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Characterization and Regulation of the Human ML_{1A} Melatonin Receptor Stably Expressed in Chinese Hamster Ovary Cells

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SUMMARY

The human ML_{1A} melatonin receptor is expressed in the suprachiasmatic nucleus of the hypothalamus and is believed to regulate circadian rhythms. We report the kinetic characteristics and pharmacological profile of 2-[^{125}I]iodomelatonin binding and the signaling pathway and agonist regulation of the human ML_{1A} melatonin receptor stably expressed in Chinese hamster ovary cells. Association of 2-[^{125}I]iodomelatonin binding was maximal by 1.5 hr at 37° and fully dissociated on the addition of 1 μM melatonin. The binding of 2-[^{125}I]iodomelatonin was saturable and of high affinity (K_D = 74 ± 14 pM, B_{max} = 679 ± 88 fmol/mg protein; three experiments). The pharmacological profile of various melatonin analogues revealed a profile (2-iodomelatonin ≥ melatonin > *N*-acetyl serotonin > luzindole) characteristic of an ML_1 subtype. Competition of melatonin for 2-[^{125}I]iodomelatonin binding to the human ML_{1A} receptor in lysed or intact cells resulted in biphasic curves revealing the existence of super high (~20%) and high (~80%) affinity states of the receptor. Guanosine-5′-*O*-(3-thio)triphosphate (100 pM–30 μM) when added alone inhibited 2-[^{125}I]iodomelatonin binding (IC_{50} = 0.87 ± 0.12 μM; three experiments), suggesting uncoupling of the receptor from G proteins. In addition, guanosine-5′-*O*-(3-thio)triphosphate (3 μM) produced a rightward shift in both the super high and high binding melatonin affinities for 2-[^{125}I]iodomelatonin resulting in monophasic curves. Melatonin (0.1 fM–1 nM) inhibited forskolin-induced cAMP formation in a concentration-dependent and biphasic manner. Low concentrations of melatonin (0.01 fM–1 pM) inhibited forskolin (100 μM)-stimulated cAMP formation with an IC_{50} of 0.1 ± 0.05 pM (four experiments) and a maximal inhibitory effect (26%) at 1 pM. Higher concentrations of melatonin (1 pM–1 nM) inhibited forskolin-induced cAMP formation with an IC_{50} of 64 ± 1.8 pM (four experiments) and a maximal inhibition (74%) at 1 nM. Luzindole (1 μM), a competitive melatonin receptor antagonist, antagonized the effect of melatonin at the higher concentrations only (IC_{50} = 1.5 ± 0.22 nM, pK_B = −7.3; three experiments). Pretreatment with pertussis toxin completely abolished melatonin-mediated inhibition of forskolin-induced cAMP formation through these receptors. Pretreatment with various concentrations of melatonin (0.1 pM–1 μM) for different periods of time (1, 6, 18, and 24 hr) did not decrease 2-[^{125}I]iodomelatonin binding. However, competition by melatonin for 2-[^{125}I]iodomelatonin binding to cells pretreated with melatonin and washed was only to a single population of super high affinity sites (IC_{50} = 1.1 ± 0.28 nM; three experiments) as revealed by monophasic curves. Cells pretreated with melatonin revealed a persistent inhibition (~20%) of forskolin-induced cAMP formation that was not reversed by extensive washes (up to 1 hr) or when luzindole (1 μM) was added together with melatonin during pretreatment. These results suggest that tight binding of melatonin to the super high affinity state of the human ML_{1A} melatonin receptor may be the mechanism by which low concentrations of circulating hormone *in vivo* regulates signaling in the suprachiasmatic nucleus of the hypothalamus.

The hormone melatonin is synthesized and secreted during the hours of darkness from the mammalian pineal gland to affect a myriad of physiological and neuroendocrine processes (1–3). In humans, melatonin regulates circadian rhythms through activation of specific melatonin receptors in the hypothalamic SCN, the site of the body clock (4–7). Membrane-associated melatonin receptors were originally classified, based on kinetic properties and pharmacological profiles, into the ML_1[1] and the *ML*$_2$ subtypes (8, 9). The ML_1 melatonin receptor binds 2-[^{125}I]iodomelatonin with picomolar affinity, shows a distinct pharmacology (2-iodomelatonin ≥ melatonin > *N*-acetyl serotonin > serotonin), and is linked to the inhibition of adenylyl cyclase by a pertussis toxin-sensitive G protein (3, 9). ML_1 receptors mediate a number of functional responses in mammals, including inhibition of dopamine release from the retina, potentiation of norepinephrine-induced contraction in arteries, and inhibition of

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[1] Receptor nomenclature as described in "Receptor and Ion Channel Nomenclature" [*Trends Pharmacol. Sci.* 17(suppl.): (1996)].

ABBREVIATIONS: SCN, suprachiasmatic nucleus; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

cAMP formation in the pars tuberalis (3, 9). 2-[^{125}I]Iodomelatonin binds to the ML_2 melatonin receptor with nanomolar affinity, displays a distinct pharmacological profile (2-iodomelatonin > N-acetyl serotonin ≥ melatonin≫serotonin), and is linked to the phosphoinositide transduction pathway (10); however, its physiological function has not been established.

Recently, cDNAs encoding ML_1-like melatonin receptors were cloned from various species, including human (5, 6, 11). These cloned receptors belong to a new family of the superfamily of G protein coupled receptors characterized by a seven transmembrane-spanning domain (5, 6, 11). Two distinct recombinant human ML_1-type melatonin receptors (ML_{1A}, ML_{1B}) were defined as unique subtypes on the basis of their molecular structure and chromosomal localization (6, 12). The ML_{1A} melatonin receptor, believed to mediate circadian function, expresses in the human SCN (5), whereas the ML_{1B}, which may be involved in retinal physiology, expresses in both the brain and retina (6). Both receptors show a pharmacological profile very similar to that of the ML_1 type and are coupled to inhibition of cAMP formation. The density of endogenous mammalian melatonin receptors is low, which has made studies on melatonin receptor regulation difficult and led to controversial results (3, 9). The use of cell lines expressing higher levels of the recombinant melatonin receptors offers, for the first time, an opportunity to study human melatonin-mediated receptor regulation and signaling in a controlled environment.

