Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3630–3634, April 1992
Pharmacology

Human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$

RICHARD L. WEINSHANK*, JOHN M. ZGOMBICK, MARY J. MACCHI, THERESA A. BRANCHEK, AND PAUL R. HARTIG

Synaptic Pharmaceutical Corporation,† Paramus, NJ 07652

Communicated by Eric R. Kandel, January 17, 1992 (received for review September 16, 1991)

ABSTRACT **The serotonin 1D (5-HT_{1D}) receptor is a pharmacologically defined binding site and functional receptor site. Observed variations in the properties of 5-HT_{1D} receptors in different tissues have led to the speculation that multiple receptor proteins with slightly different properties may exist. We report here the cloning, deduced amino acid sequences, pharmacological properties, and second-messenger coupling of a pair of human 5-HT_{1D} receptor genes, which we have designated 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ due to their strong similarities in sequence, pharmacological properties, and second-messenger coupling. Both genes are free of introns in their coding regions, are expressed in the human cerebral cortex, and can couple to inhibition of adenylate cyclase activity. The pharmacological binding properties of these two human receptors are very similar, and match closely the pharmacological properties of human, bovine, and guinea pig 5-HT_{1D} sites. Both receptors exhibit high-affinity binding of sumatriptan, a new anti-migraine medication, and thus are candidates for the pharmacological site of action of this drug.**

The serotonin 1D (5-HT_{1D}) receptor was initially characterized by radioligand binding procedures using membranes derived from the bovine caudate nucleus (1). In physiological assays, the 5-HT_{1D} receptor subtype has been shown to modulate vascular tone (2) and to inhibit neurotransmitter release through its role as a presynaptic heteroreceptor (3, 4) or as a terminal autoreceptor (5). The 5-HT_{1D} receptor is known to be a G protein-coupled receptor based upon its functional coupling to adenylate cyclase inhibition (6) and the sensitivity of high-affinity agonist ([^{3}H]5-HT) binding to guanine nucleotides (7). Sumatriptan, the only 5-HT_{1D}-selective ligand yet identified (8, 9), has been reported to effectively treat acute migraine (10).

We have recently identified a previously cloned G protein-coupled receptor that displayed high homology to the 5-HT_{1A} receptor, RDC4 (11), as a canine 5-HT_{1D} receptor subtype (12). In the current study, we have used this RDC4 gene as a probe for the isolation of its human homolog from a human hippocampus cDNA library. As described below, this human clone has been sequenced, transfected, and characterized and has been shown to encode a human 5-HT_{1D} receptor subtype. A preliminary report describing some properties of this clone has been published (13). Subsequently, a report appeared describing a human 5-HT_{1D} receptor clone that is very similar in pharmacological properties (14). We have designated this first human 5-HT_{1D} receptor gene, which is gene RDC4 in the dog, as 5-$HT_{1D\alpha}$. In addition, we have cloned a highly homologous, yet distinct, 5-HT_{1D} receptor (5-$HT_{1D\beta}$) from a human genomic library. These two 5-HT_{1D} subtypes represent two closely related members of a subfamily of 5-HT_{1D} receptors.‡

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MATERIALS AND METHODS

Cloning and Sequencing. A human placental genomic library (Stratagene) was screened with the 1.3-kilobase (kb) *Hin*dIII–*Sph* I genomic DNA fragment from the canine RDC4 clone (12). The probe was labeled with ^{32}P by random priming (15). Hybridization and Southern blot analysis were performed as described (16). For subcloning and further Southern blot analysis, DNA was inserted into plasmid pUC18. cDNA clones were isolated by screening a human hippocampal λZAPII cDNA library (Stratagene) with an oligonucleotide probe derived from the canine RDC4 sequence (17) (corresponding to amino acids 165–188 in transmembrane region IV). Overlapping oligomers were labeled with [^{32}P]dATP and [^{32}P]dCTP by synthesis with the large fragment of DNA polymerase. Hybridization was performed as described (16). DNA fragments cloned in the λZAPII vector were excised by helper phage and recircularized to generate subclones in the pBluescript (Stratagene) phagemid vector. Nucleotide sequence analysis was done by the dideoxynucleotide chain-termination method (18) on denatured double-stranded plasmid templates with Sequenase (United States Biochemical).

