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Pharmacological and Molecular Evidence that the Contractile Response to Serotonin in Rat Stomach Fundus Is Not Mediated by Activation of the 5-Hydroxytryptamine$_{1C}$ Receptor

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SUMMARY

The receptor mediating contraction in response to serotonin in the rat stomach fundus has not been characterized in light of the currently acceptable serotonergic receptor classification scheme. Several biochemical and pharmacological approaches to a characterization of this receptor have demonstrated nonidentity with the 5-hydroxytryptamine$_2$ (5HT$_2$), 5HT$_3$, 5HT$_{1A}$, and 5HT$_{1B}$ receptors, as defined by radiolabeled ligand binding studies in brain cortical membranes. Although there have been reports suggesting that the receptor in the rat stomach fundus may be analogous to the 5HT$_{1C}$ receptor, other pharmacological and biochemical studies have not been consistent with this idea. The present study utilized high affinity ligands for the 5HT$_{1C}$ receptor and the recently derived 5HT$_{1C}$ receptor cDNA clone to provide a more definitive approach to the examination of the relationship between the 5HT$_{1C}$ receptor and the serotonergic contractile receptor in the rat stomach fundus. Using three ligands with high affinity at the 5HT$_{1C}$ receptor, LY53857, ritanserin, and SCH23390, the contractile response to serotonin was inhibited by all three ligands. However, inhibition did not appear competitive nor was the inhibitory potency of these ligands consistent with their affinity at 5HT$_{1C}$ binding sites in brain cortical membranes. We further showed that SCH23390, unlike LY53857 and ritanserin, was also a partial agonist, producing a maximal contraction that was approximately 50% of the maximal response to serotonin in the rat stomach fundus. Thus, the use of these ligands did not support the contention that the receptor mediating serotonin-induced contractions in the rat stomach is identical to the 5HT$_{1C}$ receptor. In more definitive studies using a 5HT$_{1C}$ receptor cDNA probe, we were unable to detect hybridization of the probe with any mRNA species from the rat stomach fundus, whereas the 5HT$_{1C}$ receptor cDNA probe did hybridize to the 5HT$_{1C}$ receptor mRNA in rat brain. Because the cathepsin-D cDNA probe hybridized equally in rat brain and stomach fundus, ensuring the integrity of the RNA preparation from both tissues, the absence of measureable quantities of the 5HT$_{1C}$ receptor mRNA in the rat stomach was probe specific and not an artifact. Furthermore, primers specific for the rat 5HT$_{1C}$ receptor sequence did not detect significant levels of receptor mRNA in rat fundus, although the target sequence was amplified a minimum of 10^5-fold in a polymerase chain reaction. These studies do not support the contention that the receptor mediating contractile responses to serotonin in the rat stomach fundus is identical to the 5HT$_{1C}$ receptor. Rather, these studies are consistent with the idea that the receptor mediating contraction in response to serotonin in the rat stomach fundus is unique and not identical to most of the currently classified receptor binding sites.

High concentrations of serotonin are found in gastrointestinal tissue, although a physiological role for serotonin in the gastrointestinal tract has not yet been clearly elucidated. The rat stomach fundus is a tissue that markedly contracts in response to low concentrations of serotonin. Although the potent contractile response to serotonin has been known for some time (1), the receptors mediating this response in the stomach fundus have not been characterized in light of the current serotonergic receptor classification system. Recent studies have clearly documented that the contractile response to serotonin in the rat stomach fundus is not mediated by a prominent interaction with 5HT$_2$ receptors. This conclusion is based on the fact that 5HT$_2$ receptor antagonists did not antagonize the contractile response to serotonin in the rat stomach fundus (2–4) and that no correlation occurred between the ability of agonists to contract the rat stomach fundus and their ability to bind to 5HT$_2$ receptors (5). 5HT$_3$ receptors also do not mediate contraction to serotonin in the stomach fundus, because the relatively selective 5HT$_3$ receptor agonist, 2-methyl-serotonin, was not a particularly potent agonist in the rat stomach fundus (ED_{50} = 0.15 μM). Furthermore, the 5HT$_3$-selective antagonist ICS 205-930 did not block the contractile response to serotonin in the rat fundus (6).

Previous studies have considered the possibility that the

ABBREVIATIONS: 5HT, 5-hydroxytryptamine; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; kb, kilobases; DHFR, dihydrofolate reductase; 1-NP, 1-(1-naphthyl)piperazine.

receptor in the rat stomach fundus may resemble the $5HT_{1A}$ or $5HT_{1B}$ subtype (4–6). However, no correlation emerged when a series of agonists were examined for their ability to contract the rat stomach fundus and to bind to $5HT_{1A}$ receptors (5). Likewise, only a weak correlation was obtained, in a series of agonists, between their ability to contract the rat stomach fundus and to bind to $5HT_{1B}$ receptors (5). Although a weak correlation did occur, agonist affinity estimates at $5HT_{1B}$ sites did not quantitatively agree with affinities found for these agonists in the stomach fundus (5). Furthermore, with regard to antagonists, no block of serotonin-induced contractile responses in the rat stomach fundus occurred with the $5HT_{1A}$ antagonist, spiroxatrine, the $5HT_{1A}$ ligand, WB4101, or the $5HT_{1A}/5HT_{1B}$ antagonist, cyanopindolol (6).

