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ACCELERATED COMMUNICATION

Molecular Cloning, Functional Expression, and Pharmacological Characterization of a Novel Serotonin Receptor (5-Hydroxytryptamine$_{2F}$) from Rat Stomach Fundus

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SUMMARY

Using the polymerase chain reaction amplification technique in conjunction with conventional cloning techniques, we have isolated a novel member of the serotonin [5-hydroxytryptamine (5-HT)] 1C/2 receptor subfamily (designated 5-HT$_{2F}$) from rat stomach fundus. Two DNA fragments were amplified from cDNA synthesized from rat stomach fundus poly(A)$^+$ RNA using the polymerase chain reaction technique with degenerate oligonucleotide primers derived from sequence comparisons of the second, third, and sixth putative transmembrane domains of known 5-HT receptors. These fragments were used as hybridization probes to isolate full length cDNA clones from rat stomach fundus cDNA libraries. Full length cDNA clones contained one open reading frame encoding a 479-amino acid protein with seven hydrophobic domains, characteristic of members of the guanine nucleotide-binding protein-coupled receptor superfamily. Within these seven putative transmembrane domains, the 5-HT$_{2F}$ receptor shared greatest homology with the rat 5-HT$_{1C}$ and 5-HT$_2$ receptor subtypes (70% and 68%, respectively). Cell lines stably expressing the 5-HT$_{2F}$ receptor were established and demonstrated functional coupling to phosphatidylinositol hydrolysis upon 5-HT stimulation analagous to that observed for the 5-HT$_{1C}$ and 5-HT$_2$ receptors. Membranes from the stably transfected cell lines (but not the untransfected parental lines) exhibited high affinity (K_d = 7.9 nM), saturable binding of [^{3}H]5-HT. Maximum binding ranged from 0.1 to 2.4 pmol/mg of protein, depending on the clonal isolate. Using [^{3}H]5-HT as the basis for a radioligand binding assay, the relative affinities of several tryptamine and piperazine derivatives for the cloned 5-HT$_{2F}$ receptor correlated with their relative potencies to contract the rat stomach fundus. These data suggest a probable relationship between this novel 5-HT$_{2F}$ receptor and the serotonin contractile receptor of the rat stomach fundus.

Because of its exquisite sensitivity to serotonin, the rat stomach fundus was used as a bioassay for serotonin (1) before the development of more quantitative analytical assays for this biogenic amine. Although the potency for the contractile effects of serotonin has been known for some time, the receptors mediating such a response have eluded definitive characterization. Pharmacological studies attempting to characterize the contractile serotonergic receptor in the rat stomach fundus documented the lack of similarity to the 5-HT$_2$ receptor (2–4). The use of agonists and antagonists further demonstrated that the serotonin contractile receptor in the rat stomach fundus was not similar to the 5-HT$_3$, 5-HT$_{1A}$, 5-HT$_{1B}$, or 5-HT$_{1D}$ receptors (5). The similarity, but nonidentity, of the contractile serotonergic receptor in the rat stomach fundus to the 5-HT$_{1C}$ receptor was recognized, yet mRNA for the 5-HT$_{1C}$ receptor was found to be absent (6). Based on the pharmacological similarity to the 5-HT$_{1C}$ receptor and the absence of 5-HT$_{1C}$ receptor mRNA in the rat stomach fundus, efforts were initiated to clone the serotonin receptor from the rat stomach fundus.

Experimental Procedures

Chemicals. TFMPP, 5-CT, 2-Me-5-HT, 8-OH-DPAT, and quipazine were purchased from Research Biochemicals, Inc. (Natick, MA). Serotonin creatinine sulfate complex and most other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Serotonin

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; PCR, polymerase chain reaction; kb, kilobases; TFMPP, 1-(*m*-trifluoromethylphenyl)piperazine hydrochloride; 8-OH-DPAT, (±)-8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin hydrobromide; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; 5-CT, 5-carboxamidotryptamine; SSC, standard saline citrate; SSPE, standard saline phosphate EDTA; SDS, sodium dodecyl sulfate; G protein, guanine nucleotide-binding protein; PI, phosphatidylinositol; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

(A)

Transmembrane Region III

Rat 1a - III	CGTCCATCCTGCACCTGTGCGCCAT
Human 1a - III	CATCCATCTTGCACCTGTGCGCCAT
Drosophila 1 - III	CCTCGATTCTGAATCTGTGTGCCAT
Dog RDC4 - III	CCTCCATCCTGCATCTCTGTGTCAT
Rat 1c - III	CGTCCATCATGCACCTCTGCGCCAT
Rat 2 - III	CATCCATCATGCACCTCTGCGCCAT
Hamster 2 - III	CCTCCATCATGCACCTCTGCGCCAT
Mouse 1c - III	CGTCCATCATGCACCTCTGCGCCAT
	A A C
Consensus:	CGTCCATCCTGCACCTGTGCGCCAT

(B)

Transmembrane Region VI

Rat 1a - VI	TTCATCCTCTGCTGGCTGCCGTTTT
Human 1a - VI	TTCATCCTCTGCTGGCTGCCCTTCT
Drosophila 1 - VI	TTTACCGTCTGCTGGCTGCCATTCT
Dog RDC4 - VI	TTTATCGTCTGCTGGCTGCCCTTCT
Rat 1c - VI	TTTCTGATCATGTGGTGCCCGTTTT
Rat 2 - VI	TTTGTTGTAATGTGGTGCCCATTCT
Hamster 2 - VI	TTTGTCGTAATGTGGTGCCCATTCT
Mouse 1c - VI	TTTCTGATCATGTGGTGCCCGTTTT
	C C C
1a/RDC4	TTCATCGTCTGCTGGCTGCCGTTTT
	C A A C
1c/2-like	TTTGTGGTCATGTGGTGCCCGTTTT

