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Identification of cysteines involved in ligand binding to the human melatonin MT_2 receptor

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Abstract

In mammals, melatonin activates melatonin MT_1 and MT_2 receptors. Using site-directed mutagenesis and chemical modification, we investigated the role of conserved cysteines in ligand binding. Dithiothreitol inhibited 2-[^{125}I]iodomelatonin binding to the FLAG-tagged human melatonin MT_2 receptor without affecting ligand affinity. Alanine substitution of Cys^{113} or Cys^{190} resulted in a loss of specific 2-[^{125}I]iodomelatonin binding, without altering cell surface receptor expression. This suggests that a putative disulfide bond linking Cys^{113} and Cys^{190} is essential to maintain a proper human melatonin MT_2 receptor conformation for melatonin binding. *N*-ethylmaleimide alkylation of cysteines inhibited 2-[^{125}I]iodomelatonin binding, decreasing both ligand affinity and receptor density. Alkylation of Cys^{140} contributes to changes in ligand affinity, while alkylation of Cys^{143} and Cys^{219} reduced binding capacity. We suggest that a disulfide bridge is important for the proper structural conformation of the human melatonin MT_2 receptor to bind melatonin. Cysteines located in receptor regions near the ligand binding site and/or G protein coupling region are involved in *N*-ethylmaleimide-induced changes in affinity and receptor density.

Keywords: Melatonin MT_2 receptor, human; Melatonin MT_1 receptor, human; Cysteine; Site-directed mutagenesis; Thiol agents; HEK-293 cell

1. Introduction

In mammals, the hormone melatonin modulates physiological functions through activation of at least two structurally and pharmacologically distinct melatonin receptors: MT_1 and MT_2 (Dubocovich et al., 2000). The cDNAs coding the melatonin MT_1 and MT_2 receptors were cloned from several species, including humans (Reppert et al., 1994, 1995a,b). The amino acid sequences of the melatonin MT_1 and MT_2 receptors display structural characteristics of G protein-coupled receptors including seven hydrophobic segments thought to constitute the membrane spanning domains (Reppert et al., 1994, 1995a,b). The human melatonin MT_1 and MT_2 receptors share 60% homology at the amino acid level (Reppert et al., 1996), show high picomolar affinity for 2-[^{125}I]iodomelatonin binding, distinct pharmacological profiles (Dubocovich et al., 1997) and are coupled to inhibition of cAMP formation through G_i/G_o proteins (Browning et al., 2000).

G protein-coupled receptors display several conserved structural features, which suggest common mechanisms of receptor function. One of the most conserved structural features of G protein-coupled receptors is a putative disulfide bond formed by two highly conserved cysteine (Cys) residues located within the first and second extracellular loops. Mutation of these Cys residues in rhodopsin (Karnik et al., 1988), δ-opioid (Ehrlich et al., 1998), platelet-activating factor (PAF) (Le Gouill et al., 1997) and muscarinic M_3 receptors (Zeng et al., 1999) demonstrated the critical importance of this disulfide bond for the proper receptor conformation for ligand binding, receptor activation and cell surface expression. These conserved Cys residues, however, do not always participate in disulfide bonding, as shown previously for the β_2-adrenoceptor (Noda et al., 1994).

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Cysteine residues may play structural and functional roles in G protein-coupled receptors depending on their receptor domain localization. Cysteine residues localized within the extracellular domains are frequently involved in disulfide bond formation, while those found in the C-terminal intracellular tail are often involved in palmitoylation and down-regulation of receptors (Eason et al., 1994; Hayashi and Haga, 1997). Cysteines in the transmembrane domains can serve as structural residues (Gether et al., 1997) and may also be important in ligand recognition if located near or at the binding pocket (Gaibelet et al., 1997).

Many G protein-coupled receptors are sensitive to sulfhydryl reagents, including μ- and δ-opioid (Ehrlich et al., 1998; Gaibelet et al., 1997; Shahrestanifar et al., 1996; Shahrestanifar and Howells, 1996), vasopressin (Pavo and Fahrenholz, 1990) and dopamine D_2 (Javitch et al., 1994) receptors. In native tissues, 2-[^{125}I]iodomelatonin binding to melatonin receptors is also inhibited by sulfhydryl alkylating reagents, such as *N*-ethylmaleimide (Kosar et al., 1994; Ying et al., 1992). Ying et al. (1992) demonstrated the involvement of multiple sulfhydryl groups essential for ligand recognition and melatonin-induced signal transduction in chick brain membranes; however, the specific amino acids involved have not been identified.

Structural studies have focused on identifying amino acid residues in the human melatonin MT_1 receptor involved in ligand binding and receptor activation (Conway et al., 1997, 2000; Gubitz and Reppert, 2000; Kokkola and Laitinen, 1998; Nelson et al., 2001); however, little is known about the human melatonin MT_2 receptor structure–affinity relationship. This would be particularly important as the human melatonin MT_2 receptor mediates several physiological functions in mammals, including inhibition of dopamine release from the retina (Dubocovich et al., 1997) and phase shifts of circadian rhythms (Dubocovich et al., 1998; Hunt et al., 2001). Biochemical studies revealed sulfhydryl groups to be important for melatonin binding (Kosar et al., 1994; Ying et al., 1992); however, our study is the first to identify cysteine residues in the human melatonin MT_2 receptor that are involved in melatonin binding. Using chemical modification with thiol-reducing and alkylating agents and site-directed mutagenesis, we suggest that Cys^{113} and Cys^{190} form an essential disulfide bond necessary for melatonin binding to the receptor. Furthermore, Cys^{140}, Cys^{143} and Cys^{219} are involved in *N*-ethylmaleimide-induced inhibition of melatonin binding to the human melatonin MT_2 receptor.