Having ruled out several known serotonergic receptors as mediating the potent contractile activity of serotonin in the rat stomach fundus, more recently we (5) and others (7) considered the possibility that contraction in response to serotonin in the rat stomach fundus may result from its interaction with $5HT_{1C}$ receptors. In this regard, an excellent correlation (correlation coefficient = 0.93) occurred between the ability of several agonists to contract the stomach fundus and to bind to $5HT_{1C}$ binding sites (5). In brain and choroid plexis, $5HT_{1C}$ receptor activation has been associated with elevations in phosphoinositide turnover (8). However, in the stomach fundus, serotonin did not produce an increase in phosphoinositide turnover (9, 10) at concentrations well in excess of those required to contract this preparation, suggesting a dissimilarity with the $5HT_{1C}$ binding site.

The present study has explored two other approaches to determine more conclusively whether the contractile response to serotonin in the fundus is mediated by activation of $5HT_{1C}$ receptors. We have used three ligands that have documented high affinity at $5HT_{1C}$ receptors, ritanserin, LY53857, and SCH23390 (11). Our rationale for the use of these antagonists was based on the contention that, if the receptor mediating contraction in response to serotonin in the stomach fundus was analogous to the $5HT_{1C}$ receptor, then these $5HT_{1C}$ antagonists should competitively block the contractile response to serotonin with an affinity equivalent to their affinities at $5HT_{1C}$ binding sites. In the second approach of this study, we have used a mouse cDNA clone homologous to the coding sequence of the rat $5HT_{1C}$ receptor mRNA to determine, by RNA blot analysis, if rat stomach fundus contains $5HT_{1C}$ receptor-specific mRNA. If the $5HT_{1C}$ receptor is present in stomach fundus tissue, then the mouse cDNA clone should hybridize to its mRNA. Additionally, we have used primer sequences for the rat $5HT_{1C}$ mRNA in analyzing rat fundus RNA by PCR, which should reveal low levels of receptor mRNA that are not detectable by standard hybridization techniques. If very low levels of $5HT_{1C}$ receptor mRNA are present in rat fundus, then its amplification would greatly increase the probability of its detection.

Materials and Methods

Smooth Muscle Pharmacology

Isolation of smooth muscle preparations. Male Wistar rats (150–375 g; Laboratory Supply, Indianapolis, IN) were sacrificed by cervical dislocation, and longitudinal sections of the stomach fundus were prepared for *in vitro* examination. Two to four preparations were obtained from one rat fundus. Tissues were mounted in organ baths containing 10 ml of modified Krebs' solution of the following composition (mM concentrations): NaCl, 118.2; KCl, 4.6; $CaCl_2 \cdot 2H_2O$, 1.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; dextrose, 10.0; and $NaHCO_3$, 24.8. Tissue bath solutions were maintained at 37° and equilibrated with 95% O_2/5% CO_2. Tissues were placed under optimum resting force (4 g) and were allowed to equilibrate for approximately 1 hr before exposure to drugs. Isometric contractions were recorded as changes in g of force on a Beckman Dynograph with Statham UC-3 transducers.

Effect of agonists. Noncumulative contractile concentration-response curves for agonists were obtained by a stepwise increase in concentration after the preceding concentrations were washed out, every 15 to 20 min. Each agonist concentration remained in contact with the tissue for approximately 2–3 min before washout, and maximum response to each agonist concentration was measured. EC_{50} values were taken as the concentration of agonist that produced half its maximal contraction.

Effect of antagonists. Noncumulative contractile concentration-response curves for 5HT were obtained by a stepwise increase in concentration. After control responses, tissues were incubated with antagonist for 1 to 1.5 hr. Responses to agonists were then repeated in the presence of antagonist, with the readdition of the antagonist during the washout period between agonist concentrations. Concentration responses utilized only one agonist and one antagonist concentration per tissue. In each experiment, one tissue received vehicle instead of antagonist and was used to control for time-dependent changes.

Where appropriate, apparent antagonist dissociation constants (K_B) were determined for each concentration of antagonist, according to the following equation:

$$K_B = \frac{[B]}{(\text{Dose ratio} - 1)}$$

where $[B]$ is the concentration of the antagonist, and dose ratio is the EC_{50} of the agonist in the presence of the antagonist divided by the control EC_{50}. These results were then expressed as the negative logarithm of the K_B (i.e., $-\log K_B$).

Drugs used. Drugs were prepared daily in deionized distilled water or saline and kept on ice during the course of the experiments. The sources of the drugs used were as follows. 5HT-creatinine sulfate complex was from Sigma Chemical Company (St. Louis, MO). Ritanserin was a gift from Janssen Pharmaceutica (Beerse, Belgium). LY53857, 1-NP, and SCH23390 were synthesized in the Lilly Research Laboratories (Indianapolis, IN).

