin receptor signal-transduction pathway and to determine the biological activity of melatonin analogues in cells (e.g. ovine pars tuberalis, quail optic lobes) that naturally express melatonin receptors (Morgan *et al*., 1994; Spadoni *et al*., 1997; Tarzia *et al*., 1997). Such methodology relies on fresh, non homogeneous biological material, a measurement of secondary, downstream events (quantification of cyclic AMP) and is not very sensitive and reliable. Moreover, these preparations generally contain a heterogeneous receptor population and therefore do not lend themselves to the unequivocal characterization of analogues action at a specific receptor subtype. For these reasons, the pharmacology and biochemistry of the human melatonin receptor have not been adequately studied.

A cellular system stably expressing a single subtype human melatonin receptor is the only way to avoid the intrinsic problems associated with the study of the melatonin receptor signal-transduction pathway in man and the activity of new selective melatonin analogues at the receptor level. The recent cloning of the human melatonin receptor Mel_{1a} (Reppert *et al*., 1994; Mazzucchelli *et al*., 1996) has allowed us to study the pharmacology and biochemistry of this receptor subtype in isolation.

One of the first events following agonist occupation of the receptor binding domain is the guanine nucleotide exchange (Birnbaumer *et al*., 1990). GDP, bound to Gα subunit is replaced by GTP. A dissociation of the Gα-GTP from the βγ subunits follows, with the subsequent downstream events

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stimulated by both Gα-GTP and $\beta\gamma$. Coincubation with the non-hydrolyzable GTP analogue GTPγS results in permanent activation of the G-protein, because GTPγS is not readily susceptible to the intrinsic GTPase activity of Gα (Higashijima *et al*., 1987). Using [^{35}S]-GTPγS, the activation of pertussis toxin-sensitive G proteins after agonist occupation of membrane-bound receptors has been determined as an increase of the binding of the labelled nucleotide to the membranes (Hilf *et al*., 1989; Lorenzen *et al*., 1993; Lazareno & Birdsall, 1993; Tian *et al*., 1994).

Affinity and efficacy are the two key factors that determine the interaction between the receptor and its ligand. In this study, we have used NIH3T3 mouse fibroblast cell line stably transfected with the cloned Mel_{1a} human melatonin receptor to study agonist-mediated G-protein activation and the binding of melatonin ligands under conditions favouring high- and low-affinity states of the receptor. The efficacy of analogues measured by means of [^{35}S]-GTPγS binding correlated with the magnitude of ligand affinity ratio for the low *vs* high affinity states of the receptor. The increase of [^{35}S]-GTPγS binding stimulated by melatonin agonists is a ready 'functional' measure of agonist occupation of melatonin receptors and thus, the system is able to distinguish compounds of different efficacy and intrinsic activity.

Methods

Transfection

Full-length cDNAs coding for the human Mel_{1a} melatonin receptor, cloned from human cerebellum (Mazzucchelli *et al*., 1996), was subcloned into pcDNA INeo and used to transfect NIH3T3 mouse fibroblast cells by cationic liposome-mediated transfection (lipofection), by use of the transfection kit DOTAP (Boehringer Mannheim, New York, NY). In order to obtain optimal results we performed a first transfection with 8 μg/plate of cDNA and a second treatment after 24 h. The NIH3T3 cells were plated at a density yielding approximately 60% confluency at the time of transfection. Cells were cultured in Dulbecco's modified Eagle's medium containing high glucose (4,5 g^{-1}), 10% bovine calf serum, 1 mM sodium pyruvate, in 5% CO_2/95% air at 37°. Selection with G418 (1 mg ml^{-1}) was started 48 h after transfection. Transformed NIH3T3 cells were isolated and the single colonies were selected by using 2-[^{125}I]iodomelatonin binding (with a radioligand concentration of 100 pM). Colonies expressing more than 150 fmol mg^{-1} of total cellular protein were plated in 150 cm^2 flasks.

Membrane preparation

NIH3T3 cells stably expressing the cloned human Mel_{1a} receptor were grown to confluence. On the day of assay cells were detached from flasks with 4 mM EDTA in 50 mM Tris-HCl (Ph 7.4 at room temperature) and precipitated at 1000 × *g* for 10 min at 4°C. The cells were then resuspended in 2 mM EDTA/50 mM Tris-HCl, homogenized in 10–15 volumes of ice-cold 2 mM EDTA/50 mM Tris-HCl with Ultra-Turrax and centrifuged at 50000 × *g* at 4°C for 25 min. The final pellet was then resuspended in ice-cold 50 mM Tris-HCl assay buffer.

In experiments with pertussis toxin cells were treated with 100 ng ml^{-1} of pertussis toxin in culture medium for the 24 h preceding the day of assay, and then prepared as described above. Membrane protein levels were determined according to the method of Bradford (1976).

2-[^{125}I]-iodomelatonin binding

The final membrane concentration was 5–10 μg of protein per tube. The binding conditions were described in detail elsewhere (Stankov *et al*., 1991). The incubation time was 90 min. In preliminary experiments, for colonies selection, studies on the effect of $MgCl_2$ (2 mM), NaCl (300 mM), $CaCl_2$ (4 mM) and GTPγS (100 μM) and in competition experiments, total 2-[^{125}I]-iodomelatonin concentration was 100 pM. In saturation studies 2-[^{125}I]-iodomelatonin was added in the concentration range of 10 up to 1000 pM. In competition curves with NaCl (700 mM) and GTPγS (100 μM) the radioligand concentration was 200 pM (for further explanations about this method (see Spadoni *et al*., 1997 and Tarzia *et al*., 1997). 2-[^{125}I]-iodomelatonin nonspecific binding was measured in the presence of 0.1 μM cold 2-iodomelatonin.

