0026-895X/92/030439-06$03.00/0

MOLECULAR PHARMACOLOGY, 42:439-444



# Cloning and Pharmacological Characterization of a Novel Human 5-Hydroxytryptamine$_{1D}$ Receptor Subtype

SARAH A. VELDMAN and MICHAEL J. BIENKOWSKI
*Department of Cell Biology, Upjohn Laboratories, Kalamazoo, Michigan 49007*
Received March 27, 1992; Accepted June 6, 1992

**SUMMARY**

The canine RDC4 gene was used to isolate two distinct human serotonin receptor genes. The receptor encoded by clone RH-6 was the species homolog of RDC4 and was identical to a human serotonin 5-hydroxytryptamine$_{1D}$ (5-HT$_{1D}$) receptor that was recently reported [*Mol. Pharmacol.* **40**:143–148 (1991)]. The receptor encoded by RH-2 was a novel 5-HT receptor that was 61% identical to RH-6 and showed the greatest homology with the rat 5-HT$_{1B}$ receptor sequence (94%). The RH-2 gene contained an intronless, 1170-base pair, open reading frame that encoded a 390-amino acid protein that contained all of the hallmarks of a guanine nucleotide-binding protein-linked receptor. Heterologous expression of the RH-2 gene in Chinese hamster ovary cells led to the appearance of high affinity binding sites for 5-HT ($K_d$ = 2.6 nM, $B_{max}$ = 2.9 pmol/mg of membrane protein), and the receptor expressed in Chinese hamster ovary cells was coupled to inhibition of adenylyl cyclase. Competition binding experiments using compounds that are selective for various 5-HT receptor subtypes showed the highest correlation with a 5-HT$_{1D\text{-like}}$ receptor ($r$ = 0.89) and a low correlation with 5-HT$_{1B\text{-like}}$ receptors. Examples of the 5-HT$_{1D\text{-like}}$ pharmacology displayed by RH-2 include high affinity for the 5-HT$_{1D}$-selective compound sumatriptan ($K_i$ = 9.4 nM) and for the $\alpha_2$-adrenergic receptor antagonist rauwolscine ($K_i$ = 47 nM). Therefore, despite the close genetic relationship between RH-2 and the rat 5-HT$_{1B}$ receptor, our results indicate that the receptor encoded by RH-2 is best classified as a human 5-HT$_{1D}$ receptor subtype and defines a second member of the human 5-HT$_{1D}$ receptor family.

Serotonin (5-HT) is involved in numerous physiological processes in both the central and peripheral nervous systems and in the cardiovascular and gastrointestinal systems (reviewed in Refs. 1–3). 5-HT exerts its physiological effects by interacting with cellular receptors, and a minimum of seven distinct subtypes have been distinguished (4). Based on binding properties, effector coupling mechanisms, and anatomical distribution, 5-HT receptors have been classified into four distinct families, referred to as 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, and 5-HT$_4$ (5). Receptor subtypes within these various families have also been identified (5). Additional information that is useful in classifying 5-HT receptors has been derived from the molecular cloning of these receptor genes. Nucleotide and deduced amino acid sequences are now available for human and rat 5-HT$_{1A}$ (6, 7), rat 5-HT$_{1B}$ (8), human, rat, and mouse 5-HT$_{1C}$ (9–11), human 5-HT$_{1D}$ (12), human, rat, and hamster 5-HT$_2$ (9, 13, 14), and rat 5-HT$_3$ (15) receptor subtypes. In addition to these mammalian receptors, three distinct 5-HT receptor subtypes have been cloned and partially characterized from *Drosophila* (16, 17). These studies have shown that the 5-HT$_1$ and 5-HT$_2$ families are members of the superfamily of receptors that couple to intracellular effector systems via G proteins, whereas the 5-HT$_3$ receptor is a serotonin-gated ion channel.

In addition to these characterized 5-HT receptor subtypes, two orphan receptors, which are genetically most related to the 5-HT$_1$ receptor family, have been cloned (RDC4 and S31) (18, 19). The canine RDC4 receptor was originally noted to exhibit the highest homology with the human 5-HT$_{1A}$ receptor (18); however, a recent report defines it as a 5-HT$_{1D}$ subtype (20). S31 is known to be negatively coupled to adenylyl cyclase, but the details of its pharmacology are not yet known (19). In the present work, we report the cloning of two related human 5-HT$_{1D}$ receptors, which were isolated based on their homology to RDC4, and the pharmacological characterization of one of these receptor clones, RH-2. This receptor binds 5-HT with high affinity and is coupled to inhibition of adenylyl cyclase. Based on its homology to the recently reported rat 5-HT$_{1B}$ receptor (8), this receptor is classified as the species homolog of the rat 5-HT$_{1B}$ receptor. However, despite the close genetic relationship between RH-2 and the rat 5-HT$_{1B}$ receptor, its pharmacological profile correlates best with that of a 5-HT$_{1D}$ receptor. Thus, although the receptor encoded by RH-2 is the

**ABBREVIATIONS:** 5-HT, 5-hydroxytryptamine; G protein, guanine nucleotide-binding protein; PCR, polymerase chain reaction; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; 5-methoxy-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine; 5-CT, 5-carboxyamidotryptamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TM, transmembrane; bp, base pairs; Kb, kilobases; RIA, radioimmunoassay; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CHO, Chinese hamster ovary.

human homolog of the rat 5-$HT_{1B}$ receptor, it defines a novel subtype of the human 5-$HT_{1D}$ receptor family.