Expression. The entire coding region of the 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor gene was subcloned into the expression vector pcEXV3 (16) or pSVL (Pharmacia). Stable cell lines were generated by cotransfection with pcEXV3-5-$HT_{1D\alpha}$ or pSVL-5-$HT_{1D\beta}$ and pGCcos3neo, using the calcium phosphate method. Cells were grown as monolayers and selected for antibiotic resistance (16). Stable transfectants were screened to specifically bind [^{3}H]5-HT, and two clones, 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$, were selected for pharmacological characterization. No specific binding of [^{3}H]5-HT to membranes from sham-transfected cells was detected. Cells were grown as monolayers to 100% confluency before membranes were harvested for binding assays (16). Freshly prepared membranes for radioligand binding assays were prepared from stably transfected cells (12).

Radioligand Binding Studies. Binding studies were conducted with [^{3}H]5-HT (24–30 Ci/mmol; DuPont-NEN; 1 Ci = 37 GBq) as the radioligand, and 5-HT_{1D} assay conditions in the absence of masking ligands were as described (12). Occupancy studies were performed with [^{3}H]5-HT concentrations ranging from 0.25 to 50 nM, and competition studies were performed with 5 nM [^{3}H]5-HT. Binding data were analyzed by computer-assisted nonlinear regression analysis (ACCUFIT and ACCUCOMP, Lundon Software, Chagrin Falls, OH). IC_{50} values were converted to K_i values by using the Cheng–Prusoff equation (19). Drugs were obtained as described (12).

Abbreviation: 5-HT, serotonin (5-hydroxytryptamine).

*To whom reprint requests should be addressed.
†Formerly Neurogenetic Corporation.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M81589 and M81590).

Functional Studies. Intracellular cAMP levels were determined (12) by radioimmunoassay (Advanced Magnetics, Cambridge, MA). Dose–response curves for 5-HT and sumatriptan were obtained by using Y-1 cells stably transfected with the 5-$HT_{1D\beta}$ receptor gene. Functional response curves were fit to a four-parameter logistic equation to obtain EC_{50} and E_{max} values (GraphPAD InPlot, San Diego, CA).

RESULTS

We have screened a human genomic placental library (Stratagene) with a 1.3-kb *Hind* III–*Sph* I restriction fragment derived from the canine genomic clone RDC4 (12). Two of the clones obtained, hp8 and hp84, were nonoverlapping partial clones that represented human homologs of the canine RDC4 receptor. A human hippocampus cDNA library was subsequently screened with an oligonucleotide probe derived from transmembrane region IV of the canine clone RDC4. Two overlapping clones were isolated and characterized by DNA sequence analysis. Complementary portions of the clones were then ligated at an internal *Bgl* II restriction site (corresponding to nucleotides 624–629) to obtain a full-length clone, designated 5-$HT_{1D\alpha}$. Overall, 88% amino acid sequence conservation was observed between the dog RDC4 sequence and the human receptor over 377 amino acids. An additional genomic clone, hp11, was also isolated and characterized. A comparison of the deduced amino acid sequence of clone hp11 with previously characterized neurotransmitter receptors indicates that the protein product of hp11 is a member of the G protein-coupled receptor family. Clone hp11 contains an uninterrupted open reading frame that encodes a protein of 390 amino acids.

Amino acid sequence comparison shows that hp11 is closely related to, but distinct from, the 5-$HT_{1D\alpha}$ receptor (Fig. 1). The overall amino acid identity between 5-$HT_{1D\alpha}$ and hp11 is 63%, with 77% amino acid identity within the transmembrane regions alone. This high amino acid homology, particularly in the transmembrane regions, is characteristic of