[^{35}S]-GTPγS binding

Agonist-stimulated [^{35}S]-GTPγS binding was studied by using a modification of previously published methods (Hilf *et al*., 1989; Lorenzen *et al*., 1993; Lazareno *et al*., 1993; Tian *et al*., 1994). The final pellet, obtained as described above, was resuspended in ice-cold 50 mM Tris-HCl assay buffer to give a final membrane concentration of 20–30 mg ml^{-1}. Then membranes (15–25 μg of protein) were incubated for 30 min at 30°C, with and without various drugs, in assay buffer containing 0.3–0.5 nM [^{35}S]-GTPγS, 50 μM GDP, 100 mM NaCl and 3 mM $MgCl_2$. The final incubation volume was 100 μl. Basal binding was assessed in the absence of drug and nonspecific binding was measured in the presence of 10 μM GTPγS. In preliminary experiments GDP 0.1–100 μM and NaCl 1–100 mM were used. The incubation was terminated by adding 1 ml of ice-cold Tris-HCl buffer, pH 7.4, followed by rapid filtration under vacuum through Whatman GF/B glass fibre filters and by three washes with 3 ml of ice-cold Tris-HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction in 4 ml Filter-Count scintillation fluid (Packard, Downers Grove, IL).

Data analysis

Data are presented as mean ± s.e. of at least three independent experiments that were each performed in duplicates (2-[^{125}I]-iodomelatonin binding experiments) or triplicates ([^{35}S]-GTPγS binding experiments). The IC_{50}, EC_{50} and K_d values were determined by using nonlinear fitting strategies. Saturation curves were analysed with the one-site model compared with the two-site model. A two-site model was accepted only when the 'goodness-of-fit' was significantly ($P<0.05$) improved by this model, as tested using a partial *F*-test procedure (De Lean *et al*., 1978). K_i values were calculated from the IC_{50} values using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). The IC_{50} shifts induced by coincubation with GTPγS and NaCl in competition experiments were calculated as the ratio of IC_{50} in presence of GTPγS and NaCl *vs* IC_{50} in absence of GTPγS and NaCl, and the relative indexes were calculated as analogue IC_{50} shift/melatonin IC_{50} shift. The data from [^{35}S]-GTPγS binding experiments are given as percentage of basal binding, where the basal binding was fixed as 100%. The relative intrinsic efficacies are expressed as a fraction of melatonin maximal net stimulation. The analysis of competitive interaction between melatonin and *N*-[(2-phenyl-1*H*-indol-3-yl)ethyl]cyclobutanecarboxamide was also made by non-

linear fitting by using the following equation as proposed by Lew & Angus (1995):

$$\text{(a) } pEC_{50} = -\log([B] + 10^{pK_b}) - \log c$$

where the agonist pEC_{50} in presence of a given antagonist concentration is plotted against the antagonist concentration [B] and allows the estimation of the pK_b value as a fitted parameter. The parameter $-\log c$ is the difference between the antagonist pK_b and the agonist pEC_{50}. Two other equations were used (Lew & Angus, 1995) in order to test a possible deviation from a simple competitive agonist-antagonist interaction:

$$\text{(b) } pEC_{50} = -\log([B]^n + 10^{-pK_b}) - \log c$$

that allows the molecularity of the agonist-antagonist interaction to vary by allowing the exponent of [B] to vary from unity, and

$$\text{(c) } pEC_{50} = -\log([B]\ (1 + n[B]/10^{-pK_b}) - \log c$$

that allows a 'quadratic departure' equivalent to a nonlinear Shild plot. The significance of these deviations were tested by comparing the 'goodness-of-fit' of equation (a) with those of equations (b) and (c).

In order to test the possible presence of 'spare' receptors in our system (defined as the fraction of the total receptor pool not required for maximal G protein activation in NIH3T3 cells) we used the equation proposed by Venter (1997).

$$H = -K_a/K_b \Phi H + e^{ES}$$

where H is the height of the concentration-effect curve, Φ is a fixed concentration ratio [antagonist]/[agonist] and e^{ES} is an effect-stimulus parameter, related to efficacy, that may be defined as $e^{ES} = h_m/H_m$, that is maximum height of the concentration stimulus curve/maximum height of the concentration effect curve. An $e^{ES} = 1$ indicates the absence of 'spare' receptors.

Drugs

2-[^{125}I]-iodomelatonin (specific activity $\approx$2000 Ci mmol^{-1}) and [^{35}S]-GTPγS (specific activity 1070 Ci mmol^{-1}) were purchased from Amersham (Buckinghamshire, U.K.). Melatonin, N-acetyl 5′-hydroxytryptamine, pertussis toxin, GDP and GTPγS were from Sigma Chemical Co. (St. Louis, MO). 2-Iodomelatonin was obtained from RBI (Natick, MA). 6-Chloromelatonin was a gift from Ely Lilly laboratories (Indianapolis, IN). 2-Bromomelatonin, 2-phenylmelatonin and *N*-[(2-phenyl-1*H*-indol-3-yl)ethyl]cyclobutanecarboxamide were synthesized as described elsewhere (Duranti *et al*., 1992; Spadoni *et al*., 1993; Garrat *et al*., 1995). Geneticin (G418) was purchased from GIBCO (Grand Island, NY). General laboratory reagents including Tris HCl, bovine calf serum, Dulbecco's modified Eagle's medium were from Sigma.