2. Materials and methods

2.1. Materials

cDNA containing the complete coding region of the human MT_2 receptor (human Mel_{1b}; cloned into pcDNA-3) was provided by Dr. S.M. Reppert (Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA) (Reppert et al., 1995a). The oligonucleotides used for site-directed mutagenesis and DNA sequencing were synthesized in the Biotechnology Facility of Northwestern University. Restriction enzymes were purchased from Promega (Madison, WI). Effectene transfection and plasmid DNA purification kits were obtained from Qiagen (Valencia, CA). The anti-FLAG M5 monoclonal antibody was purchased from Sigma (St. Louis, MO), and the goat anti-mouse fluoroisothiocynate (FITC)-conjugated antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Cell culture products were obtained from Gibco BRL (Grand Island, NY). 2-[^{125}I]iodomelatonin (SA: 2000 Ci/mmol) was purchased from Amersham (Piscataway, NJ). Melatonin, *N*-ethylmaleimide, dithiothreitol, GTPγS and other general reagents were obtained from Sigma.

2.2. FLAG epitope tagging and site-directed mutagenesis

The coding sequence of the FLAG peptide (DYKDDDDK) was fused to the coding region of the human melatonin MT_2 receptor by subcloning the receptor cDNA into the vector pFLAG-CMV-2 from Sigma. This construct was used for expression of an N-terminal FLAG human melatonin MT_2 receptor fusion protein. Mutations were introduced into the FLAG-tagged human MT_2 using the polymerase chain reaction (PCR)-based QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Amplification of the missense mutant DNA was carried out following manufacturer instructions. The PCR cycling parameters were: one cycle of 1 min at 95 °C followed by 15 rounds of temperature cycling (95 °C for 1 min, 45 °C for 2 min, 65 °C for 15 min). The primers for introducing the alanine substitutions were as follows:

C113A:
5′ CTGGGGGAGGAGCACGCCAAGGCCAGC and
5′ GCTGGCCTTGGCGTGCTCCTCCCCCAG;
C140A:
5′ GCCATTAACCGCTACGCCTACATCTGCC and
5′ GGCAGATGTAGGCGTAGCGGTTAATGGC;
C143A:
5′ CGCTACTGCTACATCGCCCACAGCATGGCC and
5′ GGCCATGCTGTGGGCGATGTAGCAGTAGCG;
C190A:
5′ CCACGCATCTATTCCGCCACCTTCATCCA and
5′ CTGGATGAAGGTGGCGGAATAGATGCGTGG;
C219A:
5′ GTCGTGTCCTTCGCCTACCTCCGCATC and
5′ GATGCGGAGGTAGGCGAAGGACACGAC;
C263A:
5′ TTTGCCATCGCCTGGGCTCCACTTAAC and
5′ GTTAAGTGGAGCCCAGGCGATGGCAAA;
C302A:
5′ GCTTATTTCAACAGCGCCCTGAATGCCA and
5′ TGGCATTCAGGGCGCTGTTGAAATAAGC.

Successful construction of amino-terminus FLAG-tagged human MT_2 and mutant melatonin receptors was confirmed by DNA sequencing.

2.3. Transient transfections

Human embryonic kidney (HEK-293) cells were grown as monolayers in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and 1% sodium pyruvate in 5% CO_2/95% air at 37 °C. Cultures at 40–50% confluence were transiently transfected with the FLAG-tagged wild-type or mutant human MT_2 cDNAs using the Effectene transfection kit. After 48–72 h, the media was removed and the cells were washed with phosphate-buffered saline (PBS), lifted in potassium phosphate buffer (10 mM, pH 7.4) containing sucrose (0.25 M) and EDTA (1 mM), and then pelleted by centrifugation (1500 × *g*, 5 min). Cell pellets were stored at −80 °C until used.

2.4. Chemical modification

Treatments with thiol reagents were performed in whole cell lysates of Chinese hamster ovary (CHO) cells stably transfected with human MT_1 or MT_2 cDNA, or HEK-293 cells transiently transfected with either the FLAG-tagged wild-type or mutant human melatonin MT_2 receptor cDNA. Aliquots of cell lysates were incubated for 25 min at 25 °C in 50 mM Tris–HCl (pH 7.5) containing varying concentrations of either *N*-ethylmaleimide (0.01–1 mM) or dithiothreitol (50–250 mM; pH 7.4). Alkylation by *N*-ethylmaleimide was stopped by adding L-cysteine (pH 7.4) in a 10-fold molar excess. Reactions were diluted 5-fold in ice-cold 50 mM Tris–HCl (pH 7.4) and whole cell lysates were used in radioligand binding assays.

2.5. 2-[^{125}I]iodomelatonin binding studies

2-[^{125}I]iodomelatonin binding was determined in whole cell lysates (5–25 μg protein/tube) as described (Witt-Enderby and Dubocovich, 1996). Briefly, 2-[^{125}I]iodomelatonin equilibrium binding assays were performed in a final volume of 260 μl of binding buffer (50 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 0.1% bovine serum albumin), for 1.5 h at 25 °C. Incubation was terminated by rapid vacuum filtration through glass fiber filters (Schleicher and Schuell No. 30) soaked in 0.5% polyethylenimine solution. Saturation analyses were performed with 2-[^{125}I]iodomelatonin (10–4000 pM). Nonspecific binding was determined in the presence of 1 μM melatonin. K_D and B_{max} values were determined by nonlinear regression analysis. IC_{50} values were obtained from curves generated from competition of melatonin (0.1 pM to 1 μM), luzindole (0.1 nM to 1 μM) and 4-phenyl-2-propionamidotetraline (4P-PDOT) (1 nM to 1 μM) for 2-[^{125}I]iodomelatonin binding (~ 100 pM). K_i values were calculated from IC_{50} values using the Cheng and Prussof equation (Cheng and Prusoff, 1973). Analysis of ligand binding constants was performed using the Prism program (GraphPad Software, San Diego, CA).