Molecular Biology

Purification of poly(A)$^+$ RNA and RNA blot analysis. Total cellular RNA was prepared from 1–2 g of rat tissues by homogenization in 5 M guanidinium thiocyanate (12) containing 0.025 M sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% sodium *n*-lauroylsarcosine (w/v), and 0.1% antifoam A emulsion (v/v; Sigma), with several bursts of a Polytron (Brinkmann Instruments, Westbury, NY). The homogenate was layered over a cushion of 5.7 M cesium chloride, 0.025 M sodium acetate, pH 5.0, 0.002 M disodium EDTA, and centrifuged for at least 16 hr at 20° and 83,000 × *g*. Pellets were resuspended in 1–2 ml of 0.1% SDS, 0.3 M sodium acetate, pH 5.2, 0.01 M disodium EDTA, and extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The RNA was precipitated from the aqueous phase with 2.5 volumes of ethanol, and the pellet was resuspended in sterile water. An equal volume of 2× loading buffer (1× = 0.02 M Tris, pH 7.6, 0.5 M lithium chloride, 0.001 M disodium EDTA, and 0.1% SDS) was added for batch selection of poly(A)$^+$ RNA with 40 mg of oligo(dT)-cellulose (Boehringer-Mannheim, Indianapolis, IN), as outlined by Maniatis *et al.* (13). The poly(A)$^+$-enriched RNA was eluted serially with three 300-μl aliquots of 0.01 M Tris, pH 7.6, 0.001 M disodium EDTA, 0.05% SDS, and the pooled eluates were precipitated with 2.2 volumes of ethanol after being made up to 0.3 M sodium acetate. The poly(A)$^+$-enriched RNA was resuspended in sterile water.

Ten micrograms of RNA were heated to 68° for 15 min in 0.024 M sodium phosphate buffer, pH 7.0, containing 0.006 M sodium acetate,

0.0012 M disodium EDTA, 60% deionized formamide (v/v), and 7.2% deionized formaldehyde. The denatured RNA was fractionated by electrophoresis for 16 hr at 45 V, with constant recirculation of the running buffer, in a 1.5% agarose gel containing 2.2 M (6.7%, v/v) formaldehyde and 0.02 M sodium phosphate buffer, pH 7.0 (14). After electrophoresis, the gel was stained for 20 min with 30 μg/ml acridine orange in running buffer and washed twice with fresh buffer for 20 min before photography using UV light. The fractionated RNA was transferred overnight to a nitrocellulose filter by capillary blotting (15) in 20× SSC (1× buffer contains 0.15 M sodium chloride and 0.015 M trisodium citrate). The filter was baked for 2 hr at 80° and prehybridized for at least 4 hr in hybridization buffer [50% formamide (v/v), 5× SSC, 0.05 M sodium phosphate buffer, pH 7.0, 0.25% nonfat dry milk (16)] at 42°. Hybridization with the 5HT$_{1C}$ and cathepsin-D probes (2–3 × 10^6 cpm/ml) was done at 42° for 16 hr. The filter was serially washed four times for 5 min at room temperature with 2× SSC, 0.1% SDS, and twice for 30 min to a final stringency of 52° with 0.1× SSC, 0.1% SDS. After air drying, the filters were exposed to Kodak XAR-5 film with a DuPont Cronex Lightning Plus intensifying screen, at −70°.

Probe preparation. The serotonin 5HT$_{1C}$ receptor cDNA probe was prepared from a 1.42-kb DNA fragment of a mouse cDNA clone, which includes the first 868 bases of the receptor-coding sequence (17),[1] and labeled by nick translation with [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA) to a specific activity of 1–2 × 10^8 cpm/μg of DNA. A 1.0-kb fragment of a human cathepsin-D cDNA clone was similarly labeled.

The probe for the DNA hybridization analysis was an oligonucleotide, 5′-GATGAAAAACGGGCACCACATGATCAGAAACACAAAGAATACAATGC-3′, synthesized from the sixth transmembrane region of the rat 5HT$_{1C}$ receptor sequence (18) and was end labeled with [γ-^{32}P]ATP and polynucleotide kinase to a specific activity of approximately 10^6 cpm/pmol of DNA.

PCR and DNA hybridization analysis. cDNA was synthesized from 50 μg of total cellular RNA, using a first-strand reaction mix from a cDNA synthesis kit (Pharmacia LKB Biotechnology, Piscataway, NJ). After a 1-hr incubation at 37°, the cDNA mixture was diluted to 400 μl with distilled water and a 10-μl aliquot was subjected to PCR in a DNA Thermal Cycler, using reagents from a Gene Amp kit (Perkin-Elmer Cetus, Norwalk, CT). The amplification reaction consisted of 30 cycles of 96° for 15 sec, 69° for 30 sec, and 72° for 1 min. The oligonucleotide primers specific for the rat 5HT$_{1C}$ receptor sequence and the mouse DHFR sequence were synthesized at Lilly Research Laboratories from published sequences (18, 19). The 5HT$_{1C}$ receptor-specific primers were 5′-ACACCGAGGAGGAACTGGCTAATAT-3′ and 5′-GACCACATTAGAGGGGTTGACTGGC-3′ and defined the borders of a 601-base-long DNA fragment. The DHFR-specific primers were 5′-CTCAGGGCTGCGATTTCGCGCCAAACT-3′ and 5′-CTGGTAAACAGAACTGCCTCCGACTATC-3′ and defined the borders of a 446-base-long DNA fragment. The PCR products were analyzed by agarose gel electrophoresis and DNA hybridization. Ten microliters of each reaction were fractionated in a 1.5% agarose gel run for approximately 2 hr in 0.04 M Tris base, 0.002 M disodium EDTA, adjusted to pH 7.9 with acetic acid. The gel was stained with 0.5 μg/ml ethidium bromide in running buffer and photographed using UV illumination. The DNA was transferred to a nitrocellulose filter by capillary blotting (20) in 20× SSC, and the filter was processed as described for RNA hybridization analysis, except that the formamide concentration was lowered to 20% and hybridization with the labeled probe (8 × 10^5 cpm/ml) was done at 37°. The final washes were done at room temperature in 0.5× SSC, 0.1% SDS.