## Materials and Methods

A human genomic library in the vector λ FIX II was obtained from Stratagene (La Jolla, CA). Oligonucleotides used for PCR and sequencing were from Oligo's Etc (Gullford, CT) or from D. Riley (Upjohn Laboratories, Kalamazoo, MI). [$\alpha$-$^{32}$P]dATP (3000 Ci/mmol) and [$^3$H] 5-HT (11 Ci/mmol) were from Amersham (Arlington Heights, IL). The GeneAmp DNA amplification kit was from Perkin-Elmer-Cetus (Norwalk, CT), the Sequenase DNA sequencing kit was from United States Biochemicals (Cleveland, OH), and the cAMP RIA kit was from Biomedical Technologies (Stoughton, MA). Plasmid vectors were from Promega (pGEM7) or were a gift from F. Homa (p3CL-Neo) of Upjohn Laboratories. Frozen competent DH5$\alpha$ cells were from Bethesda Research Laboratories (Gaithersburg, MD). All enzymes used in cloning were from New England Biolabs (Beverly, MA). CHO-K1 cells were from the American Type Culture Collection (Rockville, MD). Geneticin (G418) was from GIBCO (Grand Island, NY). Reagents for cell culture were from Irvine Scientific (Santa Ana, CA). 3-Isobutyl-1-methylxanthine, forskolin, unlabeled 5-HT, and dihydroergotamine were obtained from Sigma (St. Louis, MO). The following compounds were obtained from Research Biochemicals Inc. (Natick, MA): 8-OH-DPAT, ritanserin, spiperone hydrochloride, LY-53,857 maleate, pindolol hydrochloride, methysergide maleate, yohimbine hydrochloride, (−)-propanolol hydrochloride, CGS-12066B dimaleate, 5-methoxy-DMT oxalate, quipazine dimaleate, ketanserin (+)-tartrate, (±)-2-methyl-5-HT maleate, 5-CT maleate, and rauwolscine hydrochloride. Sumatriptan was a gift from Glaxo, UK.

**Isolation of RH-2.** The canine RDC4 gene (1.2 kb) was amplified directly from dog genomic DNA, using two primers that flanked the coding sequence and contained unique *Hind*III sites for cloning purposes (CGCTTTAAGCTTGGAAGAGAGAGCCACCTAGA and CGCTTTAAGCTTCCAGAGTCACCAGCAAATCAGA). The PCR amplification was performed as recommended by the manufacturer, using the primers at 1 $\mu$M and 1 $\mu$g of canine genomic DNA/reaction. The reactions were heated to 94° before addition of the *Thermus aquaticus* polymerase (5 units), and the denaturing (1 min at 94°), annealing (2 min at 60°), and extension (3 min at 74°) reactions were run for 25 cycles. The product of the PCR amplification was digested to completion with *Hind*III, cloned into the plasmid vector pGEM7, and verified to be RDC4 by DNA sequence analysis. The 1.2-kb *Hind*III fragment containing the entire coding sequence of RDC4 was labeled by random priming to a specific activity of $1 \times 10^9$ dpm/$\mu$g, using Klenow DNA polymerase and [$\alpha$-$^{32}$P]dATP. Five genomic equivalents of a human genomic λFix II library were screened with the $^{32}$P-labeled RDC4 probe. Replicate Nytran plaque lifts were prehybridized for 2 hr at 65° in 5× sodium phosphate/EDTA (0.75 M sodium chloride, 50 mM sodium phosphate, 5 mM EDTA, pH 7.4), 5× Denhardt's, 0.5% sodium dodecyl sulfate, 0.1 mg/ml salmon sperm DNA. Hybridization was performed overnight in the same buffer, containing $4 \times 10^6$ dpm/ml $^{32}$P-RDC4, at 65°. After hybridization, the filters were washed twice in 2× standard saline citrate/0.1% sodium dodecyl sulfate for 1 hr at 65° and then autoradiographed for 2.5 days at −70°, with intensifying screens. Replicate positive clones were cloned by limiting dilution, followed by rescreening. Individual λ clones were grouped by restriction mapping with *Hind*III and hybridization to RDC4, as described above. The 3.0-kb *Hind*III fragment from clone RH-2 and the 1.3-kb *Hind*III fragment of RH-6 were subcloned into pGEM-7 and completely sequenced on both strands by using the dideoxy chain termination method, with Sequenase (United States Biochemical), using various universal and specific oligonucleotide primers.

**Expression.** The entire coding sequence of RH-2 contained on the 3.0-kb *Hind*III fragment of genomic DNA was subcloned into the mammalian expression vector p3CLNeo, which contains the neomycin resistance gene, under the control of simian virus 40 promoter, and a unique *Hind*III site flanked by the cytomeglovirus immediate early promoter and bovine growth hormone polyadenylation signal. The RH-2 *Hind*III fragment contains approximately 500 bp of 5′ untranslated DNA and 1300 bp of 3′ untranslated sequence. Stable cell lines were generated by transfection of the p3CL-RH-2 construct into CHO K1 cells, using calcium phosphate. CHO K1 cells were maintained in $\alpha$-modified Eagle's medium, 10% heat-inactivated fetal calf serum, 1000 units/ml penicillin G, 100 $\mu$g/ml streptomycin, in 5% $CO_2$. The p3CL-RH-2 plasmid (20 $\mu$g) was precipitated with calcium phosphate in the presence of HEPES-buffered saline and incubated with the cells overnight. Stable transfectants were selected by growth in the presence of G418, and clonal cell lines were isolated by limiting dilution, followed by receptor binding assays using [$^3$H]5-HT. A clone designated CHO7 had the highest receptor density and was chosen for further analysis.