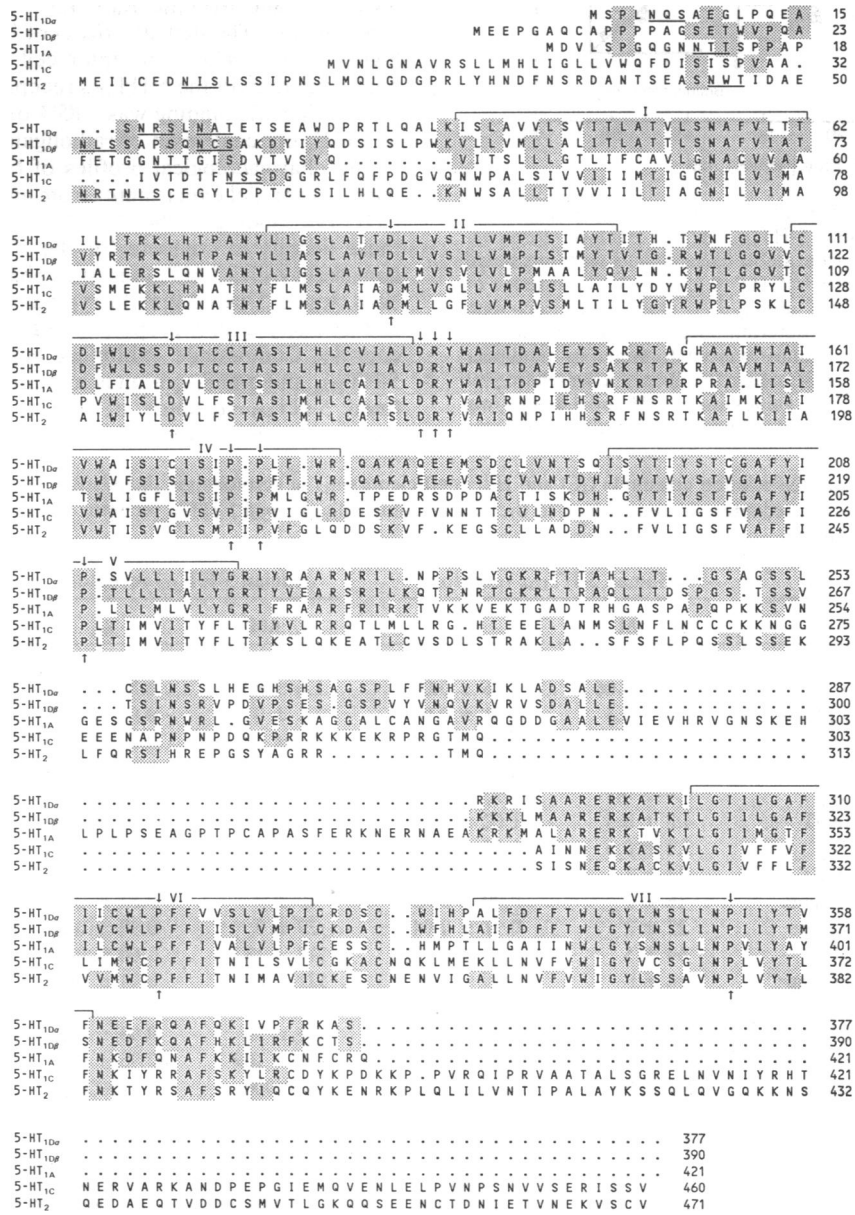

FIG. 1. Comparison of the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor deduced amino acid sequences with those of other serotonin receptors. The seven putative membrane-spanning domains (I–VII) are indicated by overbars. Homologies between the 5-$HT_{1D\beta}$ receptor and other receptors are noted by shading. In the amino-terminal region, consensus sequences for N-linked glycosylation sites are underlined. Selected invariant residues are highlighted by arrows.