Results

Effects of 5HT$_{1C}$ receptor antagonists on serotonin-induced contractions in rat stomach fundus. LY53857 has relatively high affinity at 5HT$_{1C}$ receptors (−log K_D = 8.08) (11). This compound at 10^{-9} M did not affect the contractile response to serotonin (Fig. 1) in the stomach fundus. However, as the concentration was increased to 3 × 10^{-9} and 10^{-8} M, noncompetitive inhibition of the contractile response to serotonin occurred, with a marked depression in the maximal response to serotonin (Fig. 1).

Ritanserin also possessed high affinity at 5HT$_{1C}$ sites (−log K_D = 8.64) (11). This compound at a concentration of 10^{-8} M had no effect on the contractile-response curve for serotonin in the rat stomach fundus. As the concentration was increased to 10^{-7} and 3 × 10^{-7} M, inhibition of the contraction in response to serotonin occurred, with a marked depression in the maximal response observed at 3 × 10^{-7} M ritanserin (Fig. 2).

SCH23390 also showed high affinity at 5HT$_{1C}$ sites (−log K_D

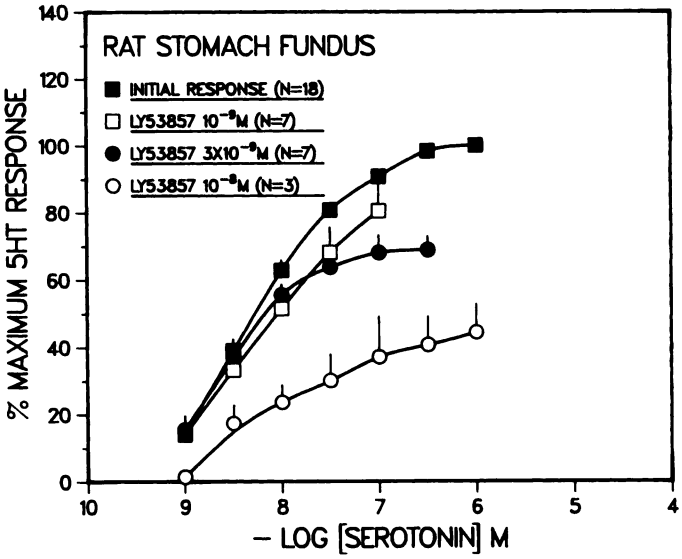

Fig. 1. Effect of LY53857 (10^{-9}, 3 × 10^{-9}, and 10^{-8} M) on the contractile concentration-response curve for serotonin in the rat stomach fundus. *Points* are mean values and *vertical bars* represent the standard error for the number of tissues indicated in parentheses.

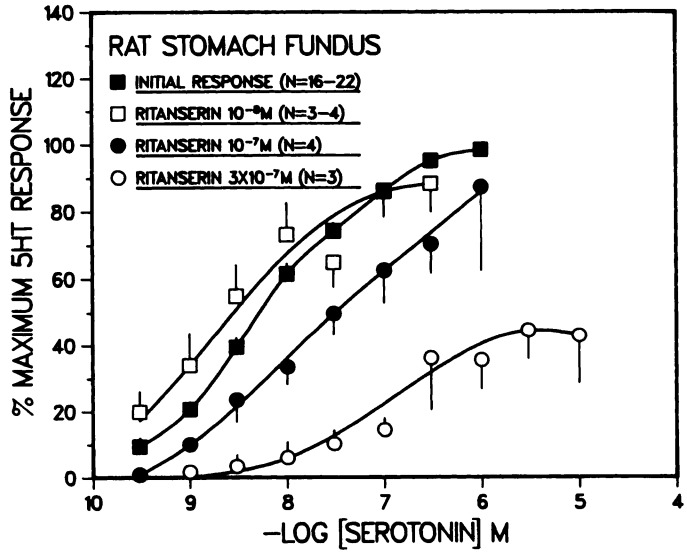

Fig. 2. Effect of ritanserin (10^{-8}, 10^{-7}, and 3 × 10^{-7} M) on the contractile concentration-response curve for serotonin in the rat stomach fundus. *Points* are mean values and *vertical bars* represent the standard error for the number of tissues indicated in parentheses.

[1] L. Yu *et al.*, unpublished data.

= 8.26) (11). Unlike the other two high affinity $5HT_{1C}$ receptor ligands, SCH23390 possessed partial agonist activity in the rat fundus (Fig. 3). This compound contracted the rat stomach fundus with an ED_{50} of $\sim 3 \times 10^{-7}$ M and a maximum response that was ~50% of the maximum contractile response to serotonin in rat stomach fundus. Contraction in response to SCH23390 was competitively antagonized by 1-NP (Fig. 4), an agent previously shown to block contractions in response to serotonin and other serotonergic agonists in the rat stomach fundus (6). The calculated −log of the "apparent dissociation constant" for 1-NP using SCH23390 as the agonist was 8.27 ± 0.11 (n = 7). At 10^{-8} M, SCH23390 did not contract the rat fundus or affect the contractile response to serotonin. As the concentration increased to 10^{-7} and 3×10^{-7} M, the contractile response to serotonin was modestly but not dose dependently shifted to the right (Fig. 5).