**Ligand binding.** [$^3$H]5-HT was used as a radioligand to detect the expression of RH-2 in membranes prepared from the transfected CHO cells. The cells were grown to approximately 90% confluence, scraped into cold 1× phosphate-buffered saline (137 mM sodium chloride, 2.7 mM potassium chloride, 5.4 mM sodium phosphate, 1.8 mM potassium phosphate), and recovered by centrifugation (600 × *g*, 10 min at 4°). Cell pellets were resuspended in lysis buffer (50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA) and homogenized with a Polytron (2 × 15 sec at full speed), and the membranes were pelleted by centrifugation (20,000 × *g*, 20 min at 4°). The membrane pellets were washed with lysis buffer and repelleted by centrifugation (20,000 × *g*, 20 min at 4°). The final membrane pellet was resuspended to approximately 1 mg of membrane protein/ml, in binding buffer (50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA). Binding reactions contained binding buffer, 50 $\mu$g of membrane protein, 5 mM ascorbate, and 2.5 nM [$^3$H]5-HT, in a final volume of 1.0 ml. Nonspecific binding was determined in the presence of a 1000-fold molar excess of unlabeled 5-HT. The reactions were incubated at 30° for 60 min and terminated by addition of 5 ml of cold 50 mM Tris·HCl (pH 7.5), followed by rapid vacuum filtration through Whatman GF/C filters that had been pretreated with 0.05% polyethyleneimine. After two 10-ml washes with cold 50 mM Tris·HCl (pH 7.5), the filter-bound $^3$H was quantified by liquid scintillation counting. For competition experiments, the unlabeled competitors were added from 100× stocks prepared in either binding buffer or dimethylsulfoxide. $K_d$ and $IC_{50}$ values were calculated from the binding data by using nonlinear least squares analysis, and $IC_{50}$ values were converted to $K_i$ values by using the Cheng-Prusoff relation (21).

**Functional studies.** Coupling of the RH-2 receptor to adenylyl cyclase was measured in intact cells. CHO7 cells were plated in six-well plates, at $5 \times 10^4$ cells/well, and were grown for 5 days. The cells were washed twice with 3 ml of prewarmed $\alpha$-modified Eagle's medium containing 10 mM HEPES and were preincubated in the same medium containing 200 $\mu$M 3-isobutyl-1-methylxanthine, with or without 1 $\mu$M 5-HT, for 5 min at room temperature. Medium containing 100 $\mu$M forskolin or control medium (1.0 ml) was then added, and the incubation was continued for an additional 15 min at room temperature. Reactions were terminated by the addition of 1.0 ml of 25% trichloroacetic acid, and the cAMP content of the various extracts was quantified by RIA, as recommended by the manufacturer.

## Results

A canine orphan G protein-linked receptor gene (RDC4) that exhibits the greatest nucleotide sequence homology with the human 5-$HT_{1A}$ receptor (45% identity) was used as a hybridization probe, to isolate the corresponding human gene. The coding sequence of RDC4 was amplified directly from canine genomic DNA, using the PCR, and the amplification product was the same size predicted by the cDNA. This result was consistent with the absence of introns in the canine coding sequence, so this probe was used to screen a human genomic library under low stringency. Thirty replicate positive clones were obtained from screening of approximately 5 genomic equivalents, suggesting either that the genes are present in

multiple copies and/or that multiple related genes were represented. Clones derived from this screening were grouped by restriction mapping, followed by hybridization to RDC4, and two distinct clones, designated RH-2 and RH-6, were selected for sequence analysis. Genomic clone RH-6 contained a single, intronless, open reading frame (1131 bp), encoding a 377-residue G protein-linked receptor protein that was 88% identical to RDC4 and that was identical to the 5-HT$_{1D}$ receptor sequence recently reported by Hamblin and Metcalf (12) (data not shown). Thus, the RH-6 clone encodes a human 5-HT$_{1D}$ receptor subtype and represents the species homolog of RDC4. The sequence of RH-2 was novel, and its nucleotide sequence and predicted translation are shown in Fig. 1. This clone contained a single, intronless, open reading frame (1170 bp) that predicted a 390-amino acid protein with 61% identity to RH-6. The sequence surrounding the predicted translation start site of RH-2 (agagccATGg) conforms to the consensus start site (gcca/gccATGg) reported by Kozak (22) to be important for initiation. The deduced amino acid sequence encoded by RH-2 contained all of the hallmarks of G protein-linked receptors, including seven hydrophobic domains (predicted membrane-spanning domains), canonical acceptor sites for asparagine-linked glycosylation (residues 24 and 32) near the amino terminus, and acceptor sites for serine/threonine phosphorylation in predicted intracellular loop III (residues 247, 252, 315, and 333). The deduced amino acid sequence of RH-2 was compared with the sequences of other 5-HT$_{1\text{-like}}$ receptors by using the CLUSTAL program (23), and the results are shown in Fig. 2. The RH-2 sequence showed the greatest amino acid identity with the recently reported rat 5-HT$_{1B}$ receptor (94%) and the human 5-HT$_{1D}$ receptor (61%) and lower homology with the human 5HT$_{1A}$ receptor (53%) and the orphan receptor S31 (59%). Based on these homologies, the receptor encoded by RH-2 appears to be best classified as a species homolog of the rat 5-HT$_{1B}$ receptor and possibly a subtype of the human 5-HT$_{1D}$ receptor.

