Conserved Helix 7 Tyrosine Acts as a Multistate Conformational Switch in the 5HT2C Receptor

IDENTIFICATION OF A NOVEL “LOCKED-ON” PHENOTYPE AND DOUBLE REVERTANT MUTATIONS*

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Studies in many rhodopsin-like G-protein-coupled receptors are providing a general scheme of the structural processes underlying receptor activation. Microdomains in several receptors have been identified that appear to function as activation switches. However, evidence is emerging that these receptor proteins exist in multiple conformational states. To study the molecular control of this switching process, we investigated the function of a microdomain involving the conserved helix 7 tyrosine in the serotonin 5HT2C receptor. This tyrosine of the NPXXY motif was substituted for all naturally occurring amino acids. Three distinct constitutively active receptor phenotypes were found: moderate, high, and “locked-on” constitutive activity. In contrast to the activity of the locked-on mutant, the high basal signaling of the locked-on Y7.53N mutant was neither increased by agonists nor decreased by inverse agonists. The Y7.53F mutant was uncoupled. Computational modeling based on the rhodopsin crystal structure suggested that Y7.53 interacts with the conserved aromatic ring at position 7.60 in the recently identified helix 8 domain. This provided a basis for seeking revertant mutations to correct the defective function of the Y7.53F receptor. When the Y7.53F receptor was mutated at position 7.60, the wild-type phenotype was restored. These results suggest that Y7.53 and Y7.60 contribute to a common functional microdomain connecting helices 7 and 8 that influences the switching of the 5HT2C receptor among multiple active and inactive conformations.

The class A or rhodopsin-like G-protein-coupled receptors (GPCRs) are one of the largest protein families identified in the human genome (1, 2). This seven-transmembrane domain protein family, which includes the visual opsins, neurotransmitter, peptide hormone, and protein receptors, includes the targets of a significant proportion of therapeutic drugs (3). In response to chemical or physical external stimuli, GPCRs undergo a conformational change leading to the activation of heterotrimeric G-proteins and other intracellular signaling mediators.

The members of this receptor family can be recognized by their high degree of amino acid conservation at homologous positions in their transmembrane domains (4). This widespread conservation pattern suggests that these amino acids are likely to contribute to structural elements that mediate common receptor functions. The various rhodopsin-like GPCRs differ in the structure of their agonists and in the classes of G-proteins that they preferentially activate. However, they all share a common transmembrane signaling function. Thus these conserved side chains have been proposed to contribute to a network of interhelical interactions that could subserve the shared requirement of these proteins to undergo conformational rearrangements during activation (5). This view has been largely substantiated by a variety of studies of various GPCRs, in particular the landmark report of the crystal structure of the inactive form of rhodopsin at 2.8-Å resolution (6, 7).

Based on spin labeling studies of rhodopsin, activation of the receptor is postulated to involve a relative rigid body movement and rotation of the helices (8). Biophysical studies indicate that activation of rhodopsin and other GPCRs causes a displacement and rotation of helix 6 (8–11) and a reduction in the distance between the cytoplasmic ends of helices 5 and 6 (12, 13). The degree of helix movement is relatively slight as a “straightjacketed” rhodopsin with four engineered disulfide links between adjacent helices can still achieve an active state (14).

Studies in many GPCRs implicate the conserved receptor side chains in a network of interactions underlying the helix rearrangement that occurs during activation (reviewed in Refs. 3, 15, and 16). In addition to the inverse agonist effect of the chromophore, the ground state of rhodopsin is also stabilized by a series of structural modules that are mostly mediated by the highly conserved GPCR residues (6). Mutagenesis studies in several receptors suggest that these loci serve related functions throughout the GPCR family (5, 17–26; for review see Refs. 3, 15, and 27). Thus the general outlines of the interhelical modules mediating activation are being resolved, and specific interactions contributing to an “activation switch” in several GPCRs have been identified (15, 21, 24, 26, 28–36).

