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Tight Association of the Human Mel_{1a}-Melatonin Receptor and G_i: Precoupling and Constitutive Activity

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ABSTRACT

If stably expressed in human embryonic kidney (HEK)293 cells, the human Mel_{1a}-melatonin receptor activates G_i-dependent, pertussis toxin-sensitive signaling pathways, i.e., inhibition of adenylyl cyclase and stimulation of phospholipase Cβ; the latter on condition that G_q is coactivated. The antagonist luzindole blocks the effects of melatonin and acts as an inverse agonist at the Mel_{1a} receptor in both intact cells and isolated membranes. This suggests that the Mel_{1a} receptor is endowed with constitutive activity, a finding confirmed on reconstitution of the Mel_{1a} receptor with G_i. Because the receptor density is in the physiological range, constitutive activity is not an artifact arising from overexpression of the receptor. In addition, the following findings indicate that the Mel_{1a} receptor forms a very tight complex with G_i which can be observed both in the presence and absence of an agonist. 1) In intact cells and in membranes, high-affinity agonist binding is resistant to the destabilizing effect of guanine nucleotides. 2) The ability to bind an agonist with high affinity is preserved even after exposure of the cells to pertussis toxin, because a fraction of G_i is inaccessible to the toxin in cells expressing Mel_{1a} receptors (but not the A_1-adenosine receptor, another G_i-coupled receptor). 3) An antiserum directed against the Mel_{1a} receptor coprecipitates G_i even in the absence of an agonist. We therefore conclude that the Mel_{1a} receptor is tightly precoupled and that its constitutive activity may play a role in pacing the biological clock, an action known to involve the melatonin receptors in the suprachiasmatic nucleus.

Melatonin, the *N*-acetyl-5-methoxy metabolite of serotonin, is a hormone known for its ability to adjust the circadian clock and to induce seasonal changes in reproductive physiology (in animals with breeding seasons). Regulation of circadian physiology by melatonin is believed to be mediated primarily by a specific receptor, the melatonin$_{1a}$ (Mel_{1a}) receptor, which is enriched in hypothalamic nuclei and in the pars tuberalis of the adenohypophyseal gland. In addition, the effect of melatonin on the circadian clock involves a second receptor that, as opposed to the Mel_{1a} receptor, has a very low expression level and does not mediate inhibition of neuronal activity in the nucleus suprachiasmaticus, the site of the master clock (Liu et al., 1997). Both the Mel_{1a} receptor and the second, unidentified receptor are G protein-coupled receptors. The small family of melatonin receptors comprises only three members, two of them, the Mel_{1a}- and Mel_{1b} receptors, are present in mammals. The melatonin receptors share little sequence homology with other receptor types, hence, they seem to have emerged early in the course of receptor evolution (for a review, see Dubocovich, 1995).

The pharmacological identification of melatonin receptors has relied largely on the labeling of receptors in brain slices with the agonist radioligand 2-[^{125}I]iodomelatonin, which is endowed with high affinity and a low level of nonspecific binding. Because of the highly discrete expression of melatonin receptors, the signaling pathways became amenable to detailed characterization only after cloning and heterologous expression of the recombinant receptor. Signaling by melatonin is sensitive to pertussis toxin (PTX) (White et al., 1987; Carlson et al., 1989) and in accordance with these findings, the recombinant Mel_{1a} receptor couples to G proteins of the G_i/G_o class, leading to inhibition of adenylyl cyclase (Witt-Enderby and Dubocovich, 1996). Additional signaling pathways activated by the Mel_{1a} receptor (arachidonic acid release, Ca^{2+}-release) were similarly suppressed by PTX, confirming the assumption that the receptor interacts predominantly with G_i (Godson and Reppert, 1997).

The ligand for the Mel_{1a} receptor, melatonin, is produced in and secreted by the pineal gland. A regular phenomenon in vertebrate physiology, melatonin levels are elevated during

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ABBREVIATIONS: PTX, pertussis toxin; OG, octylglucoside; iodomelatonin, 2-[^{125}I]iodomelatonin; HEK, human embryonic kidney; TCA, trichloroacetic acid; PCA, perchloroacetic acid; IP, inositol phosphate; NAD, nicotinamide adenine dinucleotide; R/G, receptor/G protein.

the night. The biochemical basis for the circadian changes in melatonin levels was found in the variable activity of serotonin *N*-acetyltransferase, which catalyzes melatonin production (Gastel et al., 1998). Daylight reduces the stability of the enzyme toward proteasomal degradation. Not only the melatonin production but also the melatonin receptors are regulated in a diurnal rhythm (Tenn and Niles, 1993; Gauer et al., 1994; Neu and Niles, 1997); during the day the level of iodomelatonin binding to membranes from hypothalamic nuclei increases and drops again in the evening, before the hormone level rises. Thus, the melatonin receptor-expressing cell adapts to the day-night rhythm in a fashion that is inversely related to the ambient melatonin levels. Because the melatonin receptor is considered a potential drug target, variations in the expression level may cause therapeutically relevant changes in the response to administered melatonin receptor ligands. We have therefore generated a stable human embryonic kidney (HEK)293 cell line in which the receptor is expressed to a density that presumably occurs in neuronal cells, given the cellular heterogeneity of brain tissue preparations and the distinct distribution of Mel_{1a} receptors (several hundred femtomoles per milligram of membrane protein, Barrett et al., 1996). We report that if expressed in HEK293 cells, the Mel_{1a} receptor reveals a marked degree of spontaneous activity. In addition, the Mel_{1a} receptor exhibits features that are not predicted by the classical model of receptor/G protein (R/G) coupling. For example, whereas signal transduction of the Mel_{1a} receptor is strongly reduced by PTX, the receptor retains the ability to bind an agonist ligand with high affinity after PTX treatment. We have examined this phenotype and find that the receptor forms a highly stable complex with G_i, such that the G protein α subunit is shielded from PTX. The mode of Mel_{1a} R/G coupling also results in a high basal activity of the receptor, i.e., the receptor is spontaneously active in the absence of agonists.

Experimental Procedures

Materials. [^{35}S]GTPγS (guanosine 5′-(3-*O*-thio)triphosphate) and [^{125}I]iodomelatonin were purchased from NEN (Boston, MA). (−)*N*^{6}-3[^{125}I](iodo-4-hydroxyphenyl-isopropyl) adenosine was synthesized according to Linden (1984). Guanine nucleotides, adenosine deaminase, protein A, and protein G Sepharose were from Boehringer Mannheim (Mannheim, Germany). 1-*O*-n-octyl-D-glucopyranoside (octylglucoside, OG), CHAPS (3-[3-cholaminpropyl) dimethyl-ammonio]-1-propane-sulfonic acid) and HEPES were purchased from Biomol (Munich, Germany); suramin was obtained from Research Biochemicals (Natick, MA). The materials required for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA). Fetal calf serum was purchased from PAA Laboratories (Linz, Austria), Dulbecco's modified Eagle medium (DMEM), nonessential amino acids, β-mercaptoethanol, and G418 (geneticin) were obtained from Life Technologies (Grand Island, NY). Melatonin and luzindole were purchased from Tocris (Langford, Bristol, UK). N6-cyclopentyladenosine, PTX, L-glutamine, penicillin G, and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Buffers and salts were purchased from Merck (Darmstadt, Germany).

Affinity-purified polyclonal antibodies directed against peptides derived from the carboxy-terminal domains of the α subunits of G_i(1–3) and $G_{q/11}$ were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies directed against epitopes in the amino terminus and carboxy terminus of G_o as well as a G_i-selective antiserum were a generous gift from Dr. G. Milligan, University of Glasgow (Glasgow, UK). Antiserum-536 directed against the extreme C terminus of the human Mel_{1a} receptor peptide was produced as will be described in L.B., F.R., L. Petit, P. de Coppet, M. Tissot, P. Barrett, P. J. Morgan, C.N., A.D.S., and R.J. (submitted for publication).