In order to determine the pharmacology of the receptor encoded by RH-2, a 3.0-kb *Hind*III fragment containing the entire coding sequence was cloned into the mammalian expression vector p3CL-Neo, and this construct was used to transfect wild-type CHO-K1 cells (which are devoid of 5-HT receptors). Stable transfectants were isolated by selection in G418, and membranes were prepared from pooled transfectants that exhibited specific binding with [$^3$H]5-HT. Individual cell lines cloned by limiting dilution were selected based on binding of [$^3$H]5-HT, and the cell line exhibiting the highest receptor density (CHO7) was characterized further. A saturation binding isotherm was generated by incubation of washed membranes prepared from CHO7 cells with increasing amounts of [$^3$H]5-HT, in the absence or presence of a 1000-fold molar excess of unlabeled 5-HT, and these results are shown in Fig. 3. Nonlinear least squares curve-fitting of the saturation binding iso-

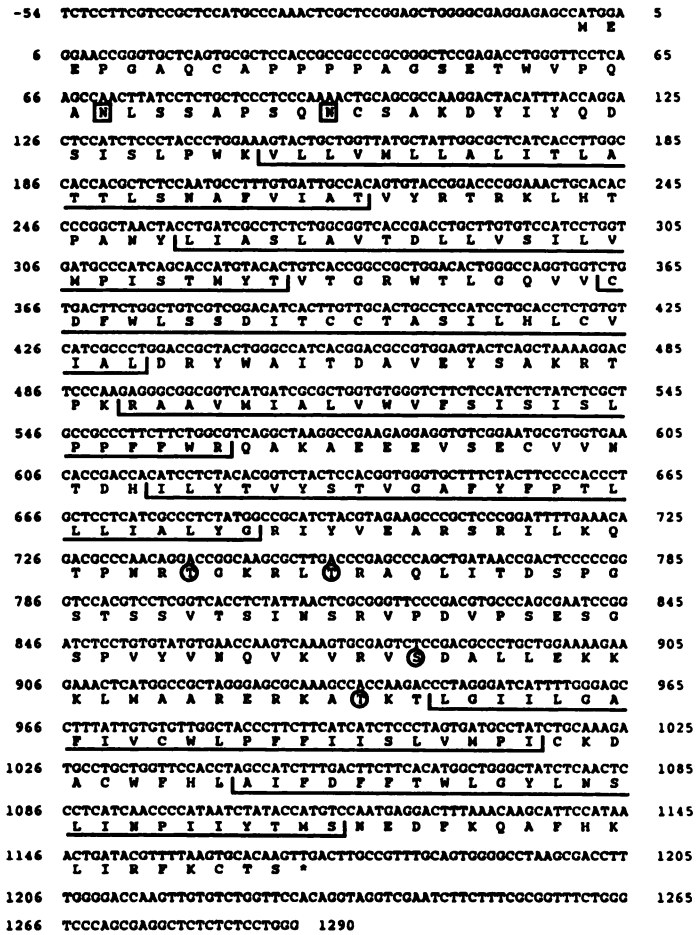

**Fig. 1.** Nucleotide and deduced amino acid sequences for clone RH-2. The numbering of the nucleotides is shown on the *left and right of each line*. *Boxed amino acids*, potential *N*-linked glycosylation sites; *circles*, potential phosphorylation sites for protein kinase A and protein kinase C. The seven predicted membrane-spanning domains are *underlined*.

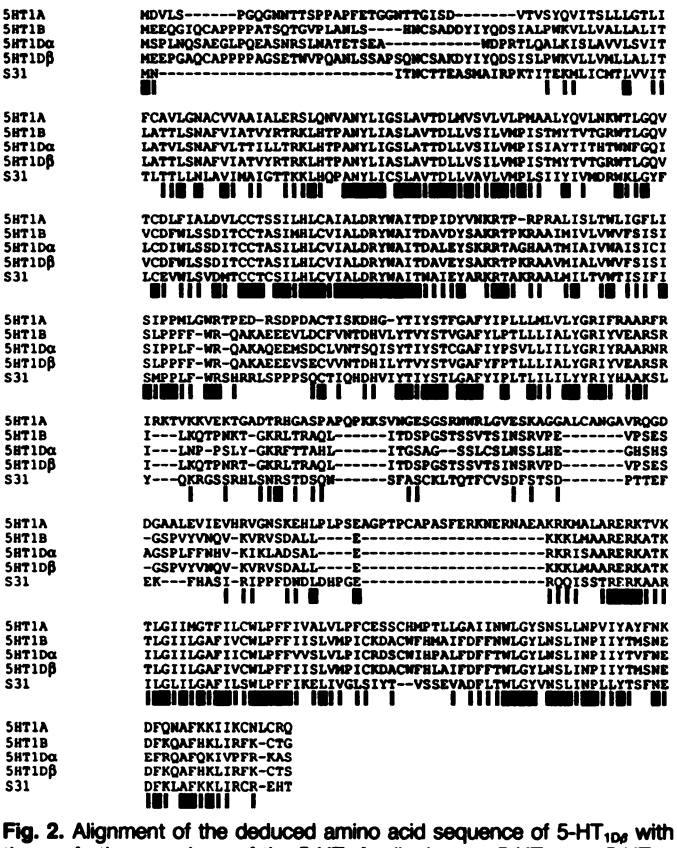

**Fig. 2.** Alignment of the deduced amino acid sequence of 5-HT$_{1D\beta}$ with those of other members of the 5-HT$_1$ family, human 5-HT$_{1A}$, rat 5-HT$_{1B}$, human 5-HT$_{1D\alpha}$, and human S31. *Solid bars*, identical residues between 5-HT$_{1D\beta}$ and the other four receptors; *double lines*, conservative substitution between these receptors. The sequences of 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D\alpha}$, and S31 were from Refs. 6, 8, 12, and 19, respectively.