However, the concept of an activating molecular switch may not completely explain the control mechanisms underlying the multistate transitions of membrane proteins. Several studies suggest that many GPCRs exhibit properties consistent with the existence of multiple conformational states. In rhodopsin, the existence of multiple conformers is evident from absorbance changes (37). Activation occurs by transition through intermediate conformations (38), with the equilibrium between these...
forms showing a characteristic pH sensitivity (39). The existence of multiple receptor conformers is also evident in single molecule spectroscopy studies of the β adrenergic receptor (40). Pharmacological studies suggest that the existence of distinct receptor conformers can have functional significance. Studies of fusion proteins of β adrenergic receptor and G-proteins suggest that partial agonists stabilize a conformational state distinct from that stabilized by a full agonist (41). Functionally, it has been shown that many agonist-activated receptors couple to multiple signaling pathways. Thus, receptor mutations in conserved TM residues have been identified that selectively disrupt one pathway of a receptor coupled to multiple pathways (29). The observation in several receptors that different agonists acting at the same receptor can direct the relative activation of downstream pathways, a phenomenon called “signal trafficking,” also suggests the presence of multiple populations of active receptor conformers (42–45). Fluorescence studies also suggest the presence of different receptor conformational populations when complexed with functionally distinct agonists (46). This emerging support for the existence of distinct, functionally relevant conformers in several GPCRs suggests that, for these receptors, the molecular activation mechanism must provide the means for switching among multiple conformations.

One conserved domain implicated in the activation mechanism of GPCRs is the conserved NXXXY domain in helix 7. Mutagenesis studies (5, 19, 22), engineered metal-ion activation sites (47), computational modeling (15), and the rhodopsin crystal structure (5) all implicate helix 7 in a network of interactions that modulate receptor activity. In the present study, we investigate the role that the conserved Y7.53 in this domain may play in switching the 5HT2C receptor among multiple states. We selected this residue for detailed study because computational simulations implicated this side chain as a key determinant in the local structure of this helix 7 domain (48, 49), and previous studies have shown that a constitutively active receptor and an uncoupled receptor could be generated with mutations at this locus in the 5HT2C receptor (50). Thus Y7.53 is a candidate to be a critical modulator of the overall conformational state of the protein.

The rhodopsin crystal structure shows that the side chain of Y7.53 is adjacent to the conserved aromatic side chain at position 7.60. Residue 7.60, although near helix 7, is actually located in a separate, cytoplasmic helix 8 domain that was first revealed in the high resolution rhodopsin structure (6). We designed the present study to explore the hypothesis that the side chain at position 7.53 in the 5HT2C receptor serves a key function in regulating the switching among distinct receptor conformers. We also explored the hypothesis that the helix 7 Y7.53 and the helix 8 Y7.60 form part of an extended functional interhelical domain. We introduced all 19 naturally occurring amino acids for Y7.53 and characterized the receptor phenotypes obtained. We also sought function-restoring revertant mutations involving positions 7.53 and 7.60 in the 5HT2C receptor. Our results identify multiple distinct receptor phenotypes and function-restoring revertant mutations, suggesting that this domain contributes to a multistate conformational switch.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled ligands, U773122, and U73343 were obtained from Sigma-RBI (St. Louis, MO).

Receptor Numbering Scheme—Receptors are numbered according to a consensus numbering scheme described in detail previously (51). The residues are numbered in reference to the most conserved residue contained in a helix. The conserved residue, Pro-385, found in helix 7 of 5HT2C receptor is designated as P7.50. Residues N-terminal to this conserved locus are numbered in decreasing order. Thus, Tyr-368 in the NXXXY motif in helix 7 has the designation Y7.53 and Tyr-375 has the designation Y7.60.

DNA Constructs and Transfection—The cDNA encoding the human 5HT2C receptor was generously supplied by Dr. Alan Saltzman (52). Mutations were introduced into the 5HT2C receptor using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing. The cDNA was subcloned into the EcoRI and XbaI site of the pcDNA3 expression vector (Invitrogen, San Diego, CA) and used to transfect COS-1 cells (American Type Culture Collection, Rockville, MD) as described previously (53).