Stable Cell Lines. Cloning of the human melatonin type 1a (Mel_{1a}) receptor cDNA using polymerase chain reaction primers selected from the reported Mel_{1a} receptor sequence and generation of stable cell lines expressing the Mel_{1a} receptor will be described in detail elsewhere. In brief, human brain mRNA was obtained through polymerase chain reaction amplification, the coding sequence was cloned into the expression vector pcDNA3 (Invitrogen, Carlsbad, CA), and confirmed by DNA sequencing. For stable receptor expression, HEK293 cells were transfected with the human Mel_{1a} receptor cDNA together with a plasmid carrying the geneticin resistance gene by liposome-mediated transfection. Clones were selected in DMEM supplemented with 10% fetal calf serum and 800 μg/ml of geneticin (G418) and screened for [^{125}I]iodomelatonin binding. For the generation of HEK293 cells expressing the human A_1-adenosine receptor see Waldhoer et al. (1998). Transfected HEK293 cells were grown in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, β-mercaptoethanol, nonessential amino acids, 100 U/ml penicillin G, and 100 μg/ml streptomycin and 0.2 mg/ml geneticin at 5% CO_2 and 37°C.

Membrane Preparation and Protein Purification. Cells were grown to confluence in 10-cm tissue culture dishes, rinsed once with ice-cold PBS, and scraped off their plastic support. After sedimentation, the cell pellet was resuspended in HME (25 mM HEPES-NaOH, pH 7.5, 2 mM $MgCl_2$, and 1 mM EDTA) and subsequently deep frozen in liquid nitrogen. Cells were thawed and disrupted by sonication. Membranes were sedimented by centrifugation at 38,000*g* for 10 min, were resuspended in HME at a protein concentration of 8 to 10 mg/ml and stored in aliquots at −80°C. For experiments in which contaminating nuclear matter had to be discarded, membranes were enriched by differential centrifugation; the supernatant obtained by centrifuging the homogenate at 9,000*g*, was sedimented at 50,000*g*.

Recombinant G_αi-1 and rG_αi-3 were produced in *Escherichia coli* and purified from bacterial lysates as described in Mumby and Linder (1994). G protein oligomers were purified from porcine brain and free $\beta\gamma$ dimers were chromatographically resolved from the α subunits as in Casey et al. (1989) with minor modifications; cholic acid was replaced by the zwitterionic detergent CHAPS and phenylsepharose was used instead of the heptylamine matrix for hydrophobic interaction chromatography (Nanoff et al., 1997).

Radioligand Binding Experiments. Equilibrium binding with [^{125}I]iodomelatonin was carried out in a final volume of 25 to 100 μl containing: 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM $MgCl_2$, and 5 to 10 μg membrane protein. The binding reaction was carried out for 60 min at 30°C and terminated by filtration over glass fiber filters using a cell harvester (Skatron, Lier, Norway). [^{125}I]Iodomelatonin binding to intact cells was carried out on cells that were detached from their growth support and resuspended in assay tubes. The binding reaction proceeded in DMEM for 90 min at 30°C. Specific [^{125}I]iodomelatonin binding was not detectable on nontransfected HEK293 cells and on HEK293 cells transfected with the A_1-adenosine receptor. Nonspecific binding was determined in the presence of 100 μM luzindole and typically amounted to less than 10% of total binding.

Mel_{1a} receptor-promoted G protein activation was determined by measuring the association rate of [^{35}S]GTPγS to Mel_{1a} receptor-expressing membranes. To suppress the spontaneous guanine nucleotide exchange, the following assay conditions were chosen. Membranes (~10 μg) were suspended in an assay volume of 30 μl buffer containing: 25 mM HEPES-NaOH (pH 7.5), 1.0 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, and 0.01 mM GDP. Following a preincubation of membranes in the presence or absence of receptor ligand (melatonin at 100 nM, luzindole at 100 μM) for 10 min at 25°C, the assay was initiated by adding [^{35}S]GTPγS to yield a final concentration of ~3

nM (specific activity = 2400 cpm/fmol). After the indicated reaction times, 0.9 ml of ice-cold stop buffer containing: 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20 mM $MgCl_2$, and 0.1 mM GTP was added. Bound and free radioactivity were separated by filtration over glass fiber filters.

Uncoupling of Mel_{1a} Receptor by G Protein-Selective Antibodies and Suramin. Membranes were prepared from HEK293 cells that had or had not been pretreated with PTX (16 h, 0.1 μg/ml), and from cells that were transfected with a $G_\alpha q$ cDNA in a pCIS plasmid (kindly provided by Dr. M. Simon, California Institute of Technology, Pasadena, CA) that drives the overexpression of $G_\alpha q$. Membranes (~10 μg protein/assay or ~25 μg protein/assay if the membranes had been prepared from PTX-treated cells) were preincubated with the indicated amounts of antibody in the presence of 0.2% OG in a volume of 20 μl for 20 min on ice. The binding reaction was started by adding [^{125}I]iodomelatonin (300 pM) to give a final OG concentration of <0.05% and was terminated as described above. The nonspecific effect of adding immune globulin was controlled for by performing the binding experiment in parallel with nonimmune IgG.

Inhibition of agonist radioligand binding through inhibition of R/G interaction by suramin was assessed as described in Waldhoer et al. (1998). To rule out that suramin (which virtually does not permeate into cells) interfered with ligand binding by combining with the binding pocket of the Mel_{1a} receptor, we evaluated the effect of suramin on iodomelatonin binding to intact cells; 100 μM suramin did not affect the affinity for iodomelatonin or the number of labeled receptors. Inhibition of iodomelatonin binding to isolated membranes by suramin then was evaluated at various radioligand concentrations. The incubation volume varied between 25 and 200 μl (for high and low radioligand concentrations, respectively) comprising between 10 and 40 μg membrane protein; membranes were used from untreated cells with or without the addition of detergent (at 0.1% OG) or from PTX-treated cells. At each radioligand concentration, suramin inhibition curves were established. The binding data were fitted to a monophasic displacement curve using nonlinear regression; IC_{50} estimates were replotted versus the relative receptor occupancy obtained at each radioligand concentration (specific binding measured in the absence of suramin relative to the B_{max} of the membrane preparation). An increase in the relative receptor occupancy increases the IC_{50} according to the equation: $IC_{50} = K_i/K_A \times A + K_i$ where K_i denotes the dissociation constant of suramin binding to the G protein, A indicates the number of activated receptors in the membrane, and K_A describes the coupling affinity of the active, agonist-liganded receptor for the G protein (Freissmuth et al., 1999).

Determination of cAMP Formation and Inositol Phosphates (IP) Accumulation in Intact Cells. HEK293 cells expressing the Mel_{1a} receptor were grown to confluence in 40-mm culture dishes in DMEM containing 10% fetal calf serum. The cellular ATP pool was labeled by incubating the cells with 2.5 μCi/ml of [^{3}H]adenine for 16 h. After removing free [^{3}H]adenine and rinsing, cells were incubated with DMEM containing rolipram (10 μM) and the assay was started by adding receptor ligands in the absence or presence of forskolin (20 μM). The reaction was stopped after 10 min by aspirating the assay medium and adding 800 μl of 2% perchloric acid with 100 μM cAMP. After neutralizing the cell extract with 80 μl of 4 M KOH, [^{3}H]cAMP was separated from ATP by sequential chromatography on Dowex AG 1X-4W and Alumina columns (Johnson and Solomon, 1991). To monitor the recovery of [^{3}H]cAMP, an internal [^{32}P]cAMP standard was prepared using recombinant adenylyl cyclase consisting of the two, separately expressed catalytic domains of the type I and type II isoforms (Mitterauer et al., 1998).

Cells were grown in 40-mm dishes and labeled for 16 h with [^{3}H]inositol (2.5 μCi/ml) in medium low in inositol (medium 199; Life Technologies). After medium exchange, cells were preincubated with 10 mM LiCl for 10 min. The assay was initiated by adding receptor ligands and carried out for 20 min. After aspirating the assay medium, cells were lysed by adding trichloroacetic acid (TCA; 5%) and the supernatant was transferred to polypropylene tubes. TCA was removed with water saturated diethyl ether; the remaining sample was diluted in 100 mM Tris, pH 8.5 and applied to ion-exchange chromatography on 0.6 ml Dowex AG 1X-8 columns. Inositol, glycerophosphoinositol, and IP were separated as described previously (Nanoff et al., 1990); IP1, which accounted for more than ~90% of the radiolabeled IPs, was eluted using 0.18 M ammoniumformiate in 0.1 M formic acid.

