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Pharmacological Characterisation of Melatonin mt_1 Receptor-mediated Stimulation of [^{35}S]-GTPγS Binding*

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ABSTRACT. The activation of G-proteins by melatonin mt_1 receptors was studied by measuring [^{35}S]-guanosine-5′-(3-thiotriphosphate) ([^{35}S]-GTPγS) binding to membranes prepared from Chinese hamster ovary (CHO) cells stably expressing human mt_1 receptors. Melatonin stimulated [^{35}S]-GTPγS binding in a concentration-dependent manner (pEC_{50}, 8.77 ± 0.02). The optimal (212 ± 4%) increase over basal levels of binding (basal = 100%) was observed following incubation of membranes (12.5 μg protein/well) for 120 min at 30° with [^{35}S]-GTPγS (0.1 nM), in the presence of GDP (10 μM), NaCl (100 mM), and $MgCl_2$ (10 mM). Melatonin analogues stimulated [^{35}S]-GTPγS binding with a rank order (2-iodomelatonin > melatonin = S20098 > GR196429 > 6-chloromelatonin = 6-hydroxymelatonin ≫ *N*-acetylserotonin ≥ GR135531 = mt_1 luzindole = 5-HT = 0), which was identical to their affinities for the high affinity state of the receptor (correlation coefficient 0.94). All agonists evoked similar maximum increases in [^{35}S]-GTPγS binding. EC_{50} values were 14- to 63-fold lower than binding affinities. The melatonin receptor antagonist luzindole (0.1–10 μM) evoked a parallel rightward shift in the melatonin concentration-response curve, with a pK_B of 7.19 ± 0.13, which is similar to its affinity in radioligand binding studies for human mt_1 receptors. Stimulation of [^{35}S]-GTPγS binding was abolished by pretreatment of cells with pertussis toxin (18 hr, 100 ng/mL) prior to preparation of membranes. Melatonin was without effect in CHO cells which lacked the mt_1 receptor. Thus, melatonin and melatonin analogues stimulate [^{35}S]-GTPγS binding with a profile which is consistent with binding to mt_1 receptors causing activation of G_i/G_o G-proteins. BIOCHEM PHARMACOL **56**;9:1167–1174, 1998.

KEY WORDS. melatonin, [^{35}S]-GTPγS, mt_1 receptor, luzindole, G-protein, pertussis toxin

The hormone melatonin, which is synthesised and secreted from the pineal gland during the hours of darkness, regulates a variety of circadian, neuroendocrine, visual, and seasonal functions (for review, see [2]). To date, three cDNAs encoding 7-transmembrane domain G-protein-coupled melatonin receptors have been cloned, two of which, termed mt_1 and mt_2, have been found in mammalian tissues (for review, see [3]). In agreement with studies of endogenous melatonin receptors (for review, see [4]), activation of both recombinant mt_1 and MT_2 receptors inhibits adenylyl cyclase activity in a PTX-‡sensitive manner, indicating an interaction with G_i/G_o G-proteins [5–7]. However, melatonin has also been shown to activate other signal transduction pathways. For example, in neonatal rat pituitary, melatonin attenuates GnRH-induced increases in cAMP and calcium via independent, PTX-sensitive pathways [8]. In NIH 3T3 cells stably expressing mt_1 receptors, melatonin both inhibits forskolin-stimulated cAMP accumulation and potentiates phospholipase C activation, again both via PTX-sensitive mechanisms [9]. In ovine pars tuberalis, melatonin-induced reduction in cAMP levels is composed of both PTX-sensitive and PTX-insensitive, cholera toxin-sensitive components, suggesting that two distinct G-proteins might mediate effects of melatonin in this tissue [10, 11]. The ability of melatonin to activate multiple G-proteins is further suggested by Yung and colleagues [12], who demonstrated that the Mel_{1c} receptor cloned from *Xenopus* [13] can couple to both PTX-sensitive G_i and PTX-insensitive G_z-proteins. Thus, both endogenous and recombinant melatonin receptors can couple to multiple signal transduction pathways and/or to multiple G-proteins.

Receptor-mediated G-protein activation can be directly investigated by determination of agonist-induced guanine

* The nomenclature and classification of melatonin receptors used here was recently approved by the Nomenclature Committee of the International Union of Pharmacology [1]. 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 molecular structure, previously referred to as ML_2.

