0026-895X/92/020180-06$03.00/0

MOLECULAR PHARMACOLOGY, 42:180-185

ACCELERATED COMMUNICATION

Human Gene S31 Encodes the Pharmacologically Defined Serotonin 5-Hydroxytryptamine$_{1E}$ Receptor

JOHN M. ZGOMBICK, LEE E. SCHECHTER, MARY MACCHI, PAUL R. HARTIG, THERESA A. BRANCHEK, and RICHARD L. WEINSHANK

Synaptic Pharmaceutical Corporation, Paramus, New Jersey 07652

Received March 6, 1992; Accepted May 12, 1992

SUMMARY

The gene encoding a human 5-hydroxytryptamine (5-HT)$_1$ receptor subtype was isolated from a human placental genomic library by using oligonucleotide probes derived from transmembrane regions of the cloned human 5-HT$_{1D\beta}$ receptor. The deduced amino acid sequence of the genomic clone hp75d is identical to that of the recently isolated, but uncharacterized, novel serotonin receptor gene S31. Transmembrane domain sequence comparison of clone hp75d with other guanine nucleotide-binding protein-coupled receptors revealed the highest degree of homology (64%) to the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ subtypes and lower degrees of homology (35–52%) to other serotonergic and catecholaminergic receptors. A stable cell line expressing this gene was established, using murine fibroblasts as the host cell, for pharmacological evaluation. High affinity (K_d = 9.7 nM), saturable (B_{max} = 2.4 pmol/mg of protein) [^{3}H]5-HT binding was detected using membranes derived from stable transfectants. Most compounds displayed low affinity (K_i > 200 nM) for the expressed gene, with the exception of 5-HT (K_i = 10 nM). The rank order of potency of ligands to compete for the [^{3}H]5-HT-labeled site best matched the binding profile of the pharmacologically defined 5-HT$_{1E}$ binding site, 5-HT > methysergide > ergotamine > 8-hydroxy-2-(di-*n*-propylamino)tetralin > 5-carboxyamidotryptamine > ketanserin. 5′-Guanylylimidodiphosphate decreased high affinity agonist ([^{3}H]5-HT) binding in a dose-dependent manner. 5-HT produced a dose-dependent inhibition of forskolin-stimulated cAMP accumulation in intact cells stably expressing the 5-HT$_{1E}$ gene. The response was blocked by the nonselective 5-HT$_1$ receptor antagonist methiothepin. The molecular biological and pharmacological data are consistent with the designation that clone hp75d encodes a functional 5-HT$_{1E}$ receptor.

The classification of serotonin receptors into four major types (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, and 5-HT$_4$) is based upon pharmacological, biochemical, electrophysiological, and molecular biological criteria (1–4). The 5-HT$_1$ receptors are the most heterogeneous, containing three accepted members, 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1D}$ (the 5-HT$_{1C}$ receptor represents a 5-HT$_2$ subtype) (3–6). Previous studies have demonstrated that these receptors mediate similar cellular responses, including negative coupling to adenylate cyclase and inhibition of 5-HT release via somatodendritic (5-HT$_{1A}$) and presynaptic (5-HT$_{1B}$ and 5-HT$_{1D}$) autoreceptors (2, 7). For the 5-HT$_{1A}$ receptor, some of the cellular responses evoked by this subtype have been attributed to potassium channel activation, resulting in neuronal hyperpolarization (8). The genes encoding these 5-HT$_1$ receptors have been cloned and functionally expressed and include the 5-HT$_{1A}$ (9, 10), 5-HT$_{1B}$ (the rat homolog of the human 5-HT$_{1D\beta}$ receptor) (11, 12), and two closely related 5-HT$_{1D}$ subtypes, designated 5-HT$_{1D\alpha}$ (13, 14) and 5-HT$_{1D\beta}$ (14). These receptors are members of the large GPCR superfamily, which are characterized by a structural motif with seven α-helical membrane-spanning regions and which functionally couple to G proteins, resulting in the activation of signal transduction mechanisms (3, 4).

Heterogeneity of the pharmacologically defined 5-HT$_{1D}$ subtype was suggested previously by radioligand binding studies. 5-CT, ergotamine, and sumatriptan competition curves of high affinity [^{3}H]5-HT binding, using 5-HT$_{1D}$ assay conditions, were biphasic, indicating binding site heterogeneity (15–17). The low affinity binding component has been termed both a 5-HT$_{1D}$ subtype (17) and a novel 5-HT$_{1E}$ receptor (15). The 5-HT$_{1E}$ binding site has been described in human cortical membranes and was defined as high affinity [^{3}H]5-HT binding in the

This research was funded in part by Grant 1R43NS27789 from the National Institutes of Health and in part by Eli Lilly and Company. The sequence of the 5-HT$_{1E}$ receptor has been deposited in GenBank (Accession Number M92826).