Thus, none of these ligands with high affinity at $5HT_{1C}$ receptors antagonized the contractile response to serotonin either in a competitive fashion or at a concentration consistent with their respective affinities at $5HT_{1C}$ binding sites.

RNA blot analysis in rat stomach fundus. A mouse serotonin receptor clone specific for the $5HT_{1C}$ subtype was hybridized to poly(A)$^+$ RNA purified from several tissues, to establish the presence of $5HT_{1C}$ receptor mRNA. The probe contains the first 868 nucleotides of the $5HT_{1C}$ receptor-coding sequence (17), which encompasses five of the seven transmembrane domains, as depicted in Fig. 6A, and shares 96% homology with the corresponding region of the rat $5HT_{1C}$ receptor sequence (18). Consequently, the mouse cDNA probe is expected to hybridize to the rat $5HT_{1C}$ receptor mRNA at high stringency (see Materials and Methods for hybridization conditions). Fig. 6B shows that the probe hybridized to a unique species of rat brain mRNA. The approximate size of this mRNA was 5.2 kb, in agreement with previous studies (18) in which an authenticated $5HT_{1C}$ receptor cDNA clone derived from rat choroid plexus hybridized to a single 5.2-kb mRNA prepared from that tissue and several other brain regions. Of special note was the absence of any hybridizing mRNA in the stomach fundus preparation. The liver, which was previously shown to lack detectable levels of $5HT_{1C}$ receptor mRNA (18), was also negative in this study. These results were consistently observed in three separate experiments.

Because the rat stomach fundus RNA preparation did not contain detectable levels of $5HT_{1C}$ receptor mRNA, we were compelled to control for the possibility of RNA degradation. A probe for cathepsin-D mRNA, which is ubiquitous in mammalian tissues (21), was combined with the $5HT_{1C}$ receptor probe for the RNA blot experiment depicted in Fig. 5B. As can be readily seen, a mRNA of the expected size (2.2 kb) was present in all three lanes, thus indicating that the mRNA preparations were intact. Both brain and stomach fundus contained similar amounts of cathepsin-D mRNA, whereas the

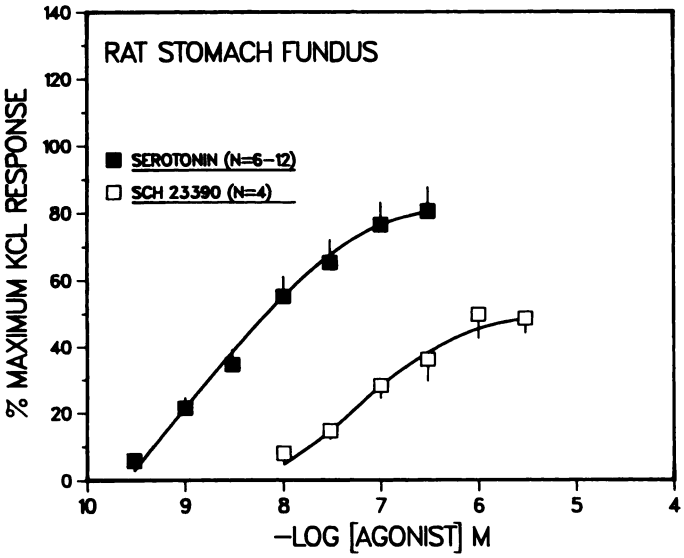

Fig. 3. Comparison of the concentration-response curve for contraction in response to SCH23390 and serotonin in the rat stomach fundus. *Points* are mean values and *vertical bars* represent the standard error for the number of tissues indicated in parentheses.

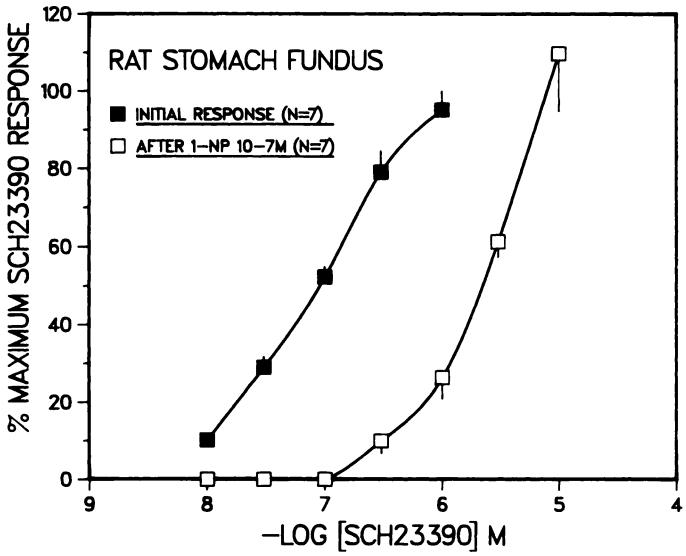

Fig. 4. Effect of 1-NP (10^{-7} M) to antagonize the contractile response to SCH23390 in the rat stomach fundus. *Points* are mean values and *vertical bars* represent the standard error for the number of tissues indicated in parentheses.

