Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3882–3886, May 1989
Neurobiology

Guanine nucleotide-binding protein regulation of melatonin receptors in lizard brain

(G proteins/solubilization)

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Communicated by Roy Hertz, January 26, 1989

ABSTRACT **Melatonin receptors were identified and characterized in crude membrane preparations from lizard brain by using ^{125}I-labeled melatonin (^{125}I-Mel), a potent melatonin agonist. ^{125}I-Mel binding sites were saturable; Scatchard analysis revealed high-affinity and lower affinity binding sites, with apparent K_d of $2.3 \pm 1.0 \times 10^{-11}$ M and $2.06 \pm 0.43 \times 10^{-10}$ M, respectively. Binding was reversible and inhibited by melatonin and closely related analogs but not by serotonin or norepinephrine. Treatment of crude membranes with the nonhydrolyzable GTP analog guanosine 5′-[γ-thio]triphosphate (GTP[γS]), significantly reduced the number of high-affinity receptors and increased the dissociation rate of ^{125}I-Mel from its receptor. Furthermore, GTP[γS] treatment of ligand–receptor complexes solubilized by Triton X-100 also led to a rapid dissociation of ^{125}I-Mel from solubilized ligand–receptor complexes. Gel filtration chromatography of solubilized ligand–receptor complexes revealed two major peaks of radioactivity corresponding to $M_r > 400,000$ and M_r *ca.* 110,000. This elution profile was markedly altered by pretreatment with GTP[γS] before solubilization; only the M_r 110,000 peak was present in GTP[γS]-pretreated membranes. The results strongly suggest that ^{125}I-Mel binding sites in lizard brain are melatonin receptors, with agonist-promoted guanine nucleotide-binding protein (G protein) coupling and that the apparent molecular size of receptors uncoupled from G proteins is about 110,000.**

The hormone melatonin is produced rhythmically by the vertebrate pineal gland (1, 2). The daily rhythm in melatonin production is synchronized by the environmental light/dark cycle, with increased production and subsequent release into blood occurring at night (1, 2).

Melatonin has two major neurobiological effects. In some species of reptiles, birds, and mammals, melatonin can entrain circadian rhythms (1, 3). In humans, melatonin can alter the entrainment of circadian rhythms and has been used to treat biological rhythm disorders, including "jet lag" (4). The second major function of melatonin is regulating the dramatic changes in reproductive function that occur in seasonally breeding mammals (1, 2, 5).

Recent evidence suggests that melatonin exerts its potent biological effects through specific receptors. With the development (6) of a biologically active (7, 8) radioiodinated agonist, ^{125}I-labeled melatonin (^{125}I-Mel), high-affinity melatonin-binding sites have been convincingly identified in nervous tissue from chicks (7), rodents (8–11), and humans (12) by radioreceptor and autoradiography techniques. Radioreceptor studies show that these putative melatonin receptors reside in the plasma membrane (7, 9, 10).

For a number of membrane-bound receptors, a family of guanine nucleotide-binding proteins (G proteins) transduces ligand-activated signals to second-messenger effector systems within the cell, ultimately leading to cellular responses (13–15). Thus, demonstration of melatonin receptor–G protein coupling is an essential step in elucidating the cellular and molecular mechanisms of melatonin action. Receptor–G protein coupling can be studied by perturbing receptor–G protein interactions with GTP and GTP analogs and examining the effects on agonist binding affinity (13–15).

In this report, we characterize high-affinity membrane-bound melatonin receptors from lizard brain, the richest source of melatonin receptors yet identified. We next provide evidence of G protein coupling to membrane-associated and solubilized receptors. Finally, we use gel filtration chromatography to reaffirm melatonin receptor–G protein coupling and to estimate the molecular size of solubilized receptors.

MATERIALS AND METHODS

Animals. Lizards (*Anolis carolensis*), mud puppies (*Necturus maculosus*), newts (*Notopthalamus viridescens*), grass frogs (*Rana pipiens*), and turtles (*Chrysemys*) were obtained from Nasco Scientific (Fort Atkinson, WI). Twelve-day-old chicks (*Gallus domesticus*) were obtained from SPAFAS (Norwich, CT). Male Sprague–Dawley rats (60 days old) were obtained from Charles River Breeding Laboratories.

