Human Serotonin$_{1B}$ Receptor Expression in Sf9 Cells: Phosphorylation, Palmitoylation, and Adenylyl Cyclase Inhibition†

Gordon YK. Ng,‡ Susan R. George,‡,§ Roman L. Zastawny,‡ Mireille Caron,∥ Michel Bouvier,⊥ Michael Dennis,∥,⊥ and Brian F. O'Dowd*,‡,§

Department of Pharmacology, University of Toronto, Toronto, Ontario M5S 1A8, Canada, Addiction Research Foundation, 33 Russell Street, Toronto, Ontario M5S 2S1, Canada, Department of Biochemistry and Pharmacology, University of Montreal, Montreal, Quebec H3C 3J7, Canada, and Biosignal Inc., 316 Carre St. Louis, Montreal, Quebec H2X 1A5, Canada

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ABSTRACT: Analysis of the primary protein structure of the human serotonin$_{1B}$ (5-HT$_{1B}$) receptor reveals consensus sites for phosphorylation and a putative site for palmitoylation. To investigate these posttranslational modifications, we have expressed a c-myc epitope-tagged 5-HT$_{1B}$ (m5-HT$_{1B}$) receptor in Sf9 cells. This strategy enabeled receptors to be detected by immunoblot analysis and purified by immunoprecipitation using a monoclonal antibody, 9E10, specific for the c-myc epitope. Agonist radioligand [^{3}H]5-HT binding studies showed that the expressed 5-HT$_{1B}$ and m5-HT$_{1B}$ receptors displayed the characteristic pharmacological profile of the neuronal 5-HT$_{1B}$ receptor. The expressed receptors displayed both high- and low-affinity states for [^{3}H]5-HT, suggesting that the receptors were coupled to endogenous G-proteins. Indeed, agonist binding to the high-affinity receptor state was regulated in the presence of GTPγS, Gpp(NH)p, and pertussis toxin. [^{32}P]ADP-ribosylation experiments identified a major ~41-kDa ADP-ribosylated protein present in Sf9 membranes that comigrated with partially purified bovine brain $G_{i\alpha}/G_{o\alpha}$ subunits. Measurements of adenylyl cyclase activity in membranes from cells expressing m5-HT$_{1B}$ receptors showed that serotonergic agonists mediated the inhibition of adenylyl cyclase activity with a rank order of potency comparable to their affinity constants. Immunoblot analysis of membranes prepared from cells expressing m5-HT$_{1B}$ receptors and photoaffinity labeling of the immunoprecipitated material revealed photolabeled species at ~95 and at ~42 kDa. Immunoprecipitated material migrating at ~95 and ~42 kDa was shown to be posttranslationally modified following whole cell metabolic labeling with [^{32}P$_i$]phosphate or [^{3}H]palmitic acid, and this provides the first evidence that the 5HT$_{1B}$ serotonin receptor is phosphorylated and palmitoylated.

The neurotransmitter serotonin or 5-hydroxytryptamine (5-HT) is postulated to have an important role in neuropsychiatric disorders such as depression, anxiety, and substance abuse (Osborne, 1982; Cross, 1988; Sellers et al., 1992). The functional diversity of 5-HT action is reflected in the four classes of 5-HT receptors: 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, and 5-HT$_4$, classified according to pharmacological and biochemical criteria [for a review, see Peroutka (1993) and Frazer et al. (1990)]. To date, cloning studies have identified six unique G-protein-coupled receptors belonging to the 5-HT$_1$ class, namely, 5-HT$_{1A}$ (Fargin et al., 1988), 5-HT$_{1B}$ (Jin et al., 1992), 5-HT$_{1C}$ (Julius et al., 1988), 5-HT$_{1D}$ (Hamblin et al., 1991), 5-HT$_{1E}$ (McAlister et al., 1992), and 5HT$_{1F}$ (Adham et al., 1993). The 5-HT$_1$ class of receptors all bind 5-HT with high affinity but are distinguished from each other by their unique rank order of affinities for typical serotonergic ligands. Each receptor appears to have a unique but overlapping distribution and possibly a distinct function in the central nervous system. The biological studies of each receptor have been limited by the nonavailability of highly selective ligands or receptor antisera. To date, there is little information regarding the purification or posttranslational modifications of the serotonin receptors except for 5-HT$_{1A}$ (El Mestikawy et al., 1989; Raymond, 1991). However, important functional roles for posttranslational modifications such as phosphorylation and palmitoylation have been demonstrated for other G-protein-coupled receptors (Bouvier et al., 1988; O'Dowd et al., 1989; Ng et al., 1993). The human 5-HT$_{1B}$ receptor that we have recently cloned has been characterized by binding assays (Jin et al., 1992), and this class of receptors has previously been shown to mediate the inhibition of adenylyl cyclase (Bouhelal et al., 1988).

Amino acid sequence analysis of the 5-HT$_{1B}$ receptor (Figure 1) revealed consensus serine and/or threonine residues in the first, second, and third intracellular loops for phosphorylation by cyclic AMP-dependent protein kinase (PKA), and protein kinase C (PKC). A putative site for palmitoylation is present at the cysteine residue in the short carboxyl tail of the 5-HT$_{1B}$ receptor (Figure 1); this site differs from other G-protein-coupled receptors shown to be palmitoylated, e.g., β-adrenergic or dopamine D1 receptors, that have long carboxyl tails (O'Dowd et al., 1989; Ng et al., 1993) but is similar to that described for the α_{2A}-adrenergic receptor (Kennedy & Limbird, 1993).

The baculovirus/Sf9 cell system was selected for our receptor characterization studies because G-protein-coupled receptors expressed in these cells appear to be biochemically, pharmacologically, and functionally similar to those in native membranes (George et al., 1989; Parker et al., 1991; Mouillac et al., 1992; Richardson & Hosey, 1992; Ng et al., 1993). Moreover, posttranslational modifications such as phosphorylation have been demonstrated for insect cell-expressed

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* Address correspondence to this author at the Department of Pharmacology, University of Toronto, Toronto, Ontario M5S 1A8, Canada. Telephone: (416) 978-7579. Fax: (416) 978-2733.

‡ University of Toronto.

§ Addiction Research Foundation.