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; 5-CT, 5-carboxyamidotryptamine; α-Me-5-HT, α-methyl-5-hydroxytryptamine; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; 5-MeO-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine; GPCR, guanine nucleotide-binding protein-coupled receptor; Gpp(NH)p, 5′-guanylylimidodiphosphate; PAPP, 1-[2-(4-aminophenyl)ethyl]-4-(3-trifluoromethylphenyl)piperazine; TFMPP, *N*-(*m*-trifluoromethylphenyl)piperazine; TM, transmembrane; G protein, guanine nucleotide-binding protein; 5-MeOT, 5-methoxytryptamine; I_{max}, maximal inhibition; E_{max}, maximal effect; DP-5-CT, *N*,*N*-dipropyl-5-carboxamidotrpyptamine.

presence of 100 nM 5-CT and 100 nM mesulergine, to block all other high affinity [^{3}H]5-HT sites (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, and 5-HT$_{1D}$). The endogenous ligand 5-HT displayed the highest affinity for this site. With the exception of methysergide (K_i = 59 nM), all other compounds bound with low affinity (K_i > 150 nM). The low affinity of 5-CT and ergotamine clearly discriminated (100–1000-fold) between the pharmacologically defined 5-HT$_{1D}$ and 5-HT$_{1E}$ receptor subtypes (15). High affinity [^{3}H]5-HT binding to the 5-HT$_{1E}$ site was sensitive to guanine nucleotides, a property characteristic of members of the GPCR superfamily. However, the functional coupling of the 5-HT$_{1E}$ site to signal transduction mechanism(s) has not been established.

A homology cloning strategy was implemented to isolate additional 5-HT$_1$ receptors. Using oligonucleotide probes targeted to the TM region of the human 5-HT$_{1D\beta}$ receptor, a GPCR gene was isolated with relatively high (64%) TM homology to the 5-HT$_{1D}$ subtypes (14). During the preparation of this manuscript, a preliminary report describing the isolation of a novel serotonin receptor gene (S31) with the exact deduced amino acid sequence of clone hp75d was published; however, the identity of this clone was not determined (18). Functional expression of clone hp75d yielded pharmacological properties that match the binding profile of the pharmacologically defined 5-HT$_{1E}$ binding site (15).

Materials and Methods

Cloning and sequencing. A human placental genomic library (Stratagene) was screened by using oligonucleotides derived from the human 5-HT$_{1D\beta}$ receptor gene as probes (14). Overlapping oligomers complementary to the 5-HT$_{1D\beta}$ sequence in TM domains III, V, and VI were labeled with [^{32}P]dATP and [^{32}P]dCTP by synthesis with the large fragment of DNA polymerase (19). Hybridization and Southern blot analysis were performed as previously described (20). For subcloning and further Southern blot analysis, DNA was inserted into pUC18 (Pharmacia, Piscataway, NJ). Nucleotide sequence analysis was done by the Sanger dideoxy nucleotide chain-termination method (21), on denatured double-stranded plasmid templates, using Sequenase (United States Biochemical Corp., Cleveland, OH).

Expression. The entire coding region of clone hp75d was cloned into the eukaryotic expression vector pcEXV-3 (22). Stable cell lines were obtained by co-transfection with the plasmid pcEXV-3 (containing the 5-HT$_{1E}$ receptor gene) and the plasmid pGCcos3neo (containing the aminoglycoside phosphotransferase gene) into LM(tk$^-$) cells, using calcium phosphate (reagents obtained from Specialty Media, Lavellette, NJ). The cells were grown in a controlled environment (37°, 5% CO_2), as monolayers, in Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY) (23). Stable clones were then selected for resistance to the antibiotic G-418, and harvested membranes were screened for their ability to bind [^{3}H]5-HT; one clone, hp75d, was used for pharmacological evaluation.

Membrane preparation. Stably transfected LM(tk$^-$) cells were grown to 100% confluency, and membranes were prepared by standard techniques (23). Membrane preparations were kept on ice and used within 2 hr for radioligand binding assays. Protein concentrations were determined by the method of Bradford (24), using bovine serum albumin as the standard.

Radioligand binding studies. [^{3}H]5-HT binding was performed using the 5-HT$_{1E}$ assay conditions reported by Leonhardt *et al.* (15), with the omission of masking ligands. Radioligand binding studies were performed as previously described (23). Saturation studies were conducted using [^{3}H]5-HT at nine different concentrations, ranging from 0.5 nM to 100 nM. Competition experiments were performed using 12 concentrations of unlabeled compound and 5 nM [^{3}H]5-HT. Incubation times were 30 min for both saturation and displacement studies, based upon initial investigations that determined equilibrium binding conditions. Nonspecific binding was defined in the presence of 10 μM 5-HT. Binding data were analyzed by 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 (25). All experiments were performed in triplicate.

Functional studies. The functional coupling of clone hp75d to adenylate cyclase was determined in Y-1 cells stably expressing this gene. The functional assay was performed according to the method described by Zgombick *et al.* (23). Intracellular cAMP was determined by radioimmunoassay (Advanced Magnetics, Cambridge, MA). Functional data were fitted to a four-parameter logistic equation, to obtain response parameters (EC_{50}, E_{max}, and n_H) (GRAPHpad Software, San Diego, CA).