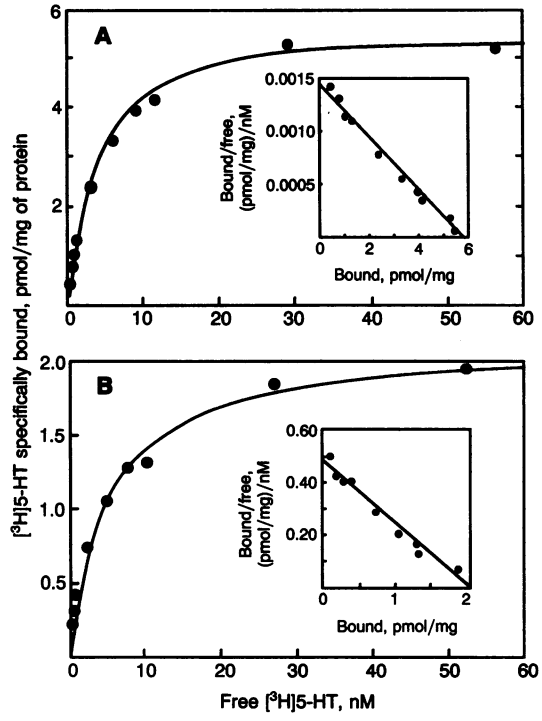

FIG. 2. Determination of equilibrium dissociation constant (K_d) of [^{3}H]5-HT for cloned human 5-HT$_{1D\alpha}$ (*A*) and 5-HT$_{1D\beta}$ (*B*) receptors. Membranes harvested from stable transfectants were incubated with 9–10 concentrations of [^{3}H]5-HT (0.25–50 nM) in absence or presence of 10 μM 5-HT for 30 min at 37°C in the dark. Each data point is the mean of triplicate determinations; standard deviations averaged <5%. K_d and B_{max} values were determined by nonlinear regression analysis, and are illustrated in the form of a Scatchard plot (*Insets*). Calculated K_d and B_{max} values are 4.5 nM and 5.8 pmol/mg for clone 5-HT$_{1D\alpha}$ and 5.0 nM and 2.0 pmol/mg for clone 5-HT$_{1D\beta}$.

the relationship between closely related subtypes. Furthermore, the pharmacological binding properties and second-messenger coupling of this clone are very similar to the properties of the 5-HT$_{1D\alpha}$ clone (see below). We have therefore named this second receptor 5-HT$_{1D\beta}$. A comparison of the human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor sequences to all other known serotonin receptors is shown in Fig. 1. All serotonin receptors contain certain structural features that are invariant, including the aspartic residues of transmembrane regions II and III, the Asp-Arg-Tyr sequence at the end of transmembrane region III, and the conserved proline residues of transmembrane regions IV–VII (20).

Clonal cell lines stably expressing the 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptor gene were generated from murine LM(tk$^-$) fibroblasts. Membranes harvested from cells transfected with either the 5-HT$_{1D\alpha}$ or the 5-HT$_{1D\beta}$ gene displayed an apparently homogeneous population of high-affinity, saturable [^{3}H]5-HT binding sites (Fig. 2). The equilibrium dissociation constants (K_d) of [^{3}H]5-HT for the 5-HT$_{1D\alpha}$ (K_d = 5.1 ± 0.4 nM; n = 5) and 5-HT$_{1D\beta}$ (K_d = 4.3 ± 0.5 nM; n = 5) receptors were similar. The density (B_{max}) of binding sites was 2-fold higher in the 5-HT$_{1D\alpha}$ receptor cell line (B_{max} = 4.0 ± 0.5 pmol/mg) than in the 5-HT$_{1D\beta}$ receptor cell line (B_{max} = 2.3 ± 0.4). Specific binding was >85% of total [^{3}H]5-HT binding at the K_d concentration of radioligand, for both clones.

The pharmacological profiles of both clones were determined in parallel experiments from analysis of competition of [^{3}H]5-HT binding (Table 1). The rank order of potency of these ligands to compete for the [^{3}H]5-HT binding site is consistent with a 5-HT$_{1D}$ receptor pharmacological profile for both clones: 5-carboxamidotryptamine > 5-HT > yohimbine > 8-hydroxy-2-(di-*n*-propylamino)tetralin > spiperone > zacopride. Compounds exhibiting high affinity for both the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ clones and the pharmacologically defined 5-HT$_{1D}$ receptor in native brain tissue included tryptamine derivatives (5-carboxamidotryptamine, 5-HT,

Table 1. Apparent dissociation constants (K_i) and Hill coefficients (n_H) of serotonergic ligands for the cloned human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors in comparison to the pharmacologically defined 5-HT$_{1D}$ receptor from native brain membrane preparations