Results

Transfection

A number of clones ($\approx$30) were obtained and tested for Mel_{1a} receptor expression with 2-[^{125}I]-iodomelatonin (100 pM). The clone expressing the highest number of receptors ($\approx$400 pM; single point assay) was chosen for subsequent studies, in order to ensure a good effect-to-noise ratio in the G protein stimulation assay.

Binding of 2-[^{125}I]-iodomelatonin to the Mel_{1a} receptor

Saturation experiments, conducted with a radioligand concentration range of 10–1000 pM showed that, in the absence of sodium ions and GTPγS, 2-[^{125}I]-iodomelatonin binds to a single class of high affinity binding sites (Table 1 and Figure 1a), with a K_d of 21$\pm$3 pM and a B_{max} of 620$\pm$35 fmol mg^{-1} protein. The effect of coincubation with Mg^{2+} (2 mM), Ca^{2+} (4 mM), Na^+ (300 mM) and GTPγS (100 μM) on 2-[^{125}I]-iodomelatonin (100 pM) binding was subsequently investigated (data not shown). Both calcium and magnesium ions were unable to affect 2-[^{125}I]-iodomelatonin binding; these findings, together with the presence of a single high affinity receptor population shown in the saturation analysis, indicate that in these conditions most of the melatonin receptors are in the high affinity, G protein-coupled state. Sodium ions caused a significant decrease (57% of total binding), while coincubation of membranes with GTPγS caused a decrease to a lesser extent than expected (20% decrease from the total binding), taking into account the effect of GTPγS on the native receptor in the human cerebellum (Fauteck *et al*., 1994). Both sodium ions and GTPγS are known to uncouple the receptor-G protein complexes, acting probably through two different sites with distinct mechanisms. Thus it appears that Mel_{1a} receptors expressed in NIH3T3 cells have retained their ability to couple efficiently with G proteins. In order to investigage further receptor-G protein coupling, a series of saturation experiments conducted in the presence of GTPγS and NaCl were performed (data are summarized in Table 1). GTPγS (100 μM) caused a significant decrease of the affinity (K_d values increasing from 21$\pm$3 to 40$\pm$5 pM) without any change in the B_{max}. The saturation curves obtained in the presence of 500 mM NaCl (Figure 1a) were significantly better described by the two-site model, with 22% of receptors being in the high affinity state, K_d = 19$\pm$2.8 pM (K_{dhigh} in Table 1) and 78% in the low affinity state, with a K_d of 325$\pm$52 pM (K_{dlow} in Table 1). GTPγS and NaCl (500 mM) showed a cumulative effect when coincubated with the membranes; in fact the % of receptors in the high affinity state further decreased (from 22% to 11%) without any change in K_{dhigh}, K_{dlow} or B_{maxtot} (the sum of $B_{maxhigh}$ and B_{maxlow}) values. An increase in the NaCl concentration (700 mM) was able to decrease further the number of high affinity binding sites (from 11% to 7.5%).

Table 1 Effect of GTPγS and NaCl on 2-[^{125}I]-iodomelatonin saturation binding isotherms

	Tris HCl	*GTPγS* (100 μM)	*NaCl* (500 mM)	*GTPγS* (100 μM) + *NaCl* (500 mM)	*GTPγS* (100 μM) + *NaCl* (700 mM)
K_{dhigh} (pM)	21$\pm$3	40$\pm$5	19$\pm$2.8	23$\pm$2.5	20$\pm$4.1
K_{dlow} (pM)	ND	ND	325$\pm$52	317$\pm$43	337$\pm$64
B_{maxtot} (fmol mg^{-1} protein)	620$\pm$35	671$\pm$41	682$\pm$68	602$\pm$50	621$\pm$22
% high affinity sites	a	a	22$\pm$2	11$\pm$2	7.5$\pm$1.2

ND = not detectable; a = in these cases the one-site model fitted better than the two-site model. This model assumes 100% high affinity binding sites.

Competition of the 2-[^{125}I]-iodomelatonin binding by melatonin and its analogues

The pharmacological profile of the transfected Mel_{1a} receptor was studied in a series of competition experiments, with a number of known melatonin analogues (Figure 1b). Melatonin showed a K_i of 291 ± 12 pM (Table 2). The pharmacological profile was: 2-phenylmelatonin ≥ 2-bromomelatonin > melatonin > 6-chloromelatonin > *N*-[(2-phenyl-1*H*-indol-3-yl)ethyl]-cyclobutanecarboxamide (we will refer to this compound as compound 6) ≥ N-acetyl 5-hydroxytryptamine (NAS) K_i values are shown in Table 2).