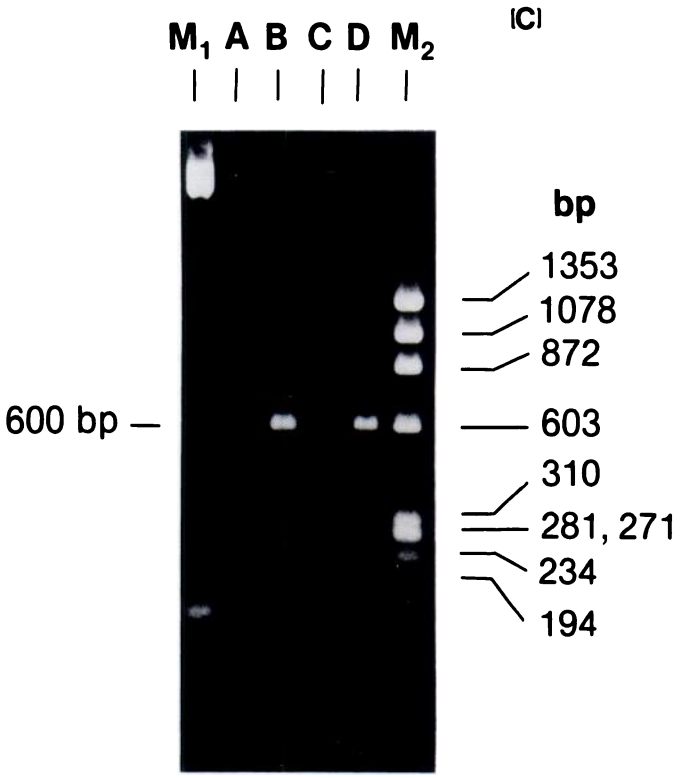

Fig. 1. Strategy for PCR primer design. A and B, Nucleic acid sequences for the third and sixth transmembrane regions of cloned serotonin receptors were aligned and compared. Consensus sequences were identified for each transmembrane region (two for transmembrane region VI) and degeneracy was incorporated at nonconsensus positions. The primer for TM-III represents a pool of eight different 25-mers, whereas the primers for TM-VI represent pools of eight and 16 different 25-mers, respectively. C, Amplification of rat stomach fundus cDNA using the TM-III/TM-VI 1a/RDC4-like primer combination (*lanes A* and *C*) and the TM-III/TM-VI 1c/2-like primer combination (*lanes B* and *D*). The TM-III/TM-VI 1C/2-like combination amplified a 600-base pair-long DNA fragment not of a size predicted from either 5-HT_{1C} or 5-HT_2 receptor sequences. M_1 represents a 123 base pair DNA ladder and M_2 a $\phi\chi$ 174/*Hac*III DNA digest for size standards.

(27.4–38.1 Ci/mmol) and all other radionuclides were obtained from DuPont-NEN (Wilmington, DE).

PCR amplification and subcloning of Initial 5-HT_{2F} receptor cDNA fragments. Total RNA was isolated from rat stomach fundus according to the guanidinium isothiocyanate/CsCl method of Chirgwin *et al.* (7). Poly(A)$^+$ RNA was selected using oligo(dT)-cellulose columns (Collaborative Research, Bedford MA) and the method of Aviv and Leder (8). First-strand cDNA was synthesized from poly(A)$^+$ RNA using the Copy Kit cDNA synthesis kit (Invitrogen, San Diego, CA). For PCR amplification of the initial 5-HT_{2F} receptor fragments, degenerate oligonucleotide primers were designed to the second, third, and sixth putative transmembrane domains based on cloned serotonin receptor sequence information. PCR amplification was carried out under the following conditions: 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM levels of each deoxynucleoside triphosphate, 0.2 μM levels of each primer, and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT), in a total volume of 100 μl, as described for the GeneAmp kit (Perkin Elmer-Cetus). The profile of 95° for 1 min, 50° for 1 min, and 72° for 2 min for 35 cycles was programmed into a model 480 Thermocycler (Perkin Elmer-Cetus). All PCR-derived fragments were separated on a 1.2% low-melting temperature agarose/Tris-acetate-EDTA gel, extracted, and ligated into the PCR-1000 vector using the T/A cloning kit (Invitrogen, San Diego, CA).

cDNA library construction, screening, and DNA sequencing. Two rat stomach fundus cDNA libraries were constructed using 10 μg of poly(A)$^+$ RNA and the Superscript lambda system (BRL, Gaithersburg, MD) for cDNA synthesis and λ cloning. Both libraries contained $>2 \times 10^6$ independent clones, with an average insert size of >1.5 kb. A pool of 1.5×10^6 independent clones from the two libraries was screened by hybridization under conditions of high stringency [50% formamide, 5× SSPE (1× SSPE = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0, 0.01 M $NaH_2PO_4 \cdot H_2O$, and 0.001 M EDTA), 5× Denhardt's (1× Denhardt's = 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% Ficoll 400), 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA, for 48 hr at 50°]. A 708-base pair PCR fragment was labeled with [^{32}P]dCTP by random priming and was used as a probe at a concentration of 2×10^6 cpm/ml. Filters were washed in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0)/0.1% SDS three times for 10 min each at 55° and in 0.1× SSC/0.1% SDS three times for 30 min each at 55°. Twenty-one clones were selected and purified to homogeneity for further characterization. PCR amplification was