Preparation of ^{125}I-Mel. Melatonin was iodinated by the method of Vakkuri *et al.* (6) and purified by high-performance liquid chromatography as described (11). ^{125}I-Mel was dissolved in 100% ethanol and diluted to the desired concentration in Tris buffer (50 mM Tris·HCl, pH 7.4) containing 0.1% ascorbic acid. ^{125}I-Mel was >95% pure (specific activity *ca.* 2000 Ci/mmol; 1 Ci = 37 GBq) and remained stable for at least 2 months.

Preparation of Membranes. All preparative steps were performed at 4°C. Tissues were sonicated (Kontes microultrasonic cell disruptor; tune = 3, power = 8, three times for 10 sec each; 200 mg of tissue per ml) in chilled Tris buffer containing 1 mM EDTA, 5 mM $MgCl_2$, and 10 μM diisopropyl fluorophosphate (Sigma) (homogenate buffer); EDTA, $MgCl_2$, and diisopropyl fluorophosphate did not affect binding. The homogenate was centrifuged at 50,000 × *g* for 20 min. Pellets (crude membranes) were washed twice with homogenate buffer and resuspended to a protein final concentration of 2 mg/ml. Protein concentration was determined by the Bradford assay with bovine serum albumin standards (16).

Radioreceptor Assay. Crude membranes (25–100 μg of protein) were incubated with ^{125}I-Mel in a total reaction volume of 200 μl; the reaction mixture was incubated in a shaker bath (25°C) for 120 min. Bound ^{125}I-Mel was separated from unbound radioactivity by pouring samples over prewetted Whatman GF/B glass fiber filters. Filters were washed

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Abbreviations: G protein, guanine nucleotide-binding protein; ^{125}I-Mel, ^{125}I-labeled melatonin; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

with 10 ml of ice-cold buffer and assayed for radioactivity. All determinations were performed in triplicate.

Solubilization. For solubilization of unoccupied receptors, crude membranes were centrifuged (50,000 × *g* for 20 min at 4°C), and pellets were resuspended in detergents (obtained from Sigma) dissolved in ice-cold homogenate buffer. The mixture was incubated with gentle agitation for 40 min at 0°C and centrifuged (150,000 × *g* for 60 min at 4°C). To assess solubilized receptors, portions of the supernate containing solubilized proteins were incubated with ^{125}I-Mel for 120 min at 25°C or 0°C. Bound radioactivity was then assessed by precipitation with 5 volumes of 0.075% bovine gamma globulin and 12% polyethylene glycol 8000 (PEG) for 10 min at 0°C, followed by filtration over prewetted Whatman GF/C filters (17).

For solubilization of prebound ligand–receptor complexes, crude membranes were first incubated with 500 pM ^{125}I-Mel for 120 min at 25°C. The mixture was then centrifuged and solubilized as above at 4°C. To assess solubilized ligand–receptor complexes, portions of the supernatant were tested by using gamma globulin/PEG. The recovery of ligand–receptor complexes by gamma globulin/PEG was confirmed by gel filtration chromatography (0.3 × 10 cm Sephadex G-50 columns; ref. 18), which yielded virtually the same number of bound counts as the gamma globulin/PEG method.

Gel Filtration Chromatography. Chromatography was performed at 4°C. A Sephacryl 300 SF column (1.5 × 75 cm; Pharmacia) was preequilibrated with 0.5% Triton X-100 in Tris buffer with 5 mM $MgCl_2$. Fractions were assayed for radioactivity and for protein content by a modification of the Lowry assay (19). The column was calibrated between experimental runs by separate, triplicate runs of known protein standards (from Sigma).

Data Analysis. Analysis of saturation and competition experiments was performed by using the EBDA/LIGAND nonlinear regression program (20). Analysis of kinetic studies was performed as described by Lanier *et al.* (21).

RESULTS

Experiment 1: Identification of an Enriched Source of Melatonin-Binding Protein. Crude membranes prepared from whole brains of rats, chicks, lizards, turtles, newts, and mud puppies and retinal tissue from chicks and frogs were tested for ^{125}I-Mel binding capacity (^{125}I-Mel at 50 pM) by radioreceptor assay. Lizard brain had by far the highest concentration of specific ^{125}I-Mel binding sites [45 ± 5 fmol/mg of protein (mean ± SEM)] and was thus selected as our source of melatonin-binding protein for subsequent characterization (Fig. 1). Note that since only a single ^{125}I-Mel concentration was used for this comparative analysis, the values represent relative amounts, not maximum number of binding sites (B_{max}).