Drug*		Human 5-HT$_{1D\alpha}$ receptor		Human 5-HT$_{1D\beta}$ receptor		Bovine membranes†
No.	Name	K_i, nM	n_H	K_i, nM	n_H	K_i, nM
1	Lysergol	0.63 ± 0.10	1.04 ± 0.12	1.19 ± 0.13	0.87 ± 0.04	ND
2	5-CT	0.70 ± 0.06	0.96 ± 0.03	1.6 ± 0.2	0.81 ± 0.03	2.5
3	CGS-12066B	2.9 ± 0.3	0.91 ± 0.04	2.1 ± 0.3	0.91 ± 0.07	32
4	Sumatriptan	3.4 ± 0.3	0.80 ± 0.04	7.7 ± 0.5	0.88 ± 0.07	29
5	Methysergide	3.6 ± 0.6	1.00 ± 0.02	25 ± 5	0.91 ± 0.03	4
6	5-HT	3.9 ± 0.3	0.93 ± 0.02	4.3 ± 0.9	0.94 ± 0.01	4
7	5-MeOT	4.8 ± 0.3	1.02 ± 0.02	34 ± 6	0.95 ± 0.01	4
8	1-NP	7.4 ± 0.6	1.05 ± 0.04	12 ± 2	1.05 ± 0.03	15
9	DP-5-CT	13 ± 1	0.97 ± 0.09	42 ± 3	0.91 ± 0.02	63
10	Rauwolscine	16 ± 2	0.90 ± 0.05	40 ± 7	0.87 ± 0.06	20
11	Yohimbine	22 ± 2	0.91 ± 0.04	27 ± 3	0.97 ± 0.04	79
12	TFMPP	64 ± 5	0.95 ± 0.03	114 ± 18	1.00 ± 0.02	282
13	Tryptamine	86 ± 6	0.95 ± 0.02	521 ± 82	0.91 ± 0.05	40
14	DPAT	120 ± 11	0.90 ± 0.05	260 ± 32	0.91 ± 0.07	1260
15	*m*CPP	216 ± 20	0.93 ± 0.05	361 ± 31	0.93 ± 0.01	1550
16	2-Methyl-5-HT	915 ± 68	0.90 ± 0.02	860 ± 109	0.95 ± 0.09	398
17	Spiperone	995 ± 96	0.86 ± 0.03	>10,000	ND	5000
18	Quipazine	>1000	ND	>1000	ND	1380
19	Pindolol	4100 ± 500	0.91 ± 0.04	4900 ± 300	0.91 ± 0.02	6300
20	Zacopride	>10,000	ND	>10,000	ND	ND

K_i values and Hill coefficients are means ± SEM from three to seven determinations. ND, not determined.
*Numbers correspond to those in Fig. 3. 5-CT, 5-carboxamidotryptamine; 5-MeOT, 5-methoxytryptamine; 1-NP, 1-naphthylpiperazine; DP-5-CT, *N, N*-dipropyl-5-carboxamidotryptamine; TFMPP, 1-[3-(trifluoromethyl)phenyl]piperazine; DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; *m*CPP, 1-(3-chlorophenyl)piperazine.
†Data from ref. 9.

5-methoxytryptamine), ergoline analogs (lysergol and methysergide), and α_2-adrenergic antagonists (rauwolscine and yohimbine). Sumatriptan was one of the most potent compounds for both clones (K_i = 3.4 nM for 5-$HT_{1D\alpha}$; K_i = 7.7 nM for 5-$HT_{1D\beta}$). With the exception of CGS-12066B, all compounds tested exhibited higher affinity for the 5-$HT_{1D\alpha}$ receptor than for the 5-$HT_{1D\beta}$ receptor (Table 1). This consistent difference in affinity values was maintained when the expression densities were reversed (i.e., B_{max} for 5-$HT_{1D\alpha}$ = 1 pmol/mg of protein; B_{max} for 5-$HT_{1D\beta}$ = 2.1 pmol/mg of protein). Structurally diverse compounds that discriminated (>7-fold) between these closely related subtypes included methysergide, 5-methoxytryptamine, tryptamine, and spiperone. High correlations were obtained between the apparent affinities of compounds at the pharmacologically defined 5-HT_{1D} receptor from native brain membranes and each of the cloned receptor subtypes (Fig. 3 *A* and *B*). In addition, a very high correlation coefficient (r = 0.96) was obtained in the comparison of the cloned 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ subtypes (Fig. 3*C*).