Reconstitution of Mel_{1a} Receptor with Purified G_i To probe the interaction of the Mel_{1a} receptor with exogenous G protein of the G_i class, membranes were washed with 6 M urea. Two milligrams of membrane protein was taken up in 10 ml 6 M urea/50 mM HEPES (pH 7.5), and after 15 min at 4°C the suspension was centrifuged at 50,000*g*. The resulting pellet was washed and resuspended in HME. In urea-washed membranes that were stripped off peripheral membrane proteins, ~80% of the iodomelatonin binding was lost. Interaction of the Mel_{1a} receptor with G_i was tested by measuring iodomelatonin binding and the receptor catalyzed G protein activation. To restore high-affinity iodomelatonin binding, recombinant $G_\alpha i$-3 and purified porcine $\beta\gamma$-dimer at the indicated concentrations were preincubated with urea-washed membranes (~3 μg membrane protein) in the presence of 0.2% OG for 20 min on ice. The binding assay was initiated by adding radioligand and by diluting the preincubation mix about 4-fold. To determine G protein activation by [^{35}S]GTPγS binding, preincubation of membranes with G_{i-1} in the presence of 0.2% OG on ice was followed by adding receptor ligands in reaction buffer. Ligands were allowed to bind for 15 min at 25°C before the reaction was carried out as described above. To control for the loss of receptor due to urea treatment we also subjected membranes carrying the A_1-adenosine receptor to urea treatment.

ADP ribosylation of α_i (10 μg) was performed with preactivated PTX (50 ng, see below) and an equimolar amount of purified brain $\beta\gamma$ as described by Carty (1994). The reaction was allowed to proceed for 1 h at 25°C in a volume of 5 μl including 50 mM HEPES-NaOH, pH 8.0, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1% Lubrol (Boehringer-Ingelheim, Heidelberg, Germany), 10 mM thymidine, 10 μM GDP, 0.4 mg *N*-(2-chloropropyl)-*N*,*N*-dimethyl-ammoniumchloride, and 0.1 mM nicotinamide adenine dinucleotide (NAD). The reaction was followed by sequential dilution with HME plus 8 mM CHAPS and by concentration over a YM 30 membrane (Amicon, Beverly, MA), resulting in a 100-fold dilution of the reaction buffer ingredients. To control for protein loss, an equal amount of α_i was processed in the same manner without PTX (sham treatment). Urea-washed membranes (10 μg) were reconstituted with a final concentration of ~0.1 μM PTX- or sham-treated α_i, and binding of [^{125}I]iodomelatonin (300 pM) was performed at a final concentration of 1 mM CHAPS.

[^{32}P]ADP Ribosylation of G_i and subsequent Immunoprecipitation Using a $G_\alpha i$-Selective Antibody. Membranes (2 mg membrane protein) from native and PTX-pretreated HEK293 cells were solubilized with 10 mM CHAPS in Tris-HCl 50 mM, pH 8.0, and EDTA 1 mM (protein/detergent ratio = 1:4) by stirring on ice for 1 h. The soluble supernatant was collected by centrifugation (75,000*g* for 15 min) and the volume was reduced over a YM 30 membrane (Amicon) to a protein concentration of ~2.0 mg/ml. PTX was dissolved in preactivation buffer (100 mM DTT, 0.8 M urea, 5 mM CHAPS, and 50 mM potassium phosphate, pH 8.0) and was activated for 25 min at 30°C. One milligram of the soluble membrane extract was subjected to ADP ribosylation by preactivated PTX (5 μg). The reaction was carried out for 1 h at 25°C in a volume of 0.8 ml including 37.5 mM Tris-HCl, 45 mM NaCl, 0.5 mM $MgCl_2$, 5 mM DTT, 10 μM GDP, 0.4 mg *N*-(2-chloropropyl)-*N*,*N*-dimethyl-ammoniumcholoride, 10 mM thymidine, 0.1 mM NAD, and 450 nCi [^{32}P]NAD at a specific activity 5.6 pCi/nmol. Proteins were precipitated by adding TCA to a concentration of 10%. The protein pellet was rinsed with acetone, dried under a stream of nitrogen, and resuspended in 250 μl RIPA buffer (50 mM Tris-HCl, pH 8.3, 1%

Nonidet P-40, 5 mM EDTA, and 150 mM NaCl). The resuspended protein pellet was incubated with 2.5 μg of an antibody directed against the carboxy terminus of $G_\alpha i1$–3 for 6 h at 4°C. Antibodies were precipitated by the addition of a 1:1 mixture of protein A/protein G-Sepharose (10% of the final volume) for 2 h at 4°C. After centrifugation, the Sepharose was washed four times with RIPA at 0.15% Nonidet P-40. Bound proteins were dissolved by boiling in 60 μl SDS-sample buffer and the entire volume was subjected to gel electrophoresis.

Immunoprecipitation of Mel_{1a} Receptor. HEK293 membranes expressing the Mel_{1a} receptor were solubilized using 1% digitonin in 75 mM Tris-HCl (pH 7.4), 12 mM $MgCl_2$, and 2 mM EDTA and protease inhibitors (5 mg/ml soybean trypsin inhibitor, 5 mg/ml leupeptin, and 10 mg/ml benzamidine). The detergent/protein ratio was 1:1; the mixture was stirred for 3 h at 4°C and centrifuged at 50,000*g*. For immune precipitation, the digitonin concentration was adjusted to 0.2% and antiserum-536 was added so that the serum made up 1/40 of the reaction volume. Immune complex formation was allowed to proceed for 18 h at 4°C with gentle agitation and during the last 6 h protein A-agarose (10%) was included. The agarose beads were sedimented by centrifugation at 5000*g* and washed five times with ice-cold buffer including 0.2% digitonin. Bound proteins were resuspended in 0.2% digitonin buffer and incubated with 50 μM guanosine-5′-(β,γ-imido)triphosphate [Gpp(NH)p] for 1 h at 37°C. The supernatant was added to SDS-sample buffer, boiled, and separated by gel electrophoresis.

Immunoblots. Membrane proteins (25 μg/lane) were separated on SDS-polyacrylamide gels (12% acrylamide and 0.16% bisacrylamide) and transferred to nitrocellulose membranes that were probed with antisera recognizing $G_\alpha i$ or a purified antibody recognizing $G_\alpha q$ (Santa Cruz Biotechnology). The immunostained bands were visualized by enhanced chemiluminescence using an anti-rabbit-IgG antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL).

Results

High-Affinity [^{125}I]Iodomelatonin Binding to Mel_{1a} Receptor on Intact Cells. When the human Mel_{1a} receptor was stably expressed in HEK293 cells and intact cells were subjected to equilibrium binding, the agonist radioligand iodomelatonin bound in a saturable manner and with high affinity (Fig. 1A); in nontransfected cells no specific binding (or cellular uptake) of iodomelatonin was detected. In membranes prepared from Mel_{1a} receptor-expressing cells (Mel_{1a} membranes), iodomelatonin bound with similar affinity (Fig. 1B). The total number of Mel_{1a} receptors detected on the surface of intact cells was about twice the number of receptors retained in isolated membranes. This is presumably due to losses during membrane preparation; furthermore, the data show that the density of receptors in the high-affinity conformation on intact cells was not less than their density in membranes. In addition, Fig. 1A depicts a saturation isotherm obtained on intact cells and in the presence of suramin (0.3 mM); suramin acts as a G protein antagonist (see Freissmuth et al., 1999), but because of its six negative charges, it is virtually membrane impermeable. Iodomelatonin binding to intact cells was not affected by suramin, indicating that the compound did not block the ligand binding site of the Mel_{1a} receptor. Thus, suramin could be used to inhibit R/G coupling in cell membrane preparations (see below; Fig. 2).

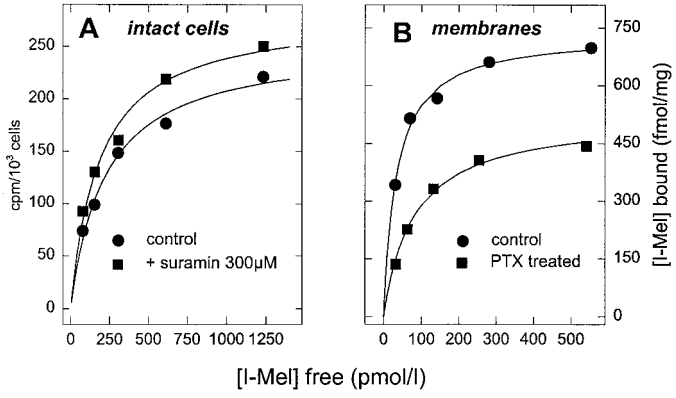

Fig. 1. Iodomelatonin binding to isolated membranes from HEK293 cells stably expressing the Mel_{1a} receptor and binding to intact cells. A, iodomelatonin binding was performed to intact cells in suspension in DMEM in the absence (●) and presence (■) of 0.3 mM suramin for 1 h at 25°C. The K_D value was estimated to be 89 ± 11 pM (n = 3). B, specific iodomelatonin binding to membranes from control (●) and PTX-treated (■) cells. B_{max} and K_D estimates were 732 ± 38 fmol/mg and 56 ± 9 pM for untreated and 526 ± 112 fmol/mg and 93 ± 17 pM for PTX-treated cells (n = 5).