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‡ *Abbreviations:* CHO, Chinese hamster ovary; PTX, pertussis toxin; and [^{35}S]-GTPγS, [^{35}S]-guanosine-5′-(3-thiotriphosphate).

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nucleotide exchange. This can be achieved by measurement of binding of the nonhydrolysable GTP analogue [^{35}S]-GTPγS to membrane preparations containing the receptor under investigation. This quantitative technique measures the primary response in the signalling pathway following receptor activation, which is the only step in the pathway which is directly regulated by ligands. Stimulation of [^{35}S]-GTPγS binding has been demonstrated following activation of a number of G-protein-coupled receptors, including bovine adenosine A_1 [14], human muscarinic [15], human dopamine D_2 [16], human somatostatin sst_5 [17], and human metabotropic glutamate [18] receptors. In the present study, we have further investigated melatonin-induced G-protein activation by characterisation of melatonin-induced stimulation of [^{35}S]-GTPγS binding in membranes prepared from CHO cells stably expressing mt_1 receptors.

MATERIALS AND METHODS

Generation of CHO-hmt$_1$ Cells

Initial 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 [13]. 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. Initial cloning was in pBluescript (Stratagene). The nucleotide sequence obtained encoded for the same amino acid sequence as described by Reppert and colleagues [5]. The sequence encoding the receptor was 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). Several G418 resistant mt_1 cell lines were selected for [^{3}H]-melatonin saturation and [^{35}S]-GTPγS binding studies. The two cell lines which gave the highest receptor expression and melatonin-induced stimulation of [^{35}S]-GTPγS binding were chosen for further [^{3}H]-melatonin radioligand binding and [^{35}S]-GTPγS binding studies, respectively.

Membrane Preparation for [^{35}S]-GTPγS Binding Assay

CHO cells stably expressing human mt_1 receptors were harvested using PBS solution containing EDTA (5 mM) and centrifuged at 700 *g* for 5 min. The pellet was resuspended in HEPES (20 mM), homogenised, recentrifuged at 700 *g* for 5 min, and the resulting supernatant centrifuged at 47,000 *g* for 20 min at 4°. The pellet was homogenised, resuspended in HEPES (20 mM), and aliquots (~2 mg protein/mL) stored at −80° until use. Protein determination was performed using the Bio-Rad-coomassie blue method with BSA as standard [19].

[^{35}S]-GTPγS Binding Assay

The assay was performed in duplicate in 96-well plates in a final assay volume of 250 μL. Drugs were incubated with membranes (50 μg/mL) in HEPES (20 mM; pH 7.4), containing $MgCl_2$ (10 mM), NaCl (100 mM), GDP (10 μM), and saponin (1 mg/mL) for 120 min at 30° in a shaking water-bath. Saponin was included to permeabilise vesicles in the membrane preparation and hence improve access of [^{35}S]-GTPγS. [^{35}S]-GTPγS (0.1 nM) was added to wells and the plates were incubated for a further 30 min at 30°. Nonspecific binding was defined using GTP (100 μM). Bound radioligand was separated by rapid filtration using a Brandel cell harvester through GF/B filters, which were washed with 4 × 1 mL distilled water. Filters were counted by liquid scintillation spectrometry.

[^{3}H]-Melatonin Binding Assay

Membranes were prepared by harvesting CHO cells using Hanks' balanced salt solution, containing EDTA (5 mM), homogenised and centrifuged at 4500 *g* for 35 min. The pellet was homogenised, resuspended in Tris–HCl buffer, containing 2 mM of $MgCl_2$, 1 mM of EDTA, 0.1% ascorbic acid, pH 7.4, and aliquots (~2 mg protein/mL) stored at −80° until use. The assay was performed in 96-well plates in a final assay volume of 500 μL. Drugs and [^{3}H]-melatonin (0.3 nM) were incubated with membranes (25 μg/mL) for 120 min at 37°. Nonspecific binding was defined by melatonin (1 μM). Bound radioactivity was determined as described above.