2.6. Immunocytochemistry and confocal microscopy

Transiently transfected HEK-293 cells were allowed to recover for 24 h before being seeded on poly-L-lysine-treated coverslips (25 mm^2) and grown for 24 h. All immunocytochemical procedures were performed at room temperature unless otherwise indicated. The cells were fixed with 4% paraformaldehyde for 7.5 min and then incubated with 10% goat serum for 45 min at 37 °C followed by a wash with PBS. Cells were treated with a

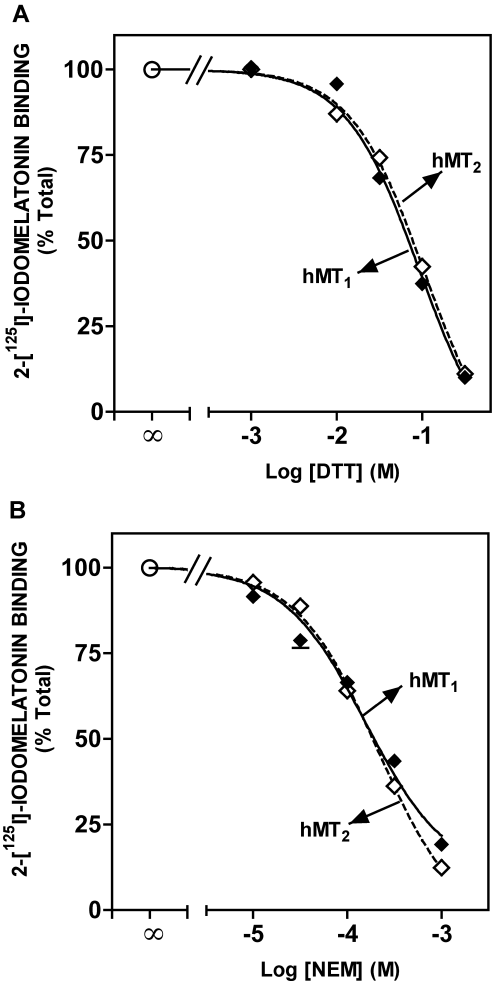

Fig. 1. Effect of *N*-ethylmaleimide (NEM) and dithiothreitol (DTT) treatment on 2-[^{125}I]iodomelatonin specific binding to CHO cells stably expressing the human melatonin MT_1 or MT_2 receptors. Specific 2-[^{125}I]iodomelatonin (150–200 pM) binding to whole cell lysates was measured after treatment with increasing concentrations of DTT (A) or NEM (B) for 25 min at 25 °C. Each curve represents the mean ± S.E.M of three independent experiments performed in duplicates.

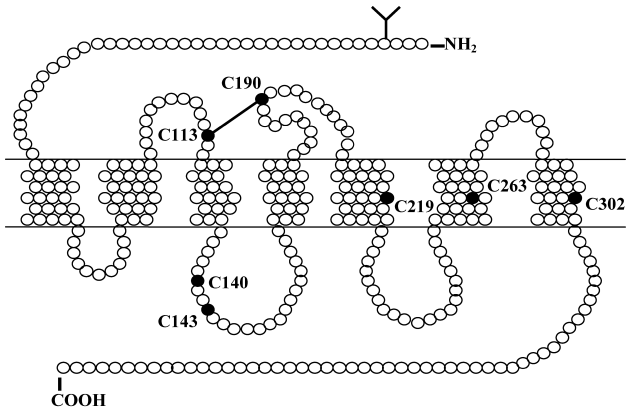

Fig. 2. Schematic representation of the putative topography of the human melatonin MT_2 receptor. The scheme shows the amino terminus is on the extracellular side and the carboxyl terminus on the intracellular side of the plasma membrane. The predicted seven transmembrane domains are shown from left to right. The position of the seven conserved cysteine residues in the human melatonin MT_1 and MT_2 receptors are indicated with a black dot. The conserved cysteines substituted with alanine are numbered. The presumed disulfide bond between Cys^{113} and Cys^{190} in putative extracellular loops 2 and 3 is shown by a black line. The potential asparagine-linked glycosylation site is represented by a Y (redrawn from Reppert et al., 1995a).

mouse anti-FLAG M5 monoclonal antibody (10 μg/ml) for 2 h at 37 °C and washed three times with PBS. After incubation with a goat anti-mouse FITC-conjugated antibody (1:100) for 1 h at 37 °C, the cells were washed with PBS and the coverslips mounted onto slides with the Slowfade antifade kit by Molecular Probes (Eugene, OR). The mounted coverslips were stored at 4 °C until images were captured using a Zeiss LSM 510 laser scanning confocal microscope.

3. Results

3.1. Dithiothreitol and N-ethylmaleimide inhibited 2-[^{125}I]iodomelatonin binding to the recombinant human melatonin MT_1 and MT_2 receptors

To identify cysteine residues involved in 2-[^{125}I]iodomelatonin binding, human melatonin MT_1 or MT_2 receptors stably expressed in CHO cells were treated with reducing (dithiothreitol: 1–300 mM) and thiol-alkylating (*N*-ethylmaleimide: 0.01–1 mM) reagents. Treatment of whole cell lysates with the thiol reagents inhibited in a concentration-dependent manner, 2-[^{125}I]iodomelatonin binding to both the human melatonin MT_1 or MT_2 receptors with identical half-maximal effective concentrations (IC_{50}: 130 mM for dithiothreitol and 160 μM for *N*-ethylmaleimide) (Fig. 1). These results suggested the presence of a disulfide bridge(s) and reactive conserved cysteine residue(s) in the human melatonin MT_1 and MT_2 receptors that are necessary for 2-[^{125}I]iodomelatonin binding. *N*-ethylmaleimide did not alter 2-[^{125}I]iodomelatonin equilibrium binding as the affinity constants (K_D) determined by kinetic analysis in control and *N*-ethylmaleimide (300 μM)-treated were similar (data not shown). These K_D values were not statistically different from the dissociation constants determined in saturation and competition studies.