Because the Mel_{1a} receptor is known to transduce signals via G proteins of the $G_{i/o}$ class, we incubated cells with PTX; membranes derived from PTX-treated cells still displayed high- affinity binding and the number of receptors amounted to about two thirds of that determined in untreated cells (Fig. 1B). These findings do not fit the widely accepted model of how receptors and G proteins interact; the high-affinity conformation of the receptor is thought to be transient in the presence of guanine nucleotides, and PTX typically eliminates the ability of G_i-coupled receptors to bind an agonist with high affinity. Thus, on intact cells and in PTX-treated membranes, iodomelatonin binding is not expected to occur.

Affinity of Mel_{1a} Receptor for Its G Protein. It is, alternatively, conceivable that the uncoupled, G protein-free form of the receptor can per se bind iodomelatonin with high affinity. Therefore, we probed the R/G interaction. Guanine nucleotide-dependent modulation of agonist binding is a sensitive index for functional R/G coupling. In membranes, guanine nucleotides apparently failed to destabilize iodomelatonin binding. However, the inclusion of detergent in the binding assay unmasked the effect of guanine nucleotides (Fig. 2A). In the presence of detergent (at "lubricant" concentrations below the critical micellar concentration ≤ 4 mM CHAPS or 0.1% OG), about 60% of the receptor population was destabilized by guanine nucleotides; GTPγS and GTP were as potent and effective as GDP in inhibiting the formation of the high-affinity complex (data not shown). The presence of low detergent concentrations did not significantly affect iodomelatonin binding affinity nor the number of labeled receptors; at higher detergent concentrations the binding was almost completely suppressed by guanine nucleotides, but the number of labeled receptors decreased (not shown). Similarly, in membranes from PTX-treated cells, guanine nucleotides suppressed iodomelatonin binding if detergent was present at low concentrations (data not shown).

The G protein antagonist suramin has been employed as an alternative to guanine nucleotides to uncouple R/G complexes in isolated membranes. Suramin blocks binding of the activated receptor to the G protein interface and has been demonstrated to be very effective in uncoupling G_i-coupled receptors (Beindl et al., 1996; Waldhoer et al. 1998). Figure 2B shows that suramin inhibited iodomelatonin binding in a dose-dependent fashion but the concentrations required (IC_{50} >10 μM) were higher when compared with those nec-

essary for uncoupling the D_2-dopamine receptor, another G_i-coupled receptor (IC_{50} ~0.2 μM; Waldhoer et al., 1998). The low potency of suramin suggested that the activated receptor had a high affinity for G_i. The affinity of the Mel_{1a} receptor for the G protein in the membranes can be estimated by determining the potency of suramin at different levels of receptor occupancy (Fig. 2B). The inhibition curves shifted to higher values as the iodomelatonin concentration increased. This was not due to suramin interfering with the ligand binding site on the receptor, because suramin did not reduce iodomelatonin binding to intact cells (see Fig. 1A); rather, the shift in suramin dose-response relationships was caused by an increase in receptor occupancy, because the concentration of active receptor in the membrane antagonized the effect of suramin. Figure 2C illustrates that the relationship between receptor occupancy and the suramin IC_{50} value is linear (within the range of experimental error). In this plot, the slope of the regression line allows an estimate of the coupling affinity as discussed in detail elsewhere (Freissmuth et al., 1999). The affinity estimate derived for the Mel_{1a} receptor was about 10 nM, thus it was in the same range as that observed for the human A_1-adenosine receptor (Waldhoer et al., 1998). If detergent was included, uncoupling by suramin occurred at lower concentrations and the slope became smaller (Fig. 2C). This confirmed that the stability of the ternary complex of the Mel_{1a} receptor is high and that the addition of detergent reduces the stability. When PTX-treated membranes were used, suramin inhibited iodomelatonin binding with higher potency than in untreated membranes; this gave a smaller slope of the suramin-regression line (Fig. 2C). The apparent potency of suramin depends on the concentration of the reaction partners that form the ternary complex, i.e., agonist, activated receptor, and G protein heterotrimer (see Freissmuth et al., 1999). The increased potency of suramin in PTX-treated membranes can therefore be accounted for by the drastic decrease in the number of available G protein moieties, which becomes limiting to the rate of complex formation, hence to complex stability. An alternative explanation is that the Mel_{1a} receptor may also combine, albeit with lower affinity, with PTX-resistant G protein subtypes and that this interaction is also inhibited by suramin.

Signaling via Mel_{1a} Receptor Is PTX Sensitive. The ability of guanine nucleotides and of suramin to decrease iodomelatonin binding indicated that high-affinity agonist binding was indeed due to the formation of a ternary complex with the Mel_{1a} receptor; this ability was retained in membranes pretreated with PTX. We therefore tested whether the Mel_{1a} receptor would interact with G protein subtypes other than G_i. Figure 3A demonstrates that melatonin accelerated the guanine nucleotide exchange reaction (association of [^{35}S]GTPγS) in isolated Mel_{1a}-membranes, and that addition of detergent enhanced the receptor-dependent association rate (Fig. 3A). PTX abolished agonist-induced G protein activation. To detect a receptor-mediated increase in G protein activation, PTX-treated membranes had to be reconstituted with G_i (not shown).

In intact cells, PTX reduced the melatonin-dependent regulation of second messenger production. Figure 3B shows that melatonin (100 nM) largely inhibited forskolin-stimulated cAMP formation and that in unstimulated cells melatonin induced a modest increase in cAMP formation. The latter effect may be due to $\beta\gamma$-mediated activation of type II/IV adenylyl cyclase, which can occur even in the absence of activated $G_\alpha s$ (Zimmermann and Taussig, 1996). Melatonin also increased the accumulation of IPs if phospholipase C was coactivated via the P_{2Y} receptor endogenously expressed in HEK293 cells (Schachter et al., 1997). All these actions of melatonin were eliminated after treating the cells with PTX. This effect is consistent with coupling of the Mel_{1a} receptor to G_i but goes against the alternative hypothesis that the Mel_{1a}

