Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 408–412, January 1993
Neurobiology

Cloning of another human serotonin receptor (5-HT_{1F}): A fifth 5-HT_1 receptor subtype coupled to the inhibition of adenylate cyclase

NIKA ADHAM, HUNG-TEH KAO*, LEE E. SCHECHTER, JONATHAN BARD, MICHAEL OLSEN, DEBORAH URQUHART, MARGARET DURKIN, PAUL R. HARTIG, RICHARD L. WEINSHANK, AND THERESA A. BRANCHEK†

Synaptic Pharmaceutical Corporation, 215 College Road, Paramus, NJ 07652

Communicated by Eric Kandel, September 28, 1992

ABSTRACT **An intronless gene encoding an additional human serotonin (5-HT) 5-$HT_{1\text{-like}}$ receptor subtype was isolated from a human genomic library with probes obtained from degenerate PCR primers used to amplify 5-HT-receptor-specific sequences. The highest degree of homology was found with the 5-HT_{1E} subtype (70%) and the 5-$HT_{1D\alpha}$ (63%) and 5-$HT_{1D\beta}$ (60%) receptors. RNA for this gene was detected in the human brain but was not detected in kidney, liver, spleen, heart, pancreas, and testes. High-affinity (K_d = 9.2 nM) ^{3}H-labeled 5-HT binding was detected. Competition studies revealed the following rank order of potencies for serotonergic ligands: 5-HT > sumatriptan >> 5-carboxyamidotryptamine > 8-hydroxy-2(di-1-propylamino)tetralin > spiperone. 5-HT produced a dose-dependent inhibition of forskolin-stimulated cAMP accumulation (EC_{50} = 7.9 nM) in transfected cells. These properties distinguish this receptor from any previously characterized and establish a fifth 5-$HT_{1\text{-like}}$ receptor subtype (5-HT_{1F}) coupled to the inhibition of adenylate cyclase.**

The diverse physiological actions of serotonin (5-HT) are mediated by at least four receptor classes: 5-$HT_{1\text{-like}}$, 5-HT_2, 5-HT_3, and 5-HT_4 (1–3). The 5-$HT_{1\text{-like}}$ class appears the most heterogeneous. Four human genes encoding 5-$HT_{1\text{-like}}$ receptors have been cloned: 5-HT_{1A} (4, 5), 5-$HT_{1D\alpha}$ (6, 7), 5-$HT_{1D\beta}$ (7–9), and 5-HT_{1E} (10–12). The 5-HT_{1B} receptor has been cloned (13–15) and is homologous to the 5-$HT_{1D\beta}$ receptor (14). These 5-$HT_{1\text{-like}}$ receptors all share an amino acid homology of at least 50%, high affinity for ^{3}H-labeled 5-HT ([^{3}H]5-HT), and coupling to the inhibition of adenylate cyclase activity as a primary coupling pathway. Many additional 5-HT-mediated functional responses do not seem to correlate with any of these cloned receptor subtypes (16, 17).

We have used a PCR cloning approach to isolate a human gene that encodes another 5-$HT_{1\text{-like}}$ receptor, termed 5-HT_{1F}, with a pharmacological profile distinct from any serotonergic receptor yet described.‡ Like the other 5-$HT_{1\text{-like}}$ receptors, 5-HT_{1F} couples to the inhibition of adenylate cyclase activity, and this response is blocked by methiothepin. Detection of the mRNA encoding this receptor in the human brain indicates that binding studies using [^{3}H]5-HT as a ligand require reevaluation. Furthermore, the high affinity of the antimigraine drug sumatriptan at this subtype indicates a possible role of the 5-HT_{1F} receptor in migraine.