There has been considerable interest in the circadian regulation by light and melatonin of melatonin receptors found in the mammalian SCN (13–15) and in the pars tuberalis of the pituitary gland (3, 16). Exposure of ewe pars tuberalis cultured cells to melatonin decreases 2-[^{125}I]iodomelatonin binding and attenuates melatonin mediated inhibition of forskolin-induced cAMP production (16). In rats, melatonin receptors in the SCN are regulated by both the light/dark cycle and melatonin (13–15, 17). Both darkness and endogenous melatonin decrease the density of melatonin receptors in the SCN with no change in binding affinity, suggesting down-regulation of melatonin receptors and/or residual melatonin bound to the receptor (Refs. 13–15 and 17 and the current study). The recombinant human ML_{1A} melatonin receptor stably expressed in CHO cells represents a model of the "body clock" receptor, allowing studies on the direct effect of melatonin on receptor regulation and signaling without the confounding influence of light input. We characterized the human ML_{1A} melatonin receptor stably expressed in CHO cells and studied the effects of melatonin exposure on receptor regulation and signaling.

Materials and Methods

Development of a stable CHO cell line expressing the human ML_{1A} melatonin receptor. A stable CHO cell line expressing the human ML_{1A} melatonin receptor was developed using lipofectamine (Life Technologies, Gaithersburg, MD). Briefly, CHO cell cultures were grown as monolayers in F12 media (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°. CHO cells were cotransfected with the human ML_{1A} melatonin receptor cDNA cloned into pcDNAI (5) and pSVneo plasmid (Clontech, Palo Alto, CA) through lipofection. Clones were selected for their resistance to the antibiotic G-418 at 300 μg/ml (GIBCO-BRL) and their ability to bind specifically 2-[^{125}I]iodomelatonin (2200 Ci/mmol; DuPont, Boston, MA). The cell line used in this study, expressing a density of ~750 fmol/mg protein of melatonin receptor, originated from a single cell selected with the use of the limited dilution protocol.

Radioligand binding assays. The binding of 2-[^{125}I]iodomelatonin to whole (intact or lysates) CHO cells expressing the human ML_{1A} melatonin receptor was determined in either cell suspensions or cells attached to the plate with the use of Tris·HCl (50 mM) or Krebs' (100 mM NaCl) solution.

Association and dissociation studies of 2-[^{125}I]iodomelatonin binding were performed on intact CHO cells grown to confluency in 24-well dishes. Briefly, cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 0.62 mM KH_2PO_4), and binding was initiated on the addition of 2-[^{125}I]iodomelatonin (80 pM) in 0.5 ml of Krebs' solution (100 mM NaCl). The cells were incubated at 37° for various periods of times in the absence (total binding) or the presence (nonspecific binding) of 1 μM melatonin (Sigma Chemical Co., St. Louis, MO). Dissociation of 2-[^{125}I]iodomelatonin binding was initiated by the addition of melatonin (1 μM) at equilibrium (1.5 hr), and the specific binding was determined at various time points. The cells were washed four times with 0.5 ml of PBS to remove unbound 2-[^{125}I]iodomelatonin, lifted in LIFT buffer (10 mM KPO_4, 1 mM EDTA, 0.25 M sucrose), and counted in a γ counter.

Saturation and selected competition studies with melatonin were performed on intact CHO cells in suspension. Cells, grown to confluency, were washed twice with 5 ml of PBS, lifted in LIFT buffer, and pelleted through centrifugation (1500 rpm for 5 min). The cells were resuspended in Krebs' solution (100 mM NaCl) and aliquoted into reaction tubes containing various concentrations of 2-[^{125}I]iodomelatonin (1–700 pM) in the absence and presence of 1 μM melatonin.

Competition studies of various agents for 2-[^{125}I]iodomelatonin binding were performed in CHO whole cell lysates in suspension. Cells, grown to confluency, were washed with PBS, lifted in LIFT buffer (minus sucrose), pelleted by centrifugation (1500 rpm, 5 min), and resuspended in Tris·HCl solution (50 mM). Aliquots (~5 μg of protein) of cell suspensions were added to tubes containing 2-[^{125}I]iodomelatonin (80 pM) and appropriate concentrations of vehicle or competing agents in a total assay volume of O.26 ml. 2-[^{125}I]Iodomelatonin binding was competed with either 2-iodomelatonin (0.01 fM–100 nM; Research Biochemicals, Inc., Natick, MA), melatonin (0.1 pM–1 μM), *N*-acetyl serotonin (10 nM–1 mM; Sigma), luzindole (1 nM–100 μM; Glaxo, Ware, UK), or GTPγS (100 pM–30 μM; Sigma). Competition of various concentrations of melatonin (0.1 pM–1 μM) for 2-[^{125}I]iodomelatonin binding was determined in the absence and presence of 0.1 or 3 μM GTPγS. Competition of melatonin for 2-[^{125}I]iodomelatonin binding was also determined in cells preincubated with vehicle or melatonin (1 μM). Cells were preincubated with F12 media containing either vehicle or melatonin (1 μM) for 1 hr and then washed four times with 5 ml of PBS at 1-min intervals to remove unbound melatonin.

Saturation and competition assays were incubated for 1.5 hr at 37°, and reactions were terminated by the addition of ice-cold Tris·HCl solution (50 mM) and rapid filtration over glass-fiber filters (Schleicher & Schuell, Keene, NH) presoaked in 0.5% polyethylenimine solution (v/v) (Sigma). Each filter was washed twice with 5 ml of cold buffer. Radioactivity was determined in a γ counter.

cAMP accumulation assays. Melatonin-mediated inhibition of forskolin-induced [^{3}H]cAMP accumulation was measured in CHO cells attached to plates endowed with the human ML_{1A} melatonin receptor. Briefly, cells were grown to confluency and then labeled with 2 μCi/ml [^{3}H]adenine (26.9 Ci/mmol; DuPont) in F12 media for 5–6 hr. The cells were then washed twice with 1 ml of PBS to remove free [^{3}H]adenine from the media. For pertussis toxin assays, cells were incubated with 60 ng/ml pertussis toxin (List Biologicals, Campbell, CA) for 16 hr in media containing serum at 37° before cAMP assays. For acute regulation experiments, cells were preincubated with F12 media and either vehicle, melatonin (1 μM), or me-

latonin plus luzindole (1 μM) for 1 hr at 37°. The cells were then washed extensively (twice for 5 min and then three times for 15 min) with media at 37°. cAMP formation was stimulated by the addition of F12 media containing 100 μM forskolin (Sigma), 30 μM rolipram (Research Biochemicals), and appropriate concentrations of vehicle or drugs. The assays were incubated for 10 min at 37° and then terminated through aspiration of the media and the addition of 1 ml of ice-cold (5%) trichloroacetic acid (16 hr at 4°) to release [^{3}H]cAMP into the solution. Before loading the columns, each well was spiked with a known amount (~1000 cpm) of [^{14}C]cAMP (52.3 mCi/mmol; DuPont) to account for recovery of cAMP eluted off the columns. [^{3}H]cAMP was separated from [^{3}H]ATP using Dowex (AG50W-X4; Bio-Rad, Hercules, CA) and alumina (Sigma) column chromatography as described previously (18–20). [^{3}H]cAMP was counted through liquid scintillation counting. For acute melatonin regulation experiments, treated cells were always run in parallel with control untreated cells.