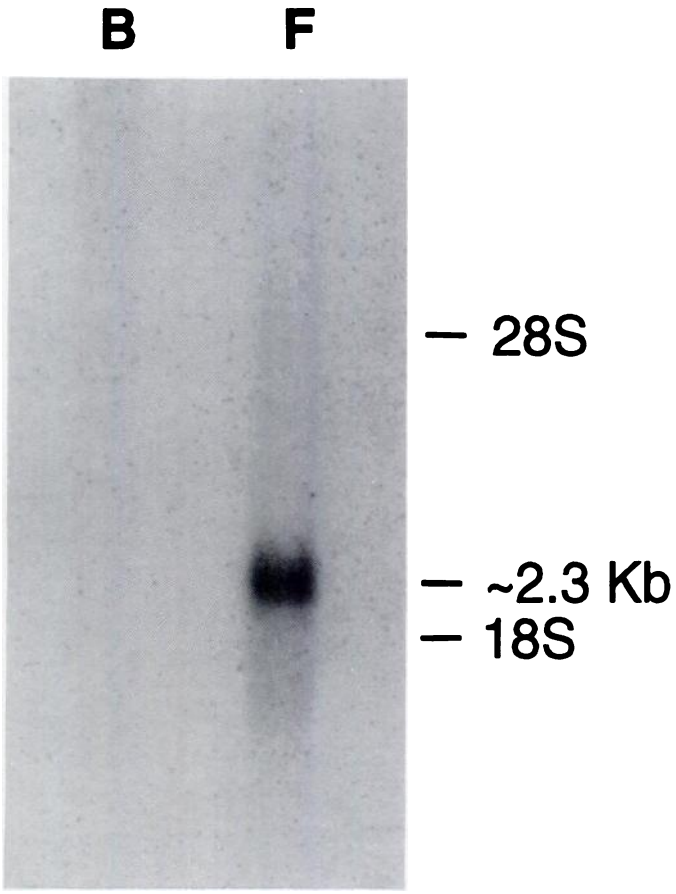

Fig. 2. Northern blot analysis of rat stomach fundus and rat brain tissues. Each lane contains 15 μg of poly(A)$^+$ RNA from either brain (*B*) or stomach fundus (*F*). The blot was probed with the 708-base pair PCR fragment labeled with [^{32}P]dCTP.

then used to identify those plaques harboring the longest cDNA inserts. Oligonucleotide primers flanking the *NotI* and *SalI* cloning sites of λgt22 arms were designed and inserts were amplified using the PCR reaction conditions described above and the following profile: 95° for 1.5 min, 50° for 1 min, and 72° for 4 min for 35 cycles. Large scale phage cultures for DNA isolation were prepared for the three clones harboring the largest cDNA inserts, using standard methods (9). The purified cDNA inserts were subcloned into the plasmid vector pSPORT-1 (BRL) for sequencing by the Sanger dideoxynucleotide chain termination method (10) and with the Taqtrack DNA sequencing kit (Promega, Madison, WI). All sequence analysis and manipulations were performed using the University of Wisconsin GCG software package and the MacVector (IBI) sequence analysis package.

Northern and Southern blot analyses. Total RNA and poly(A)$^+$ RNA were isolated from rat stomach fundus and rat whole brain, as described above. Samples of 15 μg of denatured poly(A)$^+$ RNA from each tissue were separated on a 1.0% agarose gel containing 2.2 M formaldehyde and 20 mM MOPS. The RNAs were transferred to a Nytran (Schleicher and Schuell, Keene, NH) nylon membrane using standard capillary blotting technique (9) and were prehybridized overnight at 45° in a solution containing 50% formamide, 5× SSPE, 5× Denhardt's, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was performed overnight at 45° in fresh buffer to which a randomly primed, [^{32}P]dCTP-labeled probe (708-base pair PCR fragment) was added at a concentration of 2 × 10^6 cpm/ml. The filter was washed three times for 10 min each in 2× SSC/0.1% SDS at 50° and three times for 30 min each in 0.1× SSC/0.1% SDS at 50°.

Samples of rat genomic DNA (10 μg) were digested with various restriction enzymes and separated on a 0.8% agarose/Tris-borate-EDTA gel according to standard Southern techniques (9). The samples were denatured and transferred to a nylon membrane as described above. Prehybridization, hybridization, and washing conditions were identical to those described for Northern analysis except that all were carried out at 37°.

Transfection and eukaryotic expression. A full length 5-HT$_{2F}$ receptor cDNA was modified by the addition of *SalI* linkers to the ends and was ligated into the *SalI* site of the eukaryotic expression vector p-hd (11) and designated pX5HT$_{2F}$. High levels of RNA transcription from this expression vector were achieved by the use of a hybrid regulatory element consisting of the BK virus enhancer and the adenovirus-2 major late promoter. AV-12 cells (ATCC no. CRL 9595) were transfected with linearized DNA using $CaPO_4$ co-precipitation (12). After 72 hr, stable transformants were selected by the addition of hygromycin B to the medium at a concentration of 200 μg/ml. After 3 weeks, 200 clones were selected, propagated, and assessed for levels of 5-HT$_{2F}$ mRNA expression by the "cytodot" technique (13). All cells were cultured with Dulbecco's modified Eagle medium (no. 380–2430; GIBCO, Gaithersburg, MD) supplemented with 10% fetal calf serum, 50 units/ml penicillin G, 50 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B, in a humidified atmosphere of 95% air/5% CO_2 at 37°. Two cell lines were established, one cell line expressing low levels of receptors for functional studies and one cell line expressing high levels of receptors for binding studies. To achieve high receptor levels in the second cell line, methotrexate was added to the medium to a final concentration of 1 μM, thereby inducing amplification of the integrated pX5HT$_{2F}$ DNA and consequently higher levels of RNA and protein expression.

Measurement of PI hydrolysis. Stable cell lines expressing the 5-HT$_{2F}$ receptors were labeled with 4.0 μCi of *myo*-[^{3}H]inositol/ml of cell culture medium, in 12-well plates, for 48 hr. Drugs were added to wells in 1 ml of serum-free medium containing 10 mM LiCl and 10 mM *myo*-inositol, and plates were incubated at 37° for 1 hr. The medium was aspirated and the inositol phosphates were extracted from the cells with ice-cold acetone/methanol (1:1), followed by a water wash, for analysis by ion exchange chromatography as previously described (14, 15).

Preparation of membranes from cloned cells. Suspension cells stably expressing the cloned rat 5-HT$_{2F}$ receptor were harvested by centrifugation at 2200 × *g* for 15 min at 4°. Membranes for the binding assays were prepared by vortexing the pellet in 50 mM Tris·HCl, pH 7.4 (0.5 × 10^9 cells/30 ml). The tissue suspension was then centrifuged at 39,800 × *g* for 10 min at 4°. This procedure was repeated for a total of three washes, with a 10-min incubation at 37° between the second and third washes. The final pellet was homogenized in 67 mM Tris·HCl, pH 7.4, at 12.5 × 10^6 cells/ml (original cell number) using a Tissumizer (Tekmar, Cincinnati, OH) at setting 65 for 15 sec.