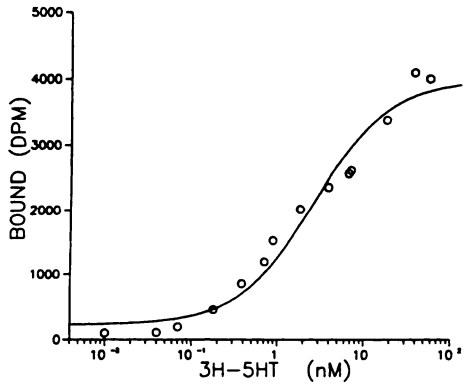


**Fig. 3.** Determination of the $K_d$ of [$^3$H]5-HT for CHO7 cells. Saturation binding analysis was performed using increasing concentrations of [$^3$H] 5-HT in the presence of a 1000-fold excess of unlabeled 5-HT, and the data were fitted to a single-site model. The *x*-axis represents the concentration of [$^3$H]5-HT used in the assay, and the *y*-axis represents the dpm of [$^3$H]5-HT specifically bound to membranes.

therm data best fit a one-site model, with a $K_d$ of 2.6 nM and a $B_{max}$ of 2.9 pmol/mg of membrane protein.

The pharmacology of the RH-2 site was investigated further, with competitive binding assays using compounds that are selective for various serotonin receptor subtypes. These results are summarized in Table 1, where the $K_i$ values obtained for CHO7 cells are compared with values for various 5-HT receptor subtypes derived from binding experiments performed using mammalian tissues (24–26). Membranes prepared from CHO7 cells were incubated with [$^3$H]5-HT in the presence of increasing concentrations of the unlabeled competitors, and binding was quantified by liquid scintillation counting. Competition curves were fitted by using nonlinear least squares analysis, and $IC_{50}$ values were converted to $K_i$ values by using the Cheng-Prusoff equation (21). The relatively nonselective 5-HT$_1$ compounds 5-HT and 5-CT showed high affinity for the RH-2 site, with $K_i$ values of 1.1 and 0.26 nM, respectively. The 5-HT$_{1A}$-selective compound 8-OH-DPAT and the 5-HT$_{1A}$/5-HT$_{1B}$/$\beta$-adrenergic-selective compounds propranolol and pindolol showed low affinity for the RH-2 site, with $K_i$ values of >2000 nM. In contrast, the 5-HT$_{1D}$-selective compounds sumatriptan, CGS-12066B, and 5-methoxy-DMT all showed high affinity for the RH-2 site, with $K_i$ values of 9.4, 9.9, and 23.2 nM, respectively. Furthermore, the $\alpha_2$-adrenergic receptor antagonist rauwolscine bound RH-2 with relatively high affinity ($K_i$ = 47 nM). Finally, compounds specific for 5-HT$_{1C}$/5-HT$_2$ receptors (ketanserin, ritanserin, spiperone, and LY-53,857) or for 5-HT$_3$ receptors (2-methyl-5-HT) all had $K_i$ values of >500 nM. The results presented in Table 1 demonstrate that the pharmacology of RH-2 shows the highest correlation with that of 5-HT$_{1D}$ receptors ($r$ = 0.89, compared with human brain) and, taken together with the sequence homology with the 5-HT$_{1D}$ receptor, suggest that RH-2 is best classified as a 5-HT$_{1D}$ receptor subtype.

Coupling of the RH-2 receptor to intracellular effector systems was investigated in CHO7 cells, and the results are shown in Fig. 4. Cells were treated with 5-HT, forskolin, or 5-HT plus forskolin, and total cellular cAMP was quantified by RIA. Treatment with 5-HT alone did not increase basal levels of cAMP, indicating that the receptor is not positively coupled to adenylyl cyclase. Forskolin stimulation resulted in a 17-fold elevation of cAMP levels, and treatment with 5-HT inhibited forskolin-stimulated adenylyl cyclase activity by 76%, demonstrating that the RH-2 receptor is negatively coupled to adenylyl cyclase in CHO cells.

## Discussion

The 5-HT$_{1D}$ receptor was originally identified, in bovine brain, as being distinct from 5HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1C}$ subtypes and was operationally defined as a population of binding sites that bound [$^3$H]5-HT in the presence of 100 nM 8-OH-DPAT and 100 nM mesulergine (27). 5-HT$_{1D}$ binding sites have been shown to be widely distributed in brain, as measured by both radioligand binding (27) and receptor autoradiography (28). 5-HT$_{1D}$ sites in human brain are enriched in the substantia nigra, the basal ganglia, and the dorsal subiculum and show a distribution very similar to that of the 5-HT$_{1B}$ sites in rat brain (29). Based on their similar distribution and pharmacology, the rodent 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes have long been considered species homologs. The results presented here provide a molecular basis for such a classification and also further subdivide the human 5-HT$_{1D}$ subtype. The deduced amino acid sequence of RH-2 is 94% identical to the rat 5-HT$_{1B}$ sequence. The amino acid sequence identity is highest in the predicted membrane-spanning domains but also occurs over the remainder of the sequence, indicating that these receptors are species homologs. Further, 5-HT$_{1D}$ and 5-HT$_{1B}$ have been shown to be negatively coupled to adenylyl cyclase in bovine (30) and rat (31) substantia nigra, respectively, and the RH-2 receptor expressed in CHO cells was coupled to inhibition of adenylyl cyclase.