Data analysis. Association and dissociation binding curves were generated through nonlinear regression analysis with Quattro Pro for Windows (Borland International, Buffalo, NY). Specific binding of 2-[^{125}I]iodomelatonin was calculated by subtracting nonspecific binding from total binding and expressed as fmol/mg protein. Nonspecific binding never exceeded 10% of the total binding, and total binding was always below 10% of the free concentration. All experiments were performed at least three times in duplicate. Saturation and competition curves were generated through nonlinear regression analysis with Prism (GraphPad, San Diego, CA). Individual [^{3}H]cAMP values were normalized through recovery of [^{14}C]cAMP with a standard amount of [^{14}C]cAMP (1000 cpm) in each column. [^{3}H]cAMP values were expressed as a percentage of the maximal forskolin response or as a percentage of control as indicated. Curves were generated through nonlinear regression analysis, and all statistical analyses were performed with 2-way analysis of variance using Prism.

Results

Development of a stable CHO cell line expressing the human ML_{1A} melatonin receptor. The human ML_{1A} melatonin receptor cDNA, cloned into the expression vector pcDNAI, was stably transfected into CHO cells. Colonies resistant to the antibiotic G418 were selected and grown. CHO cells did not show specific 2-[^{125}I]iodomelatonin binding, suggesting the absence of endogenous melatonin receptors. Eleven of 24 colonies selected bound 2-[^{125}I]iodomelatonin (500 pM) specifically. A colony selected through the limited dilution technique expressing ~700 fmol/mg protein of 2-[^{125}I]iodomelatonin binding sites was selected for further studies.

Pharmacological characteristics of 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptor. Association of 2-[^{125}I]iodomelatonin (80 pM) binding to intact CHO cells endowed with the human ML_{1A} melatonin receptor was maximal by 1.5 hr and remained stable for ≥3 hr ($t_{1/2}$ = 12.7 ± 0.3 min) at 37°, with a k_{+1} of 1.9 ± 0.06 × 10^8 min^{-1} M^{-1}. The addition of 1 μM melatonin at equilibrium completely dissociated specific 2-[^{125}I]iodomelatonin binding by 3 hr ($t_{1/2}$ = 21 ± 3 min), with a k_{-1} of 0.02 ± 0.002 min^{-1} (Fig. 1). The binding of 2-[^{125}I]iodomelatonin was saturable and of high affinity (K_D = 74 ± 14 pM, B_{max} = 679 ± 88 fmol/mg protein; three experiments) (Fig. 2). The K_D values determined in kinetic ($K_D = k_{-1}/k_{+1}$ = 92 ± 9 pM; three experiments) and saturation studies were similar.

The pharmacological characteristics of the ML_{1A} melatonin receptor was determined through competition binding to

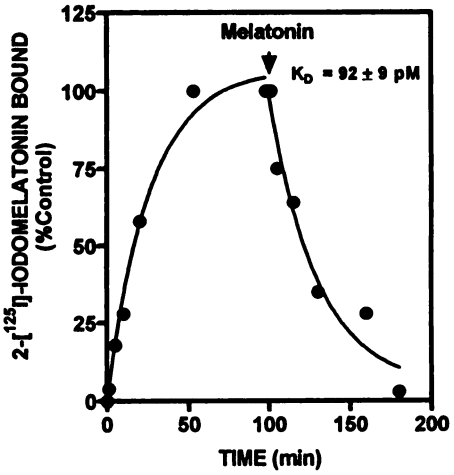

Fig. 1. Reversible 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptors on intact CHO cells at 37°C. Cells, attached to plates, were incubated with Krebs' solution (100 mM NaCl) containing 2-[^{125}I]iodomelatonin (80 pM) in the absence and presence of melatonin (1 μM) for various periods of time (1–100 min). Dissociation of 2-[^{125}I]iodomelatonin binding (80 pM) at equilibrium (100 min) was determined at various times after the addition of 1 μM melatonin (k_{+1} = 1.9 ± 0.1 pM^{-1} min^{-1}, k_{-1} = 0.018 ± 0.002 min^{-1}, $K_D = k_{-1}/k_{+1}$ = 92 ± 9 pM). Reactions were terminated by repeated washes of unbound 2-[^{125}I]iodomelatonin; then, cells were lifted, and radioactivity was directly counted. Values are mean of duplicate determinations from a representative experiment repeated twice.

CHO whole cell lysates at 37°. 2-[^{125}I]Iodomelatonin binding to the human ML_{1A} receptor was competed by various agents with the following order of affinities: 2-iodomelatonin (IC_{50SH} = 2.2 ± 0.67 fM, 39%; IC_{50H} = 7.9 ± 4.7 pM, 61%; three experiments), melatonin (IC_{50SH} = 6.5 ± 6 pM, 14%; IC_{50H} = 2.0 ± 0.47 nM, 86%; three experiments), *N*-acetyl serotonin (IC_{50} = 206 ± 18 nM; three experiments), and luzindole (IC_{50} = 1.8 ± 0.97 μM; three experiments) (Fig. 3). GTPγS (100 pM–30 μM) inhibited 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptor in a concentration-dependent manner at 37° (Fig. 4A), with an IC_{50} value of 0.87 ± 0.12 μM (three experiments) and maximal inhibition of 97% at 30 μM.

Competition of 2-[^{125}I]iodomelatonin binding to the ML_{1A} receptor by 16 concentrations of melatonin (0.1 pM–1 μM) resulted in biphasic curves suggesting binding of the radioligand to two affinity states: super high (IC_{50SH} = 6.5 ± 6 pM, 14%) and high (IC_{50H} = 2.0 ± 0.47 nM, 86%; five experiments) (Fig. 4B). In the presence of GTPγS (3 μM), competition of melatonin (0.1 pM–1 μM) for 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptor by melatonin resulted in monophasic curves (IC_{50} = 3.4 ± 0.78 nM; four experiments) (Fig. 4C). GTPγS (0.1 μM), however, did not shift the super high affinity state of the receptor (IC_{50SH} = 0.29 ± 0.18 pM, 16%; IC_{50H} = 3.8 ± 0.52 nM, 84%; three experiments) (Fig. 4B).