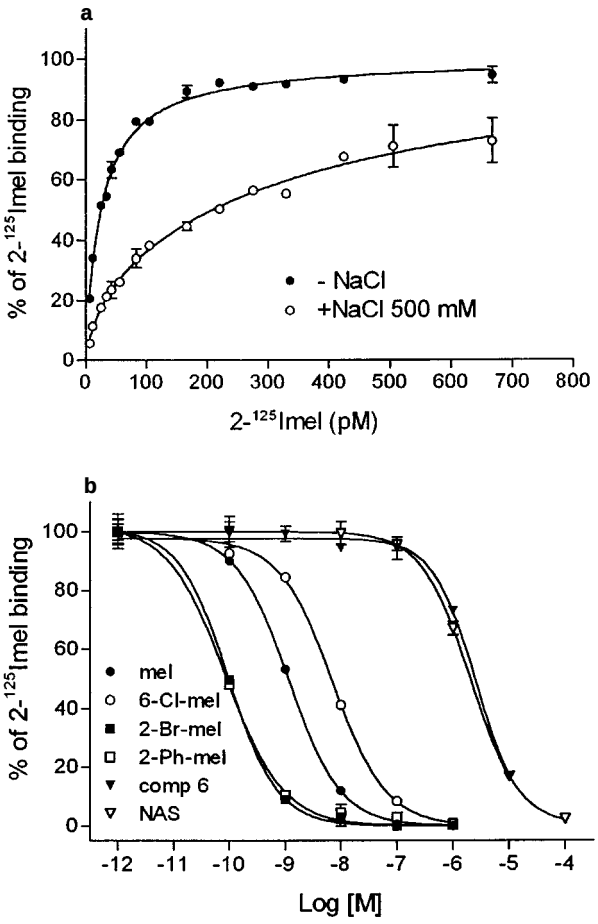

Figure 1 (a) Representative saturation isotherms of 2-[^{125}I]-iodomelatonin binding to $NIH3T3_{Mel1a}$ membranes in the absence and presence of 500 mM NaCl. The calculated B_{max} in the absence of NaCl was 596 fmol mg^{-1} protein, taken as 100%; the K_d was 24 pM (non-linear fitting). The data in the presence of NaCl were significantly better described by the two-site model, with 23% of the receptors being in the high affinity state (K_{dhigh} of 25 pM) and 73% in the low affinity state (K_{dlow} of 345 pM). (b) Competition of melatonin and its analogues to 2-[^{125}I]-iodomelatonin (100 pM) binding to $NIH3T3_{Mel1a}$ membranes. The IC_{50} values were: 2-phenylmelatonin: 8.59 E^{-11} > 2-bromomelatonin: 9.57 E^{-11} > melatonin: 1.14 E^{-9} > 6-chloromelatonin: 7.11 E^{-9} > NAS: 2.03 E^{-6} > compound 6: 2.57 E^{-6}. The experiments were carried out as described in Methods, for 90 min at 37°C. The points are the means of duplicates; the vertical lines indicate the s.e.

According to the ternary complex model the efficacy of a receptor ligand is related to the K_{dlow} K_{dhigh} ratio (Wreggett & De Lean, 1984). As seen from the saturation studies as well, even in extreme conditions (700 mM NaCl + 100 μM GTPγS) it was not possible to shift the entire receptor population into the low affinity state. Therefore it was impossible to calculate the 'real' K_{ilow} values through competition analysis. For that reason, we decided to perform a series of competition experiments conducted in the presence and absence of 100 μM GTPγS and 700 mM NaCl, using 200 pM radioligand concentration, in order to calculate the IC_{50}-shift caused by receptor-G protein uncoupling as a measure of the difference between K_{dhigh} and K_{dlow} (Spadoni *et al*., 1997; Tarzia *et al*., 1997). Data are presented in Table 2. The melatonin IC_{50}-shift value was ≈4 (Figure 2); similar shift values were calculated for 2-bromomelatonin, 2-phenylmelatonin and 6-chloromelatonin. NAS showed a shift of 1.5 while compound 6, known to be an antagonist (Garratt *et al*., 1995; Spadoni *et al*., 1997; Tarzia *et al*., 1997) showed a 12 times decrease of the IC_{50} value in the presence of GTPγS and NaCl, with a calculated IC_{50}-shift value of 0.08 (Figure 2).

Conditions for [^{35}S]-GTPγS binding

Initial studies varying GDP and NaCl concentrations were carried out to determine optimal conditions to study [^{35}S]-GTPγS binding to $NIH3T3_{Mel1a}$ membranes. Increasing GDP concentrations, from 1 nM to 0.1 mM (in the absence and presence of 10 and 100 mM NaCl) caused a dose-dependent decrease in basal [^{35}S]-GTPγS binding; the GDP IC_{50} decreased with the highest NaCl concentration (data not shown). Also NaCl (1 – 100 mM) decreased basal [^{35}S]-GTPγS binding and its effect was concentration- and GDP-dependent (data not shown). The effect of GDP and NaCl on the melatonin-induced increase in [^{35}S]-GTPγS binding was subsequently investigated, by attempting to maximize the specific stimulation. The specific melatonin stimulation (100 nM) of [^{35}S]-GTPγS binding appeared to be both, GDP- and NaCl-dependent. In the absence of either NaCl or GDP, the melatonin-induced G-protein activation was negligible (Figures 3a and b). Increasing the GDP concentration, in the presence of a fixed concentration of NaCl (100 mM), caused a decrease in both, basal and melatonin-stimulated [^{35}S]-GTPγS binding. However, the net melatonin stimulation increased with the increase of GDP concentration, maximal effect being

Table 2 Competition of [^{125}I]-iodomelatonin binding and G protein activation from melatonin and its analogues