Drugs. Drugs were obtained from the following companies: [^{3}H]5-HT (specific activity, 20.4–28.0 Ci/mmol), New England Nuclear (Boston, MA); 5-HT, ergotamine, ergonovine, oxymetazoline, (±)-pindolol, and Gpp(NH)p, Sigma (St. Louis, MO); 5-CT, DP-5-CT, 5-MeOT, 5-MeO-DMT, (±)-α-Me-5-HT, 2-Me-5-HT, tryptamine, DPAT, DOI, ketanserin, methysergide, 1-naphthylpiperazine, PAPP, spiperone, TFMPP, yohimbine, and zacopride, Research Biochemical Inc. (Natick, MA); lysergol and methylergonovine, Aldrich Chemicals (Milwaukee, WI); rauwolscine, Accurate Chemicals, (Westbury, NY); and methiothepin, Biomol Research Laboratories (Plymouth Meeting, PA). All other chemicals were the highest purity available commercially.

Results

We have screened a human genomic placental library, at low stringency, with oligonucleotide probes derived from TM domains III, V, and VI of the 5-HT$_{1D\beta}$ receptor gene. Several hundred clones were isolated, subjected to Southern blot and sequence analysis, and subsequently characterized as either previously cloned GPCRs, novel receptors, or irrelevant DNA sequences. One of these clones, hp75d, encoded what appeared to be a novel serotonin receptor, based upon its deduced amino acid sequence. A comparison of this protein sequence with all previously characterized neurotransmitter receptors indicates that hp75d encodes a new 5-HT$_1$ receptor subtype. The deduced amino acid sequence of clone hp75d is identical to the primary structure of the recently cloned serotonin receptor gene S31 (18).

The primary amino acid sequence of this receptor and a comparison with other cloned serotonin receptors are shown in Fig. 1. The amino acid sequence identity, within the TM domains, between hp75d and the 5-HT$_{1D\beta}$ receptor, its closet relative, was 64%. The relationship between hp75d and a second 5-HT$_{1D}$ subtype (5-HT$_{1D\alpha}$) was also 64%, in contrast to the 75% TM identity between the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors (14). The TM relationship between hp75d and the other cloned serotonin receptors exhibited the following order of identity: 5-HT$_{1A}$, 52%; 5-HT$_{1C}$, 40%; and 5-HT$_2$, 39%. Similar to what has been observed with the 5-HT$_{1A}$ receptor (3, 4), hp75d is more closely related to the adrenergic receptors than to the phosphoinositide-coupled 5-HT receptors (5-HT$_{1C}$ and 5-HT$_2$). These analyses also revealed a conserved aspartate residue in TM III and a single conserved serine residue in TM V, key residues characteristic of the serotonin receptor family (3, 4).

The gene encoding the human 5-HT$_{1E}$ receptor was stably expressed in murine fibroblasts for pharmacological evaluation. High affinity, saturable [^{3}H]5-HT binding was detected using membranes harvested from a clonal cell line (Fig. 2). The

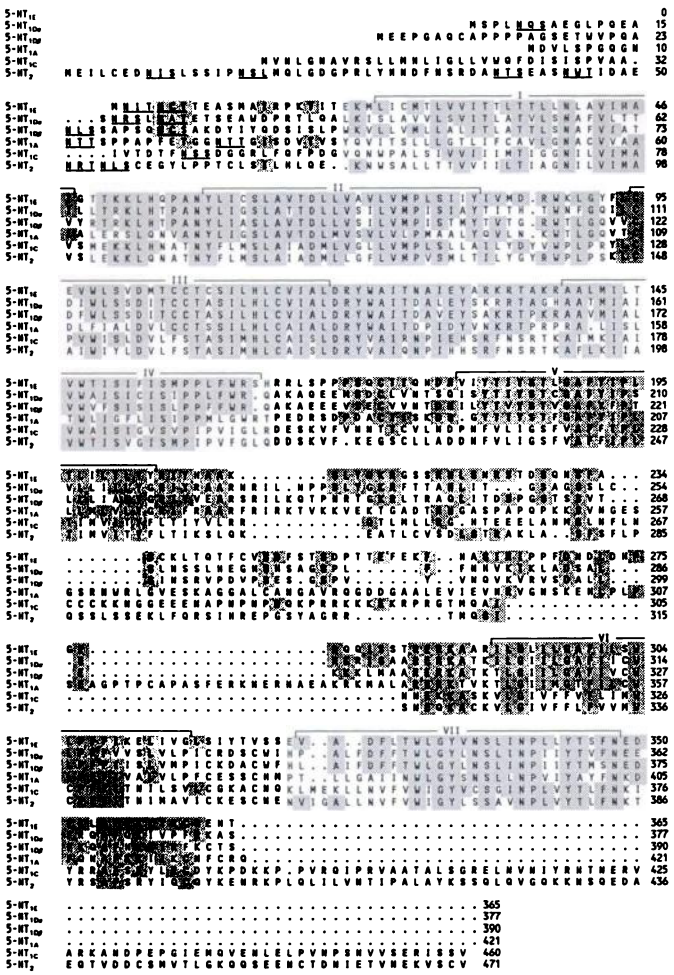

Fig. 1. Comparison of the 5-HT_{1E} receptor deduced amino acid sequences with those of other serotonin receptors. *Solid bars*, the seven putative membrane-spanning domains (TM I–VII). *Shading*, homologies between the 5-$HT_{1D\beta}$ receptor and other receptors. In the amino-terminal region, consensus sequences for *N*-linked glycosylation sites are *underlined*.