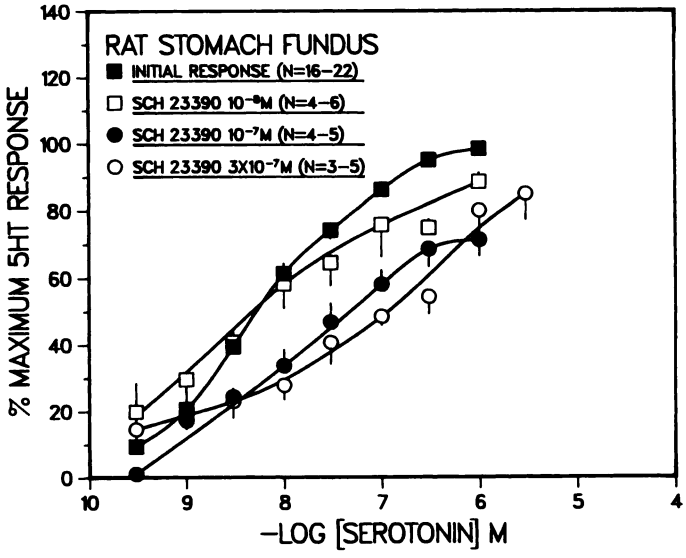

Fig. 5. Effect of SCH23390 (10^{-8}, 10^{-7}, and 3×10^{-7} M) on the contractile concentration-response curve for serotonin in the rat stomach fundus. *Points* are mean values and *vertical bars* represent the standard error for the number of tissues indicated in parentheses.

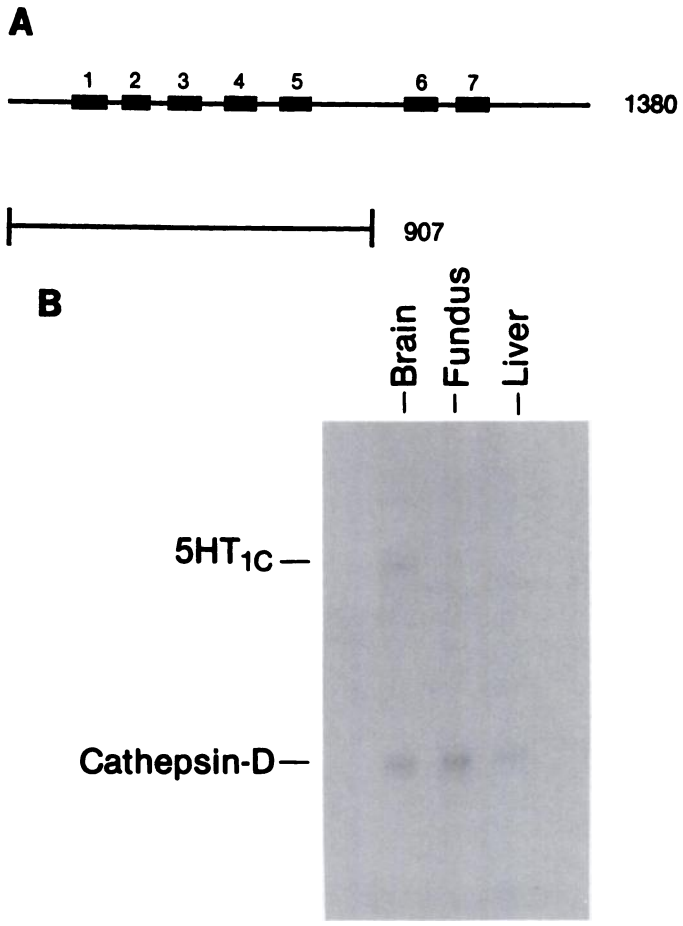

Fig. 6. Northern blot examination of rat fundus for 5HT$_{1C}$ receptor mRNA. Poly(A)$^+$ RNA (10 μg) purified from rat brain, fundus, and liver was denatured, fractionated in a 1.5% agarose gel containing 2.2 M formaldehyde, and transferred to a nitrocellulose filter that was probed with cDNA specific for mouse 5HT$_{1C}$ receptor and human cathepsin-D, as described in Materials and Methods (B). The *upper line drawing* in A depicts the rat 5HT$_{1C}$ receptor-coding sequence. *Filled rectangles* represent the relative size and position of the seven transmembrane domains of the encoded protein. The *lower line* shows the length of the mouse 5HT$_{1C}$ receptor probe and is aligned with the homologous region of the rat 5HT$_{1C}$ receptor-coding sequence.