Measurement of Inositol Phosphate Accumulation—Accumulation of [3H]inositol phosphates ([3H]IPP) was measured as previously described (53). An incubation at 37 °C for 60 min with no preincubation was used. The protein content of three to six wells on each plate was determined and used to correct for small differences in the number of cells per well among constructs.

Ligand Binding Assay—Transiently transfected COS-1 cells were harvested, and the cell pellets were stored at −70 °C. Thawed pellets were homogenized using a Dounce homogenizer, and the cell suspension was centrifuged at 35,000 × g for 10 min. The pellet was resuspended in 50 mM Tris-HCl (pH 7.4 at 25 °C) buffer. Saturation and competition assays with [3H]mesulergine (an inverse agonist with low intrinsic activity) were carried out as described previously (50), with the exception that 1 nM [3H]mesulergine was used to label 5HT2C receptor in competition binding studies. Protein content was determined by the method of Lowry et al. (54) with bovine serum albumin as the standard.

Computational Modeling—The approaches utilized to construct a three-dimensional model of the transmembrane bundle of the 5HT2 receptor based on the structure of rhodopsin have been described previously (15, 51).

Data Analysis—Parameter estimates for competition binding and saturation binding data were obtained by non-linear regression (PRISM, GraphPad, San Diego, CA, and Kaleidograph, Synergy Software, Reading, PA) as previously described (53). To facilitate the comparison of [3H]IPP accumulation among experiments, dpm/mg of [3H]IPP were normalized for the protein content, the accumulation in the presence of 10 μM SB206553 in the WT was subtracted, and the resulting values were either reported as the calculated difference or divided by the expression level (Bmax in pmol/mg) of protein determined from [3H]mesulergine saturation binding studies as indicated in the figure legends.

RESULTS

Radioligand Binding of Y7.53 5HT2C Receptor Mutants—The affinities for the 5HT2C receptor radioligand [3H]mesulergine and the levels of expression of the receptors comprising all 20 naturally occurring amino acids at position 7.53 are shown in Table I. Although the mesulergine affinity was slightly reduced for most receptor mutants, in most cases the change was modest and within severalfold of that of the wild-type receptor. Receptor density varied for the different mutant receptors. All mutant receptors were expressed at levels lower than that of the wild-type receptor. Three of the mutants, Lys, Asp, and Glu, were expressed at less than 20% of wild-type levels.

Distinct Activation Phenotypes of Y7.53 5HT2C Receptor Mutants—The levels of agonist-independent and serotonin-stimulated [3H]IPP accumulation responses were determined for the wild-type and all 19 Y7.53 mutant receptors expressed in COS-1 cells. As previously reported (50), the wild-type receptor exhibited a modest level of agonist-independent signaling that was suppressed by the inverse agonist SB206553 (Figs. 1a and 2a). The inverse agonist caused a concentration-dependent decrease in signaling, whereas the agonist serotonin (5-hydroxytryptamine (5-HT)) caused a concentration-dependent increase in signaling (Fig. 2a). The 19 mutant receptors had varying degrees of agonist-independent activity (Fig. 1). Some receptor mutants, such as Arg, Trp, and Val, showed an activation pattern similar to that of the wild-type receptor. They had detectable basal signaling that was reduced by the inverse agonist SB206553. These mutants showed a relatively large increase in signaling in the presence of the agonist 5-HT.
Table I
Summary of \[^{3}H\]mesulergine saturation binding for 5-HT2C receptor constructs