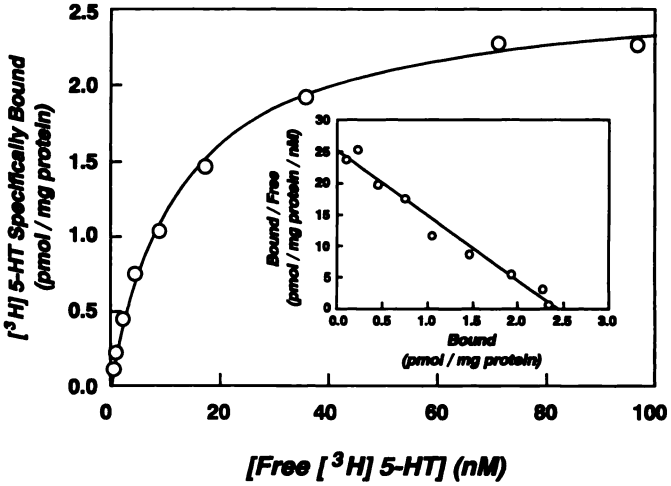

Fig. 2. Determination of the equilibrium dissociation constant (K_d) of [^{3}H]5-HT for the cloned human 5-HT_{1E} receptor. Membranes harvested from stable transfectants were incubated with nine concentrations of [^{3}H]5-HT (0.50–100 nM), in the absence and presence of 10 μM 5-HT, for 30 min at 37°, in the dark. Each data point is the mean of triplicate determinations, and standard deviations averaged <5%. K_d and B_{max} values were determined by nonlinear regression analysis, and these values are illustrated in the form of a Scatchard plot (*inset*).

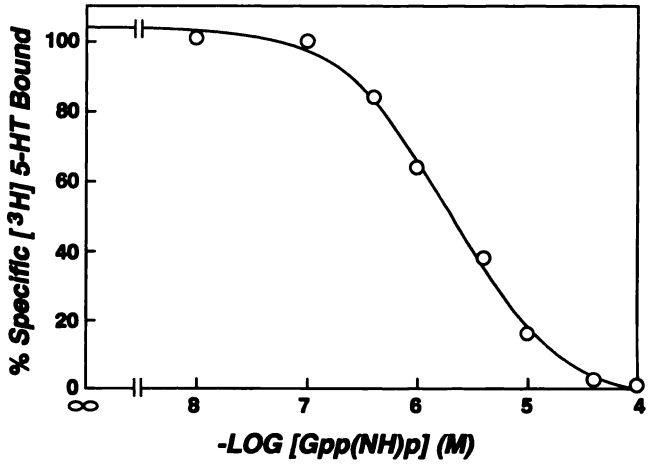

Fig. 3. Guanine nucleotide sensitivity of [^{3}H]5-HT binding to the cloned human 5-HT_{1E} receptor. Membranes harvested from stable transfectants were incubated with 5 nM [^{3}H]5-HT, in the absence and presence of eight concentrations of Gpp(NH)p, for 30 min at 37°, in the dark. Unlabeled 5-HT (10 μM) was used to define nonspecific binding. Data were normalized to 100%, relative to specific [^{3}H]5-HT binding obtained with 100 μM Gpp(NH)p. Each data point is the mean of triplicate determinations, and standard deviations averaged <5%.

equilibrium dissociation constant (K_d) of [^{3}H]5-HT derived from nonlinear analysis of saturation studies was 9.7 ± 1.5 nM. The density of binding sites (B_{max}) averaged 2.3 ± 0.4 pmol/mg of protein. Specific binding was >85% of total binding at the K_d value. No specific [^{3}H]5-HT binding was observed in untransfected host cells. Guanine nucleotide sensitivity of high affinity [^{3}H]5-HT binding was tested using membranes prepared from stable transfectants. Gpp(NH)p, a nonhydrolyzable analog of GTP, produced a dose-dependent decrease (IC_{50} = 2.4 ± 0.5 μM, I_{max} = 21 ± 2%) of high affinity [^{3}H]5-HT binding (Fig. 3).