Experiment 2: Characterization of Melatonin-Binding in Lizard Brain. *Saturability.* Crude membrane preparations were incubated with increasing concentrations of ^{125}I-Mel (10–1000 pM). Above 150 pM, specific binding plateaued. Scatchard analyses from three experiments revealed high-affinity (K_d = 2.3 ± 1.0 × 10^{-11} M) and lower affinity (K_d = 2.06 ± 0.43 × 10^{-10} M) binding sites (Fig. 2), with Hill coefficients of 1.01 ± 0.02 and 0.92 ± 0.11, respectively. B_{max} was 82 ± 10 and 118 ± 16 fmol/mg of protein for the high-affinity and lower affinity sites, respectively. Analysis of data assuming the presence of only one class of binding site yielded a Hill coefficient of 0.84 ± 0.15.

Kinetics. Kinetic studies (^{125}I-Mel at 50 pM) showed that binding reached equilibrium by 2 hr at 25°C (data not shown); association rate constant, k_1 determined from the pseudo-first-order equation was 4 × 10^8 M^{-1}·min^{-1}. With the addition of 1 μM melatonin to ligand–receptor complexes, there was displacement of *ca.* 50% of bound ^{125}I-Mel after 6 hr, indicating reversibility of radioligand binding; the rate constant for dissociation, k_2, was 0.002 min^{-1}. The K_d by kinetic analysis (k_2/k_1) was 5 × 10^{-12} M.

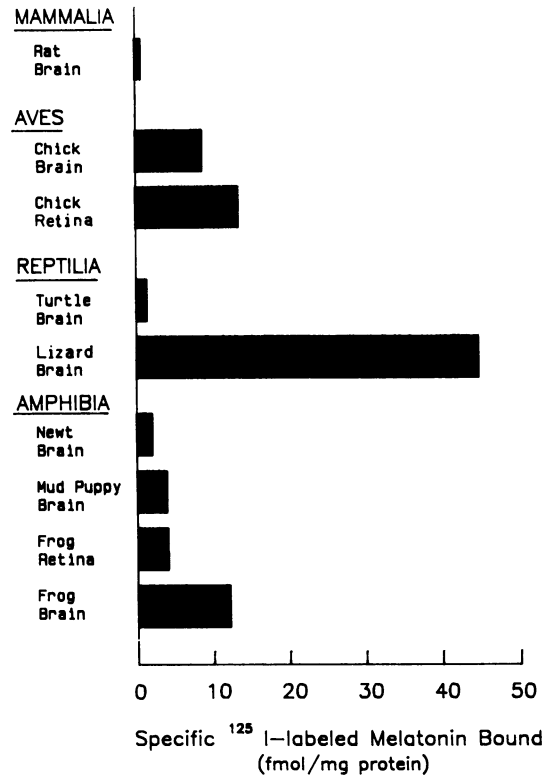

FIG. 1. ^{125}I-Mel binding to crude membranes prepared from neural tissue of selected vertebrate species. Specific binding was calculated as the difference between total binding (^{125}I-Mel at 50 pM) and nonspecific binding (^{125}I-Mel plus 1 μM melatonin). Means of triplicate determinations are presented.

Pharmacology. Membranes were incubated with ^{125}I-Mel (50–100 pM) in Tris buffer containing various concentrations of drugs dissolved in 0.25% ethanol; ethanol did not interfere with binding at concentrations up to 5%. The rank order of potency for inhibiting ^{125}I-Mel binding is depicted in Fig. 3. The same rank order of potency for inhibiting ^{125}I-Mel binding was observed at an ^{125}I-Mel concentration of 500 pM (data not shown).

Experiment 3: Melatonin Receptor–G Protein Coupling. The existence of receptor–G protein coupling was studied by perturbing receptor–G protein interactions and examining the effects on the affinity(s) of agonist binding (13–15, 22–24). Saturation studies were performed with and without the nonhydrolyzable GTP analog, guanosine 5′-[γ-thio]triphosphate (GTP[γS]; 100 μM); ^{125}I-Mel concentrations from 12.5 to 100 pM examined the high-affinity site (defined by Scatchard analysis in experiment 2, Fig. 2 *Lower*). ^{125}I-Mel at 600 pM was used to examine the effects of GTP[γS] treatment on the lower affinity site.