∥ Biosignal Inc.

⊥ University of Montreal.

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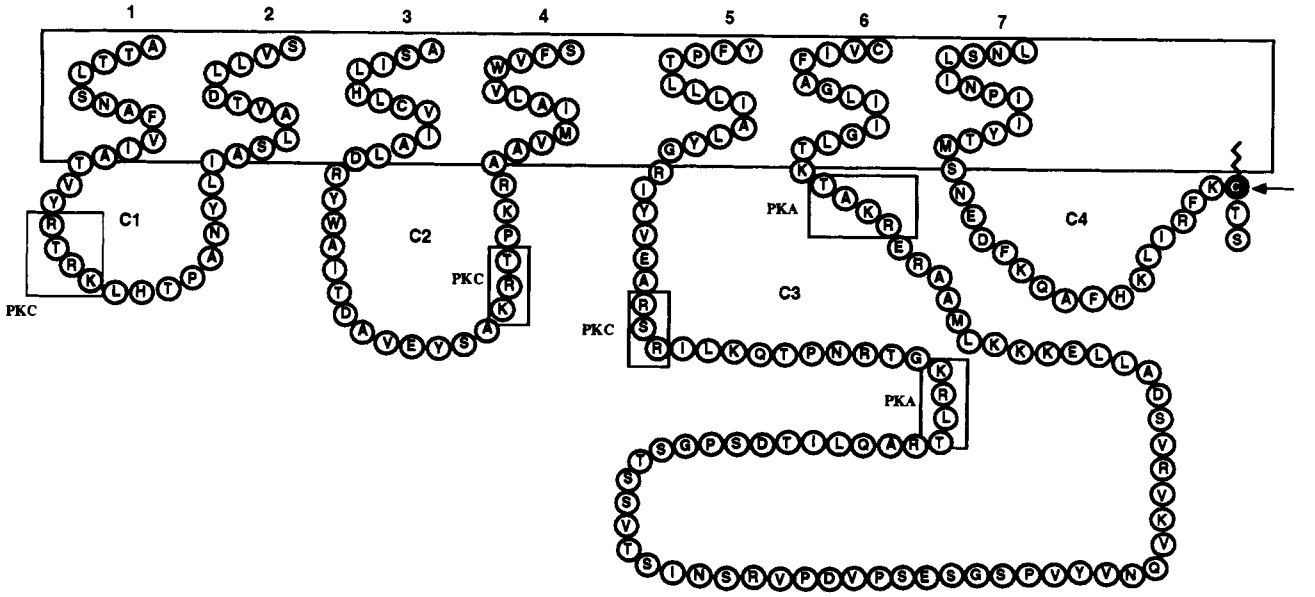

FIGURE 1: Proposed model illustrating only the intracellular domains of the human 5-HT_{1B} receptor. The seven transmembrane-spanning regions (1–7), the intracellular loops (C1–C4), and three PKC and two PKA sites are indicated. The small arrow indicates a possible palmitoylation site. Circles with letters represent amino acids in the single-letter code.

receptors such as muscarinic, β-adrenergic, substance P, and dopamine D1 receptors (Richardson & Hosey, 1992; Kwatra et al., 1993; Ng et al., 1993). The strategy chosen was to express a recombinant c-myc epitope-tagged 5-HT_{1B} (m5-HT_{1B}) receptor so that specific antibodies directed against the c-myc epitope in the amino-terminal domain of the m5-HT_{1B} receptor enabled receptor identification by immunoblotting and purification by immunoprecipitation. In addition, this strategy also allows us in future studies to express, purify, and characterize each of the other serotonin receptors. The data show that 5-HT_{1B} receptors expressed in Sf9 cells are pharmacologically similar to the human neuronal 5-HT_{1B} receptor and coupled to pertussis toxin-sensitive G-proteins that mediate the inhibition of adenylyl cyclase. Moreover, we report for the first time that the serotonin 5-HT_{1B} receptor protein is phosphorylated and palmitoylated.

EXPERIMENTAL PROCEDURES

Materials. Grace's supplemented medium, fetal bovine serum, gentamycin sulfate, fungizone, goat serum, and rabbit serum were purchased from Gibco (Toronto, Ontario). [^{3}H]5-HT, [^{125}I]MAB, carrier-free [^{32}P]P_i, [^{3}H]cAMP, [α-^{32}P]ATP, [9,10-^{3}H]palmitic acid, and [^{32}P]NAD^+ were obtained from Du Pont—New England Nuclear (NEN) (Boston, MA). Methiothepin mesylate, methysergide, and 8-OH-DPAT [(±)-8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin] were purchased from Research Biochemicals Inc. (Natick, MA), who also supplied 5-CT (5-carboxamidotryptamine) under the NIMH drug program. Sumatriptan was a generous gift from Dr. Milt Teitler (Albany Medical College, Albany, NY) and Glaxo (U.K.) 5-HT, sodium fluoride, forskolin, GTPγS [guanosine 5′-*O*-(3-thiotriphosphate)], Gpp(NH)p [guanosine 5′-(β,γ-imidotriphosphate)], ATP, GTP, cAMP, phosphoenolpyruvate, myokinase, anti-mouse IgG–agarose, leupeptin, benzamidine, soybean trypsin inhibitor, and pertussis toxin were from Sigma Chemical Co. (St. Louis, MO) and Amersham (Arlington Heights, IL). Pyruvate kinase and goat anti-mouse antibody coupled to alkaline phosphatase were from Calbiochem (San Diego, CA) and BRL (Burlington, Ontario), respectively. Digitonin was purchased from Gallard–Schlessinger (Carle Place, NY). The mouse monoclonal antibody anti-c-myc (9E10) was a generous gift from Dr. J. Park, Massachusetts General Hospital. The partially purified bovine brain G_i/G_o standards were a generous gift from J. Labrecque (Biosignal Inc., Montreal). All other chemicals for gel electrophoresis and pertussis toxin assays were purchased from Bio-Rad (Richmond, CA) and Sigma, respectively.