Sensitivity of the human ML_{1A} melatonin receptor expressed in CHO cells after pretreatment with melatonin. As described above, melatonin (0.1 pM–1 μM) competed for 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptor in whole-cell lysates and resulted in a biphasic curve. However, 2-[^{125}I]iodomelatonin binding to the ML_{1A} receptor to cells pretreated with melatonin (1 μM) for 1 hr followed by washes was to a single population of high affinity sites (IC_{50} = 1.1 ± 0.28 nM; three experiments) as

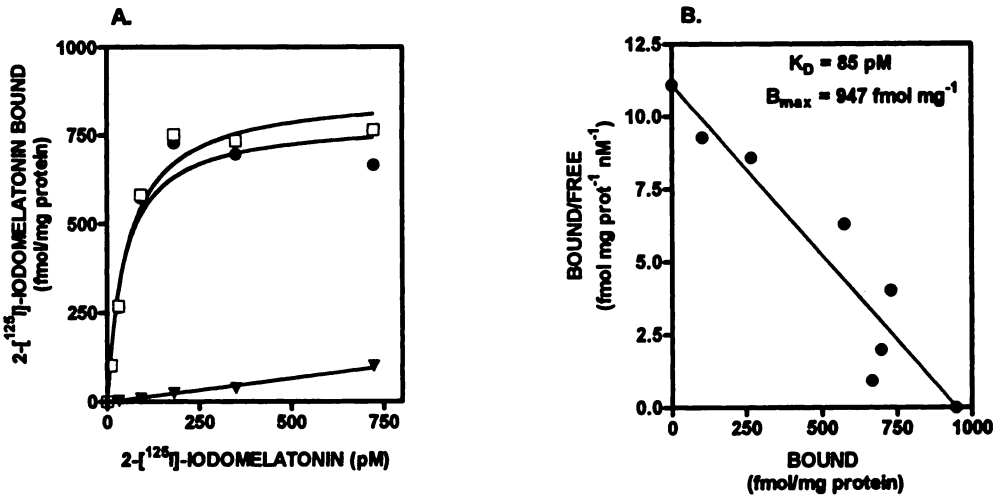

Fig. 2. 2-[^{125}I]iodomelatonin saturation binding to the human ML_{1A} melatonin receptor in intact CHO cells. Cell suspensions were incubated in Krebs' solution (100 mM NaCl) with various concentrations of 2-[^{125}I]iodomelatonin (1 pM–700 pM) for 1.5 hr at 37°. A, Nonspecific binding (▼) was measured in the presence of 1 μM melatonin. Specific binding (●) is defined as total binding (□) minus nonspecific binding. B, Scatchard plot of saturation binding: K_D = 85 pM and B_{max} = 947 fmol/mg protein; bound/free given in fmol/mg of protein/nM and bound given in fmol/mg. Values are mean of duplicate determinations from a representative experiment performed four times.

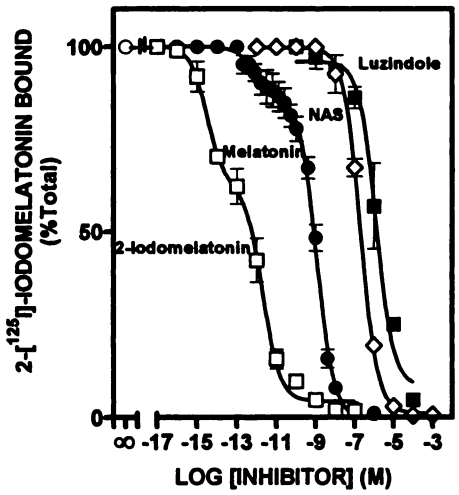

Fig. 3. Competition of various melatonin analogues for 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptor in CHO whole-cell lysates. Cells were suspended in Tris (50 mM), pH 7.4, and incubated with 2-[^{125}I]iodomelatonin (80 pM) and various concentrations (0.1–100 μM) of 2-iodomelatonin (□, IC_{50SH} = 2.2 ± 0.92 fM, 39%; IC_{50H} = 1.5 ± 0.67 pM, 61%), melatonin (●, IC_{50SH} = 6.5 ± 6.0 pM, 14%; IC_{50H} = 2.0 ± 0.47 nM, 86%), and *N*-acetyl serotonin (*NAS*) (◇, IC_{50} = 206 ± 18 nM); luzindole (■, IC_{50} = 1.8 ± 0.97 μM). Data are mean ± standard error from three independent determinations performed in duplicate.

revealed by monophasic competition of 2-[^{125}I]iodomelatonin binding by melatonin (Fig. 5A).

Cells expressing the ML_{1A} melatonin receptor were treated with various concentrations of melatonin (1 pM–1 μM) for 24 hr, washed in PBS, and incubated with 2-[^{125}I]iodomelatonin (500 pM) in Krebs' (100 mM NaCl) solution. Total 2-[^{125}I]iodomelatonin binding was not changed by the pretreatment with various melatonin concentrations (data not shown). Furthermore, melatonin (1 μM) pretreatment for various periods of time (1, 6, 18, and 24 hr) did not affect total 2-[^{125}I]iodomelatonin binding to these cells (data not shown).

ML_{1A} melatonin receptor-mediated inhibition of forskolin-induced cAMP formation in intact CHO cells. Competition of 2-[^{125}I]iodomelatonin binding by melatonin (0.1 pM–1 μM) to intact cells maintained in Krebs' (100 mM NaCl) solution at 37° was also biphasic, showing 19% of the receptors in the super high affinity state (IC_{50SH} = 2.1 ± 0.92 pM, 19%; IC_{50H} = 1.5 ± 0.67 nM, 81%; three experiments) (Fig. 6A). To assess whether the differences in affinity of the two receptor populations would result in different potencies to melatonin, we determined melatonin-mediated inhibition of forskolin-stimulated cAMP formation in CHO cells expressing the ML_{1A} melatonin receptor. cAMP formation was assessed by measuring the formation of [^{3}H]cAMP in cells labeled with [^{3}H]adenine. Forskolin (1, 10, and 100 μM) stimulated, in a concentration-dependent manner, cAMP production in CHO cells endowed with the ML_{1A} melatonin receptor prelabeled with [^{3}H]adenine (basal, 62 ± 4 pM/well; 1 μM forskolin, 93 ± 5 pM/well; 10 μM forskolin, 146 ± 18 pM/well; 100 μM forskolin, 336 ± 18 pM/well; three experiments). Because a robust and reproducible increase in cAMP formation over basal was obtained with 100 μM forskolin, this concentration was selected in the following experiments.