The addition of GTP[γS] significantly depressed specific ^{125}I-Mel binding at each ^{125}I-Mel concentration up to 100 pM (Fig. 4) in a dose-dependent manner (data not shown). GTP[γS] treatment did not change the K_d of the high-affinity binding site revealed by Scatchard analysis. However, this treatment did cause a 45% decrease in the number of high-affinity sites ($P < 0.05$). In contrast to GTP[γS] treatment, ^{125}I-Mel binding was not altered by 100 μM ATP[γS]. At the 600 pM ^{125}I-Mel concentration, GTP[γS] did not alter ^{125}I-Mel binding (data not shown), suggesting that the total number of ^{125}I-Mel binding sites (high-affinity plus lower affinity sites) was not affected by GTP.

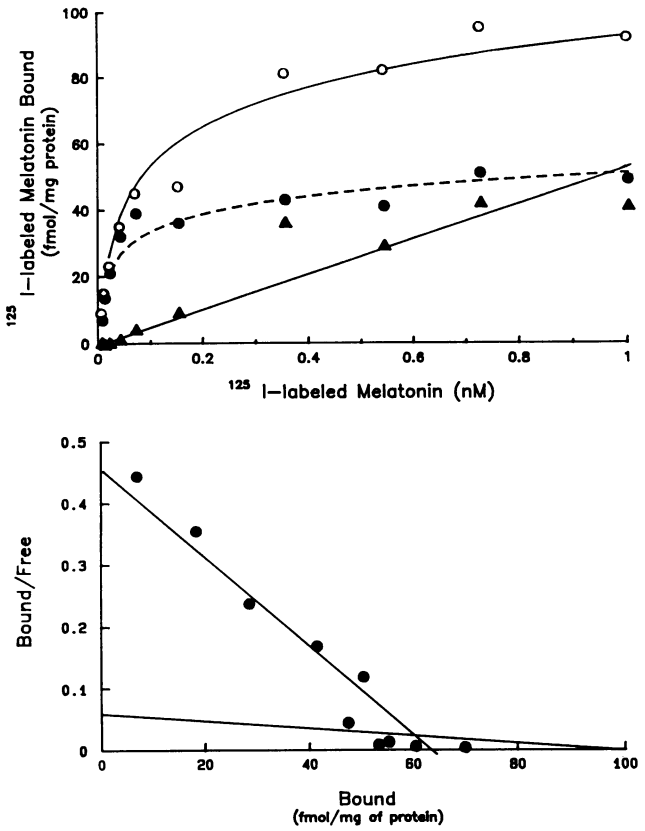

FIG. 2. Saturability of ^{125}I-Mel binding to lizard brain membranes. (*Upper*) Saturation curve. Saturation of binding sites was demonstrated by incubating crude membrane preparations with increasing concentrations of ^{125}I-Mel. Specific binding (●) was calculated as the difference between total binding (○) and nonspecific binding (▲). (*Lower*) Scatchard plot. Data points depicted are the means of triplicate determinations from one experiment and are representative of three such studies. Computer analysis revealed the presence of a high-affinity binding site ($K_d = 1.8 \times 10^{-11}$ M; B_{max} = 63 fmol/mg of protein; Hill coefficient = 1.02), and a binding site with lower affinity ($K_d = 2.2 \times 10^{-10}$ M; B_{max} = 100 fmol/mg of protein; Hill coefficient = 0.8).

We also examined the effects of GTP[γS] on the dissociation rate of ^{125}I-Mel (100 pM) from membrane-bound receptors. GTP[γS] treatment caused a 17% decrease in bound radioactivity after 5 min (Fig. 5 *Upper*) and a slower decrease over the next 55 min. Bound radioactivity did not decline over the 60-min period of study for the no-GTP[γS]-treatment group.

Experiment 4: Receptor Solubilization. We first attempted to solubilize unoccupied receptors that bind ^{125}I-Mel (100–500 pM) after solubilization. Two to four concentrations of 11 detergents representative of major detergent subclasses (deoxycholic acid, CHAPS, CHAPSO, Lubrol W-1, Brij 30, Triton X-100, Tween 80, Span 20, digitonin, octyl glucoside, hexyl thioglucoside) and chaotropic agents (1 M sodium phosphate, 3 M KCl) were tested. Although >50% of membrane proteins were solubilized with certain concentrations of detergents and chaotropic agents, binding of ^{125}I-Mel to the solubilized proteins was not detected.