Construction of 5-HT_{1B} and c-myc–5-HT_{1B} Recombinant Viruses. The plasmid vector Bluescript containing the human 5-HT_{1B} receptor gene (Jin et al., 1992) was digested with *Nco*I and *Sal*I. This fragment was isolated by agarose gel electrophoresis and subcloned into the *Nhe*I site of the plasmid pETL (a gift from C. Richardson, Biotechnology Research Institute, Montreal) by blunt-end ligation. The c-myc construct was prepared as follows: A construct consisting of a c-myc–β_2-adrenergic receptor (β-AR) in pTZ (Mouillac et al., 1992) was digested with *Nco*I and *Sal*I to remove the DNA encoding β_2-AR, and the remaining pTZ/c-myc fragment was gel-purified. A fragment obtained by digestion of 5-HT_{1B} in Bluescript with *Nco*I/*Sal*I was subcloned into the pTZ/c-myc by blunt-end ligation. A fragment containing 5-HT_{1B} with c-myc fused to the N-terminal isolated from the pTZ construct by digestion with *Eco*RI/*Hin*d III was subcloned into the pETL plasmid at the *Nhe*I site by blunt-end ligation.

Cell Culture and Membrane Preparation. Sf9 cells were grown in suspension culture using Grace's insect medium supplemented with 10% fetal bovine serum, pluronic F-68, gentamycin, and fungizone, maintained at 27 °C as described by Summers and Smith (1987). Cells at a density of 2×10^6/mL were infected with the virus at a MOI of 2–5 in 10 mL and harvested at 72-h postinfection. The preparation of membranes was done at 4 °C. Cells were centrifuged at 100*g* for 7 min and pelleted. Cells were then washed twice with PBS, centrifuged at 100*g* for 7 min (×2), and resuspended in 10 mL of buffer A [buffer A is 5 mM Tris-HCl/2 mM EDTA buffer (pH 7.4 at 4 °C) containing the following protease inhibitors: 10 μg/mL benzamidine, 5 μg/mL leupeptin, and 5 μg/mL soybean trypsin inhibitor]. The cell suspension was then Polytroned, two bursts at maximum setting for 15 s, homogenates were centrifuged at 100*g* for 7 min to pellet unbroken cells and nuclei, and supernatant was collected. The

resulting pellet was homogenized a second time in 10 mL of buffer A, centrifuged as described above, and supernatant fractions were saved. The pooled supernatant was centrifuged at high speed (27000g for 20 min), washed once with buffer A, centrifuged again at high speed and resuspended in buffer A, and stored at −80 °C or resuspended in buffer B (75 mM Tris-HCl, 12.5 mM $MgCl_2$, and 2 mM EDTA, pH 7.4) and assayed immediately for adenylyl cyclase activity. Protein content was determined by the method of Bradford (Bio-Rad) with bovine serum albumin as the standard.

Receptor Binding Assays. Saturation experiments were done with increasing concentrations of [^{3}H]5-HT (0.1–200 nM, final concentration) with 50–100 μg of membrane protein in duplicate and incubated for 2 h at 22 °C in a total volume of 1 mL of binding buffer C [50 mM Tris-HCl/4 mM $CaCl_2$ (pH 7.4)] with 0.1% ascorbic acid and protease inhibitors. Competition experiments were done using 50–100 μg of protein in duplicate with increasing concentrations (10^{-12}–10^{-3} M) of nonhydrolyzable GTP analogues: GTPγS, Gpp(NH)p, or the serotonergic ligands 5-CT, 5-HT, methiothepin mesylate, methysergide, sumatriptan, or 8-OH-DPAT in the presence of 5 nM, final concentration, [^{3}H]5-HT. Tubes were incubated for 2 h at 22 °C in a final volume of 1 mL with binding buffer C as described. For all binding experiments, nonspecific binding was defined as binding that was not displaced by 20 μM methiothepin mesylate. Bound ligand was isolated by rapid filtration through a Brandel 48-well cell harvester using Whatman GF/C filters. Filters were washed with 10 mL of cold 50 mM Tris-HCl buffer (pH 7.4) and placed in glass vials with 5 mL of Universol scintillation fluid and counted for tritium. All binding data were analyzed by nonlinear least-squares regression utilizing the computer fitting program LIGAND (Munson & Rodbard, 1980).

Treatment of Sf9 Cells with Pertussis Toxin. Pertussis toxin (1 μg/mL) was reconstituted in H_2O, added to a suspension of cell culture infected with recombinant virus, and incubated for 20 h prior to harvesting of cells at the 72-h postinfection time point.

[^{32}P]ADP-Ribosylation of Sf9 Membrane Components by Pertussis Toxin. The [^{32}P]ADP-ribosylation experiments were performed essentially as described (Ribeiro-Neto et al., 1985) with minor modifications. Pertussis toxin (20 μg/mL) was preactivated as described and added to a tube containing 50 or 100 μg of membrane protein, 10 mM Tris-HCl, pH 7.4, 25 mM DTT, 0.5 mM EDTA, 1 mM ATP, 0.1 mM GTP, 10 μM NAD^+, 5 μL of protease inhibitor cocktail, and 50 μCi of [^{32}P]NAD^+ in a final volume of 50 μL and incubated at 37 °C for 60 min. The time and temperature conditions were determined to be optimal for evaluating the stoichiometry of labeling in pilot experiments. Reactions were stopped by adding SDS sample buffer, and samples were boiled before SDS–PAGE analysis. Control samples were incubated in the absence of pertussis toxin or utilized 1–2.5 pmol of partially purified solubilized bovine brain G_i/G_o in place of Sf9 membrane protein. The number of picomoles of ADP-ribosylated protein was calculated by excising the ~41-kDa band from the gel for liquid scintillation counting.

Adenylyl Cyclase Assay. Adenylyl cyclase assays were conducted essentially as described (Salomon et al., 1974). The assay mix contained 0.02 mL of membrane suspension (25 μg of protein), 0.012 mM ATP, 0.1 mM cAMP, 0.053 mM GTP, 2.7 mM phosphoenolpyruvate, 0.2 unit of pyruvate kinase, 1 unit of myokinase, and 0.13 μCi of [^{32}P]ATP in a final volume of 0.05 mL. Enzyme activities were determined in triplicate assay tubes containing decreasing concentrations (10^{-3}–10^{-15} M) of 5-HT or 8-OH-DPAT. Adenylyl cyclase activity was also measured in the presence of H_2O (basal), 100 μM forskolin, or 10 mM sodium fluoride and incubated at 37 °C for 30 min. Reactions were stopped by the addition of 1 mL of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (25 000 cpm). cAMP was isolated by sequential column chromatography using Dowex cation-exchange resin and aluminum oxide. Data were analyzed by nonlinear least-squares regression.