[^{3}H]Serotonin binding. Binding assays were performed in triplicate in 0.8-ml total volume. Volumes of 200 μl of membrane suspension (0.07–0.10 mg of protein) and 200 μl of drug dilution in water were added to 400 μl of 67 mM Tris·HCl, pH 7.4, containing [^{3}H]serotonin, pargyline, $CaCl_2$, and L-ascorbic acid. Final concentrations of pargyline, $CaCl_2$, and L-ascorbic acid were 10 μM, 3 mM, and 0.1%, respectively. Tubes were incubated at 37° for 15 min and then rapidly filtered, using a Brandel cell harvester (model MB-48R; Brandel, Gaithersburg, MD), over Whatman GF/B filters that had been presoaked in 0.5% polyethylenimine and precooled with ice-cold 50 mM Tris·HCl, pH 7.4. The filters were then washed rapidly four times with 1 ml of ice-cold 50 mM Tris·HCl, pH 7.4. The amount of [^{3}H]serotonin trapped on the filters was determined by liquid scintillation counting (Ready Protein and Beckman LS 6000IC; Beckman Instruments, Fullerton, CA). For the saturation experiments, actual free [^{3}H]serotonin concentrations were determined by sampling the supernatant of parallel saturation experiments in which bound radioactivity had been separated by centrifugation. Serotonin (10 μM) or 1-naphthylpiperazine (10 μM) defined nonspecific binding. For competition experiments, six concentrations of displacing drugs were used, ranging from 10^{-5} to 10^{-10} M, and the

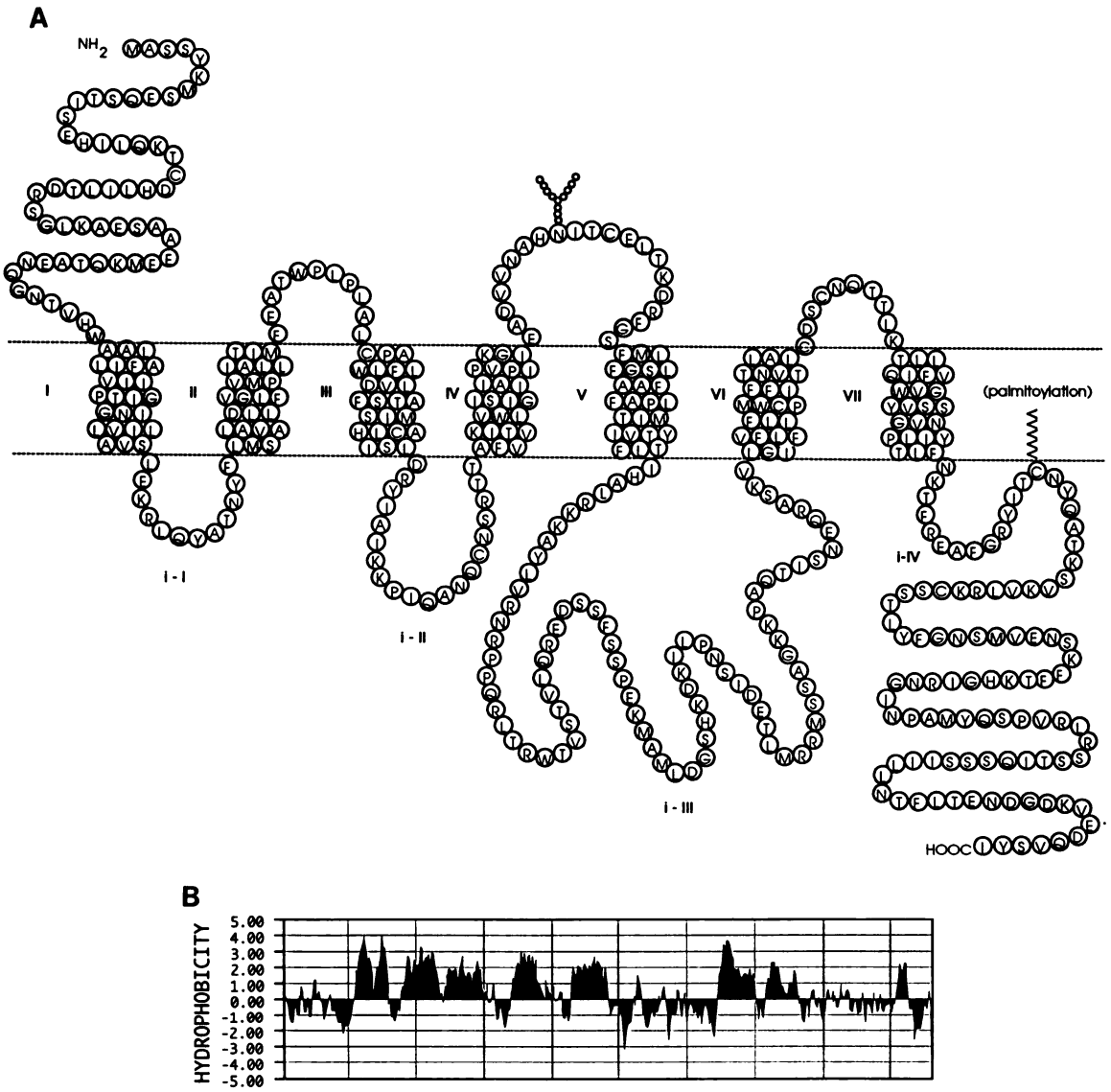

Fig. 3. Deduced amino acid sequence and putative structural model of 5-HT_{2F} receptor. A, The amino acid sequence was deduced from the one open reading frame found in the nucleic acid sequence. B, Hydropathy analysis of the sequence predicted seven putative transmembrane regions, as indicated in the model diagram.

final concentration of [^{3}H]serotonin was 2 nM. The amount of protein was determined by the method of Bradford (16), using bovine serum albumin as the standard. The K_d and B_{max} values from the saturation assays and the IC_{50} values from the competition assays were determined by nonlinear regression analysis (Systat; Systat Inc., Evanston, IL). The IC_{50} values were converted to K_i values using the Cheng-Prusoff equation (17).

Contraction of rat stomach fundus. Several tryptamine and piperazine analogues were examined for contractile efficacy in the rat stomach fundus, according to previously published procedures (3, 18). EC_{50} values were the agonist concentrations resulting in half-maximal contraction. This was determined by least squares regression analysis of the linear portion of the concentration-response curve and expressed as the negative logarithm of the EC_{50} (pEC_{50}).