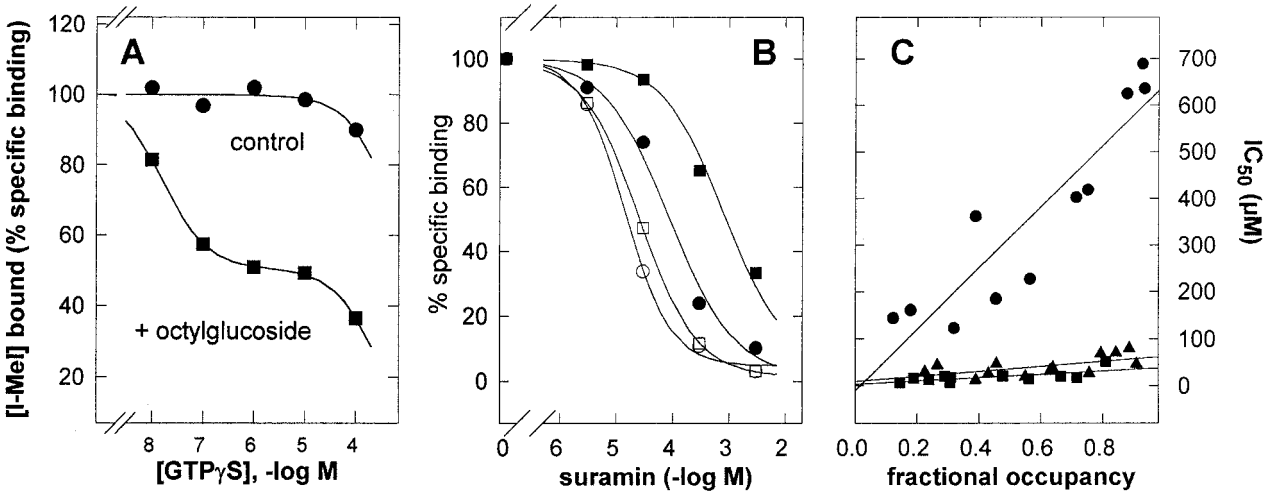

Fig. 2. Destabilization of iodomelatonin binding to the Mel_{1a} receptor by guanine nucleotides and by the G protein antagonist suramin. A, binding of iodomelatonin (300 pM) to Mel_{1a} membranes was carried out at 30°C for 60 min in the presence of GTPγS at the indicated concentrations with (■) or without (●) the addition of detergent (OG, 0.1%). B, inhibition of iodomelatonin binding to the Mel_{1a} receptor by increasing concentrations of suramin was evaluated at various degrees of receptor occupancy (75 and 99%) attained at different iodomelatonin concentrations (○, ●, 100 pM; ■, □, 500 pM). Binding data were obtained in the presence (open symbols) or absence (closed symbols) of 0.1% OG and are given in percentage of the binding in the absence of suramin. IC_{50} values were estimated by nonlinear curve fitting. C, IC_{50} estimates were obtained from individual suramin concentration-response curves as in B and were replotted versus the relative receptor occupancy. Curves were generated in native membranes (●), in native membranes resuspended in 0.1% OG (■) and in PTX-treated membranes (▲). Membranes were labeled with different concentrations of iodomelatonin (range, ~50–700 pM) in the presence of increasing concentrations of suramin, and from the inhibition curve, IC_{50} values were estimated.

receptor couples to PTX-resistant members of the $G_{q/11}$ or of the $G_{i/o}$ family (G_z), as has been suggested (Yung et al., 1995).

Specificity of G Protein Subtypes Forming a Ternary Complex with Mel_{1a} Receptor. We then tested whether the Mel_{1a} receptor engages exclusively G_i when it binds agonists with high affinity and whether it does so even after PTX treatment. We used antibodies selective for the carboxy termini of G protein α subunits to destabilize high-affinity iodomelatonin binding in membranes from untreated and PTX-treated cells (Fig. 4, A and B). The carboxy terminus of the α subunit is a site contacted by the receptor and is, in part, responsible for the selective recognition of G proteins by individual receptors (Conklin et al., 1996). The antibodies employed were directed against the carboxy terminal dekapeptides of α_i1–3, α_q, and α_o. Uncoupling of the Mel_{1a} receptor required that the antibody be preincubated with membranes in the presence of detergent; if the antibody was added in the absence of detergent a specific inhibitory effect on iodomelatonin binding was not found (data not shown). This observation is consistent with the interpretation that the interaction between receptor and G protein is tight. Figure 4, A and B show the concentration-dependent decrease in iodomelatonin binding by the antibody selective for the carboxy terminus G_αi (1–3), which inhibited agonist binding with equivalent potency and efficacy in native and in PTX-treated membranes; the effects of other antibodies were not significant except for the G_αq antibody if applied to membranes prepared from cells over-expressing G_αq (~5- to 10-fold higher α_q level than in vector-transfected controls; not shown); here, the G_αq-selective antibody was as effective as the G_αi-antibody in reducing iodomelatonin binding. Whereas overexpression of α_q led to the interaction of G_q with the Mel_{1a} receptor, it did not cause an increment in the number of receptors labeled by iodomelatonin. This suggests that there are no spare receptors but that all of the receptors on the cell surface are capable of recruiting G proteins.

Precoupling of Unliganded Receptor. Although the Mel_{1a} receptor can interact with other G protein subtypes like G_q, this coupling is of minor importance in the formation of a high-affinity ternary complex, because the bulk of ternary complexes is sensitive to disruption by the α_i-selective antibody both in control membranes and in membranes prepared from PTX-treated cells. This finding favors the interpretation that the Mel_{1a} receptor coupled to ADP-ribosylated G_i; however, this is rather unlikely. The alternative interpretation is based on previous observations that indicated that the interaction of an activated receptor with a G protein of the $G_{i/o}$ class renders the α subunit unavailable to modification by PTX (Tsai et al., 1984). If the unliganded Mel_{1a} receptor was spontaneously active, this would result in precoupling of the receptor to G_i (in the absence of a receptor agonist) and hence give rise to a sustained Mel_{1a} R/G complex in which G_i is unavailable to PTX-mediated ADP ribosylation.

To search for evidence for constitutive activity of the Mel_{1a} receptor, we first assessed the effect of the receptor antagonist luzindole on cAMP accumulation in Mel_{1a} receptor-expressing HEK293 cells. In the presence of forskolin, luzindole increased cAMP levels by 50% (from 4944 ± 1490 to 6786 ± 1950 cpm, means ± S.D., $n = 3$ and $p < .05$ by paired Student's t test) during a 20-min accumulation period. In control cells that were not transfected with the Mel_{1a} receptor, luzindole had no effect on basal or forskolin-stimulated cAMP accumulation. The luzindole effect might have resulted from displacing prebound melatonin or from restraining the constitutive activity of the Mel_{1a} receptor. Therefore, plasma membranes were prepared and washed thoroughly and the effect of luzindole was assessed on the basal associ-

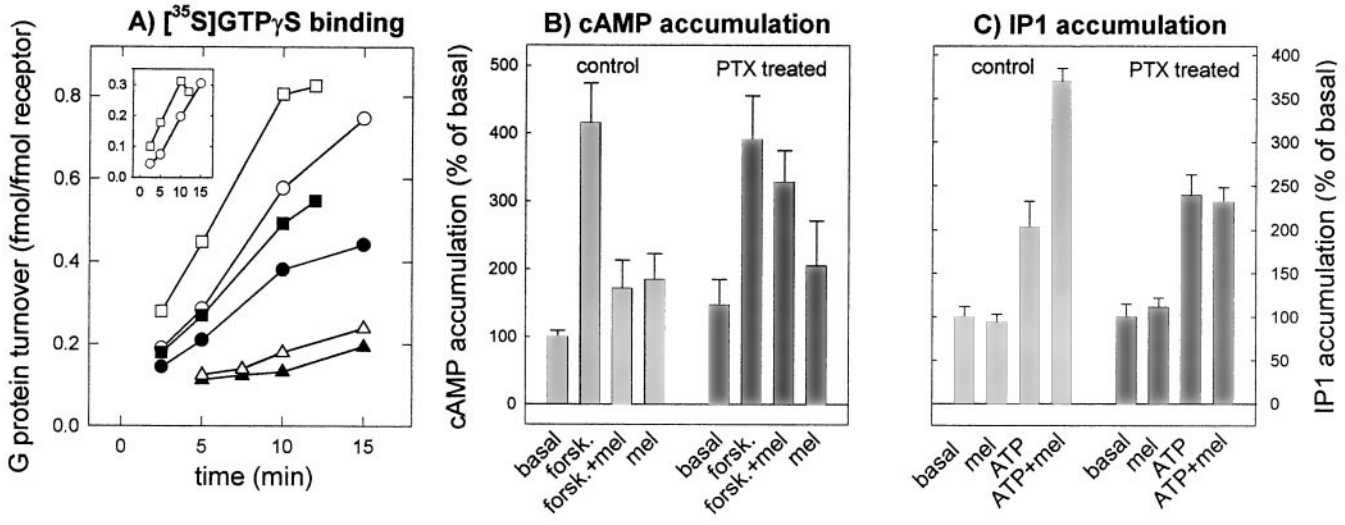

Fig. 3. G protein activation and effector regulation by the Mel_{1a} receptor stably expressed in HEK293 cells. A, G protein activation in Mel_{1a} membranes before (circles and squares) and after PTX treatment (triangles). The [^{35}S]GTPγS association rate was assessed in the presence (open symbols) and absence (full symbols) of 0.1 μM melatonin; y-axis values were obtained by normalizing GTPγS binding data to the B_{max} values from iodomelatonin saturation isotherms in PTX-treated and untreated membranes. In native membranes [^{35}S]GTPγS binding was determined with (■) and without (●) the addition of 0.1% OG; the inset shows the agonist promoted increment in [^{35}S]GTPγS binding over basal. The experiment shown is representative of three performed. B, [^{3}H]cAMP accumulation was measured in native and PTX-treated cells incubated with rolipram in the presence of the indicated agents (forsk, forskolin 10 μM; mel, melatonin 0.1 μM) for 10 min. [^{3}H]cAMP accumulation is expressed as percentage of basal accumulation, which amounted to 1300 ± 176 cpm. C, accumulation of [^{3}H]IP1 was determined in the presence of LiCl (10 mM) in native and PTX-treated cells; the indicated substances (melatonin 0.1 μM, ATP 30 μM) were added for 30 min. [^{3}H]IP1 accumulation is given in percentage of basal [^{3}H]IP1 accumulation in untreated cells (923 ± 239 cpm). In B and C data are means ± S.E. of at least five individual experiments.

ation rate of GTPγS. Luzindole inhibited GTPγS binding to Mel_{1a} membranes (but not in control membranes), indicating that G protein activation is driven by the unoccupied Mel_{1a} receptor (Fig. 5A).