SDS–PAGE Electrophoresis. Gel electrophoresis was performed with 12% polyacrylamide/SDS gels (Laemmli, 1970). Membranes were solubilized in sample buffer consisting of 50 mM Tris-HCl, pH 6.5, 10% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.003% bromophenol blue. Proteins were electrophoretically transferred using a transblot apparatus onto nitrocellulose (Schleicher & Schuell), or gels were dried and autoradiographed at −70 °C with Kodak XAR-5 film and an intensifying screen.

Solubilization and Immunoprecipitation of m5-HT$_{1B}$ Receptors. Membranes were prepared by sonication in buffer A as described. The pellet was resuspended and stirred at 4 °C for 2 h in 2 mL of freshly prepared solubilization buffer consisting of 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2% digitonin, and 5 mM EDTA with protease inhibitors. The homogenate was centrifuged at 27000g for 20 min, and the solubilized fraction was washed and concentrated in Centriprep-30 4 times with 10 mL of cold buffer C: 100 mM NaCl/10 mM Tris-HCl, pH 7.4, with protease inhibitors. The washed fraction was precleared at room temperature with a 1/20 dilution of normal goat serum and protein A–Sepharose beads for 2 h. The solubilized receptors were immunoprecipitated with the mouse monoclonal 9E10 antibody at a 1/37 dilution in buffer C for 2 h on ice and agitated gently overnight at 4 °C with a 1/40 dilution of agarose-fixed goat anti-mouse IgG. The immunoprecipitate was washed over 6 h with six changes of cold buffer C. Immunoprecipitated material was solubilized in SDS sample buffer, sonicated and electrophoresed on SDS–PAGE as described.

Photoaffinity Labeling. Membranes (2 mg of protein) were solubilized and immunoprecipitated as described. Following wash of the agarose pellet, immunoprecipitated material was resuspended by sonication in buffer D [5 mM Tris-HCl/2 mM EDTA, pH 7.4, with 0.1% ascorbic acid and protease inhibitors] and incubated in the dark with 2 μM [^{125}I]IMAB in a final volume of 1.0 mL for 3 h at 22 °C in the absence and presence of 1 mM 5-HT. Samples were placed on ice and exposed to 360-nm ultraviolet light at 2 in. from the source for 10 min. Photolabeled immunoprecipitated material was washed with buffer D and concentrated in Centricon-30 cartridges. SDS sample buffer was added to the photolabeled immunoprecipitated material and electrophoresed. Gels were dried and exposed to Kodak XAR film with an intensifying screen at −70 °C as described above.

Immunoblotting of m5-HT$_{1B}$ Receptors. Membranes (200 μg of protein) from cells expressing m5-HT$_{1B}$ receptors at 72-h postinfection were prepared, electrophoresed, and blotted onto nitrocellulose as described. The blots were washed in TBS for 10 min, blocked with 3% skim milk powder in TBS buffer for 30 min, washed with TBS for 10 min, and incubated for 1 h at 22 °C with the mouse monoclonal antibody 9E10 (Evan et al., 1985) (diluted 1/100 in TBS containing 1% skim milk powder) directed against the c-myc epitope of m5-HT$_{1B}$. Blots were then treated with 0.05% Tween 20 in TBS in 30 min, and binding of the primary antibody was detected after incubation for 1 h at 22 °C with goat anti-mouse IgG–

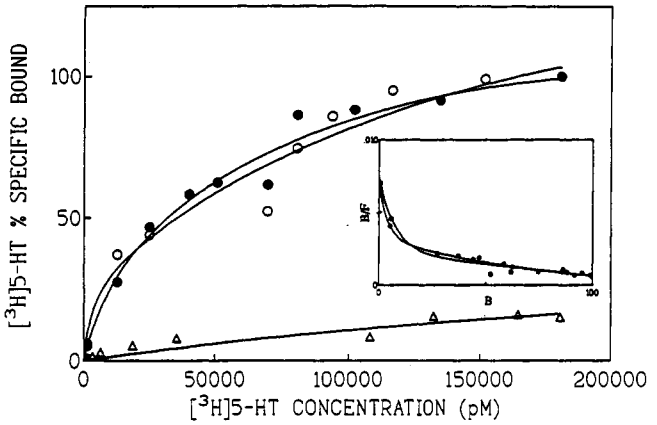

FIGURE 2: Saturation isotherms of [^{3}H]5-HT specific binding to membranes from Sf9 cells expressing 5-HT$_{1B}$ and m5-HT$_{1B}$ receptors. B_{max} (picomoles per milligram of protein) and K_d (nanomolar) values for [^{3}H]5-HT binding were determined by the nonlinear, least-squares curve-fitting program LIGAND and fitted statistically for one- or two-site models at $p < 0.05$. (Main figure) Saturation curve of [^{3}H]5-HT binding to membranes prepared at 72-h postinfection. Results shown are from one of three independent experiments, and in this experiment, B_{max} and K_d values were as follows: 5-HT$_{1B}$ receptor (○), $B_{max} = 0.5$,, $K_h = 10$, $B_{max} = 0.5$, $K_l = 100$; for m5-HT$_{1B}$ receptor (●), $B_{max} = 0.4$, $K_h = 9$, $B_{max} = 0.7$, $K_l = 120$. Inset: Scatchard plot of the same data. Specific [^{3}H]5-HT binding was defined as binding inhibited by 20 μM methiothepin. [^{3}H]5-HT binding in the presence of 20 μM methiothepin was detected in cells infected with the wild-type baculovirus (△) but represented <10% of specific binding in cells infected with recombinant virus (subscript h = high, subscript l = low).

alkaline phosphatase conjugate (BRL), diluted 1/10 in TBS containing 1% skim milk powder. Blots were then rinsed in 150 mM NaCl/50 mM Tris-HCl, pH 7.5, before developing with BCIP/NBT substrate (Bio-Rad).