Data Analysis

[^{35}S]-GTPγS binding data were analysed using a 4-parameter logistic equation (ALLFIT, [20]) to determine pEC_{50} values and Hill coefficients. Maximum response (% Emax) elicited by a drug was defined as the maximum increase in [^{35}S]-GTPγS binding over basal (basal = 100%) expressed as a percentage of the maximum increase over basal evoked by melatonin. The pK_B value for luzindole was determined using a modified form of the Schild equation [21, 22],

$$-pEC_{50} = \log_{10}(EC^c_{50}) + \log_{10}(1 + [B]^n/K_B)$$

where EC^c_{50} is the control EC_{50} value, [B] is the concentration of antagonist, K_B is the antagonist dissociation equilibrium constant, and n is the order of reaction with the receptor. If n was not significantly different from unity, consistent with simple competition, it was constrained to unity to obtain an estimate of K_B.

[^{3}H]-Melatonin competition binding data were analysed using a 4-parameter logistic equation (ALLFIT, [20]), to generate pIC_{50} values which were converted to pKi values using the Cheng–Prusoff equation [23]. Saturation analysis had previously determined the K_D and B_{max} values for [^{3}H]-melatonin to be 0.13 nM and 1.2 pmol/mg protein,

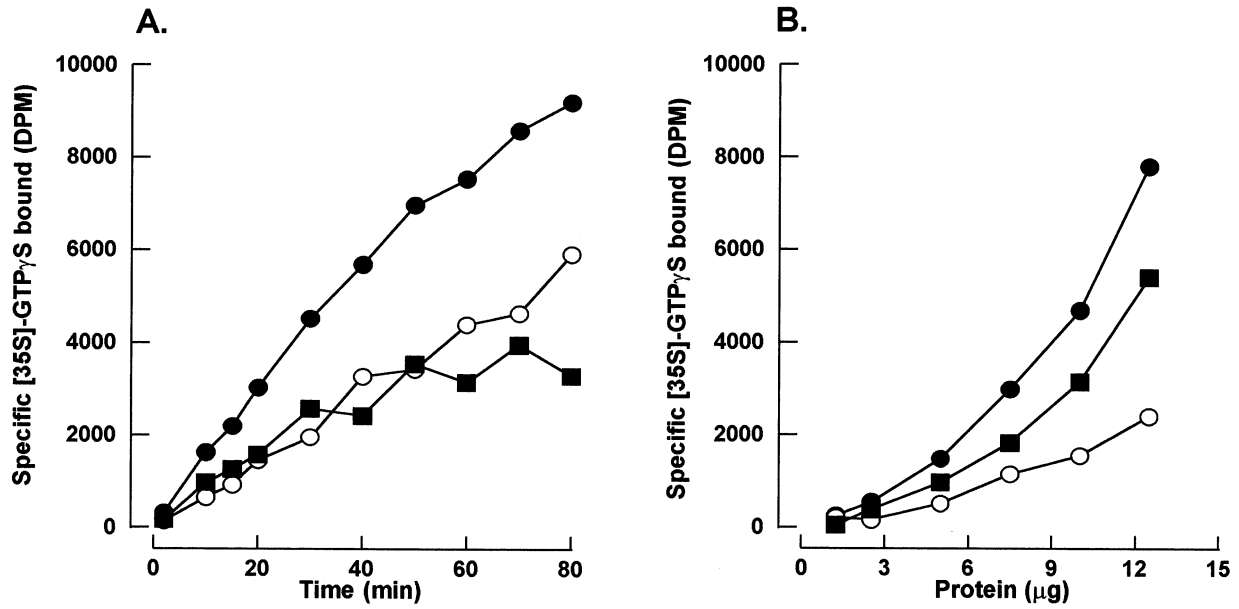

FIG. 1. Time-course of [35S]-GTPγS binding (A) and effect of protein concentration on [35S]-GTPγS binding (B) in the presence (●) and absence (○) of melatonin (1 μM) to membranes of CHO cells stably expressing human melatonin mt_1 receptors. ■ = melatonin-stimulated binding following subtraction of basal binding. Data are expressed as specific [35S]-GTPγS bound (DPM). Nonspecific binding was determined using GTP (100 μM). Data are from representative experiments, performed in duplicate on three separate occasions.

respectively [24]. Statistical comparisons were made using Student's two-tailed *t*-test.

Drugs

GR196429, S20098, GR13551, and luzindole were synthesised by Medicinal Chemistry, Glaxo Wellcome. Melatonin, 6-hydroxymelatonin, 6-chloromelatonin, *N*-acetylserotonin, 5-HT, GTP, GDP, and PTX were supplied by Sigma. 2-Iodomelatonin was purchased from RBI. [35S]-GTPγS (>1000 Ci/mmol) and [3H]-melatonin (85 Ci/mmol) were supplied by Amersham.