Pharmacological characterization of clone hp75d was determined from nonlinear analysis of competition of high affinity [^{3}H]5-HT binding. Specific [^{3}H]5-HT binding was displaced, in a monophasic manner, by a collection of structurally diverse serotonergic ligands (Fig. 4). The rank order of potency of these compounds to displace specific [^{3}H]5-HT binding (Table 1) best matches the binding profile of the pharmacologically defined 5-HT_{1E} binding site (5-HT > methysergide > ergotamine > sumatriptan > spiperone > 5-CT > ketanserin = zacopride). The endogenous ligand 5-HT, of all the compounds tested, displayed the highest affinity at this receptor. With the possible exception of three ergot alkaloids, lysergol, ergonovine, and methylergonovine (K_i = 43–89 nM), all other drugs exhibited low affinity (K_i > 120 nM) (Table 1). Serotonergic compounds that exhibit subtype selectivity and low affinity for clone hp75d include sumatriptan (5-HT_{1D}), DPAT (5-HT_{1A}), spiperone (5-HT_{1A} and 5-HT_2), ketanserin (5-HT_2), and zacopride (5-HT_3). A high correlation coefficient (r = 0.85) was calculated between the binding affinities (pK_i values) of ligands at the pharmacologically defined 5-HT_{1E} binding site (15) and clone hp75d (Fig. 5). Lower correlation coefficients were obtained with the other cloned and pharmacologically defined 5-HT_1 subtypes (r < 0.50; data not shown).

The functional coupling of clone hp75d to adenylate cyclase was tested in intact cells stably expressing the 5-HT_{1E} receptor

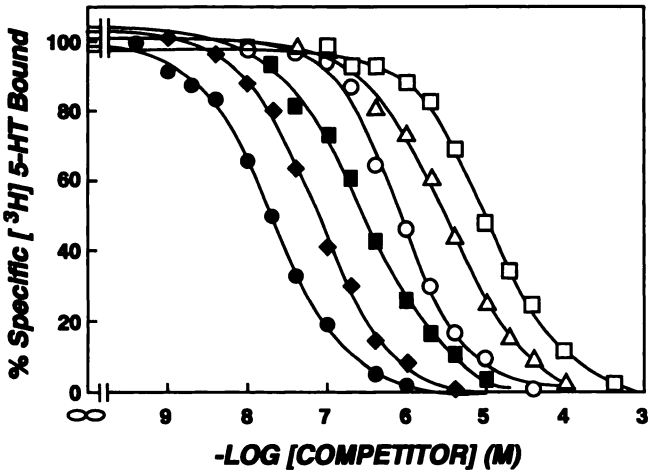

Fig. 4. Determination of affinity constants of serotonergic ligands for the cloned human 5-HT$_{1E}$ receptor. Membranes harvested from stable transfectants were incubated with 5 nM [^{3}H]5-HT, in the absence and presence of unlabeled competitor, for 30 min at 37°, in the dark. Unlabeled 5-HT (10 μM) was used to define nonspecific binding. Each data point is the mean of triplicate determinations, and standard deviations averaged <5%. ●, 5-HT; ♦, lysergol; ■, methiothepin; ○, ergotamine; △, sumatriptan; □, 5-CT.

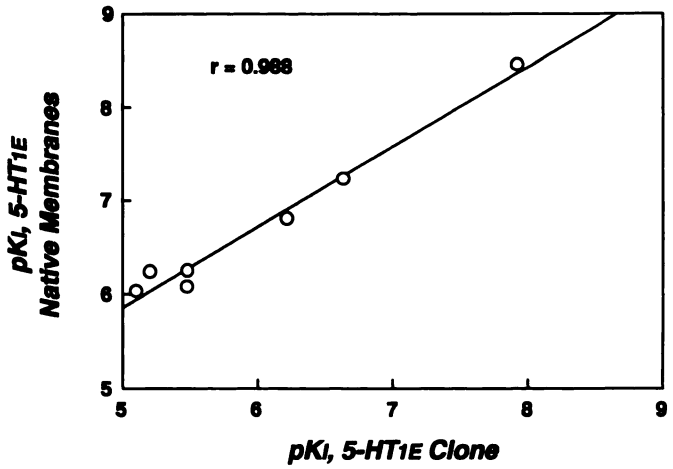

Fig. 5. Correlations between affinity constants (pK_i values) of serotonergic ligands for the cloned human 5-HT$_{1E}$ receptor and for the pharmacologically defined 5-HT$_{1E}$ binding site. Values correspond to compounds listed in Table 1. Affinity constants of the same compounds at the pharmacologically defined 5-HT$_{1E}$ binding site were taken from Leonhardt *et al.* (15). The correlation coefficient (r) is shown.

TABLE 1

Apparent dissociation constants (K_i values) and Hill coefficients (n_H) of serotonergic ligands for the cloned human 5-HT$_{1E}$ receptor

Membranes were incubated with 5 nM [^{3}H]5-HT in the presence of 12 concentrations of unlabeled competitors for 30 min at 37°. Nonspecific binding was defined with 10 μM unlabeled 5-HT. Affinity constants (K_i values) were determined from IC_{50} values by computer-assisted nonlinear curve analysis, using the Cheng-Prusoff equation (25). K_i values and Hill coefficients are expressed as mean values ± standard errors from three to seven determinations.