Results

Cloning of the 5-HT receptor. It was reasonable to postulate that the contractile serotonin receptor of the rat stomach fundus represented a novel member of the 5-HT receptor family, based on two important pieces of information. The first piece of information was the excellent correlation ($r = 0.927$) (5) exhibited between contraction of smooth muscle in the rat stomach fundus and binding affinities at the 5-HT_{1C} receptor, using 10 different agonists. This established that this receptor was most likely a member of the 5-$HT_{1C/2}$ receptor subfamily. The second piece of information indicated by both PCR and Northern blot analysis was that 5-HT_{1C} mRNA was not present in rat stomach fundus. So, although the correlation with the 5-HT_{1C} receptor was very high, the receptor was not of the 5-HT_{1C} receptor subtype. As a consequence, we designed a strategy using PCR amplification as a means of initially obtaining a portion of the 5-HT_{2F} receptor cDNA that could be subsequently used in more conventional techniques to isolate full length cDNAs. This strategy made the assumption that this novel receptor was a member of the G protein-coupled receptor superfamily.

The nucleic acid sequences of the putative transmembrane

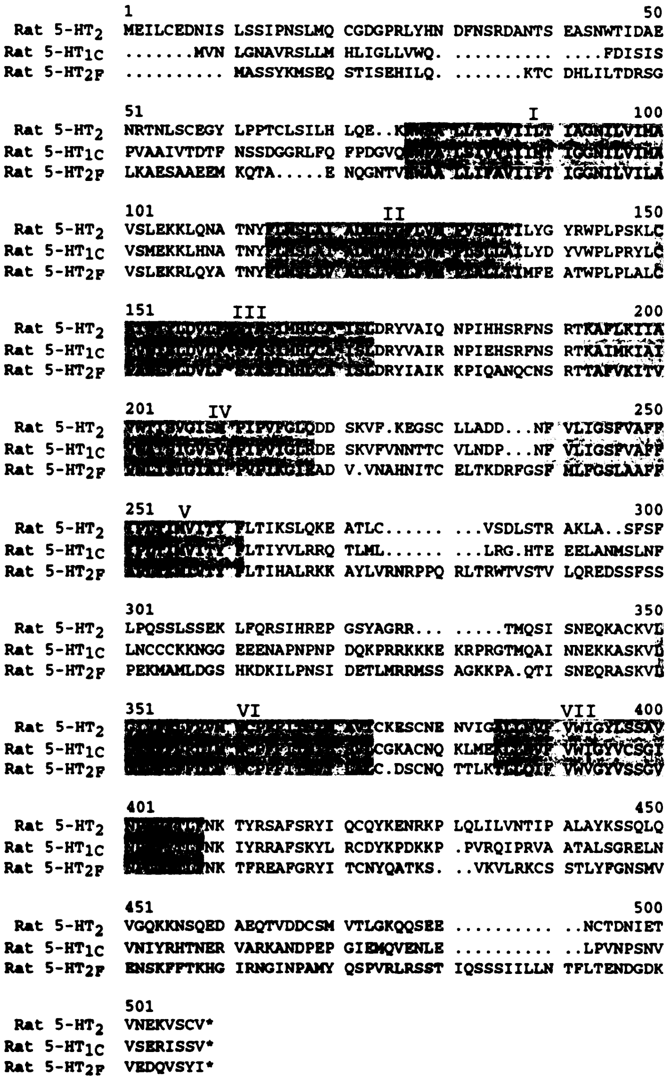

Fig. 4. Comparison of 5-HT$_{2F}$ amino acid sequence with 5-HT$_{1C}$ and 5-HT$_2$ sequences. The putative transmembrane domains have been *shaded* and indicated. Sequences were aligned using the University of Wisconsin GCG Software Package.

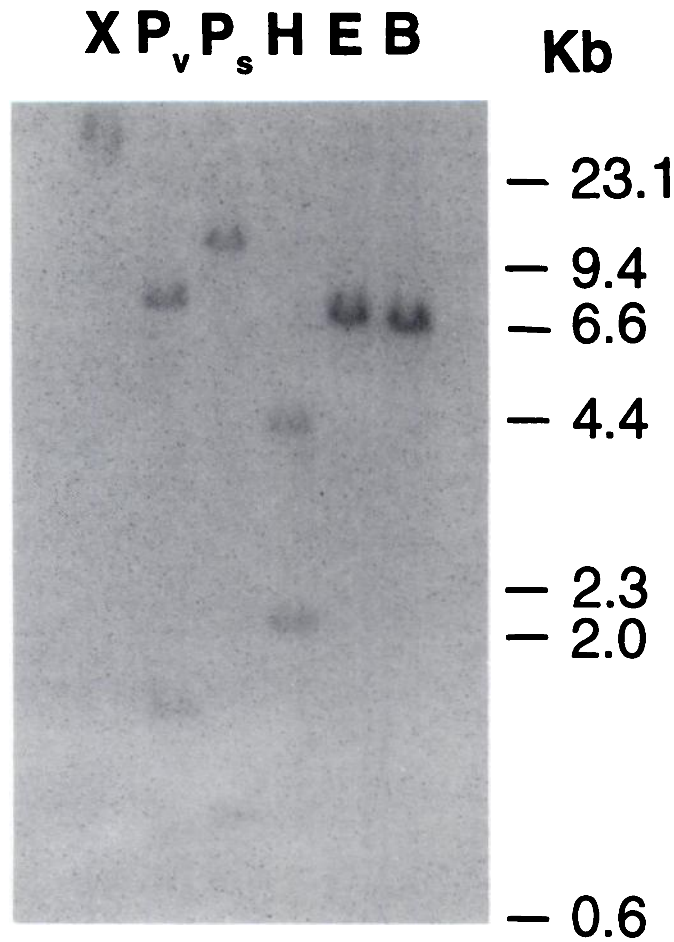

Fig. 5. Southern blot analysis of rat genomic DNA. The 708-base pair PCR fragment was used as a probe as in Northern analysis. The 10-μg samples of rat genomic DNA were digested with the following enzymes: *B, Bam*HI; *E, Eco*RI; *H, Hind*III; *P$_s$, Pst*I; *P$_v$, Pvu*II; and *X, Xba*I.

regions of the cloned serotonin receptors (19–25) as well as RDC4 (26), recently identified to be the 5-HT$_{1D\alpha}$ receptor (27), were compared and aligned. Transmembrane domains III and VI were found to be the most highly homologous. A degenerate oligonucleotide primer representing a partial consensus sequence and consisting of eight different individual sequences was designed for transmembrane region III (designated TM-III), where 22 of 25 positions were found to be strongly conserved (Fig. 1A). Because of the greater degree of degeneracy in transmembrane region VI, two degenerate oligonucleotide primers were designed (Fig. 1B), one of which was more 1C/2-like (designated TM-VI 1C/2-like) and represented a pool of 16 different sequences and one of which was more 1A/RDC4-like (designated TM-VI 1A/RDC4-like) and represented a pool of eight different sequences. Again, these primers represented partial consensus sequences in strongly conserved positions.