	Competition of [^{125}I]-iodomelatonin binding			*G protein activation*			
Compound	K_i (pM)	*GTPγS shift*	*NaCl/GTPγS index*	EC_{50} (pM)	*Maximal effect*	*Intrinsic activity*	EC_{50}/K_i
Melatonin	291 ± 12	3.95 ± 0.22	1	598 ± 45	372 ± 35.4	1	2.05
2-Br-Mel	29 ± 3	4.03 ± 0.15	1.02	47 ± 3.5	371 ± 38.6	1	1.62
2-Ph-Mel	22 ± 2.5	3.75 ± 0.35	0.95	65 ± 8	367 ± 33.1	0.98	2.95
6-Cl-Mel	1860 ± 173	3.87 ± 0.4	0.96	2593 ± 183	362 ± 34.5	0.96	1.39
NAS	484000 ± 25000	1.45 ± 0.2	0.53	526400 ± 15000	245 ± 5	0.53	1.08
Compound 6	201000 ± 16500	0.08 ± 0.01	0.02	67400[a] ± 3500	no effect	ND	0.33[b]

[a]K_b value; [b]K_b/K_i.

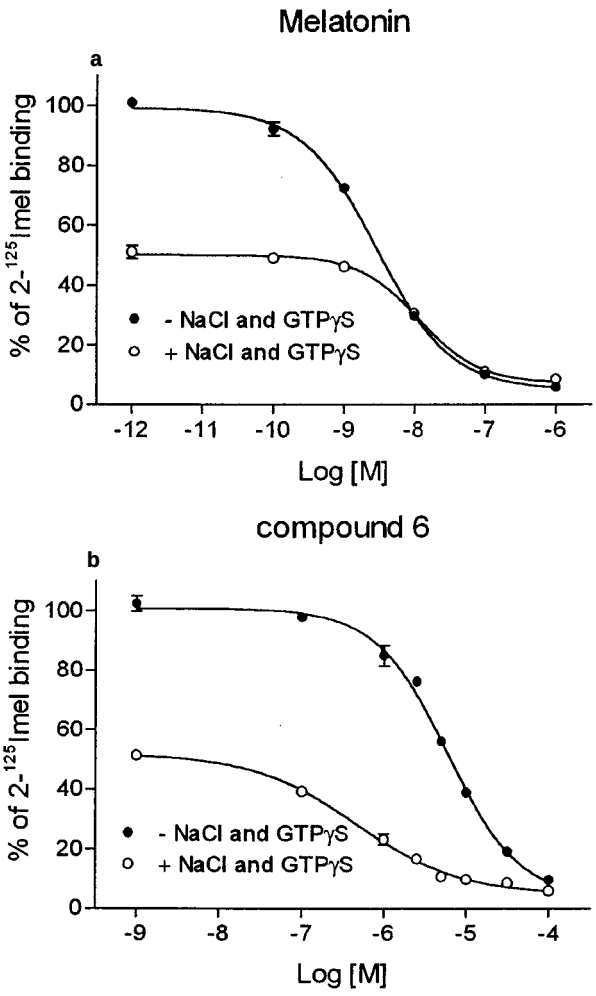

Figure 2 Representative competition curves of melatonin and compound 6 binding to $NIH3T3_{Mel1a}$ membranes, in the absence or presence of 700 mM NaCl and 100 μM GTPγS and 200 pM 2-[^{125}I]-iodomelatonin. The experiment was carried out as described in Methods, for 90 min at 37°C. The points are the means of duplicates; the vertical line indicate s.e. The melatonin IC_{50} in the absence and presence of NaCl and GTPγS were 2.92 E^{-9}, respectively; the IC_{50} shift was 4.17. The compound 6 IC_{50} in the absence and presence of NaCl and GTPγS were 5.91 E^{-6} and 4.69 E^{-7}, respectively; the IC_{50} shift was 0.079.

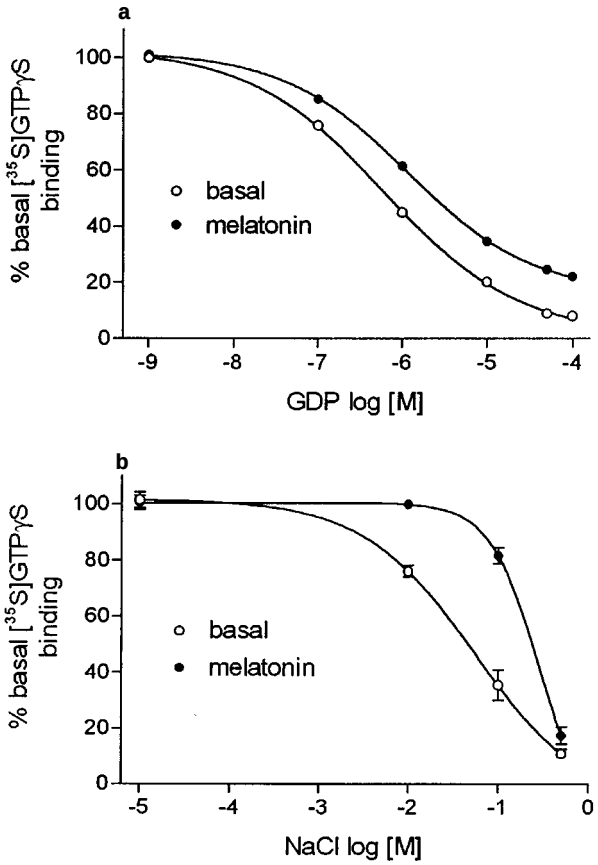

Figure 3 Effect of GDP and NaCl on the basal and melatonin-stimulated [^{35}S]-GTPγS binding to $NIH3T3_{Mel1a}$ membranes. The melatonin concentration was 100 nM. The experiment was carried out as described in Methods, for 30 min at 30°C. The points are the means of triplicates; the vertical lines indicate the s.e. (a) Representative experiment of the effect of varying GDP concentrations, in the presence of 100 mM NaCl, on basal and melatonin-stimulated [^{35}S]-GTPγS binding. (b) Representative experiment of the effect of varying NaCl concentrations, in the presence of 50 μM GDP, on basal and melatonin-stimulated [^{35}S]-GTPγS binding,