Compound	K_i	Hill coefficient
	nM	
5-HT	11 ± 1	0.92 ± 0.01
Lysergol	43 ± 5	0.92 ± 0.13
Ergonovine	88 ± 8	0.87 ± 0.05
Methylergonovine	89 ± 4	0.92 ± 0.04
α-Me-5-HT	121 ± 13	0.84 ± 0.04
Methiothepin	194 ± 4	0.87 ± 0.03
1-Naphthylpiperazine	207 ± 69	1.11 ± 0.08
Methysergide	228 ± 16	0.91 ± 0.07
Oxymetazoline	419 ± 49	0.80 ± 0.08
5-MeO-DMT	528 ± 32	1.05 ± 0.10
Ergotamine	599 ± 39	1.02 ± 0.04
2-Me-5-HT	817 ± 203	0.86 ± 0.06
Yohimbine	1270 ± 233	0.93 ± 0.11
Sumatriptan	2520 ± 135	0.92 ± 0.02
Tryptamine	2559 ± 827	1.19 ± 0.18
DOI	2970 ± 592	0.89 ± 0.06
5-Me-OT	3151 ± 1041	1.02 ± 0.06
DPAT	3333 ± 310	0.99 ± 0.11
Rauwolscine	3434 ± 102	0.85 ± 0.03
Spiperone	5051 ± 689	0.93 ± 0.04
TFMPP	6293 ± 259	0.90 ± 0.07
5-CT	7875 ± 284	0.82 ± 0.03
Ketanserin	>10,000	ND[a]
Pindolol	>10,000	ND
Zacopride	>10,000	ND
LY-165163 (PAPP)	>10,000	ND
DP-5-CT	>10,000	ND

[a] ND, not determined.

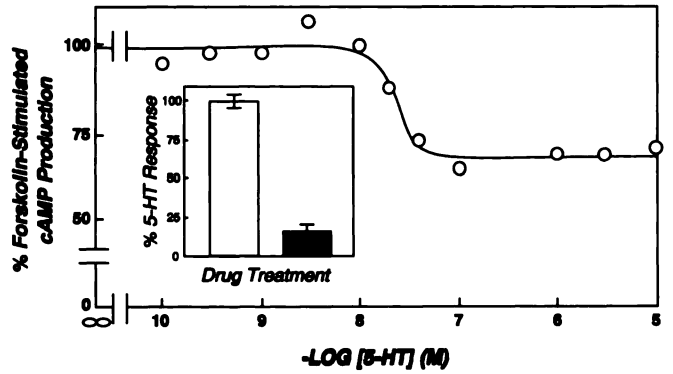

Fig. 6. Concentration-response curve for the inhibition of forskolin-stimulated cAMP production by 5-HT and antagonism of the 5-HT response by the nonselective 5-HT$_1$ antagonist methiothepin (*inset*) in stable transfectants expressing the cloned human 5-HT$_{1E}$ receptor. Forskolin (10 μM) stimulated cAMP 3–5-fold over basal levels. Concentration-response curve data were normalized to 100%, relative to forskolin-stimulated values in the absence of agonist. *Inset*, data were normalized to 100%, relative to the response obtained with 100 nM 5-HT (□). Methiothepin (1 μM) (▨) had no effect on either basal or forskolin-stimulated cAMP levels in these cells. Data represent mean values ± standard error from triplicate determinations from a representative experiment. The experiment was replicated an additional two times, with similar results.

gene. 5-HT produced a dose-dependent (EC_{50} = 20 ± 8 nM) inhibition (E_{max} = 30 ± 3%) of forskolin-stimulated cAMP accumulation (Fig. 6). This response was significantly diminished by the nonselective antagonist methiothepin (Fig. 6, *inset*). The failure of methiothepin to antagonize completely the 5-HT response can be attributed to both its low affinity (K_i = 194 nM) (Table 1) and the equiactive concentration of methiothepin used (1 μM, 5 × K_i) to block the 5-HT response (100 nM, 5 × EC_{50}). A saturating concentration of methiothepin (i.e., 10 μM, 50 × K_i) would be required for complete antagonism of this inhibitory response. 5-HT did not stimulate adenylate cyclase or phospholipase C in transfected cells. No functional 5-HT responses were observed in untransfected cells (data not shown).

Discussion

The deduced amino acid sequence of the 5-HT_{1E} receptor was analyzed to uncover relationships between it and other cloned serotonin receptor sequences. The sequence within the membrane-spanning domains was most closely related to that of the two 5-HT_{1D} receptor subtypes (64%), 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ (14). Closely related members of a "subfamily" of serotonin receptors (i.e., "subtypes") generally share a common transmitter, display similar pharmacological profiles, and activate similar effector systems (for example, 5-HT_2 and 5-HT_{1C} or 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$). Such subtypes display an amino acid identity of ~75–80% in their TM domains. Serotonin receptors that are not members of the same subfamily but are members of the serotonin "family" (in which the receptors use the same neurotransmitter, i.e., 5-HT_2 and 5-$HT_{1D\alpha}$) generally exhibit much lower TM homology (~45%). Such TM amino acid homologies, therefore, can give insight into the relationship between receptors and can be used as predictors of receptor pharmacology. Based upon these molecular analyses, the 5-HT_{1E} receptor may be viewed as a more distantly related member of the 5-HT_{1D} subfamily.