The two combinations of primers were used to probe, by PCR, cDNA synthesized from rat stomach fundus poly(A)$^+$ RNA. The TM-III/TM-VI 1C/2-like primer combination amplified a very intense 600-base pair DNA band (Fig. 1C), as visualized by ethidium bromide staining, not expected of either 5-HT$_{1C}$ or 5-HT$_2$ receptor sequences and a less intense 540-base pair DNA band suggestive in size of the 5-HT$_2$ receptor sequence. Previous studies with the rat stomach fundus had documented the presence of the 5-HT$_2$ receptor (28). The TM-III/TM-VI 1A/RDC4-like primer combination did not result in the amplification of any consistently reproducible DNA bands. The 600-base pair fragment (designated 600-K) was subcloned, sequenced, and translated in all three reading frames to determine whether it was related to serotonin receptors or any G protein-coupled receptors in general. One open reading frame was identified whose amino acid sequence shared highest homology with the serotonin receptor family.

Using another degenerate oligonucleotide primer based on transmembrane region II sequences [5′-CTGATATGCTG(G/C)TGGG(A/T)(T/C)T(A/C)CTTGTCATGCC-3′] and the TM-VI 1C/2-like primer, another DNA fragment, which was 708 base pairs long, was amplified by PCR. This fragment represented an extension of the 600-K fragment of 100 base pairs in the 5′ direction. This 708 base-pair fragment provided an excellent probe with which to screen two fundus cDNA libraries constructed in the cloning vector λgt22. A pool from these two λ libraries (1.5 × 10^6 independent clones) was screened, resulting in the identification of 21 positively hybridizing plaques. PCR amplification allowed the identification of those positive clones with the longest cDNA inserts. The long-

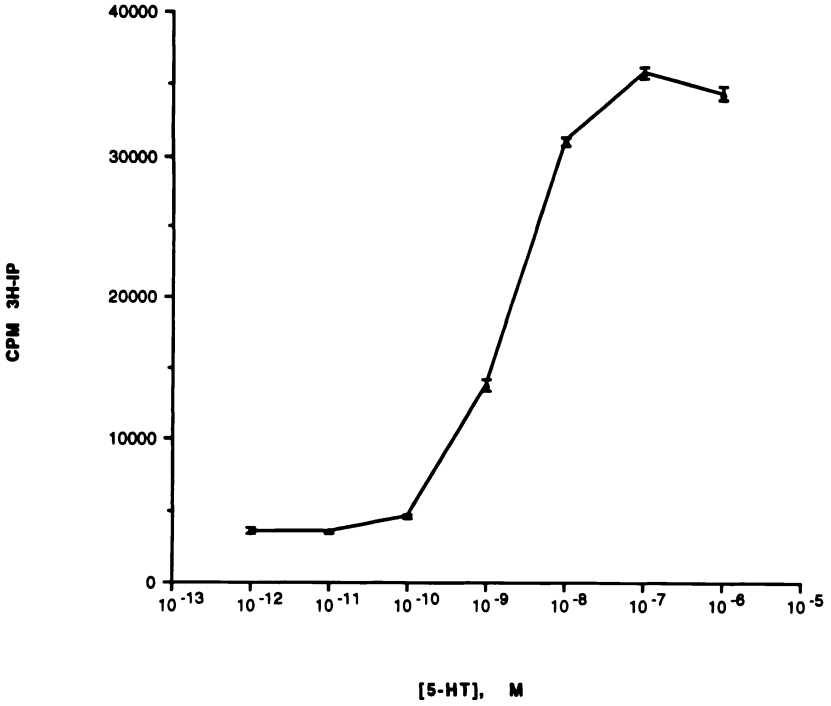

Fig. 6. Assay of phospholipase C activity in the cell line expressing the 5-HT_{2F} receptor. The clonal cell line A600K-2–3S, expressing 99–229 fmol of receptor/mg of protein, was labeled with *myo*-[^{3}H]inositol for 48 hr and stimulated with various doses of 5-HT for 1 hr at 37°. Inositol phosphates (*3H-1P*) were extracted and quantitated as described in Experimental Procedures. The dose-response curve is from a representative experiment in which each drug dose was assayed in triplicate.

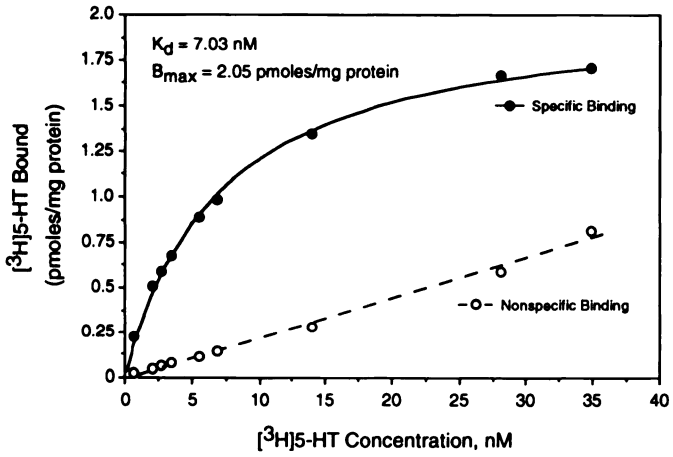

Fig. 7. Representative saturation curve for [^{3}H]5-HT binding to the 5-HT_{2F} receptor. The *curve* for specific binding was fitted to the points by nonlinear regression, and the *line* for nonspecific binding was fitted to the points by linear regression. B_{max} values for a series of curves ranged from 0.1 to 2.4 pmol/mg of protein, depending on the clonal isolate. The K_d = 7.9 ± 0.6 nM (mean ± standard error from seven separate experiments).