EXPERIMENTAL PROCEDURES

PCR. The third (III) and fifth (V) transmembrane (TM) domains of the following receptors were used to synthesize degenerate primers: 5-HT_{1A}, 5-HT_{1C}, 5-HT_2, and the 5-$HT_{1D\alpha/\beta}$ receptors. Primers 3.17 and 5.5 [(5′-TGGAATTC-TGYGYIATHKCICTGGAYMGSTA-3′) and (5′-CATIA-VIRIIARIGGDATRWARAAIGC-3′)] were used to amplify 5 μg of poly(A)+ RNA from rat brain that was reverse-transcribed by reverse transcriptase (avian myeloblastosis virus). PCR was done on single-stranded cDNA as follows: 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min for 40 cycles. After PCR, 90 μl of the reaction was phenol/chloroform-extracted and precipitated, treated with T4 DNA polymerase, and digested with *Eco*RI before separation on a 1% agarose gel. The DNA fragment was isolated from the gel, kinased, and cloned into pBluescript. Recombinant clones were analyzed by sequencing. One clone, designated S51, was chosen for further study.

Cloning and Sequencing. A human lymphocyte genomic library (Stratagene) was screened by using the rat S51 fragment as a probe. The probe was labeled with ^{32}P by the method of random priming (18) and hybridized (19). For subcloning and further Southern blot analysis, DNA was inserted into pUC18 (Pharmacia). Nucleotide sequence analysis was done by the Sanger dideoxynucleotide chain-termination method (20) on denatured double-stranded plasmid templates with Sequenase (United States Biochemical).

Expression. The coding region of clone hL16a was cloned into vector pcEXV-3 (21), and stable cell lines were obtained by cotransfection (19). One LM(tk$^-$) cell line, L-1F-3, was selected for binding studies; one NIH 3T3 stable cell line N-1F-6 was selected for functional studies.

Membrane Preparation. Membranes were prepared from stably transfected LM(tk$^-$) cells as described (19). Protein concentrations were determined by the method of Bradford (22).

Radioligand-Binding Studies. [^{3}H]5-HT-(20–30 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) binding was done as described (7). Competition experiments were done by using 10–12 concentrations of drug and 4.5–5.5 nM [^{3}H]5-HT. Nonspecific binding was defined by 10 μM 5-HT. Binding data were analyzed by nonlinear-regression analysis (7). IC_{50} values were converted to K_i values using the Cheng–Prusoff equation (23). All experiments were done in triplicate.

Adenylate Cyclase Activity. Adenylate cyclase activity was determined in initial experiments in LM(tk$^-$) cells, as described (14). A weak inhibition of forskolin-stimulated adenylate cyclase (FSAC) (25–30%) was obtained. Stable cell

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Abbreviations: TM, transmembrane; 5-CT, 5-carboxyamidotryptamine; 5-HT, serotonin (5-hydroxytryptamine); [^{3}H]5-HT, ^{3}H-labeled 5-HT; FSAC, forskolin-stimulated adenylate cyclase.

*Present address: Department of Psychiatry and Behavioral Sciences, Stanford University Medical Center, Stanford, CA 94305.
†To whom reprint requests should be addressed.

‡The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. L04962).