Because it was not possible to detect ligand binding to solubilized unoccupied receptors, we next attempted to solubilize prebound ligand–receptor complexes (^{125}I-Mel = 500 pM); this concentration of ^{125}I-Mel was used to label both the high-affinity and lower affinity binding sites. Of three detergents (octyl glucoside, digitonin, Triton X-100) that gave encouraging results, Triton X-100 was selected for subsequent studies. The greatest yield of ligand–receptor complexes (10%) was obtained at a Triton X-100 concentration of

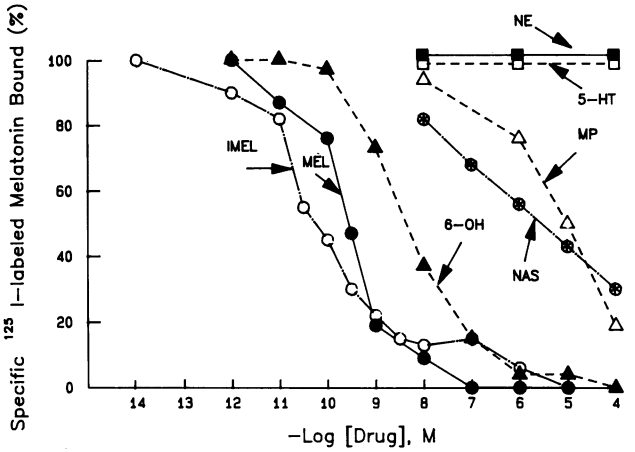

FIG. 3. Competition of indolamines and monoamines for ^{125}I-Mel binding sites in lizard brain membranes. Crude membrane preparations were incubated with 50 pM ^{125}I-Mel and various concentrations of 2-iodomelatonin (IMEL), melatonin (MEL), 6-hydroxymelatonin (6-OH), *N*-acetyl-5-hydroxytryptamine (NAS), melatonin-1-(3-proprionic acid) (MP), norepinephrine (NE), or serotonin (5-hydroxytryptamine, 5-HT). Results were analyzed as described (20) to determine K_i values: IMEL, 3.1×10^{-11} M; MEL, 2.6×10^{-10} M; 6-OH, 1.1×10^{-9} M; NAS, 5.7×10^{-7} M; MP, 8.0×10^{-7} M; NE, $>1 \times 10^{-4}$ M; and 5-HT, $>10^{-4}$ M. Additional K_i values were obtained for 6-chloromelatonin (3.2×10^{-10} M) and melatonin-1-(*p*-carboxy)benzyl (2.8×10^{-6} M) not depicted here. Results are means of triplicate determinations from one experiment per drug and are representative of two or more experiments.

0.5%, which solubilized 60% of membrane proteins. Solubilized prebound ligand–receptor complexes remained in solution after high-speed centrifugation (200,000 × *g* for 6 hr) and passed freely through 0.2-μm pore-size filters.

Experiment 5: Solubilized Receptor–G Protein Interactions. We next examined whether solubilized ^{125}I-Mel–receptor complexes include G proteins, as occurs with other G protein-coupled receptors (25–28). Experiments were performed at 4°C because solubilized ligand–receptor complexes completely dissociated within 5 min at 22°C. Membranes were pretreated with 100 μM GTP[γS] or distilled water (the vehicle for GTP[γS]) prior to the addition of 500 pM ^{125}I-Mel and solubilization with 0.5% Triton X-100. Solubilized proteins were then treated with either 100 μM GTP[γS] or distilled water; ^{125}I-Mel bound to protein was assessed by gamma globulin/PEG precipitation at 5, 15, 30, and 60 min after treatment.

GTP[γS] treatment of solubilized proteins caused a rapid dissociation of ^{125}I-Mel from receptors (Fig. 5 *Lower*). Bound radioactivity was already decreased by 70% at 5 min after incubation with GTP[γS] compared with the vehicle treatment group. Bound radioactivity continued to decrease slowly over the next 55 min after GTP[γS] treatment; bound radioactivity was relatively stable over the 60-min study period for the no-GTP[γS]-treatment group.