RESULTS

Optimisation of [35S]-GTPγS Binding Assay

Preliminary experiments (data not shown) indicated that, in order to reach equilibrium, drugs should be preincubated with membranes for 120 min prior to the addition of [35S]-GTPγS. Following 120-min preincubation, the time-course for stimulation of [35S]-GTPγS binding by a maximally effective concentration of melatonin (1 μM) was determined (Fig. 1A). While both binding in the presence and absence of melatonin continued to increase over the time-course studied, subtraction of basal from melatonin-stimulated binding indicated that melatonin stimulation of binding reached a plateau following 30-min incubation with [35S]-GTPγS. This incubation period was chosen for all future experiments.

Melatonin-induced stimulation of [35S]-GTPγS binding increased with protein concentration (Fig. 1B). A protein concentration of 12.5 μg/well was chosen for routine use.

Optimal stimulation of [35S]-GTPγS binding is critically dependent on the concentration of GDP. [35S]-GTPγS binding in the presence and absence of melatonin (1 μM) was determined in the presence of GDP (0.03–100 μM) (Fig. 2A). Maximum percentage increase in [35S]-GTPγS binding evoked by melatonin was observed using 10–30 μM GDP (Fig. 2B). Due to the higher absolute levels of counts at 10 μM GDP (Fig. 2A), this concentration was chosen for future experiments.

Under optimised conditions, melatonin stimulated [35S]-GTPγS binding in a concentration-dependent manner, with a pEC_{50} of 8.77 ± 0.02 (n = 9) and a Hill slope coefficient which was not significantly different from unity ($p > 0.05$; Table 1; Fig. 3). A maximally effective concentration of melatonin (1 μM) elicited 212 ± 4% stimulation over basal [35S]-GTPγS binding (n = 9; basal = 100%).

Pharmacological Characterisation of [35S]-GTPγS Binding

Melatonin and melatonin analogues stimulated [35S]-GTPγS binding in a concentration-related manner (Fig. 3, Table 1). Melatonin, 2-iodomelatonin, 6-chloromelatonin, 6-hydroxymelatonin, and the nonindolic agonists S20098 and GR196429 caused similar maximum increases in binding. *N*-Acetylserotonin, which has much lower affinity for mt_1 receptors, evoked a small (127 ± 9%, n = 4) increase in binding (basal = 100%). At concentrations up to 10 μM, 5-HT and GR135531, which is selective for the putative MT_3 binding site [25, 26], were without effect. With the exception of 2-iodomelatonin (Hill slope =