Melatonin (0.1 fM–1 nM) inhibited, in a concentration-dependent manner, the increase in cAMP formation induced by 100 μM forskolin (Fig. 6B) in CHO cells endowed with the human ML_{1A} melatonin receptor. The inhibition of cAMP formation by melatonin was biphasic. Low concentrations of melatonin (0.1 fM–1 pM) inhibited cAMP formation with an IC_{50SH} = 0.1 ± 0.05 pM (four experiments) and a maximal inhibitory effect (26%) at 1 pM. Higher concentrations of melatonin (1 pM–1 nM) inhibited the forskolin-induced cAMP formation in a concentration-dependent manner, with an IC_{50H} = 64 ± 1.8 pM (four experiments) and a maximal inhibitory effect (74%) at 1 nM. Melatonin-mediated inhibition of forskolin-induced cAMP formation in cells pretreated with pertussis toxin (60 ng/ml) was abolished at both low (0.1 fM–1 pM) and high (1 pM–1 nM) concentrations of melatonin (Fig. 6C). The competitive melatonin receptor antagonist luzindole (1 μM), when used alone, did not affect the forskolin-induced stimulation of cAMP formation (data not shown). However, luzindole did not affect the inhibition of forskolin-stimulated cAMP formation elicited by low concentrations of melatonin (0.1 fM–1 pM); it competitively antagonized the inhibition elicited by higher concentrations (1 pM–1 nM) of the hormone. In the presence of luzindole (1 μM), the concentration-effect curve for melatonin (1 pM–1 nM) was shifted to the right with an IC_{50} value of 1.5 ± 0.22 nM (three experiments) (Fig. 6B). The apparent dissociation constant for luzindole was pK_B = −7.3.

Melatonin (5 nM–10 μM) inhibited forskolin-induced increases in cAMP formation in untransfected CHO cells (IC_{50}

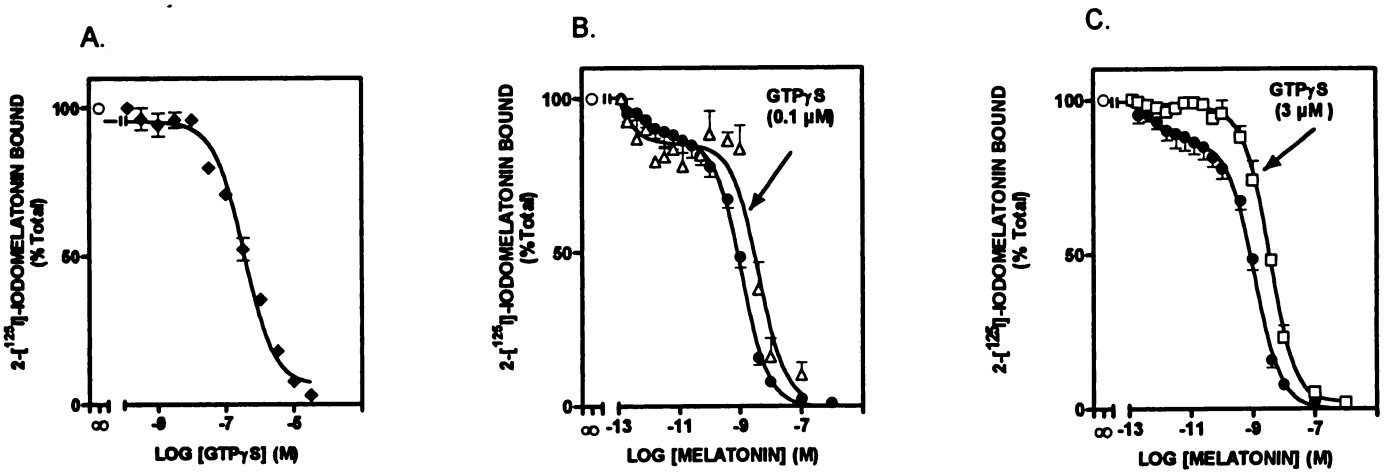

Fig. 4. GTPγS shift and melatonin competition for 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptor in CHO whole-cell lysates. Competition of melatonin (0.1 pM–1 μM) for 2-[^{125}I]iodomelatonin binding in the absence and presence of GTPγS. Cells were suspended in Tris (50 mM), pH 7.4, and incubated with 2-[^{125}I]iodomelatonin (80 pM) at 37° for 1.5 hr. A, Inhibition by GTPγS (100 pM–30 μM) of 2-[^{125}I]iodomelatonin binding (◆, IC_{50} = 0.87 ± 0.12 μM) and competition by melatonin in the absence of GTPγS (●, IC_{50SH} = 6.5 ± 6.0 pM, 14%; IC_{50H} = 2.0 ± 0.47 nM, 86%). B, Competition by melatonin in the presence of GTPγS 100 nM (Δ, IC_{50SH} = 0.29 ± 0.18 pM, 20%; IC_{50H} = 3.8 ± 0.52 nM, 80%). C, Competition by melatonin in presence of 3 μM (□, IC_{50} = 3.4 ± 0.78 nM). Data are mean ± standard error of three to six independent determinations performed in duplicate.