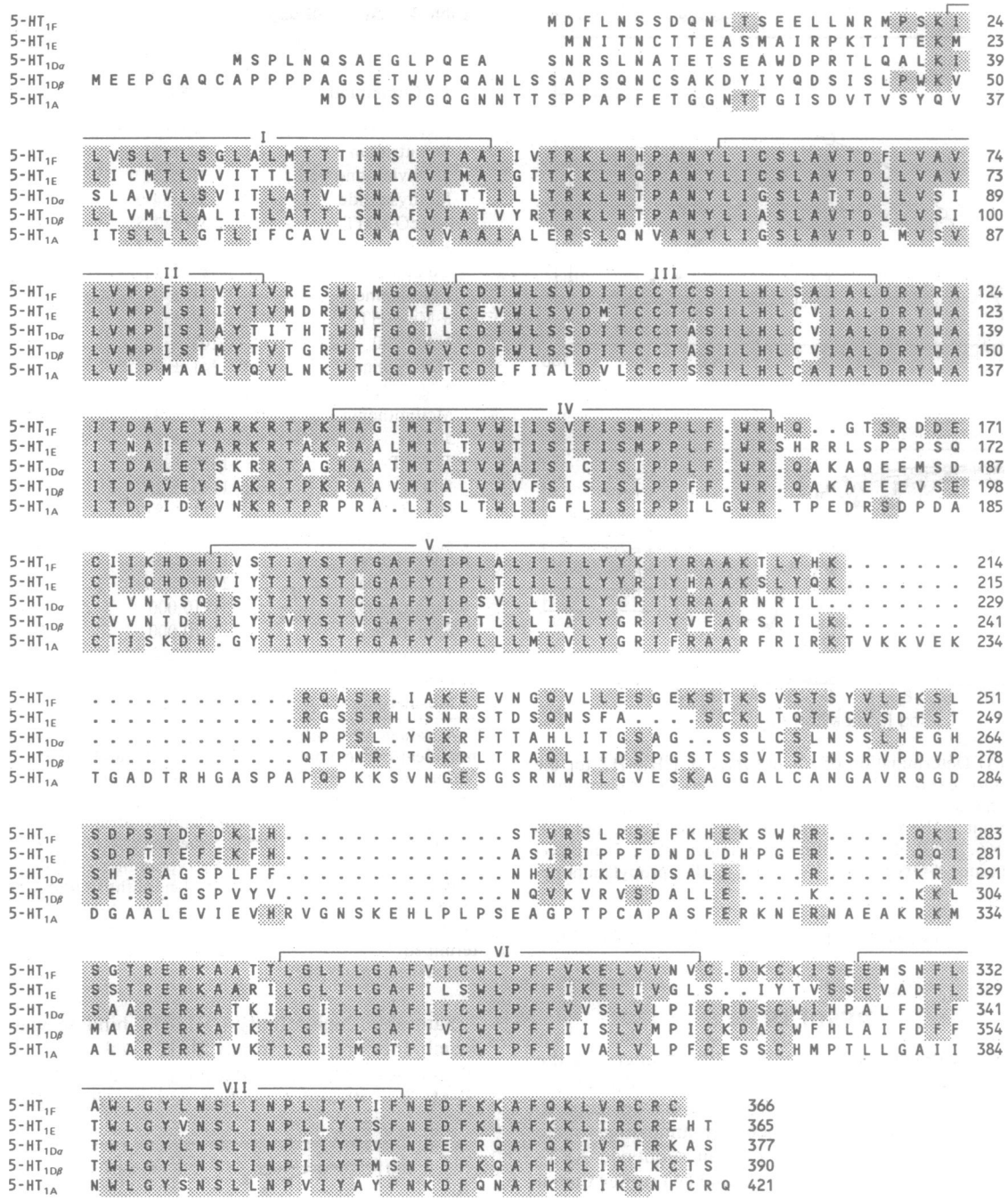

FIG. 1. Comparison of 5-HT_{1F} receptor-deduced amino acid sequences with other 5-HT receptors. The seven putative membrane-spanning domains (TM I–VII) are indicated by lines and Roman numerals. Homologies between the 5-HT_{1F} receptor and other receptors are noted by shading.

lines were produced in NIH 3T3 cells and were used for all subsequent experiments.

Tissue Localization Studies. Human tissues (National Disease Research Institute) were homogenized, and the total RNA was extracted (24). cDNA was prepared from 5 μg of total RNA with random hexanucleotide primers (500 pmol) using Superscript reverse transcriptase (BRL) in PCR reaction buffer (Cetus) containing 1 mM dNTPs, at 42°C for 1 hr. An aliquot of the first-strand cDNA was diluted (1:5) in a 50-μl PCR reaction mixture (200 μM dNTPs, final concentration) containing 1.25 units of *Taq* polymerase and 1 μM of primers from the sense strand (5′-TCTATTCTGGAGGCACCAAGGAAC-3′) and from the antisense strand (5′-TGTTGATGGGTCAGATAAAGACTT-3′). The PCR products were run on a 1.5% agarose gel and transferred to charged nylon membrane (ZetaProbe, Bio-Rad). Filters were hybridized and washed under high stringency.