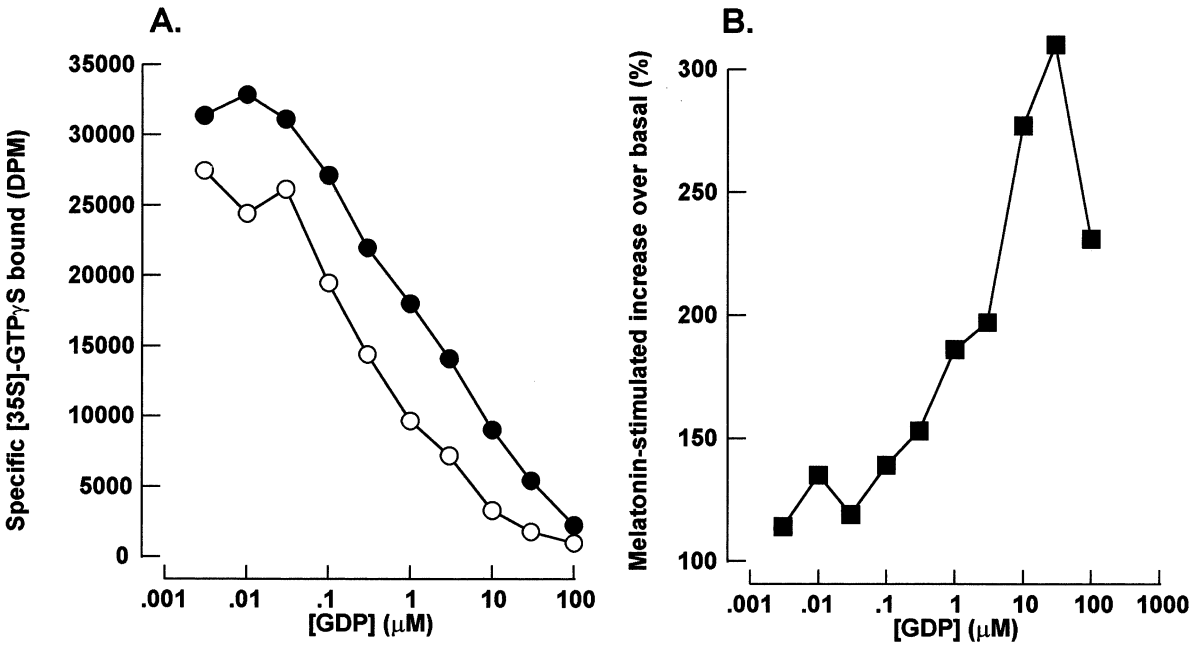

FIG. 2. Effect of GDP concentration on [^{35}S]-GTPγS binding in the presence and absence of melatonin (1 μM) to membranes of CHO cells stably expressing human melatonin mt_1 receptors. (A) Data are specific [^{35}S]-GTPγS bound (DPM) in the presence (●) and absence (○) of melatonin (1 μM). Nonspecific binding was determined using GTP (100 μM). (B) Data (■) are melatonin-stimulated increase in [^{35}S]-GTPγS binding expressed as a percentage of basal binding. Data are from a representative experiment, performed in duplicate on three separate occasions.

0.62 ± 0.07, $p < 0.05$ vs unity), all Hill slopes were not significantly different from unity ($p > 0.05$).

Potencies for stimulation of [^{35}S]-GTPγS binding were compared with abilities to compete for [^{3}H]-melatonin binding to mt_1 receptors (Table 1). Agonists stimulated [^{35}S]-GTPγS binding and competed for [^{3}H]-melatonin binding with identical rank orders of potency. Linear regression analysis yielded a correlation coefficient of 0.94. Agonists were 14- to 63-fold less active in stimulating [^{35}S]-GTPγS binding than they were to compete for [^{3}H]-melatonin binding.

The melatonin receptor antagonist luzindole [27] evoked parallel rightward shifts in the concentration-response curve for melatonin-stimulation of [^{35}S]-GTPγS binding, with no change in maximum response (Fig. 4A). The pK_B for luzindole was 7.19 ± 0.13 (n = 4), with a slope not significantly different from unity (0.87 ± 0.09, $p > 0.05$). The data is graphically displayed in a Schild plot in Fig. 4B. The affinity of luzindole in the [^{35}S]-GTPγS binding assay was similar to its affinity for mt_1 receptors (Table 1). Luzindole alone had no effect on basal [^{35}S]-GTPγS binding (Fig. 3).

Pretreatment of cells with PTX (18 hr, 100 ng/mL), prior to preparation of membranes, completely abolished mela-

TABLE 1. Comparison of potencies of melatonin analogues for stimulation of [^{35}S]-GTPγS binding with competition for [^{3}H]-melatonin binding to receptors

Compound	[^{35}S]-GTPγS			[^{3}H]-Melatonin pKi
	pEC_{50}	%Emax	Hill slope	
2-Iodomelatonin	9.11 ± 0.10	105 ± 5	0.62 ± 0.07	10.55 ± 0.11
S20098	8.55 ± 0.10	107 ± 16	1.08 ± 0.28	10.09 ± 0.09
Melatonin	8.77 ± 0.02	100	0.87 ± 0.02	9.94 ± 0.06
6-Chloromelatonin	7.50 ± 0.20	97 ± 12	1.03 ± 0.14	9.10 ± 0.05
6-Hydroxymelatonin	7.41 ± 0.13	119 ± 27	0.99 ± 0.04	9.21 ± 0.06
GR196429	7.90 ± 0.05	125 ± 18	0.99 ± 0.15	9.69 ± 0.09
N-Acetylserotonin	<5.0	0		7.08 ± 0.04
GR135531	<5.0	0		5.85 ± 0.05
Luzindole	<5.0	0		6.60 ± 0.05
5-HT	<5.0	0		<5.5

All data are means ± SEM. pEC_{50} and %Emax for stimulation of [^{35}S]-GTPγS binding were determined from 3–6 experiments performed in duplicate. %Emax was determined relative to the maximum stimulation evoked by melatonin in each experiment. pKi for competition with [^{3}H]-melatonin binding was determined in 4–12 experiments.