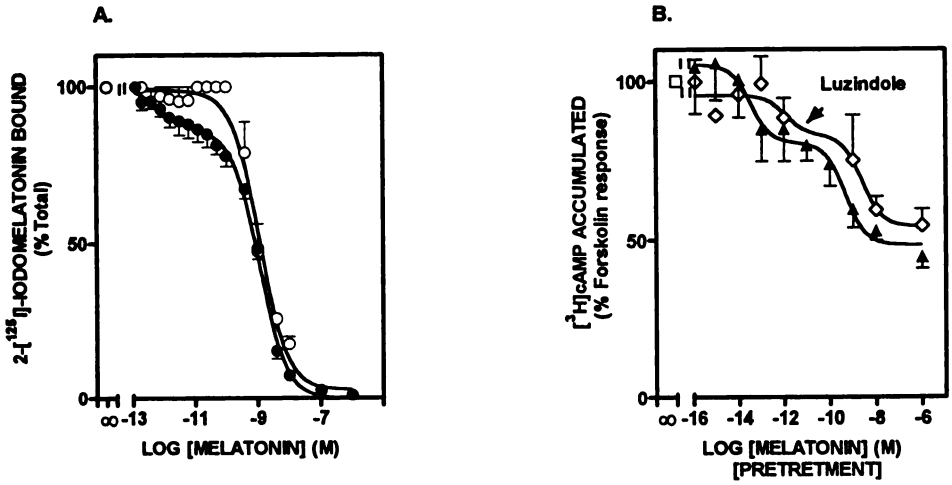

Fig. 5. Super high affinity shifts of melatonin competition for 2-[^{125}I]iodomelatonin binding are due to tight melatonin binding after acute pretreatment with melatonin. A, Acute pretreatment (1 hr) with 1 μM melatonin (○) reduces super high affinity 2-[^{125}I]iodomelatonin binding (IC_{50} = 1.1 ± 0.28 nM) compared with control (●, IC_{50SH} = 6.5 ± 6 pM and IC_{50H} = 2.0 ± 0.47 nM). B, Pretreatment with various concentrations of melatonin in the absence (▲) and presence (◇) of luzindole (1 μM) followed by extensive washes cannot remove the melatonin. An inhibition of forskolin-induced [^{3}H]cAMP formation correlated with the concentration of melatonin in the pretreatment period. Briefly, cells were incubated with media containing 1 μM melatonin for the competition assays and various concentrations of melatonin (1 fM–1 μM) in the absence and presence of 1 μM luzindole in the cAMP assays. Reactions were carried out at 37° for 1 hr where melatonin was removed by repeated washes in media for 1 hr. Cells were then either suspended in Tris (50 mM) and aliquoted into tubes containing 2-[^{125}I]iodomelatonin (80 pM) and appropriate concentrations of melatonin (0.1 pM–1 μM) (A) or intact cells attached to plates were exposed to media containing forskolin (100 μM) and 30 μM rolipram (B). Data are mean ± standard error of three or four independent determinations performed in duplicate.

= 3 ± 1 nM). However, this hormone (5 nM–10 μM) did not compete for 2-[^{125}I]iodomelatonin binding in untransfected CHO cells (data not shown).

Sensitivity of the human ML_{1A} melatonin receptor-mediated inhibition of forskolin-induced cAMP formation after acute treatment with melatonin. Cells expressing the human ML_{1A} melatonin receptor were pretreated with melatonin for 1 hr at 37° and then washed extensively for ≤1 hr, as described in Materials and Methods. In vehicle-treated cells, forskolin (100 μM) increased cAMP formation by ~500% over basal. However, cells pretreated with various concentrations of melatonin (1 fM–1 μM) and extensively washed showed reduced increases in cAMP formation when stimulated with forskolin. Melatonin (0.1 pM–1 μM) pretreatment (1 hr) led to increases in forskolin-induced cAMP production that were 20–50% lower than the increases obtained when cells were incubated with vehicle alone (Fig. 5B). The increases in cAMP induced by forskolin correlated with the concentration of melatonin used to activate the ML_{1A} receptor during the pretreatment period. In an attempt to reverse the effects of melatonin pretreatment, the melatonin receptor antagonist luzindole (1 μM) was added in combination with the various concentrations of melatonin during the pretreatment. The magnitude of forskolin-induced cAMP formation after pretreatment with melatonin and luzindole was slightly higher but not significantly different than that obtained

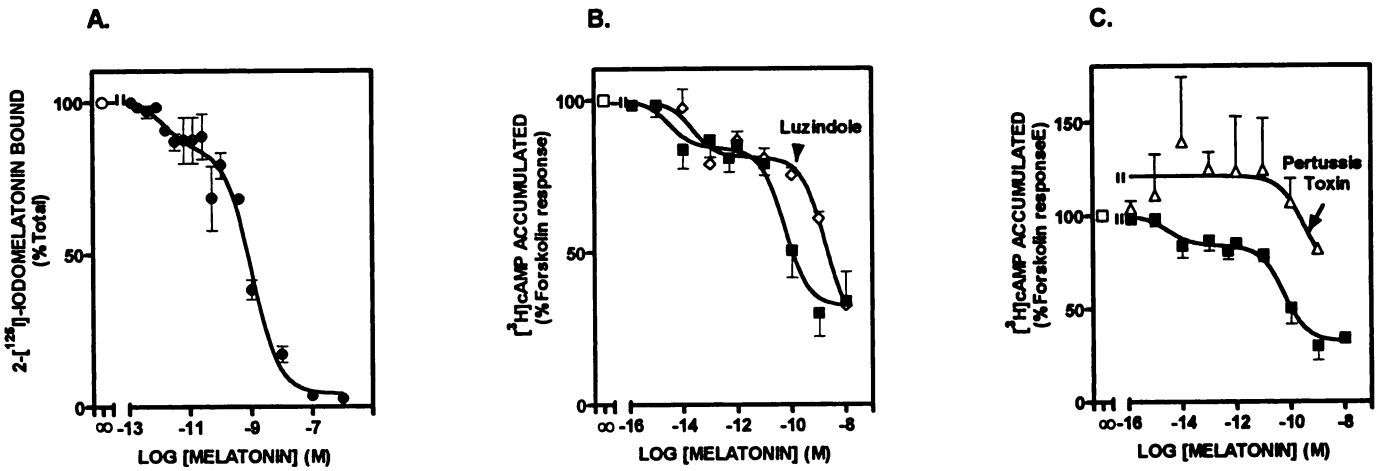

Fig. 6. Competition of 2-[^{125}I]iodomelatonin binding by melatonin and functional analysis of the human ML_{1A} melatonin receptor reveal biphasic curves on intact cells. A, Competition of melatonin (0.1 pM–1 μM) for 2-[^{125}I]iodomelatonin binding to intact cells. Cells were suspended in Krebs' (100 mM NaCl), pH 7.4, and incubated with 2-[^{125}I]iodomelatonin (80 pM) at 37° for 1.5 hr. Competition of melatonin for 2-[^{125}I]iodomelatonin binding reveals two sites: a super high affinity (●, IC_{50SH} = 2.1 ± 0.92 pM, 19%) and a high affinity (IC_{50H} = 1.5 ± 0.67 nM, 81%). B, ML_{1A} melatonin receptor-mediated inhibition of forskolin-stimulated [^{3}H]cAMP formation. Intact cells attached to plates were exposed to media containing forskolin (100 μM), rolipram (30 μM), and increasing concentrations of melatonin (0.1 fM–10 nM) in the absence and presence of 1 μM luzindole. Functional analysis reveals a biphasic curve: a super high affinity component (■, IC_{50SH} = 0.1 ± 0.05 pM with a maximal inhibition of 26%) and a high affinity component (IC_{50H} = 64 ± 1.8 pM with a maximal inhibition of 74%), where luzindole (◇) antagonizes the high affinity component (IC_{50} = 1.5 ± 0.22 nM). C, Effects of pertussis toxin (Δ) on ML_{1A} melatonin receptor-mediated inhibition of forskolin-stimulated [^{3}H]cAMP formation. Intact cells attached to plates were exposed to media containing pertussis toxin (60 ng/ml) for 16 hr at 37°. After pertussis toxin pretreatment, cAMP assays were carried out as described in B. Pertussis toxin completely abolished melatonin-mediated inhibition of forskolin-induced cAMP formation for both the super high and high affinity states. Reactions were carried out at 37° for 10 min and terminated by the aspiration of media-containing drugs and the addition of 1 ml of 5% trichloroacetic acid (16 hr at 4°). [^{3}H]cAMP was collected and counted as described in Materials and Methods. Data are mean ± standard error of three or four independent experiments performed in duplicate.