Whole Cell Phosphorylation. Sf9 cells expressing the m5-HT$_{1B}$ receptor at 72-h postinfection were incubated for 4 h with carrier-free ^{32}P (0.5 mCi/mL). Reactions were terminated by centrifugation at 100*g*, and the cells were washed 3 times with PBS before membrane preparation, solubilization, and immunoprecipitation as described previously. Following electrophoresis, the gels were fixed, dried, and exposed to Kodak XAR film at −70 °C.

Whole Cell Palmitoylation. Sf9 cells expressing the m5-HT$_{1B}$ receptor were cultured for 18 h prior to the labeling experiment in serum-free media. Following this period, cells were resuspended in Grace's insect medium supplemented with 1% FBS for 1 h at 27 °C. [^{3}H]Palmitic acid dissolved in dimethyl sulfoxide (0.01% final concentration) (0.2 mCi/mL) was then added to the culture and allowed to equilibrate over 4 h at 22 °C. The experiments were terminated by centrifugation at 100*g*, cells were washed 3 times with cold PBS, and receptors were prepared as described above. Following electrophoresis, the gels were fixed and treated with Enlightning (NEN) for 1 h, dried, and exposed to Kodak X-AR film at −70 °C.

RESULTS

Pharmacological Characterization of Expressed 5-HT$_{1B}$ Receptors. [^{3}H]5-HT bound in a specific and saturable manner to the 5-HT$_{1B}$ and m5-HT$_{1B}$ receptors expressed in Sf9 cells with receptor densities ranging from 1 to 5 pmol/mg of protein at 72-h postinfection (Figure 2). [^{3}H]5-HT binding to 5-HT$_{1B}$ and m5-HT$_{1B}$ receptors was not significantly different, and saturation data analyzed by LIGAND were best fitted to a two-site model ($p < 0.05$) similar to wild-type 5-HT$_{1B}$ receptors in neuronal tissue (Peroutka, 1993). The

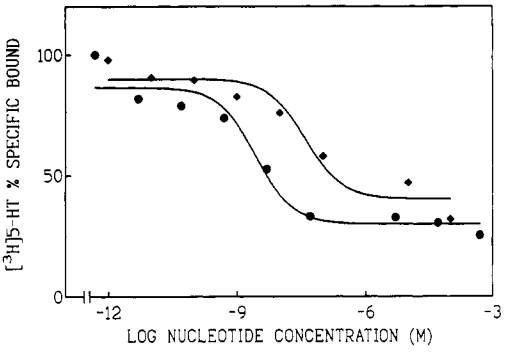

FIGURE 3: Guanine nucleotide regulation of [^{3}H]5-HT binding. Membranes from cells expressing m5-HT$_{1B}$ receptors at 72-h postinfection were incubated with ~5 nM [^{3}H]5-HT and increasing concentrations (10^{-12}–10^{-3} M) of Gpp(NH)p (♦) or GTPγS (●) in the presence and absence of 20 μM methiothepin as described under Experimental Procedures. The data were analyzed by LIGAND, and K_i values are listed in Table I. The results shown are representative of two or three independent experiments.

Table I: Affinity Constants for [^{3}H]5-HT Binding to Expressed Human 5-HT$_{1B}$ Receptors[a]

	human 5-HT$_{1B}$ receptors		
drug	Sf9 cellf K_i, nM	293 cells[b] K_i, nM	CHO cells[c] K_i, nM
5-CT	0.55	5.1 ± 0.3	0.9 ± 0.1
methiothepin	1.53	17.0 ± 2.0	3.1 ± 1.0
5-HT	3.66	16.0 ± 1.0	4.0 ± 1.9
methysgeride	18.44		7.6 ± 0.6
sumatriptan	64.15	61.0 ± 5.0	11.0 ± 0.7
8-OH-DPAT	106.9	2600 ± 100	634.0 ± 11

[a] Membranes prepared from Sf9 cells expressing the m5-HT$_{1B}$ receptor at 72-h postinfection were incubated with varying concentrations (10^{-11}–10^{-3} M) of competing drugs in the presence of ~5 nM [^{3}H]5-HT as described under Experimental Procedures. Nonspecific binding was defined by 20 μM methiothepin. Values were determined from an experiment conducted in duplicate and analyzed by LIGAND. Inhibitory constants (K_i) for the various serotonergic ligands are listed in order of their affinities for the m5-HT$_{1B}$ receptor. [b] Jin et al. (1992). [c] Demchyshyn et al. (1992).

mean K_d values for the expressed receptors and the proportions for high- and low-affinity states were 24 ± 2 nM and 62% ($n = 4$), and 104 ± 17 nM and 38% ($n = 4$), respectively. The identification of two agonist-detected affinity states of the receptor supports an interaction of expressed receptors with endogenous G-proteins in Sf9 cells. Indeed, [^{3}H]5-HT binding to the high-affinity receptor state decreased in a concentration-dependent manner in the presence of either GTPγS or Gpp(NH)p (Figure 3) with K_i values of 3.1 ± 0.1 nM ($n = 3$) and 38.8 ± 1.9 nM ($n = 2$), respectively.

Displacement of [^{3}H]5-HT binding to the high-affinity state of m5-HT$_{1B}$ receptors by competing drugs was in the appropriate rank order of potency for serotonergic ligands, consistent with the human brain 5-HT$_{1B}$ receptor profile. The 5-HT$_{1B}$ pharmacological profile is defined by higher affinity for 5-CT and a lower affinity for 8-OH-DPAT (Heuring & Peroutka, 1987; Frazer et al., 1990). In membranes prepared at 72-h postinfection, the following rank order of potency was observed: 5-CT > 5-HT ≥ methiothepin > methysergide > sumatriptan > 8-OH-DPAT, and binding constants are indicated in Table I. SCH-23390 displaced [^{3}H]5-HT binding at concentrations >1 μM (data not shown). As shown in Table I, the K_i values for the competing drugs at the expressed m5-HT$_{1B}$ receptor are in good agreement with the values we have obtained for the 5-HT$_{1B}$ receptor expressed in mammalian cells (Jin et al., 1992).