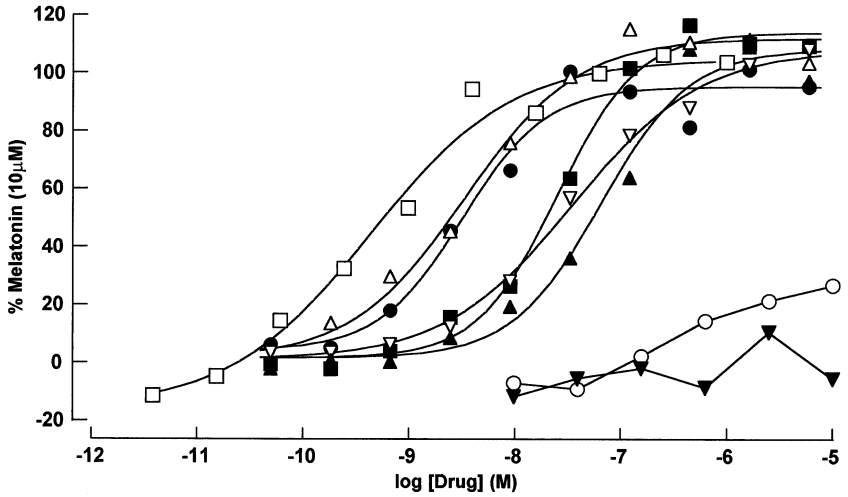

FIG. 3. Stimulation of [35S]-GTPγS binding to membranes of CHO cells stably expressing human melatonin mt_1 receptors by 2-iodomelatonin (□), melatonin (●), S20098 (△), GR196429 (■), 6-chloromelatonin (▽), 6-hydroxymelatonin (▲), *N*-acetyl-5-HT (○), and luzindole (▼). Data are expressed as % response evoked by 10 μM of melatonin. Data are from representative experiments performed in duplicate on three to six occasions. The fitted line through each data set is the result of fitting the data points to a 4-parameter logistic equation (see Materials and Methods).

tonin-stimulation of binding (n = 3; Fig. 5), with no effect on basal levels of binding (data not shown). In membranes prepared from cells transfected with the adenosine A_1 receptor, melatonin was without effect at concentrations up to 10 μM (n = 3; data not shown). In contrast, the adenosine agonist 5′-N-ethylcarboxamidoadenosine (1 μM) stimulated [35S]-GTPγS binding by 171 ± 24% (n = 3; data not shown).

DISCUSSION

This study is the first to directly investigate melatonin-mediated G-protein activation by measurement of [35S]-GTPγS binding. Melatonin was able to stimulate [35S]-GTPγS binding in membranes prepared from CHO cells stably expressing mt_1 receptors. No stimulation of binding was observed in membranes from CHO cells which expressed adenosine A_1 receptors.

Optimum conditions for melatonin-induced stimulation of [35S]-GTPγS binding were similar to those determined for other G-protein coupled receptors (e.g. [14–18]). The time-course of melatonin-stimulated [35S]-GTPγS binding demonstrated that the response was saturable following 30-min incubation, which is consistent with a receptor-mediated interaction. As previously observed, detection of agonist-stimulated [35S]-GTPγS binding required the presence of GDP, which reduced basal levels of [35S]-GTPγS binding to a greater extent than it lowered binding in the presence of agonist, enabling agonist-specific effects to be observed. Optimum signal:noise was achieved using 10 μM