registered at 50 μM GDP (Figure 3a). Sodium ions also caused a dose-dependent increase of the maximal net stimulation elicited by melatonin, with a maximal effect around 100 mM NaCl (Figure 3b). Therefore, 50 μM GDP and 100 mM NaCl were used in all further experiments.

G proteins activation by melatonin and its analogues

The potency estimates and the intrinsic activities of the analogues studied are summarized in Table 2 and in Figure 4a. In the absence of any receptor ligand the basal [^{35}S]-GTPγS binding to $NIH3T3_{Mel1a}$ membranes was $\approx$109 fmol mg^{-1} protein. The data are expressed as stimulation over the experimentally-determined basal values, arbitrarily taken as 100%. Melatonin caused a dose-dependent increase of the basal binding, to reach a plateau at $\approx$370% with an EC_{50} value of 598$\pm$45 pM (Figure 4a). 2-Bromomelatonin, 2-phenylmelatonin and 6-chloromelatonin also increased basal binding with a similar maximum, EC_{50} values being 47$\pm$3.5 pM, 65$\pm$8 pM and 2593$\pm$183 pM, respectively. NAS behaved as partial agonist increasing [^{35}S]-GTPγS binding to $NIH3T3_{Mel1a}$ membranes to a plateau of 245$\pm$5% over basal, with an intrinsic activity of 0.53 and an EC_{50} value of 526.4$\pm$15 nM. Compound 6 was without any effect on basal binding at any concentration tested.

Antagonistic properties of compound 6

The ability of compound 6 to antagonize melatonin-stimulated [^{35}S]-GTPγS binding was investigated by carrying out a series of melatonin concentration-effect curves in the presence of three different compound 6 concentrations (0.3, 1 and 10 μM). Compound 6 caused a dose-dependent shift of the melatonin concentration-effect curve (Figure 4b), showing competitive and surmountable antagonism. Analysis of these data using the equation proposed by Lew & Angus (1995) permitted us to calculate a K_b value of 67.4$\pm$3.5 nM (Table 2). The antagonism appeared consistent with a simple competitive interaction, as tested by using equations (b) and (c) (see Methods): in fact, both equations did not result in an improved 'goodness of fit', the n values being 0.998 for equation (b) and 0.002 for equation (c).

The same data were then analysed using the equation proposed by Venter (1997), in order to investigate the presence of 'spare' receptors in our system. This equation gave a straight

line with the slope being $-K_a/K_b$ and the y-intercept e^{ES}, an efficacy related parameter that measures the ratio between stimulus and effect (see Methods). Our data fitted well in the linear regression analysis ($r^2=0.97$) (Figure 4c) and we could calculate an $e^{ES}=1.006\pm0.02$, that shows the absence of 'spare' receptors, and a K_a/K_b ratio of 0.006 ± 0.001, very similar to 0.0088, the ratio between the melatonin EC_{50} and the compound 6 K_b.