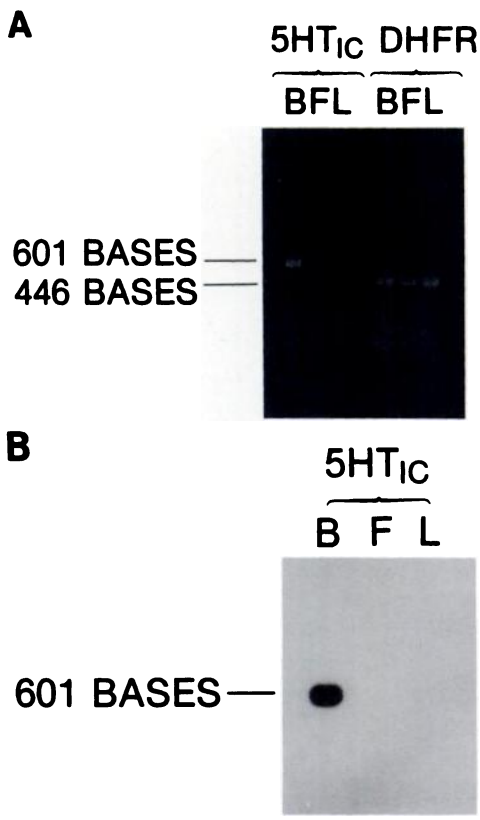

Fig. 7. PCR analysis of rat fundus for low levels of 5HT$_{1C}$ receptor mRNA. cDNAs prepared from total RNA purified from rat brain (*B*), fundus (*F*), and liver (*L*) were subjected to PCR amplification using oligonucleotide primers specific for 5HT$_{1C}$ receptor and DHFR sequences. The PCR products were fractionated in a 1.5% agarose gel and photographed with UV illumination after staining with ethidium bromide (A). The first three lanes of the gel pictured in A were further analyzed by DNA hybridization (B) using a synthetic oligonucleotide probe specific for the sixth transmembrane domain of the 5HT$_{1C}$ receptor-coding sequence, which is internal to the primer sequences that directed the amplification of the 601-base-long 5HT$_{1C}$ receptor DNA fragment (see Materials and Methods).

level in the liver was less. Based on the above observations, the absence of detectable 5HT$_{1C}$ receptor mRNA in rat stomach fundus is unlikely to be attributed to either unequal sample loading or degradation of mRNA during preparation.

PCR analysis of mRNA in rat stomach fundus. To determine whether the rat fundus contained 5HT$_{1C}$ receptor mRNA levels too low to be detected by standard hybridization techniques, we attempted to amplify the mRNA concentration by PCR. Two oligonucleotide primers specific for the rat 5HT$_{1C}$ receptor sequence were used to amplify a 601-base-long region of the receptor cDNA a minimum of 10^5-fold by 30 cycles in a DNA Thermal Cycler (22). The amplified products from the brain, fundus, and liver cDNAs were analyzed by gel electrophoresis, as depicted in Fig. 7A. Note that, in the first set of three lanes, only the brain-derived sample shows a DNA band of the expected size (601 bases) for the receptor sequence. To ascertain the integrity of the cDNAs used in the amplification reactions, primers specific for the DNA sequence of a ubiquitous enzyme, DHFR, were also used to amplify specific cDNAs. It can be readily seen in the second set of three lanes (Fig. 7A) that all samples contained approximately equal levels of amplified DNA specific for the DHFR sequence of 446 bases.

The cDNAs amplified with the 5HT$_{1C}$ receptor primers were further examined by DNA hybridization with a probe specific for a region internal to that recognized by the primers. Fig. 7B shows an autoradiogram with a single 601-base-long fragment present only in the brain sample. It should be noted that no signal was seen in the fundus- and liver-derived samples after a 16-hr exposure of the filter to film. However, prolonged exposure (6 days) of the filter revealed a weak signal in the fundus sample relative to the brain and a weaker signal yet in the liver (data not shown).

Discussion

To date, at least six serotonin receptors have been identified based on brain radiolabeled ligand binding studies. Previous efforts to characterize the receptor mediating contraction in response to serotonin in the rat stomach fundus in light of the currently accepted classification scheme revealed nonidentity with at least four of these receptors. The present study was prompted by pharmacological observations of similarity and dissimilarity between the receptor responsible for contraction

in the rat stomach fundus and the $5HT_{1C}$ binding site identified in brain tissue. The identification of high affinity ligands for the $5HT_{1C}$ receptor and the recent cloning (17, 18)[1] of the $5HT_{1C}$ receptor cDNA has permitted a definitive examination of the relationship of the $5HT_{1C}$ receptor to the serotonergic contractile receptor in the rat stomach fundus.

The use of pharmacological approaches rather than radioligand binding techniques to characterize the serotonergic contractile receptor in the rat stomach fundus has been necessary. This has been due to the limitations of high nonspecific receptor binding of serotonin to membrane preparations using the rat stomach fundus. Thus, it has not been possible to use radioligand binding techniques to characterize the serotonergic contractile receptor.[2]

We have taken advantage of three compounds reported to have high binding affinity at $5HT_{1C}$ receptors in brain membranes. Although binding affinities for ritanserin, LY53857, and SCH23390 at $5HT_{1C}$ sites have been reported (11), to date no studies of agonist or antagonist effects on biochemical parameters such as phosphoinositide turnover have been reported for these ligands. Furthermore, the kinetics for the interaction of ritanserin, LY53857, and SCH23390 with the $5HT_{1C}$ receptor have not been detailed with radioligand binding approaches using brain membranes. Nevertheless, the effects of these ligands at other receptors for which high binding affinity has been defined (i.e., $5HT_2$, D_2, and D_1) resulted in the demonstration of antagonist activity, raising the likelihood for similar antagonist effects at $5HT_{1C}$ receptors.

In the present study, although all three ligands did antagonize the contractile response to serotonin in the rat stomach fundus, we were unable to obtain clear competitive inhibition of the contractile response to serotonin. In addition, the calculation of apparent dissociation constants, if one assumed competitive antagonism, resulted in values that were not consistent with the reported affinities of these ligands at $5HT_{1C}$ receptors. Furthermore, ritanserin had about 3-fold higher affinity at $5HT_{1C}$ receptors than LY53857 (11), yet LY53857 was a more potent antagonist of serotonin-induced contractions in the rat stomach fundus. Although our conclusions with these three antagonists are limited by the minimal information available regarding the details of the interaction of these agents with $5HT_{1C}$ receptors, these data support the contention, nevertheless, that the receptor mediating serotonin-induced contraction in the rat stomach is not identical to the $5HT_{1C}$ receptor.