Constitutive activity was also demonstrated by reconstituting purified G_i with the Mel_{1a} receptor in membranes that had been stripped off peripheral membrane proteins (including heterotrimeric G proteins) by repeated washes in urea. Addition of increasing amounts of G_{i-1} to urea stripped membranes increased binding of [^{35}S]GTPγS (Fig. 5B). Both in the absence and presence of melatonin dose-response curves for G_{i-1} were not linear but hyperbolic and saturable. The apparent affinity of G_{i-1} for the unoccupied Mel_{1a} receptor was estimated to be ~50 nM and the affinity did not increase in the presence of melatonin. A linear relationship between the amount of G_{i-1} added and the binding of GTPγS was observed only after the urea-stripped membranes had first been denatured at 80°C. Accordingly, the time course shown in Fig. 5C revealed that the association rate for binding of GTPγS was accelerated in urea-stripped membranes when compared with boiled membranes. Taken together, these data demonstrate that the Mel_{1a} receptor is spontaneously active.

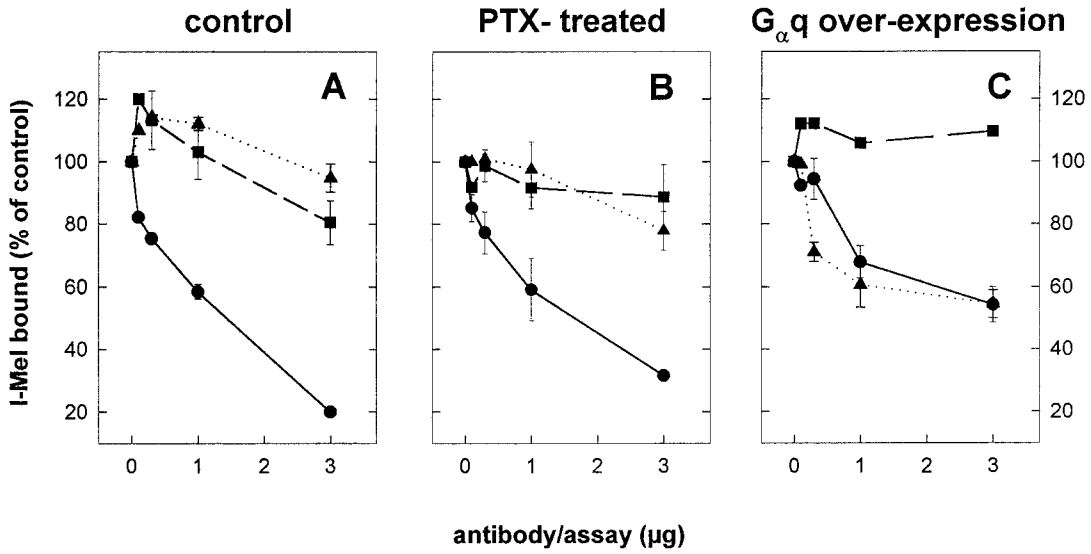

Fig. 4. Uncoupling of the Mel_{1a} receptor by G protein α subunit-specific antibodies. Membranes were prepared from HEK293 cells stably expressing the Mel_{1a} receptor before (A) and after (B) PTX treatment, and after transient transfection with the cDNA encoding for the α subunit of G_q (C). Membranes were preincubated with the indicated amounts of antibodies raised against the C terminus of $G_{\alpha}i1$–3 (●), $G_{\alpha}o$ (■), and $G_{\alpha}q/11$ (▲). Binding is expressed in percentage of the binding observed when instead of the specific antibodies, nonimmune IgG was included in the assay at the same amount. After a preincubation of membranes with the antibody in the presence of 0.2% OG for 15 min on ice, the binding reaction was performed at 30°C for 1 h at a final detergent concentration of less than 0.05%. Mean values ± S.E. are given from three to five binding experiments.

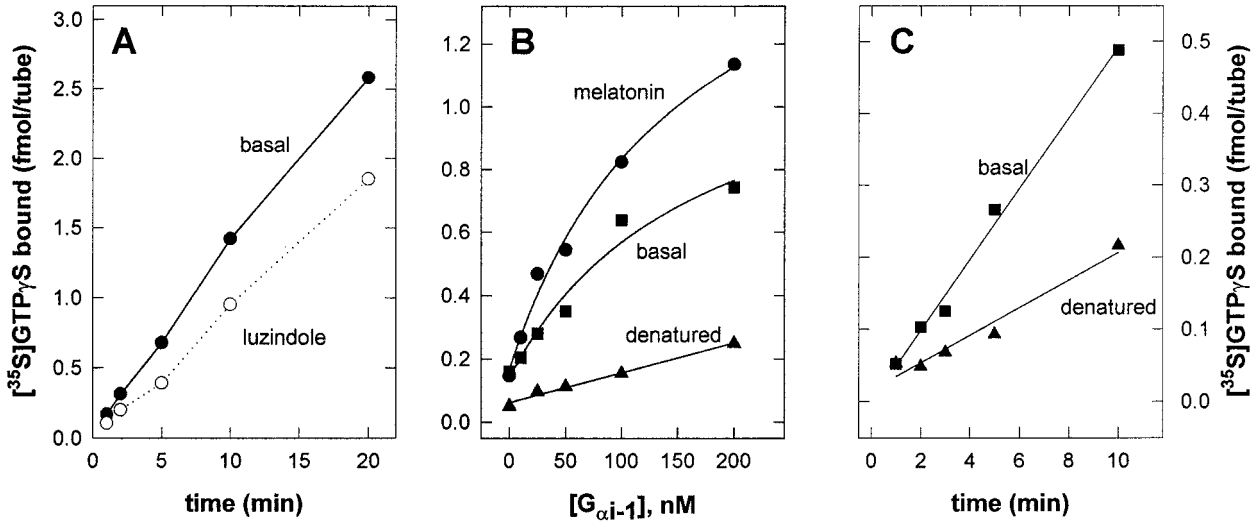

Fig. 5. Constitutive activity of the Mel_{1a} receptor in membranes of HEK293 cells. A, time course of [^{35}S]GTPγS binding to Mel_{1a} membranes in the absence (●) and presence (○) of the Mel_{1a} receptor antagonist luzindole (100 μM). The binding assay was performed at 0.175% OG in the presence of 0.3 μM GDP for the indicated intervals at 25°C. B, [^{35}S]GTPγS binding to urea-washed Mel_{1a} membranes after reconstitution with increasing concentrations of G_{i-1}. [^{35}S]GTPγS binding was determined in the absence (■) and presence (●) of melatonin (0.1 μM) and in membranes that had been heat inactivated (▲). The binding assay was initiated after preincubation of the membranes with G_{i-1} plus 0.2% OG. C, time course of [^{35}S]GTPγS binding on reconstitution of G_{i-1} with urea-washed Mel_{1a} membranes that had been (■) or had not been (▲) heat inactivated. Each figure shows a typical experiment of three performed.

Mel_{1a} Receptor Forms a Stable R/G Complex. Because the Mel_{1a} receptor displayed basal activity, we conjectured that the resistance of iodomelatonin binding to PTX resulted from the formation of a tightly associated complex of the unoccupied Mel_{1a} receptor with G_i. Direct evidence to prove this hypothesis was obtained by two experimental approaches.