3.2. 2-[^{125}I]iodomelatonin binding constants for the FLAG-tagged wild-type and cysteine mutant human melatonin MT_2 receptors

To elucidate the role of the conserved cysteines on melatonin binding to the human melatonin MT_2 receptor, the seven conserved cysteines were mutated to alanine by

Table 1
2-[^{125}I]iodomelatonin binding constants to FLAG-tagged wild-type and cysteine mutant human melatonin MT_2 receptors

Receptor mutant	2-[^{125}I]iodomelatonin binding				
	Competition, K_i (pM)			Saturation	
	MLT	Luzindole	4P-PDOT	K_D (pM)	B_{max} (pmol/mg protein)
Wild-type	515 (335–695)	14,900 (9435–20,370)	1253 (1907–2315)	128 (67–190)	2.44 ± 0.13
C113A	ND	ND	ND	ND	ND
C190A	ND	ND	ND	ND	ND
C140A	1053[a] (629–1478)	4665[a] (4131–5199)	995 (461–1529)	185 (37–333)	0.11 ± 0.02[b]
C143A	747[a] (108–1385)	16,000 (11,030–20,970)	633 (513–752)	117 (58–175)	4.50 ± 0.064[a]
C219A	370 (215–525)	15,665 (4648–26,680)	790 (268–1312)	158 (31–284)	0.52 ± 0.09[b]
C263A	385 (117–652)	9040 (7227–10,850)	825 (−330–1980)	174 (106–242)	0.08 ± 0.01[b]
C302A	360 (250–470)	8570 (4375–12,760)	555 (518–592)	112 (68–156)	0.63 ± 0.13[b]

HEK-293 cells were transiently transfected with the FLAG-tagged wild-type or cysteine mutant human melatonin MT_2 receptors. Whole cell lysates were used to determine binding constants in competition (K_i) and saturation (K_D; B_{max}) studies using 2-[^{125}I]iodomelatonin (0.01–4 nM). In saturation studies, nonspecific binding was determined with 1 μM melatonin. Values for K_i and K_D represent the mean ± S.E.M. and the 95% confidence intervals in parentheses in competition (n=3–5) and saturation (n=4–6) experiments. B_{max} values are expressed as the mean ± S.E.M. (n=4–6). MLT: melatonin. ND: Specific 2-[^{125}I]iodomelatonin binding to these mutants was not detected.

[a] $P<0.05$ when compared with the wild-type FLAG-tagged MT_2 receptor.

[b] $P<0.001$ when compared with the wild-type FLAG-tagged MT_2 receptor.

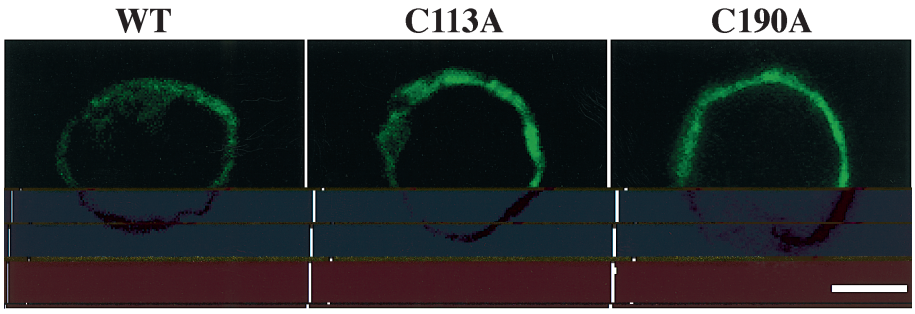

Fig. 3. Cellular localization of the FLAG-tagged wild-type and cysteine mutants (C113A and C190A) human melatonin MT_2 receptors. Fluorescent confocal microscopic images of non-permeabilized HEK-293 cells transiently expressing either the wild-type, C113A or C190A FLAG-tagged human melatonin MT_2 receptors. Expression was detected using an anti-FLAG monoclonal antibody and an FITC-conjugated secondary antibody. Each image is representative of three individual experiments. The scale bar represents 5 μm. No specific labeling was detected in untransfected HEK-293 cells (data not shown).

site-directed mutagenesis. These seven cysteines are conserved between the human melatonin MT_1 and MT_2 receptors and highly conserved across the cloned mammalian receptors to date (Reppert et al., 1995a,b; Roca et al., 1996; Weaver et al., 1996). These cysteines are located within the first (Cys^{113}) and second (Cys^{190}) extracellular loops, the second (Cys^{140} and Cys^{143}) intracellular loop and the fifth (Cys^{219}), sixth (Cys^{263}) and seventh (Cys^{302}) transmembrane domains (Fig. 2). FLAG-tagged wild-type and mutant human melatonin MT_2 receptors were transiently transfected into HEK-293 cells, and tested for melatonin binding. The FLAG-tagged sequence introduced at the N-terminus of the human melatonin MT_2 receptor did not affect 2-[^{125}I]iodomelatonin binding or receptor function (data not shown).