est inserts were 2.3 kb in length, which when compared with the 2.2–2.4-kb signal observed by Northern blot analysis of rat stomach fundus poly(A)$^+$ RNA (Fig. 2) suggested that these were full length cDNAs. The inserts from each of three clones were isolated and subcloned into the vector pSPORT-1 (BRL) for sequencing and further analysis. Sequence analysis using the University of Wisconsin GCG software package identified one open reading frame of 479 amino acids in all three clones (Fig. 3A). A hydrophobicity plot (Fig. 3B) revealed seven aggregations of hydrophobic amino acids large enough to span a plasma membrane, which is a definitive feature of G protein-coupled receptors. Because of the presence of two ATG codons within the first 21 nucleotides of the open reading frame, neither of which fit exactly the Kozak consensus sequence (29) for translational initiation, we could not determine precisely the translational start site.

Comparison of the amino acid sequence of this open reading frame with the Swiss-Protein Database demonstrated highest homology being shared with 5-HT receptors. Among this family, the 5-HT_{2F} receptor exhibited greatest homology with 5-HT receptor subtypes 1C and 2 (70% and 68%, respectively, within the transmembrane domains and 44% and 48%, respectively, overall) (Fig. 4). The high homology in the putative transmembrane regions of these three receptors strongly suggests a distinct subfamily of 5-HT receptors. One potential *N*-linked glycosylation site was identified in the second extracellular loop at residue Asn^{203}. Numerous potential phosphorylation sites for various protein kinases were also noted in the third intracellular loop and the carboxyl-terminal tail. Several other features generally characteristic of G protein-coupled receptors were also identified in the 5-HT_{2F} receptor. These included the DRY motif found at the end of transmembrane region III, a potential palmitoylation site at residue Cys^{396} in the carboxyl tail, aspartate residues 99 and 134 in the second and third transmembrane regions, and proline residues 188, 228, 338, and 376 in transmembrane regions IV, V, VI, and VII, respectively (30). Southern blot analysis of rat genomic DNA using the 708-base pair PCR fragment as a probe under conditions of high stringency revealed a pattern consistent with a single-copy gene (Fig. 5). These Southern data coupled with PCR data using 5-HT_{2F} receptor-specific primers and rat genomic DNA (data not shown) indicated that, unlike most members of the G protein-coupled receptor superfamily, the 5-HT_{2F} receptor gene contains introns within its coding sequence.

Transfection and stable expression. A full length cDNA was subcloned into the p-hd eukaryotic expression vector and subsequently transfected into the AV-12 cell line. After selection with the antibiotic hygromycin B, a number of stable transformants were expanded and assessed for levels of 5-HT_{2F} receptor mRNA expression by cytodot hybridization. Those

TABLE 1

Comparison of the affinity of agonists and partial agonists at the 5-HT$_{2F}$ receptor with their potency in the rat stomach fundus

Compound	5-HT$_{2F}$ receptor pK_i[a]	Rat stomach fundus pEC_{50}[b]	5-HT$_{1C}$ receptor pK_i[c]	5-HT$_2$ (agonist-labeled) pK_i[d]	5-HT$_2$ (antagonist-labeled) pK_i[c]
5-HT	8.02 ± 0.10 (4)	8.07 ± 0.06 (11)	7.48[e]	8.12[f]	5.53[e]
TFMPP	7.07 ± 0.09 (4)	7.54 ± 0.09 (6)	7.21[g]	7.79[f]	6.57[g]
5-CT	6.88 ± 0.07 (3)	7.64 ± 0.14 (5)	6.21[e]	7.06[h]	4.66[g]
Quipazine	6.90 ± 0.12 (3)	7.46 ± 0.16 (6)	6.73[e]	7.78[f]	6.19[e]
2-Me-5-HT	6.55 ± 0.02 (3)	7.03 ± 0.07 (4)	6.19[i]		>5[i]
8-OH-DPAT	5.39 ± 0.03 (3)	5.64 ± 0.06 (4)	5.14[e]	6.20[f]	5.15[e]
		r^2 = 0.917[j]	r^2 = 0.889	r^2 = 0.859	r^2 = 0.091
		p < 0.003	p < 0.005	p < 0.02	p < 0.561

[a] Values are expressed as the mean ± standard error from the number of separate experiments given in parentheses. pK_i is the negative logarithm of the dissociation constant.
[b] Values are expressed as the mean ± standard error from the number of separate tissues given in parentheses. pEC_{50} is the negative logarithm of the concentration of compound required to produce 50% of its maximal response.
[c] pK_i values were calculated against antagonist radioligands.
[d] pK_i values were calculated against agonist radioligands.
[e] Taken from Ref. 36.
[f] Taken from Ref. 38.
[g] Taken from Ref. 33.
[h] Taken from Ref. 39.
[i] Taken from Ref. 37.
[j] r^2 = correlation coefficient from the linear regression with the 5-HT$_{2F}$ receptor.

clones expressing the highest levels of mRNA were expanded further and used for functional and binding studies.

Functional coupling to PI hydrolysis. Due to its structural homology to the 5-HT$_{1C}$ and 5-HT$_2$ receptors, functional coupling of the 5-HT$_{2F}$ receptor to phospholipase C was also investigated. The expressed 5-HT$_{2F}$ receptor elicited dose-dependent increases of PI hydrolysis when stimulated with 5-HT and had an apparent EC_{50} of 1.6 nM (Fig. 6). A maximum stimulation of PI hydrolysis of 10-fold over basal (where basal PI turnover yields approximately 3500 cpm of [^{3}H]inositol phosphates) was noted with 100 nM 5-HT. Similar dose responses were also noted in subsequent assays (data not shown).