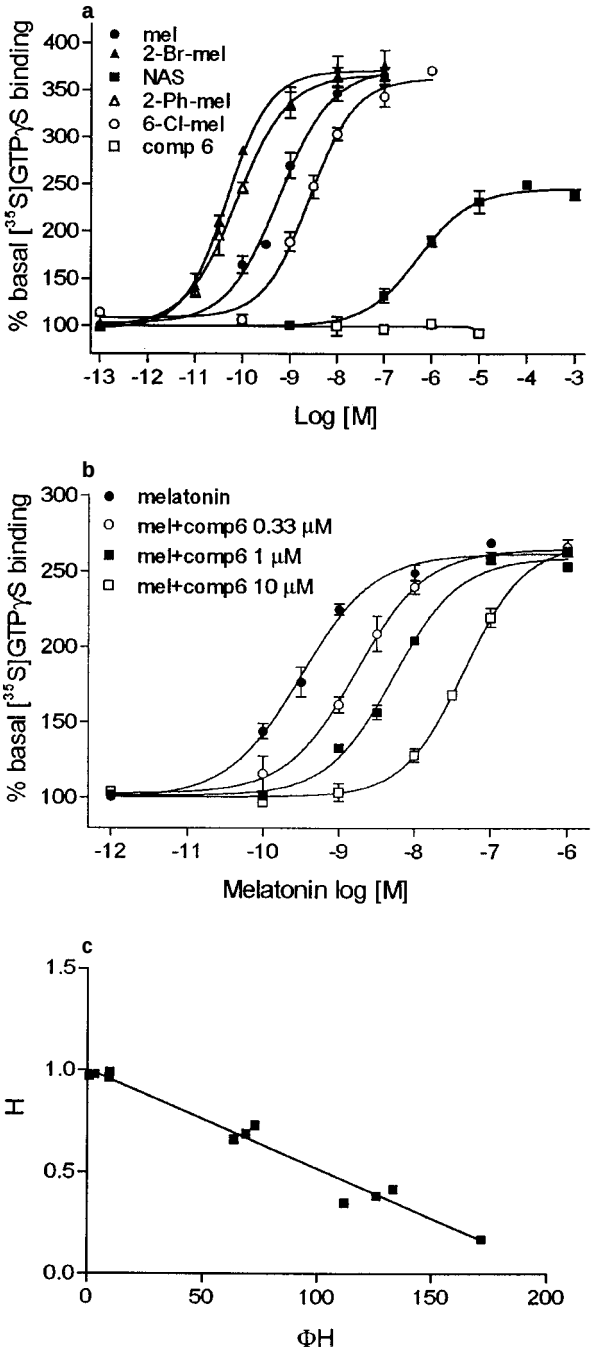

Figure 4 (a) Comparison of the stimulation of [^{35}S]-GTPγS binding to NIH3T3$_{Mel1a}$ membranes by melatonin, 6-chloromelatonin, 2-bromomelatonin, 2-phenylmelatonin, NAS and compound 6. (b) Representative experiment on the stimulation of [^{35}S]-GTPγS binding to NIH3T3$_{Mel1a}$ membranes by melatonin in the absence or presence of different compound 6 concentrations. (c) Analysis of the data presented in (b) by means of linear regression analysis, performed with the equation $H=-K_a/K_b\Phi H+e^{ES}$, as described in Methods. H=height of the point relative to maximal melatonin stimulation; Φ=ratio of compound 6 *vs* melatonin concentrations. The experiments were carried out as described in Methods, for 30 min at 30°C. The points are the means of triplicates; the vertical lines indicate the s.e. Values represent percentage of the basal binding, taken as 100%.

Comparisons of binding affinities with potencies for stimulating [^{35}S]-GTPγS binding

Competition and G protein activation studies gave similar pharmacological profiles, the order of potencies being: 2-phenylmelatonin $\geqslant$ 2-bromomelatonin > melatonin > 6-chloro-melatonin > compound 6 $\geqslant$ NAS. For the full agonists, the EC_{50} values were higher than the K_i values (Table 2). This is not surprising given that competition experiments for the K_i calculation were carried out without NaCl and so the calculated apparent K_i was essentially the K_i at the high-affinity state of the receptor. On the contrary [^{35}S]-GTPγS binding studies were conducted in the presence of GDP and NaCl and therefore with a mixed receptor population. Indeed, the EC_{50}/K_i ratio for the partial agonist NAS was close to unity. Furthermore, for the antagonist compound 6 the K_b value was lower than the K_i (K_b/K_i ratio of 0.33), consistent with the data of IC_{50} shift by NaCl and GTPγS (0.08) that suggest a higher affinity of compound 6 for the low-affinity state of the receptor.

Pertussis toxin sensitivity

A 24 h pretreatment of NIH3T3$_{Mel1a}$ cells with 1 μg ml^{-1} pertussis toxin (PTX) completely abolished the melatonin-induced increase in [^{35}S]-GTPγS binding. Basal [^{35}S]-GTPγS binding was significantly lower in membranes prepared from PTX-pretreated cells ($40\pm3\%$) than in membranes prepared from control cells (100%). Melatonin-stimulated binding was $398\pm17\%$ in control cells, while it was not significantly different from basal values in PTX-pretreated cells.

Discussion

Ligand binding and activation of G protein were accomplished in membranes from NIH3T3 mouse fibroblast cells stably transfected with the human Mel_{1a} melatonin receptor (NIH3T3$_{Mel1a}$). The melatonin receptor incorporated in the membranes of NIH3T3$_{Mel1a}$ bound melatonin and its analogues with high affinity and activated in a dose-dependent manner PTX-sensitive G-proteins (G_i or G_o), thus providing a suitable model for the study of Mel_{1a} signal transduction mechanisms and for evaluation of new melatonin analogues.

In absence of Na^+ ions and GTPγS, 2-[^{125}I]-iodomelatonin bound to a single class of high affinity binding sites, as it does in brain and PT membranes from a number of species (Morgan *et al*., 1994), including the human cerebellum (Fauteck *et al*., 1994), the source of Mel_{1a} full length cDNA coding region (Mazzucchelli *et al*., 1996) used for transfection in the present study. Furthermore Mg^{2+} and Ca^{2+} ions known to confer higher affinity to the melatonin receptor (Fauteck *et al*., 1994; Morgan *et al*., 1994), were not able to increase 2-[^{125}I]-iodomelatonin binding to NIH3T3$_{Mel1a}$ membranes. These data indicate that, in the experimental conditions used, most of the melatonin receptors expressed in NIH3T3$_{Mel1a}$ cells were in its high affinity, presumed G protein-coupled state. Similar results were obtained with the human 5-HT_{1a} receptor transfected in NIH3T3, that showed the presence of the only high affinity state, even with very high receptor numbers (up to 700 fmol mg^{-1} protein) (Varrault *et al*., 1992).