Pertussis Toxin-Mediated [^{32}P]ADP-Ribosylation of Membrane Components from Sf9 Cells. To demonstrate the

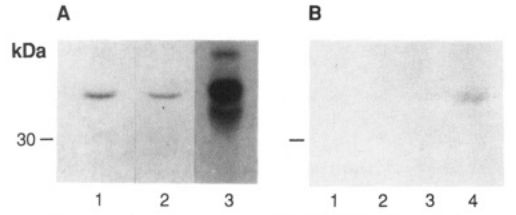

FIGURE 4: Pertussis toxin assays. [^{32}P]ADP-ribosylation reactions were done as described under Experimental Procedures, and proteins were resolved in 12% SDS–PAGE gels under constant current for 18 h. (A) Autoradiograph of [^{32}P]ADP-ribosylated Sf9 membrane proteins resolved by SDS–PAGE analysis, as shown for (lane 1) 100 μg of Sf9 membrane protein, (lane 2) 100 μg of membrane protein from Sf9 cells expressing m5-HT$_{1B}$ receptors, and (lane 3) 2.5 pmol of bovine brain G_i/G_o. Under our assay conditions, no other pertussis toxin substrate could be resolved. Lanes 1 and 2 are autoradiographs from a 2-h exposure, and lane 3 is from a autoradiograph from a 15-min exposure. The data shown are representative of three independent experiments. (B) Autoradiograph of pertussin toxin-mediated [^{32}P]ADP-ribosylated Sf9 membrane proteins from cells pretreated with varying concentrations of pertussis toxin, as follows: lane 1, 1.5 μg/mL; lane 2, 1.0 μg/mL; lane 3, 0.5 μg/mL; lane 4, control, nontreated. The data shown are representative of two independent experiments with 50 μg of protein per lane.

presence of pertussis toxin-sensitive proteins in Sf9 cells, toxin-mediated [^{32}P]ADP-ribosylation was studied. Figure 4A shows an autoradiograph of pertussis toxin-mediated [^{32}P]-ADP-ribosylation of membrane components resolved on SDS–PAGE. A phosphorylated protein band at ~41 kDa was present in membrane from wild-type baculovirus-infected cells and in membranes from cells expressing m5-HT$_{1B}$ receptors. This band migrated identically with the ~41-kDa bovine brain G_i/G_o standard, which was also [^{32}P]ADP-ribosylated by pertussis toxin under the same reaction conditions. No labeling was observed in Sf9 membranes in the absence of pertussis toxin (data not shown). Bands were excised, and liquid scintillation counting was determined the presence of 6.5 ± 0.15 pmol (n = 3) of ADP-ribosylated protein/mg of Sf9 membrane protein. The results clearly indicate the presence of a pertussis toxin substrate in Sf9 membranes.

Pertussis Toxin Sensitivity of Sf9 Cells and Effect on [^{32}P]-ADP-Ribosylation. To determine whether intact Sf9 cells were pertussis toxin-sensitive, cells were incubated with different concentrations of pertussis toxin prior to toxin-catalyzed [^{32}P]ADP-ribosylation experiments on membrane preparations (Figure 4B). Autoradiographs of the pertussis toxin-mediated [^{32}P]ADP-ribosylated proteins resolved on SDS–PAGE show that pretreatment of intact cells with the toxin resulted in a dose-dependent decrease in labeling of the ~41-kDa substrate present in untreated cells. These results show that Sf9 cells are pertussis toxin sensitive and preincubation with a concentration of 1 μg/mL toxin is required to inactivate endogenous pertussis toxin substrates.

Effect of Pertussis Toxin on the Binding of [^{3}H]5-HT. The [^{3}H]5-HT binding isotherm in nontreated Sf9 cells expressing m5-HT$_{1B}$ receptors is best fitted by LIGAND to a two-site model ($p < 0.05$) with mean K_d values and proportion of receptors in high- and low-affinity states of 40 ± 14 nM and 62% and 106 ± 6 nM and 38% (n = 2), respectively. In contrast, the [^{3}H]5-HT binding isotherm in membranes from Sf9 cells expressing m5-HT$_{1B}$ receptors treated with pertussis toxin for 20 h was shifted toward the right and best fitted by LIGAND to a single-affinity site with a K_d of 185 ± 9 nM (n = 2). These results suggest that the m5-HT$_{1B}$ receptor is coupled to a pertussis toxin-sensitive G-protein in Sf9 cells.

m5-HT$_{1B}$ Receptor Coupling to Endogenous Adenylyl Cyclase. The agonist-detected receptor high-affinity state

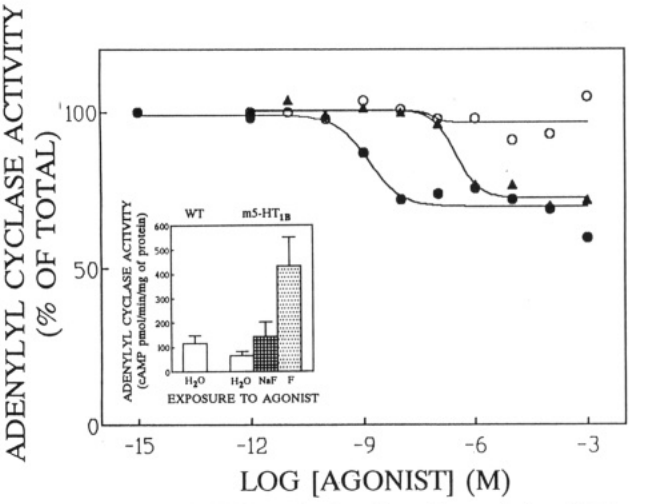

FIGURE 5: Agonist inhibition of adenylyl cyclase activity. (Main figure) effect of increasing concentrations of 5-HT (●) and 8-OH-DPAT (▲) to inhibit basal adenylyl cyclase activity in cells infected with recombinant m5-HT$_{1B}$ baculovirus at 72-h postinfection, and the effect of increasing concentrations of 5-HT (○) on adenylyl cyclase activity in cells infected with wild-type baculovirus. Adenylyl cyclase activity was measured as described under Experimental Procedures and is expressed as percent maximal response for comparison. The data were analyzed using nonlinear least-squares regression. Inset: Basal (H_2O) adenylyl cyclase activities in membranes from wild-type (WT) baculovirus-infected cells and from cells expressing the m5-HT$_{1B}$ receptor, and NaF- and forskolin (F)-stimulated activities are shown. The results shown are representative of three independent experiments.