When agonist-activated, receptor–G protein coupling was prevented by pretreating membranes with GTP[γS] before solubilization, GTP[γS] treatment of solubilized proteins did not cause a reduction in bound radioactivity (*Inset* of Fig. 5 *Lower*). For both postsolubilization treatment groups, bound radioactivity decreased by *ca.* 60% over the 60-min period of study.

Experiment 6: Estimation of Receptor Size by Gel Filtration Chromatography. When solubilized ligand–receptor complexes (with 500 pM ^{125}I-Mel) were applied to a Sephacryl column, two major peaks of radioactivity were observed (Fig. 6). The first peak was eluted close to the void volume, which corresponded to a M_r > 400,000. The second peak of radioactivity was eluted at a volume corresponding to a M_r *ca.*

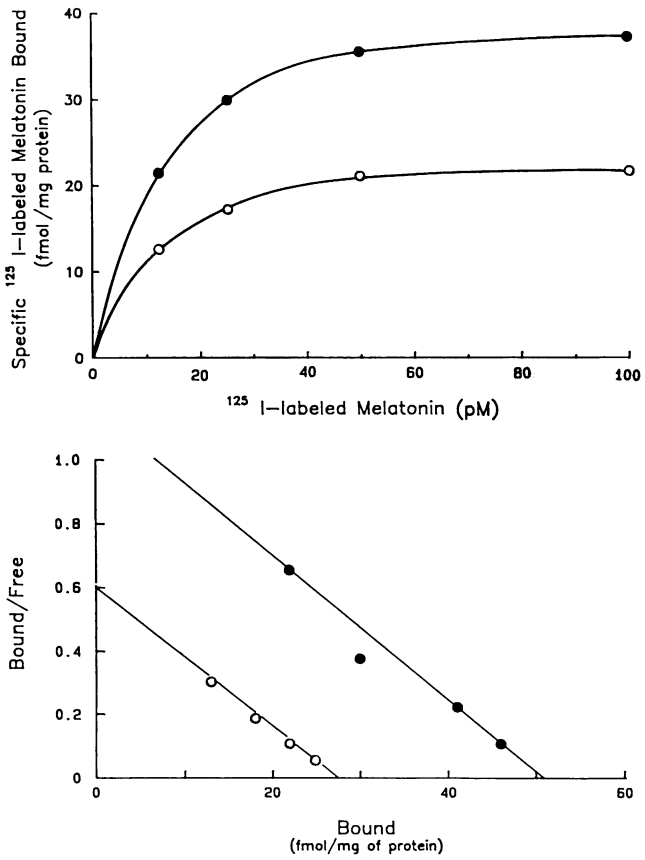

FIG. 4. GTP[γS] effects on high-affinity ^{125}I-Mel binding sites in lizard brain membranes. (*Upper*) Saturation curves. Crude membrane preparations were incubated in the presence (○) or absence (●) of 100 μM GTP[γS]. Specific binding was calculated as the difference between total and nonspecific binding. Data presented are from one experiment and are representative of three such studies. (*Lower*) Scatchard plots. Computer analysis showed the presence of a high-affinity site, whose affinity (K_d) was not affected by GTP[γS] [$9.6 \pm 3.8 \times 10^{-12}$ M (mean ± SEM) without GTP; $9.9 \pm 2.0 \times 10^{-12}$ M with GTP]. GTP[γS] decreased the number of high-affinity sites by 45% (B_{max} without GTP, 51 ± 9.5 fmol/mg of protein; B_{max} with GTP, 28 ± 5.5 fmol/mg of protein; $P < 0.05$, Student's t test). Data presented are means of three experiments.