Saturation analysis of 2-[^{125}I]iodomelatonin (0.01–4 nM) binding to the FLAG-tagged wild-type human melatonin MT_2 transiently expressed in HEK cells showed high ligand affinity (K_D) and receptor density (B_{max}) (Table 1). Melatonin (0.1 pM–100 μM) competed for 2-[^{125}I]iodomelatonin binding to the FLAG-tagged wild-type human melatonin MT_2 receptor with high affinity (Table 1). Mutation of either Cys^{113} or Cys^{190} to alanine rendered human MT_2 mutants devoid of specific 2-[^{125}I]iodomelatonin binding (0.01–4 nM) (Table 1). However, confocal microscopy revealed cell surface expression of both the C113A and C190A mutants, suggesting that the loss of binding was not a result of inadequate plasma membrane expression (Fig. 3).

The apparent affinity of 2-[^{125}I]iodomelatonin determined by saturation analysis (K_D) to the FLAG-tagged C140A, C143A, C219A, C263A and C302A mutants were similar to those determined for the wild-type receptor (Table 1). However, the number of 2-[^{125}I]iodomelatonin binding sites was slightly increased in mutant C143A and significantly reduced in the other four cysteine mutants (Table 1). Melatonin and the melatonin receptor ligands luzindole and 4P-PDOT competed for 2-[^{125}I]iodomelatonin binding to both FLAG-tagged wild-type and C140A, C143A, C219A, C263A and C302A mutant receptors with similar affinities (Table 1). Melatonin and luzindole showed a small but statistically significant difference in affinity for the C140A receptor mutant when compared with the wild-type receptor. 2-[^{125}I]Iodomelatonin binding to both the FLAG-tagged wild-type and C140A, C143A, C219A, C263A and C302A mutant receptors was inhibited by increasing concentrations of the non-hydrolyzable GTP analogue GTPγS (IC_{50} = 6 μM). The half-effective concentrations (IC_{50}) of GTPγS for wild-type and mutant receptors were almost identical (data not shown).

3.3. Effect of dithiothreitol on specific 2-[^{125}I]iodomelatonin binding to the FLAG-tagged human MT_2 receptor transiently expressed in HEK cells

Treatment of the FLAG-tagged wild-type human MT_2 receptors transiently expressed in HEK cells (K_D = 158 ± 23 pM; B_{max}: 1.9 ± 0.4 pmol/mg protein) with 100 mM dithiothreitol significantly reduced the total number of binding

Table 2
N-ethylmaleimide (NEM) treatment decreased 2-[^{125}I]iodomelatonin binding affinity and density of the FLAG-tagged human melatonin MT_2 receptor

Treatment	2-[^{125}I]iodomelatonin binding		
	Competition	Saturation	
	K_i for MLT (pM)	K_D (pM)	B_{max} (pmol/mg protein)
None	365 (217–513)	171 (125–218)	2.73 ± 0.35
NEM 30 μM	528 (254–801)	227[a] (169–284)	3.00 ± 0.45
NEM 100 μM	960[a] (218–1702)	524[b] (32–1017)	2.55 ± 0.34
NEM 300 μM	1568[a] (438–2697)	847[b] (137–1558)	2.31 ± 0.45
NEM 1000 μM	2160[a] (615–3705)	1191[b] (91–2290)	1.33 ± 0.33[a]

HEK-293 cells transiently transfected with the FLAG-tagged wild-type human melatonin MT_2 melatonin receptor were lysed and treated with increasing concentrations of *N*-ethylmaleimide (30–1000 μM) for 25 min at 25 °C. Whole cell lysates were used to determine binding constants in competition (K_i) and saturation (K_D; B_{max}) studies using 2-[^{125}I]iodomelatonin (0.01–4 nM). In saturation studies, nonspecific binding was determined with 1 μM melatonin. K_i and K_D values represent the mean ± S.E.M. and the 95% confidence intervals in parentheses in competition (n = 3–6) and saturation (n = 3–6) experiments. B_{max} values are expressed as the mean ± S.E.M. (n = 4–6). MLT: melatonin.

[a] $P < 0.05$ when compared with the untreated control.

[b] $P < 0.001$ when compared with the untreated control.