***In Situ* Hybridization.** *In situ* hybridization was done as described (25) by using male Hartley guinea pigs (300–350 g). A fragment of the guinea pig 5-HT_{1F} receptor gene was cloned by homology and sequenced. Forty-five-base oligoprobes synthesized to the 4,5 loop and 5′-untranslated regions were 3′-end-labeled with deoxyadenosine 5′-[γ-[^{35}S]thio]triphosphate to a specific activity of 4×10^9 Ci/mmol. The nucleotide sequences were as follows: 5′-GTGATGCTTGATGATGCACTCATCATCTCGGCTTGTCCCCTGGTG-3′ and 5′-TAGCAGTTCCTCTGAGGTCAAGTTTTGATCAGAAGAGTTTAAGAA-3′. Sense probes, melting temperature, and RNase pretreatment were used as controls. Sections were exposed to Kodak X-Omat AR film for 1 week or

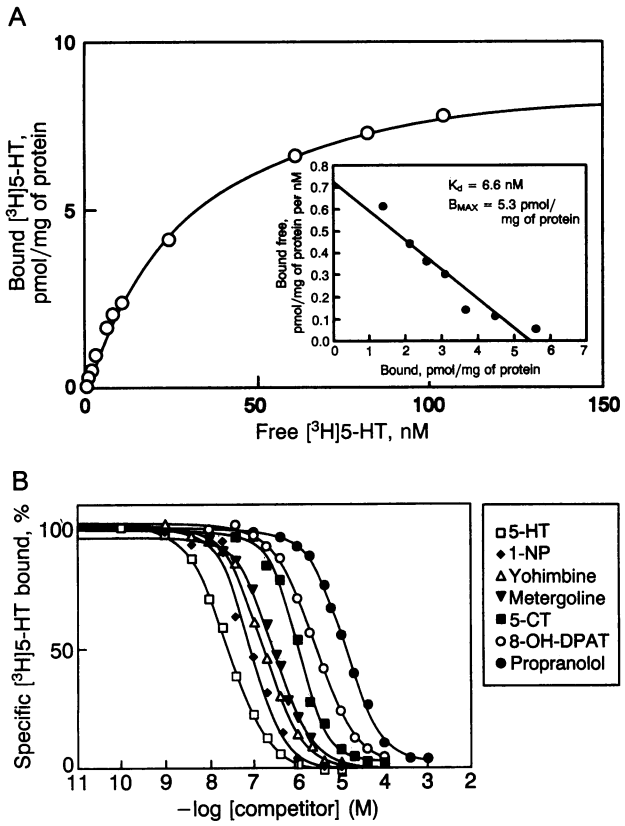

FIG. 2. (A) Determination of K_d and B_{max} of [^{3}H]5-HT for the 5-HT$_{1F}$ receptor expressed in LM(tk$^-$) cells. (B) Determination of affinity constants of serotonergic ligands for the 5-HT$_{1F}$ receptor. Each data point is the mean of triplicate determinations, and SDs averaged <5%. 1-NP, 1-(1-naphthyl)piperazine; 5-CT, 5-carboxyamidotryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-1-propylamino)tetralin.

coated with Kodak NTB-2 emulsion/2% glycerol, 1:1, for 2 weeks.