First, the R/G association was explored with an antiserum (antiserum-536) raised against a peptide corresponding to the 19 C-terminal amino acids of the human Mel_{1a} receptor; the antiserum-536 immunoprecipitates the Mel_{1a} receptor from digitonin-solubilized membrane extracts (L.B., F.R., L. Petit, P. de Coppet, M. Tissot, P. Barrett, P. J. Morgan, C.N., A.D.S., and R.J., submitted for publication). For immunoprecipitation experiments membranes were prepared from naive cells or cells pretreated with PTX; the membranes were incubated with or without melatonin (100 nM) and solubilized with digitonin. The soluble extracts were incubated with antiserum-536 (dilution 1/40) and sedimented with protein A-agarose. G proteins that had been dissociated from immune complexes by treatment with Gpp(NH)p were resolved on an SDS gel, transferred to nitrocellulose, and the blots were developed with a G_i-selective antiserum. Figure 6 shows that antiserum-536 indeed coprecipitated G_i; preincubation with melatonin markedly increased the yield of G_i, indicating that the agonist ligand stabilizes the complex and that the coprecipitation reflects the result of a specific R/G interaction. Similarly, if membranes from $G_\alpha q$-transfected cells were employed, α_q was recovered from receptor precipitates, consistent with interpretation that the receptor can also bind to G_q (data not shown). Figure 6 also shows that immunoprecipitates from PTX-treated membranes also contained $G_\alpha i$. The result was similar to that in untreated membranes, except that the yield was smaller; even after PTX, melatonin increased the amount of precipitated G_i , although the agonist-induced increment was less than in the controls. This experiment indicates that the Mel_{1a} receptor has the ability to combine with G_i in the absence of an agonist, and that the spontaneous association is preserved on PTX exposure. This association is tight enough to be retained after detergent solubilization, although the addition of detergents lowers the affinity of the interaction (see Fig. 2). Conversely, it is not unexpected that the addition of the agonist stabilizes the R/G complex and thereby increases the yield of coimmunoprecipitated $G_\alpha i$.

We confirmed this result by subjecting a soluble membrane extract to PTX-mediated "back ADP ribosylation" with radioactive substrate [α-^{32}P]NAD. Cells were treated with PTX and soluble membrane extracts were prepared and re-exposed to PTX in the presence of [α-^{32}P]NAD. After the reaction, G_i was immunoprecipitated, separated by gel electrophoresis, and exposed to X-ray film. The following controls were performed. First, A_1-adenosine receptor-expressing cells were treated in an identical manner and detergent extracts were subjected to back ADP ribosylation with [α-^{32}P]NAD. Second, membrane extracts from non-PTX-exposed $Me1_{1a-}$ and A_1-adenosine receptor-expressing cells were also subjected to in vitro ADP ribosylation. The results shown in Fig. 7A demonstrate that in the Mel_{1a} cells a radioactive G_i band was detected after the cells had been pretreated with PTX. This band was not present in PTX-treated cells expressing the A_1-adenosine receptor. The radioactivity incorporated into the α_i band is quite modest compared with precipitates from untreated controls. Matched amounts of membrane protein were applied to the in vitro ADP ribosylation procedure. We therefore obtained a semiquantitative estimate for the detected radioactivity as follows. The densities of the α_i bands in lane 2 [Fig. 7, Mel_{1a}, 1/50 representing the 50th part of the sample in lane 1 (Mel_{1a})] and lane 3 (from PTX-treated Mel_{1a} cells (PTX)] were comparable. Because about 0.2% (or 50 pmol/mg) of the membrane-bound protein in the HEK293 cells we used is G_i (Waldhoer et al., 1998), the 50th part being ~1 pmol/mg, the amount of labeled G_i is roughly equivalent to the amount of receptor. To complement this finding, we tested the alternative hypothesis, namely that the Mel_{1a} receptor coupled to G_i in the ADP-ribosylated form. To this end, purified G_{i-3} was subjected to PTX treatment in vitro and, after buffer exchange, was reconstituted with urea-stripped membranes. Figure 7B shows that in urea-stripped membranes reconstitution of iodomelatonin binding with G_{i-3} was saturable (K_A ~ 40 nM) but only a few receptors were shifted into the high-affinity conformation. Figure 7C shows the result of reconstitution of urea-stripped Mel_{1a} membranes and control membranes carrying the human A_1-adenosine receptor with PTX- and sham-treated G_{i-3} (~100 nM). The efficiency of reconstitution with G_{i-3} was similar for the Mel_{1a} and the A_1-adenosine receptor. Neither the Mel_{1a} nor the A_1-adenosine receptor coupled to PTX-treated G_{i-3}.

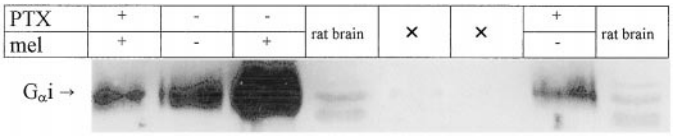

Fig. 6. Recovery of $G_\alpha i$ by immunoprecipitation of the Mel_{1a} receptor. Membranes from native and PTX-treated HEK293 cells expressing the Mel_{1a} receptor were incubated with or without melatonin (0.1 μM) for 1 h at 25°C. After solubilization with 1% digitonin, extracts were subjected to immune precipitation using antiserum-536 and protein A-agarose; the precipitates were resuspended and G proteins were dissociated with Gpp(NH)p. The supernatants were subjected to gel electrophoresis and immunoblotted using an antiserum specific for G_i protein α subunits. Membranes prepared from rat brain cortex (20 μg/lane) were applied as a source of G protein standards.

Discussion

The mode of G protein coupling of the Mel_{1a} receptor is at variance with the predictions of the classical ternary complex model (Hepler and Gilman, 1992), because agonist high-affinity binding is stable in the presence of guanine nucleotides both in intact cells and in cell membranes. High-affinity binding of iodomelatonin to intact cells has previously been observed in various cell types, hence is not due to an artifact arising from clonal selection of a stable cell line (Witt-Enderby and Dubocovich, 1996). Even following pretreatment of HEK293 cells with PTX, which completely abolishes agonist high-affinity binding to typical $G_{i/o}$-coupled receptors, a major proportion of the Mel_{1a} receptor population retains the ability to bind iodomelatonin with unaltered affinity. In the present work, we show that these unusual features arise from a highly stable association of the Mel_{1a} receptor with G_i.

This conclusion is based on several findings. First, high-affinity agonist binding to the Mel_{1a} receptor is strictly de-

pendent on G protein coupling; uncoupling (by guanine nucleotides, suramin or G protein-directed antisera) results in a loss of high-affinity agonist binding. In membranes, guanine nucleotides destabilize this R/G complex only on the addition of detergent, which reduces the G protein affinity of the receptor (by about 20-fold at the detergent concentration employed) and enhances receptor-mediated G protein activation. Secondly, in HEK293 cells the Mel_{1a} receptor interacts preferentially with G_i; this is true even after treatment with PTX, because an antiserum directed against the carboxy terminus of $G_\alpha i$ eliminates high-affinity binding in membranes from PTX-treated cells. Thirdly, the receptor is spontaneously active, a property, which, by definition, gives rise to a precoupled state. In the precoupled state, the association of the unliganded receptor with G_i is also tight and this makes the cysteine residue in the α_i C terminus unavailable to PTX-mediated ADP ribosylation. The formation of the complex between unliganded Mel_{1a} receptor and G_i is governed by an affinity of 50 nM. This estimate represents the lower limit of the affinity because it was obtained in reconstitution experiments in the presence of detergent. Nevertheless, the complex between unliganded receptor and G protein is sufficiently stable such that it can be recovered by immunoprecipitation with an antiserum directed against the receptor.

There is an apparent discrepancy in the effects of PTX; whereas pretreatment with the toxin does not abolish high-affinity agonist binding, it completely suppresses agonist-promoted GTPγS binding and effector regulation. However, the sensitivity of agonist-promoted GTPγS binding is limited by the basal rate of ligand binding to the panoply of other GTP binding proteins that are present in cell membranes. This basal rate of binding is typically reduced by the addition of excess GDP. Under these assay conditions, agonist-promoted binding can, however, only be detected if the receptor acts catalytically (to activate several G proteins) or if the expression level of the receptor is very high. Pretreatment with PTX renders the bulk of G_i unavailable to the receptor leaving only stoichiometric amounts (which escape detection). More importantly, these observations also indicate that (unmodified) G_i has to be present in excess over the receptor to support efficient signaling from the Mel_{1a} receptor to an effector (e.g., to adenylyl cyclase inhibition).