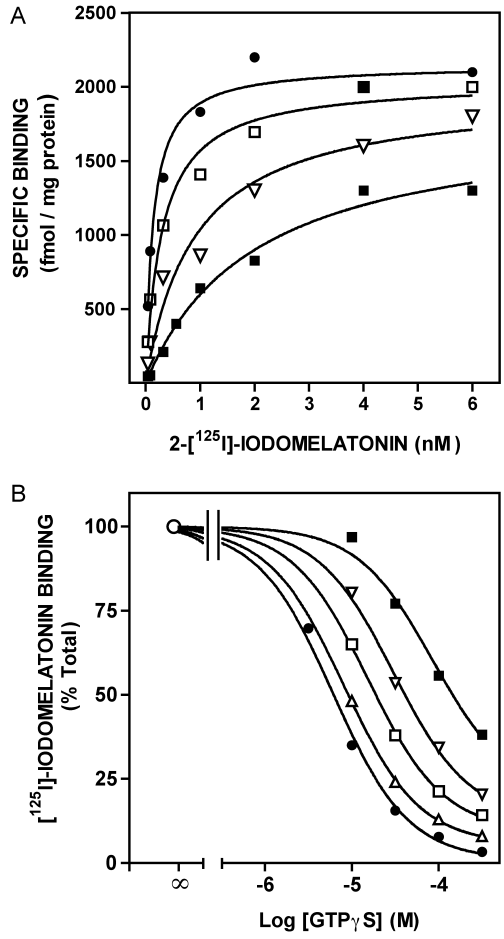

Fig. 4. Effect of *N*-ethylmaleimide (NEM) treatment on affinity, receptor density and GTPγS inhibition of 2-[^{125}I]iodomelatonin binding to the FLAG-tagged human MT_2 receptor. HEK-293 cells transiently transfected with the FLAG-tagged wild-type human MT_2 receptor were lysed and treated with increasing concentrations of NEM (0.03–1 mM) at 25 °C for 25 min. (A) Saturation analysis of 2-[^{125}I]iodomelatonin binding was performed as described in 'Experimental procedures'. Representative saturation curves are shown for each treatment. Experiments were performed in duplicate and repeated three to six times with similar results. Corresponding binding parameters (K_D, B_{max}) are shown in Table 2. (B) 2-[^{125}I]iodomelatonin binding (200 pM) to whole cell lysates in absence or presence of GTPγS (3–300 μM). The IC_{50} values for GTPγS were as follows: (●) control, IC_{50} = 6 μM; (△) NEM (30 μM), IC_{50} = 8 μM; (□) NEM (100 μM), IC_{50} = 15 μM; (▽) NEM (300 μM), IC_{50} = 30 μM; (■) NEM (1000 μM), IC_{50} = 85 μM. Data are means from one to three independent experiments performed in duplicate.

sites (B_{max}: 0.83 ± 0.09 pmol/mg protein, n = 3; P < 0.05) without affecting 2-[^{125}I]iodomelatonin affinity (K_D = 134 ± 19 pM).

3.4. Effect of N-ethylmaleimide treatment on specific 2-[^{125}I]iodomelatonin binding to the FLAG-tagged human melatonin MT_2 receptor cysteine mutants

N-ethylmaleimide (0.03–1 mM) decreased, in a concentration-dependent manner, the equilibrium affinity constant (K_D) of 2-[^{125}I]iodomelatonin binding for the human melatonin MT_2 receptor (Table 2; Fig. 4A) and the apparent affinity (K_i) of melatonin for competition with the radioligand (Table 2). The thiol-alkylating agent, *N*-ethylmaleimide, also significantly reduced the density of 2-[^{125}I]iodomelatonin binding sites, but only at concentrations higher than 300 μM *N*-ethylmaleimide (Table 2). This differential effect on 2-[^{125}I]iodomelatonin binding sug-

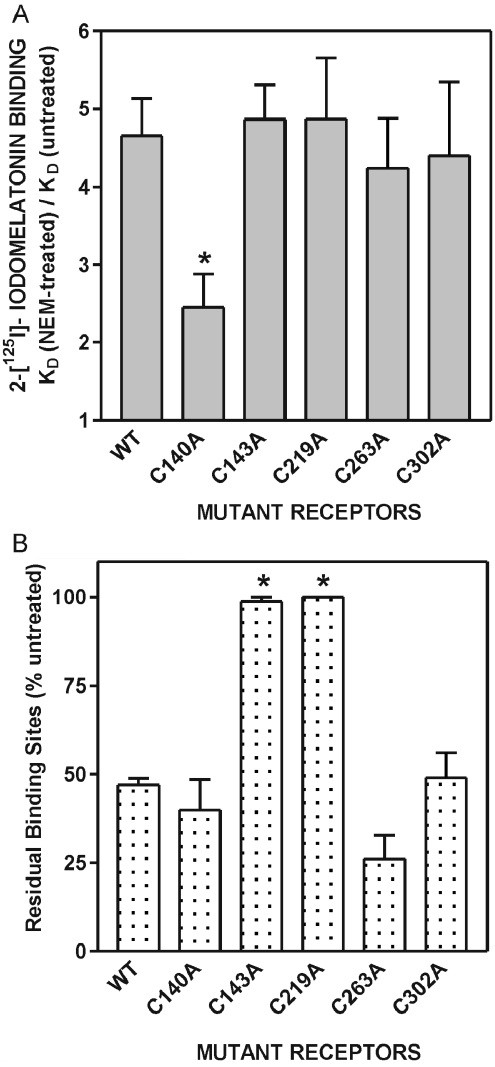

Fig. 5. Effect of *N*-ethylmaleimide (NEM) on affinities and density of 2-[^{125}I]iodomelatonin binding to the wild-type and mutant human melatonin MT_2 receptors. The K_D and B_{max} values for 2-[^{125}I]iodomelatonin were determined by saturation binding studies using transiently transfected HEK-293 cells, as described under 'Experimental procedures'. (A) The ordinate represents the fold change in K_D values for mutant receptors with respect to the K_D for the wild-type following treatment with 300 μM NEM at 25 °C for 25 min. Data are means from three to five independent experiments performed in duplicate. * P < 0.05 when compared with the wild-type FLAG-tagged MT_2 receptor. (B) The ordinate represents the percentage residual 2-[^{125}I]iodomelatonin binding to mutant receptors when compared with the wild-type after alkylation with 1 mM NEM at 25 °C for 25 min. The B_{max} values determined for each receptor transiently expressed in HEK-293 cells prior NEM treatment was taken as 100%. Data are means from three to five independent experiments performed in duplicate. * P < 0.05 when compared with the wild-type FLAG-tagged MT_2 receptor.

gested the possible existence of at least two alkylation sites with differing sensitivity for *N*-ethylmaleimide. The reactive cysteine sensitive to lower concentrations of *N*-ethylmaleimide might be involved in determining melatonin receptor affinity and/or receptor/G protein coupling. The cysteines sensitive to higher concentrations of *N*-ethylmaleimide may determine binding capacity (Fig. 4A). *N*-ethylmaleimide (30–1000 μM) also decreased in a concentration-dependent manner the potency of GTPγS to inhibit 2-[^{125}I]iodomelatonin binding to the FLAG-tagged human MT_2 receptor (Fig. 4B).