RESULTS

We synthesized degenerate oligonucleotide primers on the basis of sequences corresponding to the third and fifth TM segments of 5-HT receptors. The primers were designed to amplify only 5-HT-specific sequences by annealing to the nucleotide sequence encoding the protein sequence AFY-(F)IP in TM V. The presence of an alanine (A) rather than a serine (S) at the amino end of this sequence distinguishes the closely related adrenergic and dopaminergic receptor families from the serotonergic receptor family. After 30 amplification cycles, agarose gel electrophoresis revealed a clear pattern of cDNA species of ≈250 base pairs. Individual cDNAs were cloned into pBluescript and subjected to sequence analysis. One clone, designated S51, was observed to encode a distinctive receptor. We then screened a human genomic lymphocyte library with the PCR fragment S51. Isolation of the full-length coding region was obtained from a genomic clone designated hL16a. The primary amino acid sequence of clone hL16a is shown in Fig. 1. A long open reading frame can encode a protein 366 amino acids in length, having a relative molecular weight (M_r) of ≈42,000. Comparison of this protein sequence with previously characterized neurotransmitter receptors indicates that hL16a is most closely related to the recently characterized 5-HT$_{1E}$ receptor (10–12) (Fig. 1). The TM amino acid identity of hL16a to other known 5-HT$_1$ receptors is as follows: 5-HT$_{1E}$ (70%), 5-HT$_{1D\alpha}$ (63%), 5-HT$_{1D\beta}$ (60%), and 5-HT$_{1A}$ (53%).

Table 1. Apparent dissociation constants (K_i values) of various drugs for cloned 5-HT$_{1F}$ and 5-HT$_{1E}$ receptors.

Compound	K_i, nM 5-HT$_{1F}$	K_i, nM 5-HT$_{1E}$*
5-HT	10± 2.0	11 ± 1.0
Sumatriptan	23 ± 11	2520 ± 135
Methylergonovine	31 ± 11	89 ± 4.0
Methysergide	34 ± 4.9	228 ± 16
5-MeO-DMT	37 ± 1.5	528 ± 32
1-NP	54 ± 3.8	207 ± 69
Yohimbine	92 ± 11	1270 ± 233
Ergotamine	171 ± 28	599 ± 39
α-Me-5-HT	184 ± 35	121 ± 13
NAN 190	203 ± 13	ND
Metergoline	341 ± 71	ND
2-Me-5-HT	413 ± 5.6	817 ± 203
Methiothepin	652 ± 41	194 ± 4.0
5-CT	717 ± 71	7875 ± 284
TFMPP	1002 ± 85	6293 ± 259
5-MT	1166 ± 197	3153 ± 1041
DP-5-CT	1613 ± 817	>10,000
DOI	1739 ± 84	2970 ± 592
8-OH-DPAT	1772 ± 38	3333 ± 310
Tryptamine	2409 ± 103	2559 ± 827
Ketanserin	>10,000	>10,000
Spiperone	>10,000	5051 ± 689
Zacopride	>10,000	>10,000
Pindolol	>10,000	>10,000
Mesulergine	>10,000	ND

K_i values were determined from IC_{50} values by computer-assisted nonlinear-curve analysis using the Cheng–Prusoff equation (23). Values are means ± SEM from at least three separate experiments. DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; 8-OH-DPAT: 8-hydroxy-2-(di-1-propylamino)tetralin; 5-MeOT, 5-methoxytryptamine; 5-MeO-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine; α-Me-5-HT, α-methyl-5-hydroxytryptamine; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; 1-NP, 1-(1-naphthyl)piperazine; TFMPP, *N*-(*m*-trifluoromethylphenyl)piperazine; 5-MT, 5-methoxytryptamine; DP-5-CT, dipropyl-5-carboxyamido-tryptamine. ND, not determined.
*Published values (12).