GTPγS is known to bind to the catalytic site of the Gα subunit and promote its active, receptor-uncoupled state,

decreasing the affinity of agonists to the receptor (Birnbaumer *et al*., 1990). Sodium ions are also known to destabilize the receptor-G protein interaction, but the site of sodium binding and the exact mechanism of action are still unclear. A number of studies suggest that the site of sodium ions action could be on the receptor protein itself (Jagadeesh *et al*., 1990; Horstman *et al*., 1990; Tian *et al*., 1994). Our data show that GTPγS decreased the affinity of 2-[^{125}I]-iodomelatonin but failed to show an effective uncoupling of the melatonin receptor-G protein complex. On the contrary, the presence of NaCl, although in high concentrations (500 – 700 mM), alone or coincubated with GTPγS caused the appearance of binding sites in the low affinity state. With maximal concentrations of both GTPγS (100 μM) and sodium ions (700 mM), we obtained the maximum number of receptors in the low affinity state, but we could not induce a complete shift of all receptors to a homogeneous population of receptors in their low-affinity state (the two-site model still fitted significantly better than the one-site model). This unusually tight, GTPγS-insensitive coupling, is not characteristic either for the native Mel_{1a} receptor in human cerebellum (Fauteck *et al*., 1994), or in the tissues of other species, as the quail brain, ovine and bovine pars tuberalis (Morgan *et al*., 1994; Nonno *et al*., 1995a; Spadoni *et al*., 1997; Tarzia *et al*., 1997), though GTPγS-insensitivity has been obtained for the melatonin receptor in bovine (Nonno *et al*., 1995b) and ovine hippocampus (Barrett *et al*., 1994). However similar data have been reported for other PTX-sensitive G protein-coupled receptors such as A_1 adenosine (Nanoff *et al*., 1995). Wreggett and De Lean (1984) reported that with the D_2 receptor (another inhibitory G protein-coupled receptor), in contrast to the findings with the β-adrenoceptor (G_s coupled), Gpp(NH)pp was not able to shift all the receptors toward low affinity state and they suggested that this could be a common feature for receptors interacting with inhibitory G proteins. Furthermore, the essential role of sodium to bring about complete dissociation of the R/G complex was reported for the α_2-adrenoceptor (Jagadeesh *et al*., 1990) and for the D_2 dopamine receptor (Grigoriadis & Seeman, 1983), both $G_{i/o}$ coupled receptors. In that sense our data confirm the existence of a general tendency of the $G_{i/o}$ coupled-receptors to form very stable R/G complexes (Wregget & De Lean, 1984). There is also evidence that different $G_{i/o}$ coupled-receptors exist in a pre-coupled state even in the absence of an agonist (Wreggett & De Lean, 1984; Costa *et al*., 1990; 1992; Varrault *et al*., 1992).

In preliminary experiments for choosing [^{35}S]-GTPγS binding conditions we noted that in the absence of sodium ions or GDP there was no melatonin-induced increase of basal [^{35}S]-GTPγS binding. The necessity for the presence of NaCl and/or GDP for the expression of agonist-dependent G protein stimulation is not a common feature but has been already reported in studies on $G_{i/o}$ activation by other receptors, such as the A_1 adenosine receptor (Lorenzen *et al*., 1993) and the μ-opioid receptor (Traynor & Nahorski, 1995).

Melatonin and its analogues increased basal [^{35}S]-GTPγS binding in a concentration-dependent manner, with the exception of compound 6 that was without any effect, behaving as antagonist. The antagonistic properties of compound 6 have already been reported (Garrat *et al*., 1995; Spadoni *et al*., 1997; Tarzia *et al*., 1997), and here we show for the first time the calculation of antagonist K_b. The analysis of the concentration-effect curves carried out in presence of melatonin and compound 6, furthermore, permitted us to show that in our system there are no 'spare' receptors. The absence of 'spare' receptors is an obvious advantage if one wants to evaluate the efficacy of analogues, in that sense a direct confrontation of maximal effect (intrinsic activity) obtained with an agonist compared with that of the reference-agonist gives a measure of the relative efficacy.

2-Bromomelatonin, 2-phenylmelatonin and 6-chloromelatonin increased basal binding to the same extent as melatonin, showing full agonism at the Mel_{1a} receptor, while NAS behaved as a partial agonist and its effect was calculated to be around 50% of that of melatonin. NAS has already been described as a partial agonist at the *Bufo ictericus* melatonin receptor in a skin-lightening test (Filadelfi & Castrucci, 1996). The ternary complex model predicts that the efficacy of a ligand is positively related to the ratio between its affinity to the low affinity (K_{low}) state and to the high affinity state (K_{high}) (Wreggett & De Lean 1984; Costa *et al*., 1992); in terms of ternary complex model interpretation of receptor-G protein interactions GTPγS and sodium ions reduce the affinity between receptor and G protein (Costa *et al*., 1992), thus inducing a shift of the receptors toward the low affinity state (as seen in saturation studies with 2-[^{125}I]-iodomelatonin). Therefore, our IC_{50} shift value, calculated in competition experiments carried out in the absence and presence of NaCl and GTPγS, must be positively related to the K_{low}/K_{high} ratio. Our data fit very well with the ternary complex model prediction in that GTPγS and sodium ions induced an IC_{50} shift in a manner related to the intrinsic activity of the analogues, as measured in the G protein activation assay. Also, we have recently demonstrated an excellent correlation between the GTPγS-induced IC_{50} shift (GTPγS index) and the intrinsic activities of various analogues determined by their effects on forskolin-stimulated cyclic AMP accumulation in quail brain explants (Spadoni *et al*., 1997; Tarzia *et al*., 1997).

In conclusion, Mel_{1a} stably transfected NIH3T3 cells appear to be a promising model for studying in detail the melatonin receptor signal-transduction pathway. The G-protein activation assay introduced in the present work bears all the prerequisites for a reliable method for determination of the biological activity and efficacy of melatonin analogues.

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