after pretreatment with melatonin alone (Fig. 5B). These results suggest that luzindole did not antagonize these persistent inhibitory effects of low concentrations of melatonin.

Discussion

We report the development of a CHO cell line expressing a high density of the recombinant human ML_{1A} melatonin receptor that expresses in mammalian SCN (4) and pars tuberalis (5). This human melatonin receptor subtype shows the pharmacological characteristics of the ML_1 receptor (3, 8, 9), and it is coupled to inhibition of cAMP formation (Refs. 5 and 6 and current study) via pertussis toxin-sensitive G proteins (Refs. 3 and 9 and current study). The ML_{1A} melatonin receptor expressed in CHO cells exists in two affinity states: super high and high. Activation by melatonin inhibited forskolin-induced cAMP formation with super high (IC_{50SH} = 0.1 ± 0.05 pM) and high (IC_{50H} = 64 ± 1.8 pM) potency. The inhibition of cAMP formation elicited by higher concentrations of melatonin was antagonized by luzindole, a competitive melatonin receptor antagonist (21), showing 10 times higher affinity for the ML_{1B} than ML_{1A} recombinant melatonin receptor.[1] Luzindole, however, did not antagonize the inhibition of cAMP formation elicited by lower concentrations of melatonin, suggesting tight binding of the hormone to the receptor.

The recombinant human ML_{1A} melatonin receptor expressed in CHO cells is pharmacologically identical to the ML_1 melatonin receptor described in neural tissues of various species (4, 8, 9, 21). In this cell line, the ML_{1A} melatonin receptor expresses at a density ≥10–100 times higher than in mammalian tissues endowed with the endogenous receptor [rat SCN, 50 fmol/mg protein (17); rabbit retina, 9 fmol/mg protein (9)]. Competition of melatonin and 2-iodomelatonin for 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptor of cell lysates and intact cells maintained under physiological conditions resulted in biphasic curves, which is indicative of binding to super high and high affinity states of the receptor. Biphasic competition profiles of 2-[^{125}I]iodomelatonin binding to melatonin receptors in native tissues are observed in retina (9), chick brain (22), ovine pars tuberalis (23), and human malignant melanoma cells (24). Although in these studies 2-[^{125}I]iodomelatonin seems to bind to two affinity states of the high affinity ML_1 receptor, binding of the radioligand to multiple melatonin receptor subtypes or receptors for other neuromodulators cannot be excluded.

Activation of the human ML_{1A} receptor with melatonin inhibited forskolin-induced stimulation of cAMP in a concentration-dependent and biphasic manner. This inhibition occurred in the same concentration range at which the hormone competed for binding of 2-[^{125}I]iodomelatonin binding to both the super high and high affinity states of the receptor. It is likely that inhibition of forskolin-induced cAMP formation by low concentrations of melatonin (0.1 fM–1 pM) occurred through activation of the super high affinity state of the ML_{1A} melatonin receptor. Similarly, in Syrian hamster hypothalamus, melatonin at picomolar concentrations inhibited forskolin-stimulated adenylyl cyclase to a maximum of 20% (25). In the current study, melatonin, at concentrations of >1 pM, further inhibited in a concentration-dependent manner forskolin-induced cAMP formation. In rat brain, melatonin inhibited forskolin-induced adenylyl cyclase activity in a biphasic manner. However, the inhibitory effects of this

[1] M. L. Dubocovich, unpublished observations.

hormone occurred over a broader range and at higher concentrations of melatonin (26) than those necessary to inhibit cAMP through activation of the recombinant ML_{1A} melatonin receptor expressed in CHO cells.

The recombinant human ML_{1A} receptor in CHO cells seems to display varying magnitudes of coupling to G proteins, as demonstrated by the sensitivity of 2-[^{125}I]iodomelatonin binding for GTPγS. GTPγS (0.1 μM) shifted the high affinity state of the receptor to lower affinities; however, a concentration of the GTPγS analogue 30 times higher was required to shift the receptors in the super high affinity state (Fig. 4, B and C). Uncoupling of ML_1-type melatonin receptors from their G proteins by nonhydrolyzable GTP analogues was demonstrated in chicken brain and retina (9, 22), pars tuberalis (3), and human malignant melanoma (M6) cell line (24). Furthermore, both the super high and high affinity states of the ML_{1A} receptor seem to be coupled to G proteins because pertussis toxin completely blocked the ability of melatonin to inhibit forskolin-induced cAMP formation through activation of both the super high and high affinity states of the receptor. Although coupling to stimulatory G proteins in native and recombinant melatonin receptors, resulting in increases in cAMP formation, has been observed (27, 28), our results suggest that ML_{1A} melatonin receptors in both the super high and high affinity states may couple to either different pertussis toxin-sensitive G_i proteins or to the same pertussis toxin-sensitive G_i protein with varying magnitudes (29, 30).

The inhibitory effect of melatonin on forskolin-induced cAMP formation in untransfected CHO cells seemed to be mediated through a receptor-independent mechanism. Inhibition of forskolin-induced cAMP formation by high concentrations of melatonin through a pertussis toxin-insensitive mechanism was shown in ovine pars tuberalis cells (31). It is conceivable that the inhibitory actions of melatonin on forskolin-induced cAMP formation at concentrations of >5 nM were mediated through a direct action of the hormone as a calmodulin antagonist (32). Thereby, through this mechanism, melatonin should inhibit calmodulin-dependent adenylyl cyclase, leading to inhibition of cAMP formation (33).