The pharmacological binding profile (Table 1) obtained with clone hp75d best matches the binding properties of the pharmacologically defined 5-HT_{1E} binding site (15). The high affinity of [^{3}H]5-HT for clone hp75d (Fig. 2) is a pharmacological property common to 5-HT_1 subtypes (1). The natural ligand 5-HT exhibited the highest affinity (K_i = 10 nM) for the cloned receptor (Table 1). The low affinity of a collection of structurally diverse serotonergic compounds for clone hp75d (Table 1) is consistent with a previous report on the pharmacology of the 5-HT_{1E} site (15). The lack of high affinity ligands is unique to the 5-HT_{1E} receptor and contrasts with the pharmacological profiles observed with other serotonergic receptors. Compounds that effectively discriminate (100–1000-fold selectivity) between 5-HT_{1D} receptors and the 5-HT_{1E} site are ergotamine and the 5-HT analogs 5-CT and sumatriptan (14–17) (Table 1). These three ligands exhibited very low affinity (>600 nM) for clone hp75d. In contrast, these compounds displayed high affinity (K_i < 10 nM) for the cloned human 5-HT_{1D} subtypes (14). The binding properties of clone hp75d support the molecular biological evidence, suggesting that this human clone represents a 5-HT_{1E} receptor, rather than another 5-HT_{1D} subtype.

The signal transduction pathway(s) activated by the endogenous 5-HT_{1E} receptor *in vivo* is not presently known; investigation is hampered by the lack of selective ligands and of a model system expressing only this receptor. In the present study, the cloned human 5-HT_{1E} receptor stably expressed in Y-1 cells coupled to the inhibition of adenylate cyclase (Fig. 6), congruent with the functional response observed with the transfected S31 gene in murine fibroblasts (18). This second messenger coupling is consistent with responses obtained with the other cloned 5-HT_1 receptors, including the 5-HT_{1A} receptor (26), the 5-HT_{1B} receptor (12), and two 5-HT_{1D} subtypes (14). These cloned 5-HT_1 receptors inhibited forskolin-stimulated cAMP accumulation in intact cells transfected with the appropriate gene, mimicking their accepted functional responses in model tissue preparations (27–29). The modest inhibition elicited by clone hp75d (E_{max} = 35%) (Fig. 6) may reflect a less efficacious coupling between receptor and the native G protein α_i subunit(s) present in Y-1 cell membranes. A similar response was also obtained in a LM(tk$^-$) cell line stably expressing the 5-HT_{1E} receptor gene (data not shown). Further studies are required to establish the signal transduction mechanism(s) activated by the 5-HT_{1E} receptor *in vivo*.

The pharmacologically defined 5-HT_{1E} binding site (15) may still represent a heterogeneous population of 5-HT_1 sites. A 5-HT_1-like receptor has been described in several isolated tissue preparations (30–32). The pharmacological properties of the 5-HT_1-like receptor(s) mediating these functional responses are similar, but not identical, to the 5-HT_{1D} receptors. Diversity of the 5-HT_1 receptor subfamily is also suggested, based upon recent radioligand binding studies (33, 34). The current receptor classification scheme cannot adequately describe this 5-HT_1-like receptor, even when the 5-HT_{1E} receptor is included in these analyses. These data are suggestive of additional heterogeneity of the 5-HT_1 receptors. The identification of additional 5-HT_1 receptors by molecular cloning techniques will be instrumental in the resolution of this question.

Acknowledgments

The authors wish to thank Mr. Harvey Lichtblau, Ms. Anastasia Kokkinakis, Ms. Lisa Gonzalez, Mr. Marc Dizon, and Ms. Jenny Xanthos for their excellent technical assistance and Mr. Ernest Lilley and Mr. George Moralishvili for assistance with the illustrations.