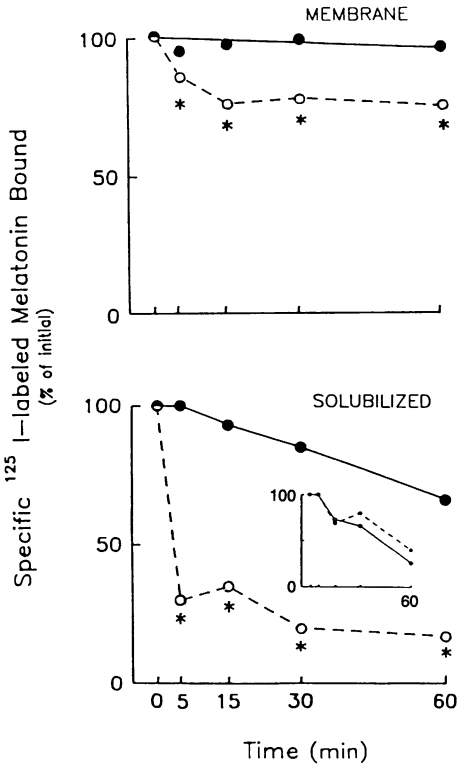

FIG. 5. Effect of GTP[γS] on the dissociation rate of ^{125}I-Mel from membrane-bound and solubilized receptors. (*Upper*) Crude membranes were prepared and incubated with 100 pM ^{125}I-Mel at 25°C for 2 hr. Total bound radioactivity was assessed at several intervals after the addition of distilled H_2O (●) or GTP[γS] (○). Nonspecific binding was assessed by using membranes incubated with 1 μM melatonin. Specific bound radioactivity is shown as a percentage of initially bound radioactivity. Data are means of three experiments. *, $P < 0.05$ (Student's t test). (*Lower*) Solubilized agonist–receptor complexes were prepared (4°C) after incubating membranes with ^{125}I-Mel (500 pM) and distilled H_2O or 100 μM GTP[γS] (*Inset*). Total bound radioactivity was assessed at several intervals after the addition of distilled H_2O (●) or GTP[γS] (○) to solubilized samples. Nonspecific binding was determined from samples treated identically as above except that 1 μM melatonin was included in the pretreatment incubation before solubilization. Specifically bound radioactivity is shown as a percentage of initially bound cpm (736 ± 51 cpm without GTP[γS] pretreatment; 789 ± 113 cpm with GTP[γS] pretreatment; see *Inset*). Data are means of four experiments. *, $P < 0.05$ (Student's t test).

110,000, as determined from a calibration curve of known protein standards. When membranes were treated with GTP[γS] prior to solubilization, only one major peak of radioactivity was eluted, corresponding to a M_r *ca.* 110,000. Pretreatment of membranes with 100 μM ATP[γS] prior to solubilization resulted in the two-peak elution profile similar to that observed when membranes were solubilized in the absence of GTP[γS]. The protein content of eluted fractions was relatively constant from the void volume to volumes corresponding to M_r 10,000.

To examine if the peak of radioactivity corresponding to M_r > 400,000 represents agonist–receptor complexes coupled to G proteins, fractions from this peak were incubated with or without 100 μM GTP[γS] for 15 min at 0°C; bound radioactivity was then assessed by gamma globulin/PEG. GTP[γS] treatment resulted in a 49% decrease ($P < 0.05$; Student's t test) in specifically bound radioactivity, compared with control treatment (385 ± 29 cpm vs. 761 ± 27 cpm). In contrast, GTP[γS] treatment of pooled fractions from the M_r 110,000 peak obtained from solubilized membranes incubated with GTP[γS] prior to solubilization did not result in a further significant decrease in specifically bound radioactivity compared with control incubations (155 ± 4 cpm vs. 184 ± 11 cpm). Gel filtration resulted in a *ca.* 10-fold enrichment of receptor proteins.

DISCUSSION

Lizard brain is a greatly enriched source of high-affinity melatonin receptors compared with neural tissues from other vertebrate species. ^{125}I-Mel binding in lizard brain is 4-fold higher than in the next highest tissue, chicken retina. Interestingly, the concentration of melatonin receptors in whole lizard brain exceeds that observed in whole rat brain by *ca.* 600-fold. Thus, lizard brain is an abundant source of ^{125}I-Mel binding protein and an ideal tissue for receptor characterization and purification studies.

The results of saturation, kinetic, and pharmacologic studies with crude membranes strongly suggest that the ^{125}I-Mel binding sites in lizard brain are melatonin receptors. The K_d value of the high-affinity site is well within the physiologic range of melatonin concentrations found in the circulation of lizards (*ca.* 5–600 pM; ref. 29). Competition studies with various indoleamines and monoamines reveal the same rank order of potencies for inhibiting ^{125}I-Mel binding in lizard brain as that observed in chicken retina (7), where high-affinity melatonin receptors have been recently identified.