was sensitive to both guanine nucleotides and pertussis toxin, suggesting that 5-HT$_{1B}$ receptors were coupled to endogenous guanine nucleotide binding proteins. As seen in Figure 5, m5-HT$_{1B}$ receptor activation mediated a 30% inhibition of adenylyl cyclase activity, with a rank order of potency highest for 5-HT followed by 8-OH-DPAT. The IC_{50} values for 5-HT and 8-OH-DPAT inhibition of adenylyl cyclase activity by m5-HT$_{1B}$ receptors were (expressed as an average ± SD) 1 ± 8 nM (n = 2) and 12 ± 3 μM (n = 2), respectively. Adenylyl cyclase activity was not affected by increasing concentrations of 5-HT in cells infected with the wild-type baculovirus (Figure 5). Interestingly, basal adenylyl cyclase activity in membranes from cells expressing the 5-HT$_{1B}$ receptor was 26% lower than in membranes from wild-type baculovirus-infected cells (Figure 5). Thus, the expressed receptors appeared to be fully functional and coupled to an endogenous G-protein.

Biochemical Characterization of the m5-HT$_{1B}$ Receptor. Immunoblot analysis of membranes prepared from cells expressing m5-HT$_{1B}$ receptors, using the anti-c-myc 9E10 monoclonal antibody, specifically identified immunoreactive species of ~42 and ~95 kilodaltons (kDa) (Figure 6A); similar patterns have been identified for other G-protein-coupled receptors expressed in Sf9 cells (Parker et al., 1991; Mouillac et al., 1992; Ng et al., 1993). Since the molecular mass of the 5-HT$_{1B}$ receptor from the deduced amino acid sequence is ~40 kDa (Jin et al., 1992), it is possible that the higher molecular mass immunoreactive species (~95 kDa) represents receptor dimers. Receptor dimer formation has also been suggested for the muscarinic M1 and M2 receptors expressed in Sf9 cells (Parker et al., 1991) and for the cloned metabotropic glutamate receptor expressed in hamster kidney cells (Pickering et al., 1993). Specific immunoreactive material was absent in membranes prepared from cells infected with the wild-type baculovirus or with the untagged 5-HT$_{1B}$ receptors (Figure 6A).

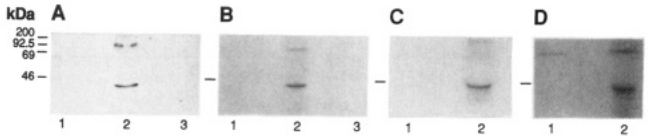

FIGURE 6: Biochemical characterization of m5-HT_{1B} receptors. (A) Two milligrams of protein was solubilized from membranes prepared from wild-type and m5-HT_{1B} baculovirus-infected cells at 72-h postinfection and immunoprecipitated as described under Experimental Procedures. Immunoprecipitated material was photolabeled with [^{125}I]MAB and visualized by autoradiography. Lane 1, sample from cells infected from wild-type baculovirus; lane 2, sample from cells infected with m5-HT_{1B} receptors in the absence and, lane 3, in the presence of 1 mM 5-HT. The autoradiograph shown is from a 7-day exposure and is representative of two independent experiments. The mobility of protein molecular mass standards (Amersham) is indicated on the left. (B) Identification of immunoreactive m5-HT_{1B} receptors in Sf9 membranes. Membranes (200 μg of protein) were prepared at 72-h postinfection from cells infected with wild-type baculovirus (lane 1), cells infected with m5-HT_{1B} baculovirus (lane 2), and cells infected with the untagged 5-HT_{1B} baculovirus (lane 3). Membranes were solubilized in SDS buffer, electrophoresed, and electroblotted onto nitrocellulose as described under Experimental Procedures. Immunoreactivity was revealed with a goat anti-mouse IgG conjugated to alkaline phosphatase. (C) Phosphorylation of m5-HT_{1B} receptors. The figure shows an autoradiogram (7-day exposure) of 9E10-immunoprecipitated m5-HT_{1B} receptor solubilized from membranes prepared from Sf9 cells prelabeled with carrier-free ^{32}P, expressing m5-HT_{1B} receptors (lane 2) or infected with wild-type baculovirus (lane 1). This figure is representative of two independent experiments. (D) Palmitoylation of m5-HT_{1B} receptors. Fluorograph (3-week exposure) following immunoprecipitation from cells prelabeled with [^{3}H]palmitic acid at 72-h postinfection. Shown are a sample from cells infected with recombinant m5-HT_{1B} baculovirus (lane 2), and a sample from cells infected with wild-type baculovirus (lane 1). This figure is representative of four independent experiments.

The specificity of the 9E10 monoclonal antibody for c-myc has previously been well characterized by us in biochemical and desensitization studies utilizing a c-myc epitope-tagged D1 dopamine receptor and a c-myc epitope-tagged β-adrenergic receptor (Ng et al., 1993; Mouillac et al., 1992). We previously showed that the 9E10 antibody specifically recognized and immunoprecipitated over 70% of the c-myc-tagged receptors (Mouillac et al., 1992; Ng et al., 1993). Immunoprecipitated material from cells expressing m5-HT_{1B} receptors was shown to be specifically and reversibly photoaffinity-labeled with the benzazepine [^{125}I]MAB [3-methyl-2-(4-azidophenyl)-2,3,4,5-tetrahydro-2*H*-3-benzazepine] (Figure 6B). Upon exposure to UV light, [^{125}]MAB was specifically photoincorporated into two major protein species at ~42 and ~95 kDa as resolved under reducing conditions using SDS–PAGE. Receptor labeling was prevented by incubation with 1 mM 5-HT and was absent in immunoprecipitated material from cells infected with the wild-type baculovirus (Figure 6A). Thus, both the ~95- and ~42-kDa proteins were capable of binding ligand.