References

1. Peroutka, S. J. 5-Hydroxytryptamine receptor subtypes: molecular, biochemical, and physiological characterization. *Trends Neurosci.* **11**:496–500 (1988).
2. Frazier, A., S. Maayani, and B. B. Wolfe. Subtypes of receptors for serotonin. *Annu. Rev. Pharmacol. Toxicol.* **30**:307–348 (1990).
3. Hartig, P. R., H.-T. Kao, M. Macchi, N. Adham, J. Zgombick, R. Weinshank, and T. Branchek. The molecular biology of serotonin receptors: an overview. *Neuropsychopharmacology* **3**:335–347 (1990).
4. Julius, D. Molecular biology of serotonin receptors. *Annu. Rev. Neurosci.* **14**:335–360 (1991).
5. Hamon, M., M. B. Emerit, S. El Mestikawy, M. C. Gallissot, and H. Gozlan. Regional differences in the transduction mechanisms of 5-hydroxytryptamine receptors in the mammalian brain, in *Cardiovascular Pharmacology of 5-Hydroxytryptamine: Prospective Therapeutic Applications* (P. R. Saxena, D. I. Wallis, W. Wouters, and P. Bevan, eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands, 41–59 (1990).
6. Schmidt, A. W., and S. J. Peroutka. 5-Hydroxytryptamine receptor "families." *FASEB J.* **3**:2242–2249 (1989).
7. Hoyer, D., and D. N. Middlemiss. Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. *Trends Pharmacol. Sci.* **10**:130–132 (1989).
8. Andrade, R., R. C. Malenka, and R. A. Nicoll. A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. *Science* (*Washington, D. C.*) **234**:1261–1265 (1986).
9. 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).
10. Fargin, A., J. Raymond, M. Lohse, B. Kobilka, M. Caron, and R. Lefkowitz. The genomic clone G-21 which resembles the β-adrenergic receptor sequence encodes the 5-HT_{1A} receptor. *Nature* (*Lond.*) **335**:358–360 (1988).
11. Voight, 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).
12. Adham, N., P. Romanienko, P. Hartig, R. Weinshank, and T. A. Branchek. The rat 5-hydroxytryptamine$_{1B}$ receptor is the species homologue of the human 5-hydroxytryptamine$_{1D\beta}$ receptor. *Mol. Pharmacol.* **41**:1–7 (1992).
13. 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).
14. Weinshank, R. L., J. M. Zgombick, M. Macchi, T. A. Branchek, and P. R. Hartig. The human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. *Proc. Natl. Acad. Sci. USA* **89**:3630–3634 (1992).
15. Leonhardt, S., K. Herrick-Davis, and M. Titeler. Detection of a novel serotonin receptor subtype (5-HT_{1E}) in human brain: interaction with a GTP-binding protein. *J. Neurochem.* **53**:465–471 (1989).
16. Waeber, C., P. Schoeffter, J. M. Palacios, and D. Hoyer. Molecular pharmacology of 5-HT_{1D} recognition sites: radioligand binding studies in human, pig and calf membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **337**:595–601 (1988).
17. Sumner, M. J., and P. P. A. Humphrey. 5-HT_{1D} binding sites in porcine brain can be sub-divided by GR43175. *Br. J. Pharmacol.* **98**:29–31 (1989).
18. Levy, F. O., T. Gudermann, M. Birnbaumer, A. J. Kaumann, and L. Birn-

baumer. Molecular cloning of a human gene (S31) encoding a novel serotonin receptor mediating inhibition of adenylyl cyclase. *FEBS Lett.* **296**:201–206 (1992).
19. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
20. Weinshank, R. L., J. M. Zgombick, M. Macchi, N. Adham, H. Lichtblau, T. A. Branchek, and P. R. Hartig. Cloning, expression, and pharmacological characterization of a human α_{2B}-adrenergic receptor. *Mol. Pharmacol.* **38**:681–688 (1990).
21. Sanger, S. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467 (1977).
22. Miller, J., and R. N. Germain. Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. *J. Exp. Med.* **164**:1478–1489 (1986).
23. 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).
24. Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
25. 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 enzyme reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
26. Fargin, A., J. R. Raymond, J. R. Regan, S. Cotecchia, R. J. Lefkowitz, and M. G. Caron. Effector coupling mechanisms of the cloned 5-HT1A receptor. *J. Biol. Chem.* **264**:14848–14852 (1989).
27. De Vivo, M., and S. Maayani. Characterization of the 5-hydroxytryptamine$_{1A}$ receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity in guinea pig and rat hippocampal membranes. *J. Pharmacol. Exp. Ther.* **238**:248–253 (1986).
28. 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).
29. 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 calf substantia nigra. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **337**:602–608 (1988).
30. Perren, M. J., W. Feniuk, and P. P. A. Humphrey. Vascular 5-HT$_1$-like receptors that mediate contraction of the dog isolated saphenous vein and carotid arterial vasoconstriction in anaesthetized dogs are not of the 5-HT$_{1A}$ or 5-HT$_{1D}$ subtype. *Br. J. Pharmacol.* **102**:191–197 (1991).
31. Villalon, C. M., A. H. Bom, J. P. C. Heiligers, O. Den Boer, and P. R. Saxena. Constriction of porcine arteriovenous anastomoses by indorenate is unrelated to 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, or 5-HT$_{1D}$ receptor subtypes. *Eur. J. Pharmacol.* **190**:167–176 (1990).
32. Buzzi, M. G., M. A. Moskowitz, S. J. Peroutka, and B. Byun. Further characterization of the putative 5-HT receptor which mediates blockade of neurogenic plasma extravasation in rat dura mater. *Br. J. Pharmacol.* **103**:1421–1428 (1991).
33. Zemlan, F. P., and E. F. Schwab. Characterization of a novel serotonin receptor subtype (5-HT$_{1S}$) in rat CNS: interaction with a GTP binding protein. *J. Neurochem.* **57**:2092–2099 (1991).
34. Mahle, C. D., H. P. Nowak, R. J. Mattson, S. D. Hurt, and F. D. Yocca. [^{3}H] 5-Carboxamidotryptamine labels multiple high affinity 5-HT$_{1D}$-like sites in guinea pig brain. *Eur. J. Pharmacol.* **205**:323–324 (1991).

Send reprint requests to: John M. Zgombick, Synaptic Pharmaceutical Corporation, Paramus, NJ 07652.