The distinction between these two receptor subtypes is apparent when the binding specificities of compounds that distinguish between 5-HT$_{1D}$ and 5-HT$_{1B}$ are compared. Although there are no ligands that specifically bind to either the 5-HT$_{1D}$ or 5-HT$_{1B}$ receptor subtype, several trends have been documented in the literature. 5-HT$_{1B}$ receptors exhibit relatively high affinity for the $\beta_2$-adrenergic receptor antagonists pindolol and propranolol, with approximately 1000- and 100-fold selectivity for 5-HT$_{1B}$ over 5-HT$_{1D}$, respectively (24, 32). Conversely, 5-HT$_{1D}$ receptors bind the $\alpha_2$-adrenergic receptor antagonist rauwolscine with relatively high affinity, with approximately a 250-fold selectivity for 5-HT$_{1D}$ receptors, compared with 5-HT$_{1B}$ receptors (24, 32). Consistent with these trends, the binding affinity of rauwolscine for the receptor encoded by RH-2 is 106-fold higher than the reported values for 5-HT$_{1B}$ receptors, whereas pindolol and propranolol have affinities that are 522- and 54-fold lower, respectively, than the values reported for 5-HT$_{1B}$ receptors (Table 1). These results, taken together with the current dogma that 5-HT$_{1B}$ receptors are rodent specific, indicate that the RH-2 gene encodes a human 5-HT$_{1D}$ subtype, and we refer to it as 5-HT$_{1D\beta}$, because it was the second human 5-HT$_{1D}$ receptor to be cloned and characterized. The receptor encoded by the RH-6 gene is identical to the human 5-HT$_{1D}$ receptor recently described by Hamblin and Metcalf (12) and has been designated the 5-HT$_{1D\alpha}$ receptor. Comparison of the binding affinities of compounds at the 5-HT$_{1D\alpha}$ versus the 5-HT$_{1D\beta}$ receptor site (Table 1 and Ref. 12) reveals that, despite their relatively low degree of sequence homology, these receptors exhibit very similar pharmacological profiles and should be classified as 5-HT$_{1D}$ receptor subtypes. Additional evidence in support of the classification of the receptor encoded by RH-2 as a 5-HT$_{1D}$ receptor is its high affinity for sumatrip-

TABLE 1

**Summary of drug affinities for cloned human RH-2 receptor**

Apparent dissociation constants ($K_i$ values) of serotonergic ligands for the cloned human RH-2 receptor are shown in comparison with $K_i$ values for other 5-HT$_1$ receptors, determined in mammalian tissue (24–26). Competition binding was performed using [$^3$H]5-HT in the presence of increasing amounts of unlabeled competitors. $IC_{50}$ values were generated using computer-assisted linear least squares analysis and were fit to a single class of binding sites. $IC_{50}$ values obtained in this fashion were converted to $K_i$ values by using the Cheng-Prussoff equation (21).

| Selectivity | Drug | $K_i$ | | | |
|---|---|---|---|---|---|
| | | RH-2 | 5-HT$_{1D}$[a] | 5-HT$_{1B}$[a] | 5-HT$_{1A}$[a] |
| | | | nM | | |
| | 5-HT | 1.1 | 6.4[b] | 25.0 | 3.0 |
| | 5-CT | 0.26 | 0.93[b] | 5.0 | 0.32 |
| 5-HT$_{1A}$ | 8-OH-DPAT | 2,800 | 1,260[b] | 63,095 | 1.0 |
| 5-HT$_{1B}$ | Propranolol | 2,720 | 5,500[b] | 50.0 | 158.0 |
| | Pindolol | 7,842 | 16,200[b] | 15.0 | 19.0 |
| 5-HT$_{1D}$ | 5-Methoxy-DMT | 23.2 | 32.0[c] | 1,260[c] | |
| | Sumatriptan | 9.4 | 31.0[c] | 398[c] | 794[c] |
| | CGS-12066B | 9.9 | 2.9[c] | 130[c] | |
| | Methysergide | 163.0 | 3.0 | 1,584 | 25 |
| | Rauwolscine | 47.0 | 759.0[b] | 5,011 | 126 |
| | Dihydroergotamine | 2.3 | 19.0 | 0.8 | 1.3 |
| 5-HT$_2$/5-HT$_{1C}$ | Ketanserin | >10,000 | 50,000[b] | 1,995 | 1,258 |
| | LY-53,857 | >1,000 | | 3,160 | 398 |
| | Ritanserin | >1,000 | 1,584 | >10,000 | 6,310 |
| | Spiperone | >10,000 | 5,010 | 5,010 | 63 |
| 5-HT$_3$ | 2-Methyl-5-HT | 514 | 398[c] | >10,000[c] | |
| | Quipazine | >1,000 | 3,720[b] | 316 | 3,160 |

[a] From Hoyer and Schoeffter (24).
[b] From Waeber *et al.* (26).
[c] From Hoyer (25).

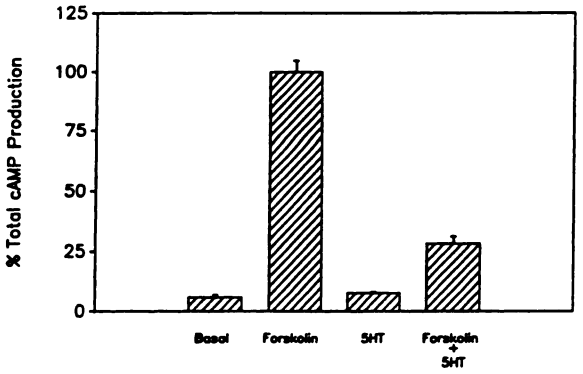


**Fig. 4.** Inhibition of cAMP accumulation by 5-HT in cells stably expressing 5-HT$_{1D\beta}$ (CHO7 cells). cAMP measurements on intact cells were performed as described in Materials and Methods. Forskolin and 5-HT were used at final concentrations of 10 $\mu$M and 1 $\mu$M, respectively. The reaction conditions are indicated *below each bar*, and basal values reflect cAMP accumulation in the absence of forskolin or added drug. Data represent mean values ± standard deviations from triplicate determinations derived from a representative experiment.

tan, a compound currently regarded as a 5-HT$_{1D}$-selective agonist. After the completion of this work, the relationship between the rat 5-HT$_{1B}$ receptor sequence and a human 5-HT$_{1D}$ receptor subtype was reported (33). However, neither the nucleotide sequence nor the pharmacology of the human 5-HT$_{1D}$ receptor was presented.