Following metabolic labeling of the cells with [^{32}P]P_i, m5-HT_{1B} receptors were immunoprecipitated using the 9E10 antibody. As shown in Figure 6C, autoradiography of an SDS–PAGE of the immunoprecipitated material revealed a major phosphorylated species of ~42 kDa and a lesser phosphorylated species at ~95 kDa, corresponding to the size of the photolabeled receptors. No phosphorylated immunoreactive proteins were immunoprecipitated from cells infected with the wild-type baculovirus processed in parallel.

Following metabolic labeling with [^{3}H]palmitic acid of Sf9 cells expressing m5-HT_{1B} receptors, immunoprecipitated material was electrophoresed on SDS–PAGE, and subsequent fluorography revealed the presence of ~42 and ~95-kDa radiolabeled bands migrating at sizes equivalent to those of the photolabeled and phosphorylated receptor species (Figure 6D).

DISCUSSION

Using a c-myc epitope-tagged receptor, we have employed the baculovirus/Sf9 cell system to successfully express and study the pharmacology, function, and posttranslational modifications of the human 5-HT_{1B} receptor. Because of the recent cloning of distinct 5HT_{1B} and 5-HT_{1D} receptors in human brain which are pharmacologically indistinguishable and for which no specific antibodies are available, this strategy of epitope-tagging has provided an opportunity to investigate the characteristics of the isolated 5-HT_{1B} receptor, in the absence of the 5-HT_{1D} receptor.

Analysis of the saturation binding isotherm of [^{3}H]5-HT to membranes from Sf9 cells infected with recombinant virus fitted a two-site model, suggesting that the expressed m5-HT_{1B} receptor, detected in two affinity forms, interacted with an endogenous G-protein. This was evidenced by the guanine nucleotide regulation of [^{3}H]5-HT binding to the high-affinity state of the receptor with loss of the high-affinity binding site. The binding of guanine nucleotides to G-proteins is thought to cause dissociation of the G-protein from the receptor, thus converting the receptor to a state with low agonist affinity. Similarly, guanine nucleotide sensitivity of agonist binding has been described for other G-protein-coupled receptors expressed in Sf9 cells (Kwatra et al., 1993; Mills et al., 1993; Ng et al., 1992).

Further confirmation of receptor/G-protein interaction was provided by the decrease (loss) of [^{3}H]5-HT binding to the high-affinity receptor state by pertussis toxin and suggested the presence of a pertussis toxin-sensitive G-protein in Sf9 cells. Pertussis toxin has been shown to ADP-ribosylate and uncouple the α subunits of G_i and G_o from receptors (Sternweis & Robishaw, 1984; Neer et al., 1984; Van Dop et al., 1984). The relationship between 5-HT_{1B} receptors and G-proteins in the brain shows that activation of 5-HT_{1B} receptors inhibits adenylyl cyclase (Bouhelal et al., 1988). The exact identity of the G-protein involved in this coupling has not been determined but is likely to be G_i or G_o. The m5-HT_{1B} receptor expressed in Sf9 cells appears to be coupled to endogenous pertussis toxin-sensitive G-proteins that mediate the inhibition of adenylyl cyclase activity. In fact, we have isolated a partial cDNA clone predicted to encode a G_o protein from Sf9 cells (data not shown). The data suggest that 5-HT_{1B} receptors inhibit adenylyl cyclase activity via a pertussis toxin-sensitive G-protein in Sf9 cells.

In contrast to our findings, Quehenberger et al. (1992) recently reported the expression of an uncoupled recombinant *N*-formyl peptide receptor in Sf9 cells, and were unable to demonstrate the presence of a major G-protein that served as a substrate for pertussis toxin-mediated [^{32}P]ADP-ribosylation in Sf9 cells. However, an important finding of our present study is the definitive identification of a ~41-kDa substrate for pertussis toxin in Sf9 cells that comigrated with partially purified bovine brain $G_{i\alpha}/G_{o\alpha}$ standards. Another group has recently expressed the muscarinic M3 receptor in Sf9 cells and, in confirmation of our findings, has demonstrated electrophysiological evidence for functional M3 receptor coupling to endogenous pertussis toxin-sensitive G-proteins (Vasudevan et al., 1993).

Of the ligands tested for the expressed 5-HT_{1B} receptor, 5-CT (a 5-HT_1 agonist) had the highest affinity and 8-OH-DPAT (a 5-HT_{1A} subtype-selective agonist) had the lowest affinity of the ligands tested. The above data indicated that

the expressed 5-HT$_{1B}$ receptors were pharmacologically similar to the cloned human neuronal 5-HT$_{1B}$ receptors (Jin et al., 1992).

Consensus sequences for PKA and PKC phosphorylation are present in the primary structure of the 5-HT$_{1B}$ receptor (Figure 1), and immunoprecipitated material (comigrating with the photolabeled receptor species) from [^{32}P]P$_i$ metabolically labeled Sf9 cells expressing m5-HT$_{1B}$ receptors was shown to be phosphorylated. Thus, the 5-HT$_{1B}$ receptor is phosphorylated, and this posttranslational modification may be involved in receptor processes such as desensitization.

We have shown that the m5-HT$_{1B}$ receptor in Sf9 cells is posttranslationally modified with palmitic acid. Palmitoylation may involve the cysteine residue (Cys^{388}) in the carboxy tail, since we have previously shown that the equivalent cysteine, Cys^{341}, located in the carboxy tail of the β_2-adrenergic receptor is the site for palmitoylation (O'Dowd et al., 1989). According to the proposed model (Figure 1), anchorage of a palmitate residue attached to Cys^{388} in the receptor would impose a loop structure of the amino-terminal region of the carboxy tail forming a small fourth intracellular loop. For the 5-HT$_{1B}$ receptor, although shown to be palmitoylated, it remains to be determined whether this modification is involved in the mechanisms relating to receptor function.

In summary, we have provided evidence that m5-HT$_{1B}$ receptors are coupled to a major pertussis toxin-sensitive G-protein in Sf9 cells and mediate the inhibition of adenylyl cyclase. We have also provided the first evidence that this serotonin receptor can be purified by immunoprecipitation and posttranslationally modified by phosphate and palmitate residues. The baculovirus/Sf9 cell system has proven to be a useful system to study the biology of this important class of G-protein-coupled receptors.

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