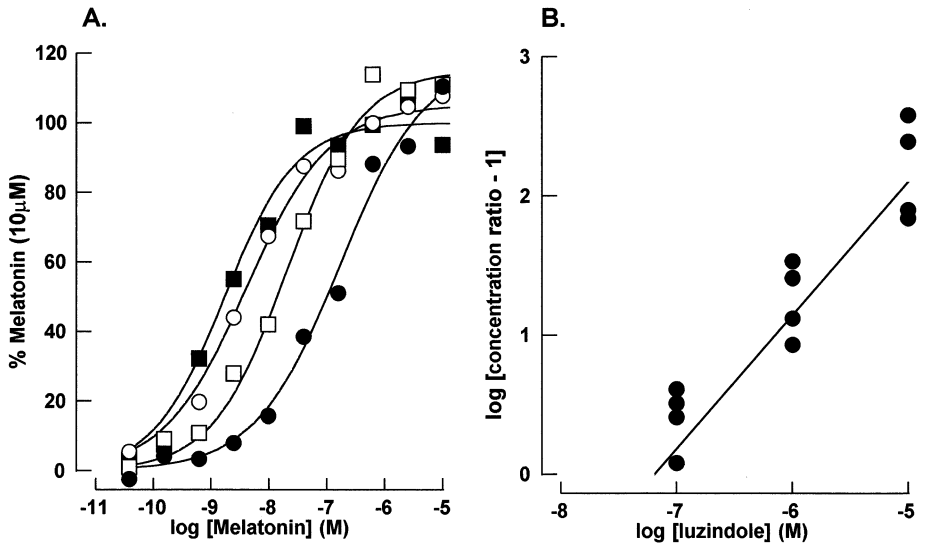

FIG. 4. (A) Antagonism by luzindole (zero (■), 0.1 μM (○), 1 μM (□), and 10 μM (●)) of melatonin-induced stimulation of [35S]-GTPγS binding to membranes of CHO cells stably expressing human melatonin mt_1 receptors. Data are from a representative experiment performed in duplicate on four occasions. The fitted line through each data set is the result of fitting the data points to a 4-parameter logistic equation (see Materials and Methods). (B) Schild plot for antagonism of melatonin-induced responses by luzindole. The line through the data was calculated as described in Materials and Methods. pK_B = 7.19 ± 0.13; slope = 0.87 ± 0.09. Data are individual values from four experiments performed in duplicate.

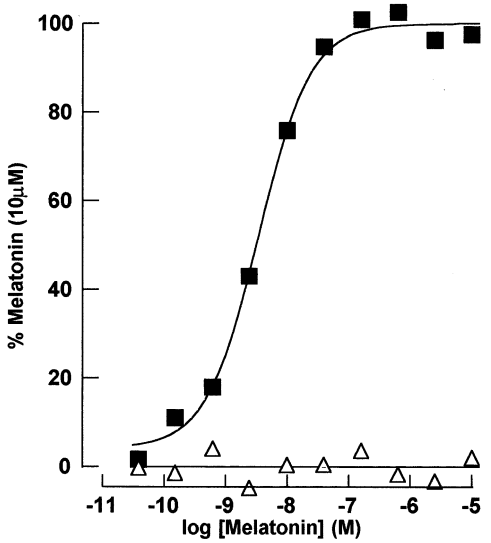

FIG. 5. Melatonin-induced stimulation of [^{35}S]-GTPγS binding to control (■) or pertussis toxin-treated (△) membranes of CHO cells stably expressing human melatonin mt_1 receptors. Data are expressed as % response evoked by 10 μM of melatonin. Data are from a representative experiment performed in duplicate on three separate occasions. The fitted line through each data set is the result of fitting the data points to a 4-parameter logistic equation (see Materials and Methods).

of GDP. GDP probably reduces basal binding by competing for the GTP binding site on all G-proteins present in the cells.

A maximally effective concentration of melatonin evoked a twofold stimulation of [^{35}S]-GTPγS binding, which is of similar magnitude to that observed for other receptors (e.g. [14–18]). A range of melatonin analogues, including the nonindolic agonists S20098 [28] and GR196429 [29], stimulated [^{35}S]-GTPγS binding with a rank order of potency which was identical to their abilities to compete for [^{3}H]-melatonin binding to the same recombinant cell line (correlation coefficient 0.94). GR135531, which binds to the putative MT_3 binding site [25, 26], and 5-HT were without effect. With the exception of 2-iodomelatonin, Hill slope parameters were not significantly different from unity, suggesting either an interaction with a single G-protein or an interaction with multiple G-proteins, for which the receptor has similar affinities. The reason for the low Hill slope parameter for 2-iodomelatonin-evoked stimulation of binding is unknown. Whilst this may be because 2-iodomelatonin interacts with multiple G-proteins, we have observed that 2-[^{125}I]-iodomelatonin has very slow kinetics of association and dissociation to mt_1 and MT_2 receptors,§ suggesting that equilibrium may not have been achieved in the [^{35}S]-GTPγS binding assay. The absolute potencies of melatonin agonists to stimulate [^{35}S]-GTPγS binding were 14- to 63-fold lower than their affinities for competition with [^{3}H]-melatonin binding to the same recombinant cell line. Similarly, somatostatin analogues were substantially less potent at evoking [^{35}S]-GTPγS binding in response to somatostatin sst_5 receptor stimulation than they were at competing with binding of the agonist ligand [^{125}I]-Tyr^{11}-somatostatin-14 [17]. In contrast, dopaminergic agonists stimulated [^{35}S]-GTPγS binding to dopamine $D_{4.4}$ receptors with potencies similar to their affinities for competition for binding of the antagonist ligand [^{3}H]-spiperone [30]. These data suggest that the low affinity state of the receptor probably mediates the functional response, rather than the high affinity state of the receptor, which is preferentially labelled by agonist ligands. As expected, the absolute potency of melatonin in the [^{35}S]-GTPγS binding assay was somewhat lower than its subnanomolar potency to inhibit forskolin-stimulated cAMP in other mt_1 cell lines [5, 7, 9], reflecting the fact that [^{35}S]-GTPγS binding measures the initial response in the signalling pathway following receptor activation.