It has long been known that an activated receptor prevents ADP ribosylation of G protein α subunits by PTX (Tsai et al., 1984). To the best of our knowledge, it has, however, not yet been documented that an unliganded receptor can effectively prevent access of PTX to $G_\alpha i$. The stoichiometric levels of unmodified $G_\alpha i$ that persist after PTX treatment suffice to support high-affinity agonist binding to the Mel_{1a} receptor. In contrast, an abundant pool of G proteins is indispensable for productive signaling of the receptor because regulation of

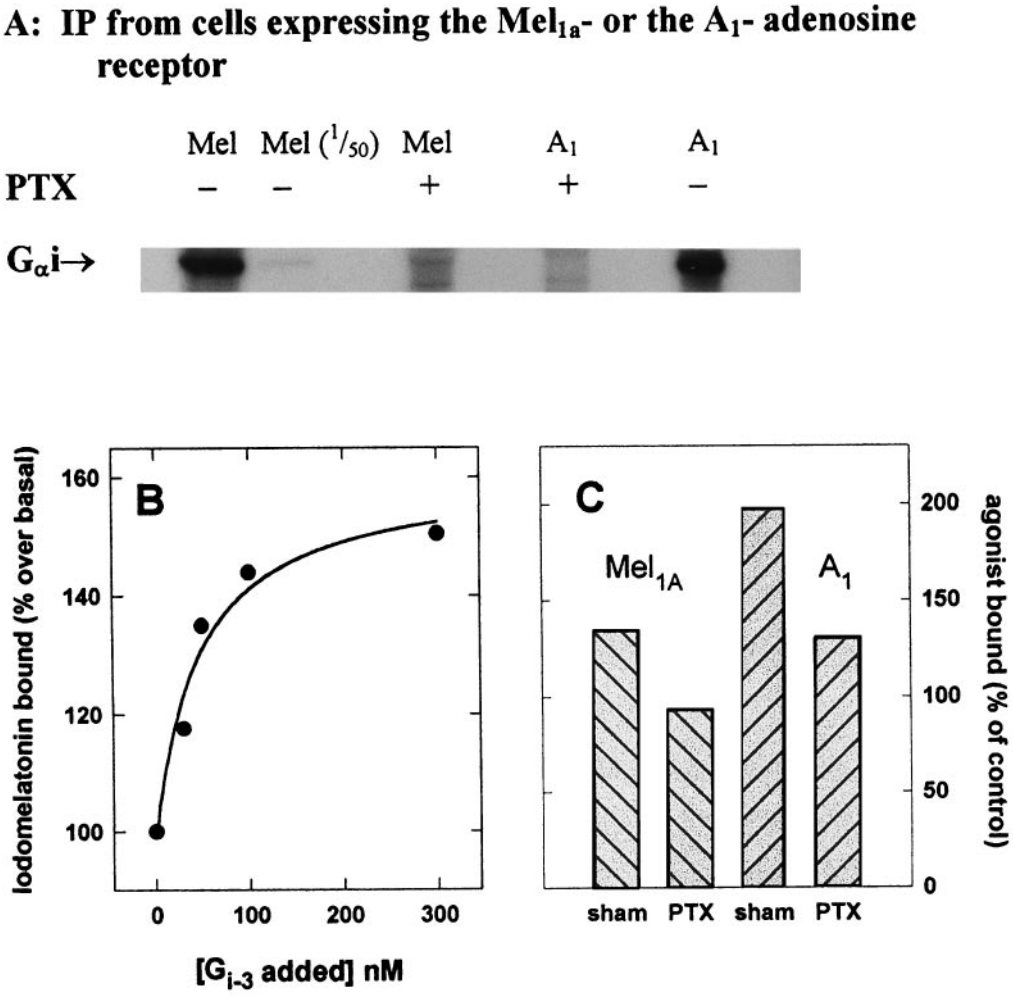

Fig. 7. [^{32}P]ADP ribosylation by PTX of G proteins extracted from HEK293 cell membranes. A, soluble extracts from native and PTX-treated HEK293 cells expressing either the human Mel_{1a} or the A_1-adenosine receptor were incubated with preactivated PTX using [^{32}P]NAD as a substrate. From the soluble extracts (~1 mg protein), G proteins were quantitatively immunoprecipitated with a $G_\alpha i$-selective antibody and resolved by gel electrophoresis. Half of the immune precipitates, obtained from PTX-exposed (PTX+) or from nonexposed (PTX−) cells, were loaded onto the gel, except in the second lane, where only the 50th part of the sample to the left was applied. Shown is a representative autoradiography of three experiments performed. B, reconstitution of the Mel_{1a} receptor with $G_{i\text{-}3}$. Iodomelatonin binding to urea-washed Mel_{1a} membranes was performed after reconstitution with increasing amounts of $rG_\alpha i\text{-}3$ in the presence of a 4-fold molar excess of $\beta\gamma$-dimer purified from porcine brain. This experiment was repeated twice with similar results. C, reconstitution of the Mel_{1a} and A_1-adenosine receptor with PTX- and sham-treated $G_{i\text{-}3}$ (100 nM).

adenylyl cyclase or phospholipase C is abolished by PTX treatment. We stress that the marked avidity with which the Mel_{1a} receptor binds to G_i is not a consequence of a very high receptor expression level in HEK293 cells. First, the levels of Mel_{1a} receptor in our HEK293 cell clone (~0.7 pmol/mg) is not substantially higher than that observed in membranes prepared from brain nuclei (~0.3 pmol/mg; Barrett et al., 1996) if the cellular heterogeneity in these preparations is taken into account. Secondly, a similar resistance of the Mel_{1a} receptor to guanine nucleotide modulation has been observed in membranes prepared from various brain regions (Rivkees et al., 1989; Morgan et al., 1996), as well as in membranes from NIH3T3 cells expressing the cloned receptor (Nonno et al., 1998). Thirdly, we have previously examined the A_1-adenosine and the D_2-dopamine ($G_{i/o}$-coupled) receptors in HEK293 cells that were expressed to levels that were either lower or higher (between 0.2 and 4.0 pmol/mg) than that obtained with the Mel_{1a} receptor cell clone. High-affinity agonist binding to these receptors was readily suppressed at low guanine nucleotide concentrations and was completely abolished by PTX (Waldhoer et al., 1998). Finally, even a fusion protein composed of the human A_1-adenosine receptor and G_αi in which the receptor is forced into close contact with the α subunit does not reproduce the features of Mel_{1a} receptor/G protein coupling (Waldhoer et al., 1999). We therefore conclude that the tight association of the Mel_{1a} receptor and G_αi is caused by intrinsic properties of the receptor and that it is independent of the expression level.

Several receptors endowed with constitutive activity (e.g., the α_2-adrenergic receptor, the 5-hydroxytryptamine$_{2C}$ receptor, and a mutated form of the α_{1B}-adrenergic receptor) form ternary complexes that display limited sensitivity to guanine nucleotides (Neubig et al., 1988; Jagadeesh et al.,1990; Westphal and Sanders-Bush, 1994, Kjelsberg et al., 1992) However, a guanine nucleotide-resistant ternary complex does not necessarily result in constitutive activity of the receptor. In the avian β-adrenergic receptor, for example, a stretch of the extended carboxy terminus confers guanine nucleotide resistance and restrains the spontaneous activity of the receptor; a truncated form of this receptor is constitutively active and becomes sensitive to guanine nucleotide modulation (Hertel et al., 1990; Parker and Ross, 1991). Similarly, guanine nucleotide modulation of the brain A_{2A}-adenosine receptor is restored by partial proteolysis of the receptor protein (Nanoff et al., 1991). Like these receptors, the Mel_{1a} receptor possesses an extended carboxy terminus; based on this analogy, it is attractive to speculate that the carboxy terminus is the candidate domain mediating the high stability of the R/G complex.

The level of Mel_{1a} receptor stably expressed in HEK293 cells is reasonably similar to the density that is expected to occur in individual hypothalamic nerve cells; hence, it is highly probable that the constitutive activity of the receptor is physiologically relevant because the neuronal activity will vary with the fluctuations in the receptor level, independently of the ambient melatonin concentration. Because the receptor expression follows a circadian rhythm (Tenn and Niles, 1993; Neu and Niles, 1997), the effect of melatonin receptor ligands in adjusting the internal clock will be different with the time of the day; in rodents, for example, melatonin or melatonin agonists advance the phase of the circadian clock only when administered during a time window preceding the onset of locomotor activity (Van Reeth et al., 1997). Based on our observations, we postulate that melatonin antagonists rather than agonists will alter the circadian rhythm at other time points, e.g., at noon, when the Mel_{1a} receptor density in target cells increases, and during the night, when the hormone levels rise. We also propose that inverse agonists will be effective in adjusting the circadian clock because they not only block the actions of melatonin but also eliminate signaling resulting from the spontaneous receptor activity.

Acknowledgments

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