Saturable, high-affinity [^{3}H]5-HT binding was seen with membranes prepared from LM(tk$^-$) cells (Fig. 2A). Analysis of saturation data indicated a K_d of 9.2 ± 0.99 nM (mean ± SEM, n = 4) and a binding density (B_{max}) of 4.4 ± 0.36

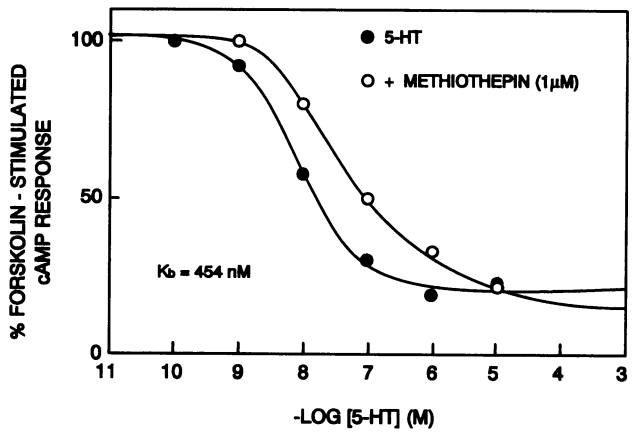

FIG. 3. 5-HT concentration–effect curves are represented without (●) and with (○) methiothepin (1.0 μM). Data were normalized to 100% relative to forskolin-stimulated values in the absence of agonist to derive E_{max} and EC_{50} values. The antagonist K_b was estimated by the method of Furchgott (26): K_b = dose of antagonist/[(EC_{50} in the presence of antagonist/control EC_{50}) − 1].

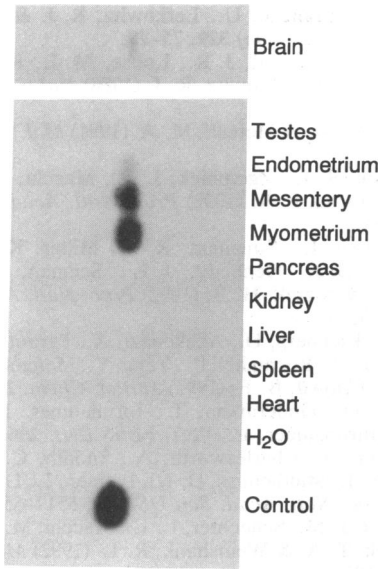

FIG. 4. Human tissue distribution of RNA coding for 5-HT_{1F} receptor gene. Total RNA was converted to single-stranded cDNA by random priming with reverse transcriptase. cDNAs were amplified by PCR using 5-HT_{1F}-specific PCR primers. PCR products were run on a 1.5% agarose gel, blotted onto nylon membranes, hybridized to internal gene-specific oligonucleotides, and washed under high stringency. Positive controls represent gene-specific recombinant plasmids; water (H_2O) served as a negative control. PCR amplification and Southern blotting of RNA samples not treated with reverse transcriptase were negative.

pmol/mg of protein (mean ± SEM, n = 4). No specific [^{3}H]5-HT binding was seen in untransfected host cells. 5′-Guanylyl imidodiphosphate produced a dose-dependent inhibition (IC_{50} = 243 ± 115 nM, I_{max} = 56 ± 3.2%; mean ± SEM, n = 3) of the specific [^{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. 2*B*). 5-HT displayed the highest affinity (Table 1). 5-CT and other indole derivatives, excluding sumatriptan, had poor affinity. Other compounds including 8-hydroxy-2-(di-1-propylamino)tetralin (5-HT_{1A}), spiperone (5-HT_{1A}/5-HT_2), ketanserin (5-HT_2), 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (5-HT_{1C}/5-HT_2), pindolol (5-$HT1_A$/5-$HT1_B$), and zacopride (5-HT_3) had very poor affinity.