Ligand-binding studies. Using membranes from cells stably transformed with 5-HT$_{2F}$ receptor cDNA, it was found that [^{3}H]5-HT bound in a saturable fashion and with high affinity (Fig. 7). Nonlinear regression analysis of the saturation curves was consistent with binding to a single class of sites. No specific [^{3}H]5-HT binding was found in the untransfected parental cell line (data not shown). The high affinity for [^{3}H]5-HT (K_d = 7.9 ± 0.6 nM) was consistent with the structural data suggesting that this was indeed a 5-HT receptor. A series of full and partial agonists were examined at the rat stomach fundus receptor and at the 5-HT$_{2F}$ receptor (Table 1). The affinities of these compounds were in agreement with the molecular data placing this receptor in the 5-HT$_2$ receptor subfamily. As shown in Table 1, there were statistically significant correlations between the pK_i values for the compounds at the 5-HT$_{2F}$ receptor and values previously reported for both the antagonist-labeled 5-HT$_{1C}$ receptor and the agonist-labeled 5-HT$_2$ receptor. There was, however, no correlation between pK_i values at the 5-HT$_{2F}$ receptor and at the antagonist-labeled 5-HT$_2$ receptor. The finding that the profile for the antagonist-labeled 5-HT$_{1C}$ receptor correlated well with that for the agonist-labeled 5-HT$_{2F}$ receptor is consistent with previous work showing little difference between agonist affinities regardless of whether the 5-HT$_{1C}$ receptor was labeled by an agonist or an antagonist (31).

Table 1 also shows the high degree of correlation between the pK_i values for the 5-HT$_{2F}$ receptor and the pEC_{50} values for agonists in contracting the rat stomach fundus. The agonists and partial agonists chosen for this preliminary profile were not sufficiently selective to differentiate the 5-HT$_{2F}$ receptor from either the 5-HT$_{1C}$ or the 5-HT$_2$ receptor. However, the very low affinity of ketanserin for the 5-HT$_{2F}$ receptor (pK_i = 5.45 ± 0.02) clearly differentiates this receptor from the 5-HT$_2$ receptor (pK_i = 9.4) (32). Pharmacological differentiation could also be seen between the 5-HT$_{2F}$ and 5-HT$_{1C}$ receptors. Thus, ketanserin had much higher affinity for the 5-HT$_{1C}$ receptor (pK_i = 7.01) (33) than it did for the 5-HT$_{2F}$ receptor. Mesulergine also showed higher affinity for the 5-HT$_{1C}$ receptor (pK_i = 8.79) (33) than it did for the 5-HT$_{2F}$ receptor (pK_i = 7.45 ± 0.05).

Discussion

The presence in rat stomach fundus of a contractile receptor with extreme sensitivity to serotonin has been well documented for over 30 years. A large body of data accumulated over the last few years has defined more precisely the relationship of the contractile 5-HT receptor in the rat stomach fundus to the better known and more thoroughly characterized serotonin receptors. These data have established a close relationship, but nonidentity, with the 5-HT$_{1C}$ receptor. By taking advantage of the power of the PCR amplification technique and the high degree of evolutionary conservation of the nucleic acid sequences of the cloned serotonin receptors, we have successfully cloned and characterized a new member of the serotonin receptor family.

Northern analysis has revealed that this receptor is expressed at low levels in rat stomach fundus but is absent in rat whole brain. Its absence in brain may simply reflect the limited sensitivity of this particular assay and does not exclude the possibility of its presence at the level of protein expression on neuronal processes that form synapses in the brain but whose cell bodies lie outside the brain. In addition, the receptor may be expressed in discrete nuclei within the brain, which would require more thorough and precise dissection. Current experiments are addressing all of these possibilities.

The rat 5-HT$_{2F}$ receptor has 96% homology, at the amino acid level, to a partial amino acid sequence recently reported

to have been cloned from mouse stomach fundus (34). No data regarding the binding and/or second messenger-coupling characteristics of this receptor were available for more extensive comparisons with the 5-HT_{2F} receptor. Analysis of the primary structure of the 5-HT_{2F} receptor provides very strong evidence for a close evolutionary relationship between the 5-HT_{2F} receptor and the 5-HT_2 receptor subfamily. The presence of introns within the coding sequence of 5-HT_{2F} receptor and the ability of the receptor to couple functionally *in vitro* to PI hydrolysis in a heterologous cell line also support this evolutionary relationship. The human 5-HT_2 (35) and mouse 5-HT_{1C} receptors (34) have recently been reported to contain introns within their coding regions, which is consistent with our Southern and PCR data using rat genomic DNA.

The pharmacological characterization of the 5-HT_{2F} receptor is consistent with the molecular data that classified this receptor as a member of the 5-HT_2 receptor subfamily. The nanomolar affinity of the receptor for 5-HT makes it highly unlikely that this receptor is anything but a 5-HT receptor. The significant pharmacological correlations with both the 5-HT_{1C} and 5-HT_2 receptors (Table 1), as well as its coupling to PI hydrolysis, argue for its inclusion into the 5-HT_2 receptor subfamily. The low affinity of the 5-HT_{2F} receptor for both 8-OH-DPAT and 5-CT rules out a close relationship with the 5-HT_{1A} receptor, and the low affinity for 5-CT also excludes other 5-HT_1 receptor subtypes, such as the 5-HT_{1B} and 5-HT_{1D} receptors, as close relatives. Although the structural and pharmacological data illustrate the similarity of the 5-HT_{2F} receptor to both the 5-HT_2 and 5-HT_{1C} receptors, these data also emphasize that the 5-HT_{2F} receptor is distinct from these other two members of the 5-HT_2 receptor subfamily. The only other known 5-HT receptor that shows a close pharmacological correlation with the 5-HT_{2F} receptor is the 5-HT receptor that mediates contractions in the rat stomach fundus (Table 1). The current data do not constitute proof that the 5-HT_{2F} receptor is identical to the contractile serotonin receptor in the rat stomach fundus, nor is there one single piece of overwhelming evidence that these two receptors are likely to be one and the same. However, the combination of evidence from several different sources is at least suggestive that these two receptors may be equivalent. This is based on 1) the presence of mRNA for the 5-HT_{2F} receptor in the rat stomach fundus, and 2) the highly significant pharmacological correlation between agonist affinities at the 5-HT_{2F} receptor and at the fundus receptor. In addition, the 5-HT_{2F} receptor can couple to PI hydrolysis, a primary mechanism by which G protein-coupled receptors can stimulate smooth muscle contraction. Thus, the 5-HT_{2F} receptor appears to be a likely candidate for mediating 5-HT-induced contraction of the rat stomach fundus.

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