A partial explanation for the difference in the binding properties between the rat 5-HT$_{1B}$ receptor and its human species homolog 5-HT$_{1D\beta}$ may be predicted from a recent study concerning site-specific mutagenesis of the human 5-HT$_{1A}$ receptor (34). Mutation of Asn-385, which is present in TM-VII of the human 5-HT$_{1A}$ receptor, resulted in a mutant receptor that had binding affinities for agonists similar to those of the wild-type receptor but that had a 100-fold lower affinity for the antagonist pindolol. Alignment of the sequences for TM-VII in the 5-HT$_1$ family (Fig. 2) reveals that both the human 5-HT$_{1A}$ and rat 5-HT$_{1B}$ sequences have asparagine at a similar position, whereas the 5-HT$_{1D}$ subtypes and S-31 all have threonine at that position. Clearly, Asn-385 is important in antagonist binding; however, differences in agonist binding suggest that there are other differences between these receptors. This result represents an unusual pharmacological distinction between species homologs and underscores the importance of cloning the human receptors if development of drugs for human use is the ultimate goal.

The original classification of the 5-HT$_1$ receptor family was based on high receptor affinity for 5-HT (5). Molecular cloning of the receptors in this family has added another dimension to this classification system. The members of this family currently include the 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D\alpha}$, 5-HT$_{1D\beta}$, and recently reported S31 genes (19). These receptor sequences have several features in common, and these features provide an additional basis for inclusion of receptor sequences into the 5-HT$_1$ family. First, all five receptors are encoded by genes that are intronless over their coding sequence. Second, they all have relatively large predicted intracellular loops between TM-V and TM-VI (approximately 90 residues) and short predicted carboxyl-terminal tails (approximately 18 residues). The latter features are peculiar to receptors that couple to inhibition of adenylyl cyclase and, indeed, all five of these 5-HT$_1$ receptors couple negatively to adenylyl cyclase. Also, all five of these receptors exhibit the highest degree of sequence homology with other 5-HT$_1$ family members and significantly less homology with other 5-HT receptors, such as those defined by the 5-HT$_2$ family (5-HT$_2$ and 5-HT$_{1C}$ receptors). In an effort to elucidate features that are unique to 5-HT$_1$ receptors, we compared the amino acid sequences of these five distinct 5-HT$_1$ receptors with those of other members of the 5-HT family. In the comparison, we

scored residues that are absolutely conserved in all 5-$HT_1$ receptors but that are divergent in the 5-$HT_{1C}$/5-$HT_2$ family. Examples of residues that satisfy these criteria include a cysteine residue in TM-III that is changed to a phenylalanine in both 5-$HT_{1C}$ and 5-$HT_2$, two arginines, an isoleucine, and an alanine that are found in intracellular loop III of 5-$HT_1$ receptors and are deleted in both 5-$HT_{1C}$ and 5-$HT_2$, and an asparagine residue in TM-VII that is changed to a cysteine in 5-$HT_{1C}$ and to a serine in 5-$HT_2$. The importance of these residues in receptor function is unknown but can now be tested experimentally, using the cloned receptors. The availability of uniform populations of appropriately coupled serotonin receptors prepared by heterologous expression can play an important role in the design and evaluation of subtype-specific compounds. In addition, access to cloned receptors will allow the distinction between 5-$HT_{1D}$ receptors that have been pharmacologically characterized in both the central nervous system and cardiovascular systems.

**Acknowledgments**

We would like to thank Mary Shuck for providing the dog genomic DNA and the final sequence of RH-6 and Jim McGuire for helping with the adenylyl cyclase assay.