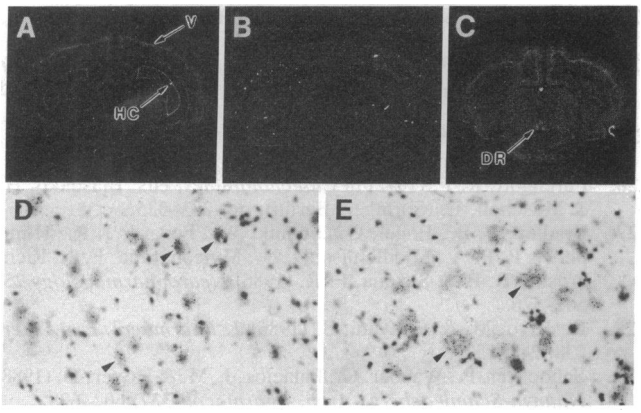

FIG. 5. 5-HT_{1F} receptor mRNA in guinea pig brain coronal sections. (*A*) Antisense oligonucleotide probe (4,5 loop) was used. An identical pattern was seen with the 5′-untranslated probe (data not shown). Hybridization densities are high in layer V of cerebral cortex (V) and in CA1–CA3 of the hippocampus (HC). (*B*) Control contralateral hemisphere of a section adjacent to that in *A*. No hybridization was seen by using a sense probe of identical specific activity. (*C*) Section hybridized with the antisense probe. The dorsal raphe (DR) is densely labeled. (*D*) Hybridization (antisense probe) is detected in layer V of sensorimotor cortex. Arrowheads indicate heavily labeled pyramidal cells. (*E*) Hybridization (antisense probe) in layer V of sensorimotor cortex sectioned through the dorsal raphe. Arrowheads indicate large, heavily labeled neurons. (×130 in *D* and *E*.)

The functional coupling of clone hL16a to adenylate cyclase inhibition was tested in intact NIH 3T3 cells ($B_{max} \approx 1$ pmol/mg of protein). 5-HT (10 μM) had no effect on either basal activity or FSAC in untransfected or mock transfected NIH 3T3 cells, indicating an absence of endogenous cyclase-coupled 5-HT receptors in untransfected cells. Addition of 10 μM forskolin increased the basal cAMP release (0.036 ± 0.0050 pmol/ml per 10 min) by ≈10-fold. 5-HT inhibited the response (Fig. 3) with an EC_{50} value of 7.9 ± 0.76 nM and a maximum percentage inhibition of FSAC (E_{max} value) of 74 ± 4.6 (mean ± SEM, n = 10). The 5-HT response (1 μM) was completely blocked by the nonselective antagonist methiothepin (10 μM). This antagonism was surmountable (Fig. 3), indicating probable competitive antagonism. The dose shift produced by methiothepin yielded an apparent K_b of 438 ± 14 nM, consistent with the K_i for this compound (Table 1). No direct effect of methiothepin was seen. No other compound tested in this study was an antagonist. In addition, no evidence for coupling of this receptor to inositol phospholipid turnover was detected at a dose of 10 μM 5-HT.

Expression of the 5-HT_{1F} transcripts was analyzed from PCR–RNA blots and *in situ* hybridization studies. By PCR, we detected 5-HT_{1F} receptor mRNA in the human brain, uterus (endometrium and myometrium), and mesentery (Fig. 4) but did not detect it in kidney, liver, spleen, heart, pancreas, or testes. In *in situ* hybridization experiments, we observed 5-HT_{1F} transcripts in lamina V of frontal cortex (Fig. 5*A*) in large pyramidal cells (Fig. 5*D*). Moderate labeling was also detected over layer VI nonpyramidal neurons. In both layer V and layer VI, the labeling was most evident in dorsal sensorimotor neocortex and in cingulate and retrosplenal cortices (Fig. 5*C*). The pyramidal cells in the piriform cortex were heavily labeled, as were large neurons in the raphe nuclei (Fig. 5*E*). Hippocampal pyramidal cells in CA1–CA3 were moderately labeled, as were the granule cells in the dentate gyrus, and some neurons in the nucleus of the solitary tract. Little labeling was found in the thalamus and hypothalamus.