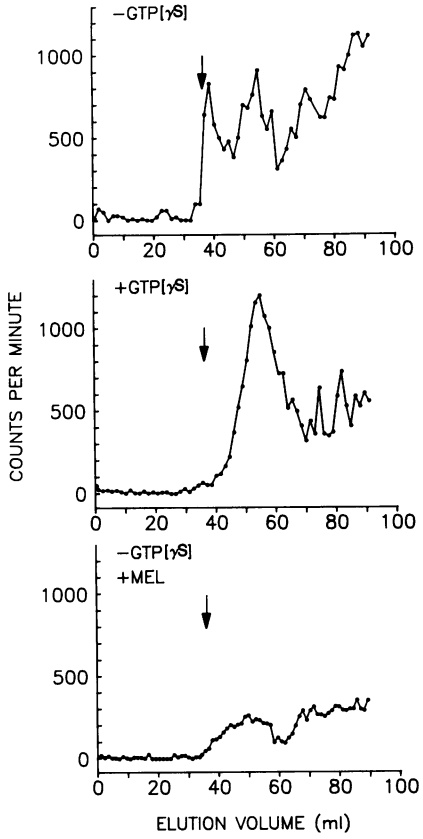

FIG. 6. Elution profiles from gel filtration chromatography of solubilized ^{125}I-Mel–receptor complexes. Crude membrane preparations (3 mg) were incubated with 500 pM ^{125}I-Mel (*Top*), ^{125}I-Mel plus 100 μM GTP[γS] (*Middle*), or ^{125}I-Mel plus 1 μM melatonin (nonspecific binding) (*Bottom*). Membrane proteins were solubilized with 0.5% Triton X-100, applied to a Sephacryl SF column (70 × 1.5 cm), and eluted with Tris buffer containing 0.5% Triton X-100 and 5 mM $MgCl_2$. Flow rate was 15 ml/hr; 1.5-ml fractions were collected. Arrows denote void volume. Data are representative of three experiments; two other experiments were performed with 3 mg and 12 mg of membrane protein.

We provide several lines of evidence suggesting that melatonin receptors in lizard brain are coupled to G proteins. First, GTP[γS] markedly decreases the number of high-affinity receptors in crude membranes. Our data suggest that this decrease is the result of conversion of high-affinity receptors to the lower affinity state because the total number of binding sites does not appear to be affected by GTP[γS]. Second, GTP[γS] increases the dissociation rate of ^{125}I-Mel from its receptor, as expected if GTP induces conversion of receptors from the high-affinity state to a lower affinity state. For several well-documented G protein-coupled receptors (22–24), agonist-induced receptor activation of G proteins, which involves exchange of complexed GDP for GTP, causes dissociation of G proteins, converting high-affinity receptors to the lower affinity state.

Solubilization of unoccupied receptors that bind ^{125}I-Mel was not successful despite trials with different concentrations of several detergents and chaotropic agents. However, we were able to solubilize stable ligand–receptor complexes that remain coupled to G proteins. When solubilized ligand–receptor complexes were treated with GTP[γS], ^{125}I-Mel binding decreased quickly and dramatically. Conversely, pretreatment of lizard membranes with GTP[γS] to block receptor–G protein coupling before solubilization prevents the formation of soluble ligand–receptor complexes that are sensitive to subsequent GTP[γS] treatment.

Gel filtration chromatography of ligand–receptor complexes provides compelling evidence of melatonin receptor–G protein coupling. Agonist binding to membranes prior to solubilization results in elution of two major peaks of radioactivity ($M_r > 400,000$ and $M_r \approx 110,000$). GTP[γS] treatment of both peaks after chromatography markedly decreases ^{125}I-Mel binding only in the M_r >400,000 peak, suggesting that this peak contains receptor–G protein complexes. Furthermore, preincubation with GTP[γS] before agonist binding and solubilization results in elution of only one major peak of radioactivity at M_r 110,000.

Our size estimate of free melatonin receptors (M_r *ca.* 110,000) compares favorably with size estimates for well-characterized G protein-coupled receptors (e.g., β-adrenergic and muscarinic acetylcholine) by gel filtration (18, 30). This size estimation, along with our data showing G protein regulation of receptor affinity, strongly suggests that the melatonin receptor is a member of the growing family of G protein-coupled receptors.

We thank Richard Conron for expert assistance. This work was supported by Grants HD14427 and DK38116 to S.M.R.; S.A.R. is an Association of Medical School Pediatric Chairman Inc., Pediatric Scientist Training Program Fellow supported by Grant HD-00850; and S.M.R. is an Established Investigator of the American Heart Association.

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