Comparison of IC_{50} values obtained from both competition and functional analyses revealed the existence of spare receptors. The potency of melatonin to inhibit forskolin-stimulated cAMP formation was higher than the affinity of the hormone for competition of 2-[^{125}I]iodomelatonin binding to the human ML_{1A} melatonin receptor. In a CHO cell line stably transfected with the human ML_{1A} melatonin receptor, expressing 10-fold fewer receptors (B_{max} = 80 fmol/mg protein), only the super high affinity state of the receptor is expressed (K_i = 9 ± 1 nM). The potency of melatonin to inhibit forskolin-induced cAMP formation was IC_{50} = 0.2 ± 0.09 pM. Consistent with the presence of a receptor reserve population in the CHO cell line used in these studies, the potency of melatonin to inhibit forskolin-induced cAMP formation was similar to that observed in the CHO cell line expressing lower density of ML_{1A} melatonin receptors. Although the existence of spare ML_{1A} melatonin receptors in native tissues has not been reported, spare receptors exist for other G protein-coupled receptors (34).

Melatonin is released during the hours of darkness, and its physiological effects are dependent on the length of the melatonin exposure (3). The functional consequence of prolonged melatonin exposure was studied both *in vitro* (pars tuberalis) and *in vivo* (SCN). Activation of melatonin receptors by either endogenous or exogenous melatonin decreases specific 2-[^{125}I]iodomelatonin binding in the SCN of rat (13–15, 17) and cultured ovine pars tuberalis cells (16), respectively. The ability of melatonin to inhibit forskolin-induced cAMP formation was attenuated after prolonged pretreatment of ovine pars tuberalis cultured cells with melatonin (16). We exposed melatonin (0.1 pM–1 μM) for ≤24 hr without an effect on 2-[^{125}I]iodomelatonin-specific binding. Interestingly, although melatonin treatment did not affect the density of melatonin receptors, it shifted the biphasic 2-[^{125}I]iodomelatonin binding competition by melatonin curve to a monophasic curve (Fig. 5A). These results may suggest either uncoupling of or residual melatonin bound to the super high affinity state of the melatonin receptor. Uncoupling of the super high affinity state of the receptor by melatonin is unlikely because this receptor did not desensitize in that melatonin-mediated inhibition of forskolin-stimulated cAMP formation after melatonin pretreatment for 1 hr was not attenuated (Fig. 5B). These data suggest that a small proportion of exogenously added melatonin remained tightly associated with the ML_{1A} receptor. It is interesting to note that the persistent inhibition was not antagonized by added luzindole, even in combination with melatonin, during the pretreatment period (Fig. 5B). It is conceivable that melatonin-mediated signal transduction is regulated by tight melatonin binding to the super high affinity state of the receptor. This "locking" of agonist to its receptor has been shown in other G protein-coupled receptors, including the A_1 adenosine receptors (35), muscarinic acetylcholine receptors (36), β-adrenergic receptors (37, 38), and endothelin receptors (39–41).

The super high affinity state of the human ML_{1A} receptor expressed in CHO cells seems to bind melatonin by forming very tight complexes with its G protein. Tight ternary complexes of agonist/receptor/G protein have been reported (42) for the melatonin receptor as well as other receptor systems (41). In the absence of a chemical modification, 2-[^{125}I]iodomelatonin/melatonin receptor/G protein complexes can be visualized with the use of sodium dodecyl sulfate gel electrophoresis (42). The endothelin A receptor colocalize in caveolae, a small invagination of the plasma membrane known to contain G proteins, with endothelin still bound (41). In our study, the presence of melatonin/ML_{1A} receptor/G protein complexes in the CHO cells endowed with the human ML_{1A} melatonin receptor was inferred by demonstrating that low concentrations of GTPγS (0.1 μM), which minimally inhibited 2-[^{125}I]iodomelatonin binding, promoted the uncoupling of the high affinity state ML_{1A} receptors from their G protein while leaving those in the super high affinity state fully coupled (Fig. 4, B and C). These super high affinity state receptors, with tightly bound melatonin, uncoupled from their G protein only with higher concentrations of GTPγS. The combined results of these studies support the concept that some receptors belonging to the family of G protein-coupled receptors are capable of forming very tight complexes with G proteins in the presence of agonist.

It is conceivable that the main physiological effects of melatonin are mediated through activation of the super high affinity state of melatonin receptors via tight coupling. This may begin to explain the mechanism or mechanisms by

which circulating picomolar concentrations of melatonin in humans may regulate circadian function (7, 43). Because melatonin binding to its receptor was shown to be dependent on G protein coupling, then perhaps the ability of melatonin to signal is regulated at the level of receptor/G protein coupling (Ref. 3 and current study). The melatonin receptor in rat SCN undergoes changes in affinity states in a diurnal fashion. During the light phase, when melatonin levels are lowest, the "high affinity state" of the melatonin receptor predominates (17). Conversely, during darkness, when melatonin levels are highest, the "low-affinity state" of the melatonin receptor predominates (17). Although in the rat SCN increases in melatonin levels during darkness correlated with decreases in melatonin receptor levels, suggesting down-regulation of the melatonin receptor (13, 14), the possibility of residual melatonin tightly bound to the "high affinity state" of the receptor cannot be ruled out. Whether a down-regulation of or residual melatonin bound to the melatonin receptor occurs after melatonin exposure, both phenomena would display similar 2-[^{125}I]iodomelatonin binding characteristics (i.e., no change in K_D and a decrease in B_{max}). Because a diurnal rhythmicity in melatonin receptor affinity states occurs in rat SCN (17), it is likely that mechanisms such as regulation of G_i protein levels, which may affect melatonin receptor/G protein coupling, may also regulate melatonin-mediated signaling through its receptor.

In conclusion, we demonstrated that under physiological conditions, the human recombinant ML_{1A} melatonin receptor, which expresses in the SCN, exists in super high and high affinity states. The super high affinity state of the human ML_{1A} melatonin receptor couples tightly to G proteins and melatonin, and melatonin-mediated signaling is not reversed with luzindole. Therefore, it is likely that *in vivo*, very low levels of circulating melatonin during the day regulate function by tight binding to the super high affinity state of the melatonin receptor via tight G protein coupling. It is of interest to note that melatonin receptors in rat SCN follow a diurnal rhythm where the "high affinity state" of the receptor, which should correspond to the super high affinity state described here, predominates during the day (17). Thus, it is conceivable that mechanisms affecting the coupling state of the receptor/G protein complex produce dramatic effects on melatonin-mediated signal transduction in the circadian system.

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