N-Ethylmaleimide reactive sites were identified by assessing the sensitivity of the cysteine mutant receptors to this alkylating agent. Treatment of the FLAG-tagged wild-type and C143A, C219A, C263A and C302A mutant receptors with *N*-ethylmaleimide (300 μM) decreased 2-[^{125}I]iodomelatonin binding affinity (Fig. 5A). However, the decrease in 2-[^{125}I]iodomelatonin affinity (K_D) of the C140A mutant receptor induced by *N*-ethylmaleimide treatment was significantly smaller when compared with that of the wild-type FLAG-tagged human MT_2 receptor (Fig. 5A). A similar result was obtained for competition of melatonin for 2-[^{125}I]iodomelatonin (K_i) (data not shown). *N*-Ethylmaleimide (1 mM) treatment did not modify the total number of 2-[^{125}I]iodomelatonin binding sites (B_{max}) of C143A and C219A cysteine mutants when compared with control (Fig. 5B).

4. Discussion

In this study, we demonstrate for the first time the presence of conserved cysteines involved in the structure–affinity of the human melatonin MT_2 receptor using site-directed mutagenesis, chemical modification analysis and biochemical strategies. The thiol-reducing agent, dithiothreitol, reduced the density of the FLAG-tagged human MT_2 receptor expressed in HEK-293 cells without affecting ligand affinity. A putative disulfide bond linking two cysteines appears to be essential to maintain a proper receptor conformation to bind melatonin as alanine substitution of Cys^{113} or Cys^{190} resulted in loss of specific 2-[^{125}I]iodomelatonin binding, without altering cell surface receptor expression. Alkylation with *N*-ethylmaleimide affected cysteine residues mediating decreases in 2-[^{125}I]iodomelatonin ligand affinity (Cys^{140}) and receptor density (Cys^{143} or Cys^{219}).

The thiol-reducing agent, dithiothreitol, decreased the density of FLAG-tagged human MT_2 receptors expressed in HEK-293 cells probably by altering the three-dimensional molecular structure of the receptor preventing ligand recognition at the binding site. This decrease in human MT_2 melatonin receptor density appears to result from a reduction of a putative disulfide bridge as mutations of the extracellular Cys^{113} or Cys^{190} to alanine rendered a receptor protein devoid of specific 2-[^{125}I]iodomelatonin binding. This loss of binding suggests that the putative disulfide bridge between Cys^{113} and Cys^{190} is essential for melatonin binding. These residues could either form part of the melatonin binding pocket or more likely be essential to maintain the structural conformation of the receptor. It has been proposed that melatonin fits within a hydrophilic binding pocket formed by the seven transmembrane helices of the receptor (Navajas et al., 1996). Therefore, Cys^{113} and Cys^{190} located in the first and second extracellular loops are unlikely to form part of the melatonin binding pocket. The absence of specific binding is not due to a lack of membrane expression as both the C113A and C190A mutant receptors traffik and express at the cell surface membrane. Mutations of equivalent conserved extracellular cysteines in rhodopsin (Karnik et al., 1988), the β_2-adrenoceptor (Dohlman et al., 1990), and the VIP receptor 1 (Knudsen et al., 1997) produced mutants with a significant reduction in ligand binding. However, measurable residual binding provided evidence of receptor expression and insertion into the plasma membrane. In contrast, mutations of analogous cysteines in the PAF receptor resulted in receptors that were defective in ligand binding and were not detected on the cell surface (Le Gouill et al., 1997). These PAF receptor mutants were expressed but remained in the intracellular compartment, indicating that the disulfide bridge may form during the early stages of receptor synthesis and be necessary for the normal folding and insertion into the plasma membrane (Le Gouill et al., 1997). Our data clearly demonstrated that the Cys^{113} and Cys^{190} residues play a significant role on the structure–affinity relationship of the human melatonin MT_2 receptor. The putative extracellular disulfide linkage between Cys^{113} and Cys^{190} is essential to form and maintain the active conformation of the human MT_2 receptor and most likely the human MT_1 receptor for melatonin binding.

The alkylating agent *N*-ethylmaleimide altered ligand binding and coupling, suggesting free cysteines are important in the structure–affinity of the human melatonin MT_2 receptor. *N*-Ethylmaleimide at concentrations below 300 μM decreased the affinity of 2-[^{125}I]iodomelatonin for the human melatonin MT_2 receptor. G Protein/receptor uncoupling could be a potential mechanism mediating this decrease in agonist affinity as shown in other systems (Vanhauwe et al., 2000; Witt-Enderby and Dubocovich, 1996; Ying et al., 1992). It is likely that the thiol group modified by *N*-ethylmaleimide in the human melatonin MT_2 receptor modulating the affinity for 2-[^{125}I]iodomelatonin may lie within the intracellular domain between the receptor and the G protein. In support of this hypothesis, the potency of GTPγS to inhibit 2-[^{125}I]iodomelatonin binding to the FLAG-tagged human MT_2 was less pronounced following *N*-ethylmaleimide treatment. Alkylation of the most sensitive thiol group may promote uncoupling of the FLAG-tagged human MT_2 receptor and the G protein. Similarly, it was suggested a reactive sulfhydryl group lies between melatonin receptors and G protein (Kosar et al., 1994; Ying et al., 1992). Indeed, alanine substitution of Cys^{140} in the

human MT_2 receptor partially protected against the *N*-ethylmaleimide induced decrease in 2-[^{125}I]iodomelatonin binding affinity. Consistent with this finding is the fact that Cys^{140} is located in the second intracellular loop of the human MT_2 receptor, a domain believed to be involved in receptor coupling with the G protein (Kokkola and Laitinen, 1998; Navajas et al., 1996). *N*-Ethylmaleimide modification of Cys^{140} probably creates steric hindrance resulting in receptor and G protein uncoupling. However, another reactive site besides Cys^{140} may be involved in the *N*-ethylmaleimide-induced decrease in affinity because mutation of this residue produced only partial protection against the *N*-ethylmaleimide-induced decrease in affinity. This site could be an amino acid residue in the FLAG-tagged human MT_2 that was not mutated in this study, or it could be an *N*-ethylmaleimide-reactive site located within the G protein. In fact, evidence suggests that *N*-ethylmaleimide can modify critical cysteine residues of pertussis toxin-sensitive G proteins, promoting G protein uncoupling from the receptor (Kitamura et al., 1990; Winslow et al., 1987).