The functional coupling of these cloned 5-HT_{1D} receptor subtypes to second-messenger responses was determined in intact cells stably expressing each one of these genes (Fig. 4). Forskolin (10 μM) produced a 3- to 5-fold increase in cAMP above basal values. 5-HT (1 μM) inhibited forskolin-stimulated cAMP accumulation by 70 ± 4% in transfected cells expressing the 5-$HT_{1D\alpha}$ receptor and by 92 ± 1% in transfected cells expressing the 5-$HT_{1D\beta}$ receptor. The EC_{50} for 5-HT was 2.49 ± 0.65 nM for 5-$HT_{1D\alpha}$ and 1.8 ± 1.2 nM for 5-$HT_{1D\beta}$ (Fig. 4). Sumatriptan was found to produce agonist responses (EC_{50} = 3.2 ± 0.67 nM for 5-$HT_{1D\alpha}$ and 5.2 ± 1.2 nM for 5-$HT_{1D\beta}$). The responses to 5-HT or sumatriptan were completely blocked by the nonselective antagonist methiothepin (10 μM) in both transfected cell lines (data not shown). In the absence of forskolin, 5-HT did not stimulate adenylate cyclase in these transfected cells. In addition, 5-HT (1 μM) was unable to produce any alterations in phosphotidylinositol metabolism. In untransfected cells, 5-HT did not stimulate or inhibit cAMP accumulation.

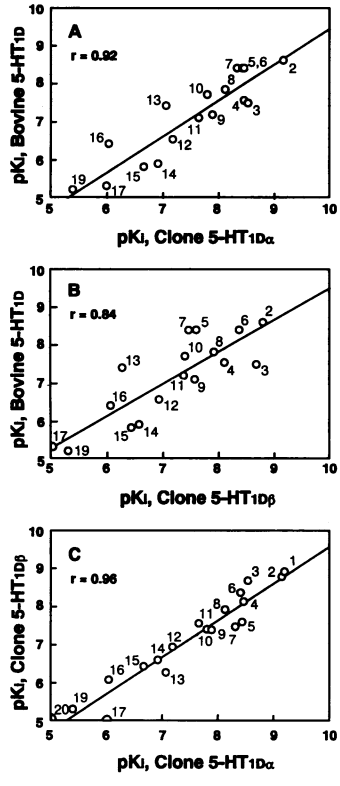

FIG. 3. Correlations between affinity constants (pK_i values) of serotonergic ligands for the cloned human 5-$HT_{1D\alpha}$ (*A*) or 5-$HT_{1D\beta}$ (*B*) receptor and the pharmacologically defined 5-HT_{1D} receptor. Correlation between pK_i values of the same ligands for the cloned human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors is also shown (*C*). Numbers correspond to compounds listed in Table 1. The correlation coefficient (r) is listed in each panel.

DISCUSSION

In the current study, two separate human 5-HT_{1D} receptor genes (5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$) have been isolated and characterized. The dog homolog of the 5-$HT_{1D\alpha}$ receptor, known as the RDC4 sequence (11), was the first 5-$HT_{1D\alpha}$ receptor gene to be isolated, although its identity as a 5-HT_{1D} receptor was only recently recognized (12). The second human 5-HT_{1D} receptor to be cloned and characterized, the 5-$HT_{1D\beta}$ receptor, exhibits pharmacological and functional properties that are strikingly similar to those of the 5-$HT_{1D\alpha}$ receptor. The rat homolog of the human 5-$HT_{1D\beta}$ receptor was recently cloned, characterized, and shown to encode a 5-HT_{1B} receptor site (21, 22). This result confirmed the predicted relationship of the rat 5-HT_{1B} and human 5-HT_{1D} sites (23). The fact that sufficient pharmacological differences were present to have originally led to the naming of the 5-HT_{1D} and 5-HT_{1B} sites as separate receptor subtypes is a reflection of the unusual degree of species variation shown by the 5-$HT_{1D\beta}$ receptor.

The binding properties of these two 5-HT_{1D} receptor subtypes are so similar that a linear correlation coefficient (r) of 0.96 was calculated in the comparison of log K_i values of 19 compounds at these two receptor sites. It is interesting that the 5-$HT_{1D\alpha}$ receptor displayed higher affinity for all structural classes of compounds tested than did the 5-$HT_{1D\beta}$ receptor. Although the 5-$HT_{1D\alpha}$ cell line had a higher expression level than did the 5-$HT_{1D\beta}$ cell line, this difference does not account for the differences in binding affinity. Specifically, when the receptor densities of two cell lines were reversed and studied in parallel for a selection of compounds showing the largest differences in affinity values, the 5-$HT_{1D\alpha}$ receptor still showed higher affinity for these compounds