Melatonin-evoked stimulation of [^{35}S]-GTPγS binding was antagonised by the melatonin receptor antagonist, luzindole [27], in a competitive manner. The affinity of luzindole for antagonism of melatonin-stimulated binding was similar to its affinity for competition for [^{3}H]-melatonin binding to mt_1 receptors and also to its affinity for competition for 2-[^{125}I]-iodomelatonin binding and antagonism of melatonin-induced inhibition of forskolin-stimulated cAMP in CHO cells expressing mt_1 receptors [7, 31].

As the [^{35}S]-GTPγS binding assay measures the initial response in the signalling cascade following receptor activation, there is less amplification in the system compared to measurement of downstream responses, for example second messengers. Thus, the assay may be suitable for determination of relative efficacies of ligands. For example, Newman–Tancredi and colleagues [32] used the assay to identify neutral antagonists and full, partial, and inverse agonists at human 5-HT_{1A} receptors. With the exception of *N*-acetylserotonin, which, in radioligand binding studies, has low affinity for mt_1 receptors, all the melatonin agonists examined evoked similar maximum levels of [^{35}S]-GTPγS binding, suggesting that they were all full agonists under the conditions of this study. Luzindole alone had no effect on basal levels of [^{35}S]-GTPγS, indicating either that luzindole is a neutral antagonist or, under the conditions used in this study, there was no constitutive activity in this cell line. Indeed, conditions were designed to minimise basal levels of binding and optimise agonist effects, i.e. high sodium and high GDP concentrations. In contrast, inverse agonism is more likely to be detected under conditions of high basal levels of [^{35}S]-GTPγS binding, i.e. low sodium and low GDP concentrations. The lack of effect of PTX on basal levels of [^{35}S]-GTPγS binding also suggests that there is little or no constitutive activity of G_i/G_o G-proteins under these assay conditions. Thus, it is possible that manipulation of assay conditions would reveal inverse agonist activity of luzindole.

Pretreatment with PTX completely abolished melatonin-induced [^{35}S]-GTPγS binding, indicating an involvement of G_i/G_o G-proteins in this response. Similarly, PTX has

§ Unpublished observations.

been shown to prevent mt_1 receptor-mediated inhibition of forskolin-evoked cAMP formation [5, 7]. However, while the present study provides no evidence for coupling of mt_1 receptors to multiple G-proteins, this possibility cannot be excluded, since an important caveat is that, to date, the technique of [^{35}S]-GTPγS binding has been used most successfully for receptors which couple to G_i/G_o G-proteins, e.g. adenosine A_1 [14], μ-opioid [33], dopamine D_2 [16], dopamine $D_{4.4}$ [30], 5-HT_{1A} [32], and somatostatin sst_5 [17] receptors. This is likely to be because G_i/G_o G-proteins exhibit a much higher level of guanine nucleotide exchange relative, at least, to G_q and G_z G-proteins [34–36].

In summary, we have developed an assay to measure melatonin-mediated stimulation of [^{35}S]-GTPγS binding. We have demonstrated that melatonin and melatonin analogues stimulate [^{35}S]-GTPγS binding to mt_1 receptors with a pharmacological profile which is consistent with mt_1 receptor-mediated activation of G_i/G_o G-proteins. This assay will be useful to determine the relative efficacies of novel melatonin ligands and in the development of subtype specific melatonin receptor agonists and antagonists with which to probe the functions of native melatonin receptors.

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