The kinetics seen with the $5HT_{1C}$ high affinity ligands in the rat stomach fundus were complex and apparently noncompetitive for LY53857 and ritanserin, whereas SCH23390 appears to be a partial agonist at the serotonergic contractile receptor. The agonist effects of SCH23390 could be antagonized by 1-NP, an agent that has been reported to competitively block serotonergic contractile responses in the rat stomach fundus (6), consistent with the agonist properties of SCH23390 being mediated by serotonergic receptors in the stomach fundus. The ability of these three ligands to antagonize, albeit noncompetitively, the contraction in response to serotonin in the rat stomach fundus raises the possibility that these ligands may be useful tools to probe with radioligand binding techniques in brain membranes the possibility that they can interact with yet another 5HT receptor that might be analogous to the one found in the rat stomach fundus.

Studies using the mouse $5HT_{1C}$ receptor cDNA probe provided additional support for a distinction between the contractile receptor in the fundus and the $5HT_{1C}$ site. Although other investigators have used a cDNA probe to determine the distribution of $5HT_{1C}$ receptor mRNA in several rat tissues (18), we are the first to examine rat stomach fundus by this method in an attempt to correlate the serotonin-mediated contraction of this tissue with the presence of the $5HT_{1C}$ receptor mRNA. Our cDNA probe includes 868 bases of the mouse $5HT_{1C}$ receptor-coding sequence and spans five of seven transmembrane domains that are structural features common to the family of GTP-binding protein-coupled receptors (23). Comparison of our probe sequence with the analogous region of the rat $5HT_{1C}$ receptor sequence reveals a homology of 96% (data not shown) and, thus, indicates that the mouse $5HT_{1C}$ receptor should hybridize at high stringency to the rat $5HT_{1C}$ mRNA.

Using this $5HT_{1C}$ receptor cDNA probe, hybridization was obtained with the probe and mRNA prepared from rat brain, a tissue known to contain the $5HT_{1C}$ receptor (8). A mRNA species of approximately 5.2 kb is clearly visible in Fig. 5B, even though the rat brain was not dissected to enrich for regions of high receptor density before the preparation of mRNA. This indicates that a high density of $5HT_{1C}$ receptors, such as that found in choroid plexus (24), is not essential for detection of receptor mRNA by our hybridization technique. Nevertheless, the rat stomach fundus did not contain detectable levels of $5HT_{1C}$ receptor mRNA.

We further established that the lack of detectable hybridization of the $5HT_{1C}$ receptor cDNA with the rat stomach fundus mRNA was probe specific and not limited by the mRNA utilized, because a cDNA probe for cathepsin-D (21) showed comparable hybridization with both brain and stomach fundus mRNA preparations. In an attempt to detect low levels of $5HT_{1C}$ mRNA in rat fundus, cDNAs were synthesized and amplified by PCR under conditions whereby a minimum increase of 10^5-fold in the target sequence was expected. Again, only the brain-derived cDNA revealed a specific signal when analyzed by gel electrophoresis, thus indicating that, if functional levels of $5HT_{1C}$ receptor are present in the fundus, their density is probably 10^5-fold lower than that in the brain. The DNA hybridization analysis of the PCR products revealed weak autoradiographic signals in fundus and liver only after prolonged exposure (6 days) of the labeled samples to the X-ray film. This suggests that the extremely low levels of $5HT_{1C}$ receptor mRNA detected in the fundus (and liver) samples may not be significant and probably result from illegitimate transcription. The detection of ectopic gene transcripts by PCR analysis has been previously documented for several other genes (25). Although we cannot rule out the possibility of an exceptionally low concentration of $5HT_{1C}$ receptor mRNA in the rat stomach fundus, it is most likely that the $5HT_{1C}$ receptor gene as expressed in brain is not expressed in the stomach fundus and, therefore, is not responsible for serotonin-induced contraction in this tissue.

Thus, although there are some pharmacological similarities (primarily based on agonist affinities) between the receptor mediating contraction in response to serotonin in the rat stomach fundus and the $5HT_{1C}$ receptor identified in brain tissue and recently cloned, these receptors appear to be distinct. This

[2] M.L. Cohen and R.J. Secrest, unpublished observations.

conclusion is based on 1) different second messenger systems responsible for activation, 2) the inability of 5HT$_{1C}$ ligands to inhibit 5HT-induced contractions in the stomach at concentrations consistent with their reported affinity at 5HT$_{1C}$ receptors, and 3) lack of 5HT$_{1C}$ receptor mRNA in concentrations sufficient to be measured and/or meaningful in stomach fundus. The possibility that contraction in response to serotonin in the rat fundus may be mediated by interaction with 5HT$_{1D}$ sites remains to be explored. This will require the development of more selective and potent 5HT$_{1D}$ ligands or identification of a cDNA probe for this receptor. Alternatively, identification of selective agonists and antagonists with high affinity and selectivity for the serotonergic contractile receptor in the rat fundus will be necessary to prove the uniqueness of this receptor, relative to other serotonergic receptors defined by radioligand binding studies. Furthermore, the cloning and sequencing of the receptor responsible for the serotonin-mediated contraction in the rat fundus and comparison of the sequence with those of the previously cloned receptors, together with a characterization of the ligand binding profile of the expressed receptor clone, would unequivocally establish the identity of the rat stomach fundus serotonin receptor.

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