**References**

1. Leysen, J. E., and P. J. Pauwels. 5-$HT_2$ receptors, roles and regulation. *Ann. N. Y. Acad. Sci.* **600**:183–193 (1990).
2. Fozard, J. R. *The Peripheral Effects of 5-Hydroxytryptamine*. Oxford University Press, Oxford, UK (1989).
3. Sanders-Bush, E. *The Serotonin Receptors*. Humana Press, Clifton, NJ (1989).
4. Frazer, A. S., S. Maayani, and B. B. Wolfe. Subtypes of receptors for serotonin. *Annu. Rev. Pharmacol. Toxicol.* **30**:307–348 (1990).
5. Peroutka, S. J., A. W. Schmidt, A. J. Sleight, and M. A. Harrington. Serotonin receptor "families" In the central nervous system: an overview. *Ann. N. Y. Acad. Sci.* **600**:104–113 (1990).
6. Kobilka, B. K., T. Frielle, S. Collins, T. Yang-Feng, T. S. Kobilka, U. Francke, R. J. Lefkowitz, and M. G. Caron. An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. *Nature* (*Lond.*) **329**:75–79 (1987).
7. Albert, P. R., Q. Y. Zhou, H. H. M. Von Tol, J. R. Bunzow, and O. Civelli. Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine$_{1A}$ receptor gene. *J. Biol. Chem.* **265**:5825–5832 (1990).
8. Voigt, M. M., D. J. Laurie, P. H. Seeburg, and A. Bach. Molecular cloning and characterization of a rat brain cDNA encoding a 5-hydroxytryptamine$_{1B}$ receptor. *EMBO J.* **10**:4017–4023 (1991).
9. Saltzman, A. G., B. Morse, M. M. Whitman, Y. Ivanshchenko, M. Jaye, and S. Felder. Cloning of the human serotonin 5-$HT_2$ and 5-$HT_{1C}$ receptor subtypes. *Biochem. Biophys. Res. Commun.* **181**:1469–1478 (1991).
10. Julius, D., A. B. MacDermott, R. Axel, and T. M. Jessell. Molecular characterization of a functional cDNA encoding the serotonin 1C receptor. *Science* (*Washington D. C.*) **241**:558–564 (1988).
11. Yu, L., H. Nguyen, H. Le, L. J. Bloem, C. A. Kozak, B. J. Hoffman, T. P. Snutch, H. A. Lester, N. Davidson, and H. Lubbert. The mouse 5-$HT_{1C}$ receptor contains eight hydrophobic domains and is X-linked. *Mol. Brain Res.* **11**:143–149 (1991).
12. Hamblin, M. W., and M. A. Metcalf. Primary structure and functional characterization of a human 5-$HT_{1D}$-type serotonin receptor. *Mol. Pharmacol.* **40**:143–148 (1991).
13. Pritchett, D. B., A. W. J. Bach, M. Wozny, O. Taleb, R. Dal Toso, J. C. Shih, and P. H. Seeburg. Structure and functional expression of cloned rat serotonin 5-$HT_2$ receptor. *EMBO J.* **7**:4135–4140 (1988).
14. Chambard, J. C., E. Van Obberghen-Schilling, R. J. Haslam, V. Vouret, and J. Pouyssegur. Chinese hamster serotonin (5-HT) type 2 receptor cDNA sequence. *Nucleic Acids Res.* **18**:5282 (1990).
15. Maricq, A. V., A. S. Peterson, A. J. Blake, R. M. Myers, and D. Julius. Primary structure and functional expression of the 5-$HT_3$ receptor, a serotonin-gated ion channel. *Science* (*Washington D. C.*) **254**:432–437 (1991).
16. Witz, P., N. Amlaiky, J. L. Plassat, L. Maroteaux, E. Borrelli, and R. Hen. Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **87**:8940–8944 (1990).
17. Saudou, F., U. Boschert, N. Amlaiky, J. L. Plassat, and R. Hen. A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. *EMBO J.* **11**:7–17 (1992).
18. Libert, F., M. Parmentier, A. Lefort, C. Dinsart, J. Van Sande, C. Maenhaut, M. J. Simons, J. Dumont, and G. Vassart. Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science* (*Washington D. C.*) **244**:569–572 (1989).
19. Levy, F. O., T. G. Gudermann, M. Birnbaumer, A. J. Kaumann, and L. Birnbaumer. Molecular cloning of a human gene (S31) encoding a novel serotonin receptor mediating inhibition of adenyl cyclase. *FEBS Lett.* **296**:201–206 (1992).
20. Zgombick, J. M., R. L. Weinshank, M. Macchi, L. E. Schechter, T. A. Branchek, and P. R. Hartig. Expression and pharmacological characterization of a canine 5-hydroxytryptamine$_{1D}$ receptor subtype. *Mol. Pharmacol.* **40**:1036–1042 (1991).
21. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
22. Kozak, M. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNA's. *Nucleic Acids Res.* **15**:8125–8148 (1987).
23. Higgins, D. G., and P. M. Sharp. CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. *Gene* **73**:237–244 (1988).
24. Hoyer, D., and P. Schoeffter. 5-HT receptors: subtypes and second messengers. *J. Recept. Res.* **11**:197–214 (1991).
25. Hoyer, D. Biochemical mechanisms of 5-HT receptor-effector coupling in peripheral tissues, in *The Peripheral Actions of 5-Hydroxytryptamine* (J. Fozard, ed.). Oxford University Press, Oxford, UK, 72–99 (1989).
26. Waeber, C., P. Schoeffter, D. Hoyer, and J. M. Palacios. The serotonin 5-$HT_{1D}$ receptor: a progress review. *Neurochem. Res.* **15**:567–582 (1990).
27. Heuring, R. E., and S. J. Peroutka. Characterization of a novel $^3$H-5-hydroxytryptamine binding site subtype in bovine brain membranes. *J. Neurosci.* **7**:894–903 (1987).
28. Waeber, C., M. M. Dietl, D. Hoyer, A. Probst, and J. M. Palacios. Visualization of a novel serotonin recognition site (5-$HT_{1D}$) in human brain by autoradiography. *Neurosci. Lett.* **88**:11–16 (1988).
29. Palacios, J. M., C. Waeber, D. Hoyer, and G. Mengod. Distribution of serotonin receptors. *Ann. N. Y. Acad. Sci.* **600**:36–53 (1990).
30. Schoeffter, P., C. Waeber, J. M. Palacios, and D. Hoyer. The 5-hydroxytryptamine 5-$HT_{1D}$ receptor subtype is negatively coupled to adenylate cyclase in rat substantia nigra. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **337**:602–608 (1988).
31. Bouhelal, R., L. Smounya, and J. Bockaert. 5-$HT_{1B}$ receptors are negatively coupled with adenylate cyclase in rat substantia nigra. *Eur. J. Pharmacol.* **151**:189–196 (1988).
32. Schoeffter, P., and D. Hoyer. 5-Hydroxytryptamine 5-$HT_{1B}$ and 5-$HT_{1D}$ receptors mediating inhibition of adenylate cyclase activity. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**:285–292 (1989).
33. Adham, N., P. Romanienko, P. Hartig, R. L. Weinshank, and T. Branchek. The rat 5-hydroxytryptamine$_{1B}$ receptor is the species homologue of the human 5-hydroxytryptamine$_{1D\beta}$ receptor. *Mol. Pharmacol.* **41**:1–7 (1991).
34. Guan, X. M., B. K. Kobilka, and S. J. Peroutka. Alteration of ligand binding profiles of the 5-$HT_{1A}$ receptor with a single point mutation. *Soc. Neurosci. Abstr.* **17**:720 (1991).

**Send reprint requests to:** Michael J. Bienkowski, 301 Henrietta, Kalamazoo, MI 49007.