N-Ethylmaleimide at concentrations above 300 μM decreased human melatonin MT_2 receptor density. Mutation of Cys^{143} or Cys^{219} in the human MT_2 receptor fully protected against the *N*-ethylmaleimide-induced decrease in receptor density (B_{max}), indicating that these residues are potential sites for *N*-ethylmaleimide alkylation. The *N*-ethylmaleimide-induced decrease in receptor density suggests that this thiol group may be located at or close to the ligand binding site. Potentially, interaction of *N*-ethylmaleimide at a site remote from the ligand binding pocket could also decrease binding due to conformational changes of the receptor. The location of Cys^{219} within the human melatonin MT_2 receptor putative structure suggests that this residue could be at or near the binding pocket. In fact, Navajas et al. (1996) proposed a model for melatonin recognition at its receptor, in which the hormone fits within a hydrophilic binding pocket formed by the seven transmembrane helices. Based on this model, several conserved amino acid residues in transmembrane five, including Cys^{219}, face towards the hydrophilic binding pocket. Although Cys^{219} is not essential for melatonin binding, *N*-ethylmaleimide-induced alkylation of Cys^{219} may decrease the number of 2 [^{125}I]iodomelatonin binding sites due to steric hindrance. However, it should be acknowledged that reaction at a site remote from the ligand binding site could lead to a loss of binding capacity due to modification-induced conformational changes of the receptor. Cys^{143} is located in the second intracellular loop far from the proposed binding pocket, therefore mutation of Cys^{143} may promote a conformational change in the human MT_2 receptor preventing alkylation of Cys^{219} by *N*-ethylmaleimide.

The function of conserved cysteines in G protein-coupled receptors depends on their location within the receptor structure. Cysteines Cys^{113} and Cys^{190} in the extracellular loops of the human melatonin MT_2 receptor appear to be essential for melatonin recognition. These cysteines are modified by dithiothreitol, suggesting the presence of a disulfide bridge that maintains a proper receptor structure. In the human melatonin MT_2 receptor, as in most G protein-coupled receptors, cysteines within the transmembrane segments (Cys^{219}, Cys^{263} and Cys^{302}) are probably located either at or near the binding pocket. The two cysteines (Cys^{140} and Cys^{143}) in the intracellular loops, however, could be in contact with the G protein coupling domain as the intracellular loops have been proposed to be involved in receptor/G protein association (Kokkola and Laitinen, 1998). Alanine substitution of cysteines residues 140, 143, 219, 263 and 302 in the FLAG-tagged human MT_2 receptor yielded mutant receptors that exhibited high affinity for 2-[^{125}I]iodomelatonin, melatonin and the melatonin receptor ligands luzindole and 4P-PDOT, and showed similar affinities compared to the wild-type receptor. This suggests that these cysteines are not essential for ligand–receptor interactions. The affinity of melatonin or luzindole to compete for 2-[^{125}I]iodomelatonin binding to the C140A mutant receptor was slightly but significantly altered, suggesting a possible effect of this residue in binding of these indole ligands to the human MT_2 receptor. Interestingly, both melatonin and luzindole also bind the human melatonin MT_1 receptor, indicating this conserved cysteine may also be an important residue in the binding pocket of the human melatonin MT_1 receptor. In contrast, the amidotetraline 4P-PDOT, which has a more rigid structure and shows higher selectivity for the melatonin MT_2 receptor (Dubocovich et al., 1997, 1998), competed for 2-[^{125}I]iodomelatonin binding with similar affinity to both mutant and wild-type receptors. These mutant human melatonin MT_2 receptors (C140A, C143A, C219A, C263A and C302A) are fully sensitive to GTPγS and therefore able to couple to G proteins. The coupling efficiency of G protein-linked receptors correlates with the ability of GTP analogues to inhibit agonist binding to native and recombinant receptor systems. This has previously been shown for melatonin receptors in chick brain (Ying et al., 1992), human melatonin MT_1 receptor expressed in CHO cells (Witt-Enderby and Dubocovich, 1996) and neurotensin NTR_1 receptors expressed in HEK cells (Martin et al., 1999). Together, our results suggest that cysteine residues probably located near or at the binding site ($Cys^{219,263,302}$) and the G protein coupling domain (Cys^{140} and Cys^{143}) are not required for either ligand binding, agonist/antagonist recognition or for proper G protein coupling to the FLAG-tagged human MT_2 receptor. Therefore, the inhibitory effect of *N*-ethylmaleimide on ligand binding to the receptor is probably due to steric hindrance of large moieties of this alkylating reagent attached to the sulfhydryl groups of the cysteine residues. This suggests some of these conserved cysteines are in regions of the receptor that can affect melatonin binding.

Taken together, our results with dithiothreitol and *N*-ethylmaleimide suggest that conserved cysteines in the human melatonin MT_1 and MT_2 receptors are involved in structure–affinity relationships. Our study extends previous

biochemical observations suggesting the involvement of sulfhydryl groups in melatonin binding as we identify cysteine residues necessary for melatonin binding (Cys^{113} and Cys^{190}) as well as cysteines regulating affinity (Cys^{140}) and binding capacity (Cys^{143}, Cys^{219}) to the human melatonin MT_2 receptor. These findings will contribute to future efforts to map the melatonin receptor binding pocket as well as for the development of drugs to treat sleep, circadian and neuroendocrine disturbances.

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