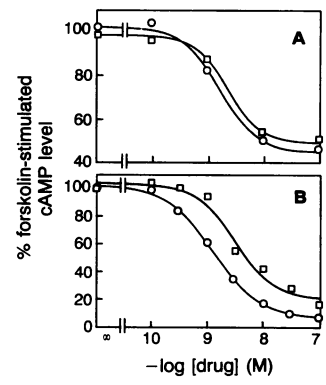

FIG. 4. Concentration–response curve for the inhibition of forskolin-stimulated cAMP production by 5-HT (○) and sumatriptan (□) in clonal cell lines stably expressing the 5-$HT_{1D\alpha}$ (*A*) or 5-$HT_{1D\beta}$ (*B*) receptor gene. Curves shown were fitted to a four-parameter logistic equation to obtain response parameters. Calculated EC_{50} values for 5-HT and sumatriptan in the experiments shown were 1.7 nM and 2.4 nM in the 5-$HT_{1D\alpha}$ cell line and 1.3 nM and 3.3 nM in the 5-$HT_{1D\beta}$ cell line. Each data point is the mean of triplicate determinations; standard deviations averaged <5% of the mean. The point preceding the break in the curve represents the mean of triplicate measurements of forskolin-stimulated cAMP accumulation in the absence of agonist. Basal cAMP levels were subtracted from all cAMP values obtained by using forskolin and were normalized to 100% relative to forskolin-stimulated cAMP levels in the absence of agonist. The experiment was replicated twice with similar results.

than did the 5-$HT_{1D\beta}$ receptor. The strong similarity in properties of the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors makes it difficult to determine whether any of the multiple physiologically or pharmacologically defined 5-HT_{1D} subtypes matches the properties of these genetically defined receptors. That these two clones are not sufficient to explain the known range of 5-HT_{1D} subtypes means that additional 5-HT_{1D}-like receptor subtypes may remain to be cloned.

Similar to the 5-HT_{1D} receptor in native tissues, both cloned human 5-HT_{1D} receptors were found to couple to adenylate cyclase inhibition. Neither produced a phosphatidylinositol response. The EC_{50} values obtained for compounds at each receptor subtype closely matched the affinity constants for binding. These results contrast with the similar comparison of agonist affinities for binding and function of the 5-HT_{1D} receptor in the calf substantia nigra (9). It is likely that the high degree of agonist activity in these heterologous expression systems relative to those reported in the literature can be attributed to differences in receptor reserve. Since the 5-HT_{1A} receptor has been shown to activate potassium channels in hippocampal neurons through a pertussis toxin-sensitive G-protein (24), it will be interesting to determine whether the 5-HT_{1D} receptor subfamily shares this property.

The presence in the human genome of two different 5-HT_{1D} receptor genes that encode receptors which are remarkably similar in their pharmacological selectivity, second-messenger coupling, and amino acid sequence cannot be attributed to the trivial explanation that only one gene is expressed. The 5-$HT_{1D\alpha}$ receptor was isolated from both human (present study) and dog (11) cDNA libraries. The 5-$HT_{1D\beta}$ receptor was expressed in the human brain, since mRNA encoding this receptor was detected by PCR amplification of cDNA derived from human cortex (data not shown). The possibility that these two 5-HT_{1D} receptors are expressed in different brain regions or in different cells within the same region needs to be investigated. Other possible explanations include different rates of desensitization, coupling to different G proteins, or different ratios of high- and low-affinity agonist states in native tissues.

The 5-HT_{1D} receptor has been implicated in a variety of important clinical problems, but only one partially selective ligand for the 5-HT_{1D} receptor site, sumatriptan, is available. This new anti-migraine compound has been shown to interact with 5-HT_{1D} (and 5-HT_{1B}) sites in animal preparations, with apparent dissociation constants ranging from 17 to 251 nM (8, 25, 26). The two cloned human 5-HT_{1D} receptor subtypes both exhibit high-affinity dissociation constants (3.4 nM and 7.7 nM) for sumatriptan, and therefore both the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors are likely targets for the therapeutic actions and side effects of this drug.

We acknowledge the excellent technical assistance provided by Mr. Marcelino Dizon, Ms. Barbara Dowling, Ms. Anastasia Kokkinakis, Mr. Harvey Lichtblau, and Ms. Michelle Smith. We thank Drs. Nika Adham, Jonathan Bard, and Lee Schechter for experimental support. We thank Mr. Ernest Lilley and Mr. George Moralishvili for producing the illustrations. This research was funded in part by Grant 1R43NS27789 from the National Institutes of Health and in part by Eli Lilly and Company.

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