DISCUSSION

The pharmacological profile obtained from cells expressing gene hL16a (Table 1) indicates that it encodes a member of the 5-$HT_{1\text{-like}}$ receptor subfamily, as 5-CT is an agonist, and methiothepin is an antagonist (27). As for the 5-HT_{1E} receptor, its closest genetic relative (Fig. 1), the affinity for these compounds is relatively low. Nonetheless, the high affinity for sumatriptan, 5-methoxy-*N*,*N*-dimethyltryptamine, methysergide, and other compounds from diverse structural classes (Table 1) distinguishes this additional receptor from the 5-HT_{1E} subtype (r = 0.45). We have classified it as a 5-HT_{1F} receptor on the basis of its distinctive pharmacology: the low affinity of the 5-HT_{1F} receptor for 5-CT but the high affinity of 5-HT_{1F} receptor for sumatriptan. This activity clearly distinguishes it from any other 5-$HT_{1\text{-like}}$-binding site or functional response model.

The cloned human 5-HT_{1F} receptor was found to couple to the inhibition of adenylate cyclase, as do other cloned members of the 5-$HT_{1\text{-like}}$ receptor family. This coupling mimics their accepted functional responses in native tissue preparations (28–30, except the native 5-HT_{1E} receptor for which a functional correlate has not been reported). The 5-HT_{1F} receptor represents the fifth cloned 5-HT receptor that shows coupling to the inhibition of adenylate cyclase.

The only "5-HT_1 receptor" that does not couple to adenylate cyclase is the 5-HT_{1C} receptor (31), which is now considered a member of the 5-HT_2 subfamily (2). Why so many different 5-HT_1 receptor subtypes have been maintained in the genome is not at all clear. Possible explanations include different midpoints for activation by 5-HT under physiological conditions, different desensitization rates, different efficiencies or kinetics of coupling to adenylate cyclase inhibition, coupling to diverse adenylate cyclases, different developmental regulation, coupling to separate ion channels, or coupling to ion-channel regulation rather than adenylate cyclase regulation as a primary physiological pathway.

Localization of transcripts for this receptor indicates a relatively selective tissue distribution. Of tissues reported here, the 5-HT_{1F} receptor was detected only in the brain, uterus, and mesentery. The possible role of this receptor in uterine or vascular function is intriguing. Future studies defining the specific cell type(s) in these tissues that express the receptor may provide insight into its function in the periphery. In the brain, the expression had a limited distribution compared with that of other 5-HT receptors (9). In the neocortex, labeling of layer V pyramidal neurons may indicate a functional role for the 5-HT_{1F} receptor protein in the integration of sensorimotor (somatodendritic; frontal cortex) or afferent information associated with limbic functions (somatodendritic; cingulate/retrosplenial cortex), or in spinal cord processes (axonal). Strong labeling was also detected in hippocampal pyramidal cells, in several thalamic nuclei, and in the dorsal raphe. The detection of transcripts for this gene in the dorsal raphe nucleus indicates a possible role as an autoreceptor. This localization is shared by both the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors (9). Such heterogeneity of autoreceptors has recently been described (32).

The cloned 5-HT_{1F} receptor does not appear to be any of the 5-HT_1-like receptor subtypes defined in a variety of isolated tissue preparations by *in vitro* response assays. However, such preparations contain heterogeneous populations of 5-$HT_{1\text{-like}}$ receptors and are primarily studied in nonhuman tissues. The elucidation of the physiological role of the 5-HT_{1F} receptor, including its possible autoreceptor function, awaits the discovery of selective compounds, most likely through the use of heterologous expression systems expressing each individual subtype.

We thank Drs. M. Macchi, E. Gustafson, and D. Wang for helpful discussions; B. Dowling, B. Ellerbrock, L. Gonzalez, A. Kokkinakis, J. McHugh, M. Smith, H. Lichtblau, and S. Nawoschik for their excellent technical assistance; and J. Xanthos for sequence comparison studies. We thank E. Lilley and G. Moralishvili for assistance with the illustrations and E. Marton for assistance with the manuscript. Human samples were obtained from the National Disease Research Institute. This work was partially funded by Small Business Innovation Research Grants 1R43NS28616 and 2R44NS27789 and by Eli Lilly and Company.

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