| Amino acid at 7.53 | $K_d$ (mM) | $B_{max}$ (% of WT) |
|-------------------|-----------|---------------------|
| Tyr (WT)          | 0.75      | 100                 |
| Asn               | 1.40      | 54 ± 4              |
| Phe               | 4.95      | 53 ± 10             |
| Gln               | 1.15      | 74 ± 4              |
| Met               | 1.02      | 82 ± 1              |
| Thr               | 1.88      | 42 ± 10             |
| Ser               | 0.95      | 31 ± 5              |
| Cys               | 2.62      | 56 ± 10             |
| Gly               | 0.64      | 62 ± 6              |
| His               | 0.88      | 67 ± 13             |
| Arg               | 1.37      | 38 ± 5              |
| Trp               | 1.67      | 61 ± 4              |
| Val               | 1.55      | 74 ± 9              |
| Pro               | 1.54      | 61 ± 6              |
| Leu               | 1.05      | 94 ± 6              |
| Ile               | 1.48      | 86 ± 15             |
| Ala               | 1.44      | 75 ± 15             |
| Lys               | 1.53      | 17 ± 4              |
| Asp               | 1.76      | 17 ± 4              |
| Glu               | 0.74      | 11 ± 3              |

 contrast, several of the mutants (Gln, Met, Thr, Ser, Cys, Gly, and His) had very high constitutive signaling and showed little or no further accumulation of \[^{3}H\]IP in the presence of 5-HT (Fig. 2B). The basal signaling of these mutants was reduced in the presence of SB206553. The Lys, Asp, and Glu mutants appear to show some agonist-independent and 5-HT-stimulated activity but could not be classified due to their low levels of expression. The Y7.53F mutant had no evidence of either basal or 5-HT-stimulated signaling, as previously reported (50). The Y7.53N mutant showed a unique activation phenotype. This receptor had a very high level of constitutive signaling that was neither increased by 5-HT nor suppressed by SB206553 (Fig. 2C).

Characterization of a “Locked-on” Y7.53N Mutant Receptor—The novel phenotype of the Y7.53N mutant was characterized in more detail and compared with that of the wild-type and other constitutively active mutant receptors. To determine whether the apparent phenotype of this mutant was generalizable to structurally distinct agonists and inverse agonists, we determined the signaling in the presence of diverse ligands (Fig. 3). Nine ligands showed inverse agonist activity at the wild-type receptor and reduced the degree of constitutive signaling at the high constitutive activity Y7.53C mutant receptor. In contrast, none of the inverse agonists decreased the signaling of the Y7.53N receptor. All agonists studied increased \[^{3}H\]IP accumulation at the wild-type receptor but caused little or no increase in \[^{3}H\]IP accumulation when complexed with the Y7.53C or Y7.53N receptors (Fig. 3). Thus, although both the Y7.53C and Y7.53N receptors appear to achieve nearly maximal activity in the absence of agonist, the activity of only the Y7.53N receptor is not diminished in the presence of any of the inverse agonists tested.

The 5HT2C receptor stimulates phosphatidylinositol hydrolysis via G$_i$-mediated activation of phospholipase C. The phospholipase C inhibitor U73122, but not its inactive stereoisomer U73343, reduced basal \[^{3}H\]IP accumulation in both the wild-type and the Y7.53N mutant receptors to the level observed for the wild-type in the presence of SB206553. We also confirmed that the agonist-independent signaling mediated by the Y7.53N mutant resulted from enzymatic activity by measuring the accumulation of \[^{3}H\]IP over time. Basal \[^{3}H\]IP accumulation showed a linear increase for all constructs over the 60 min duration of the experiment. 5-HT increased the rate of \[^{3}H\]IP accumulation in both the wild-type and Y7.53L receptors (Fig. 4, B and E). The small basal and 5-HT-stimulated increases observed over time with the Y7.53F receptor were equivalent to those seen in cells transfected with vector alone (Fig. 4, A and C). The basal accumulation of \[^{3}H\]IP for the Y7.53N mutant receptor was linear over time and was not further increased by 5-HT (Fig. 4D). These data indicate that the high levels of \[^{3}H\]IP accumulation generated by the Y7.53N mutant receptor arise from persistent stimulation of signaling that is unaffected by either agonists or inverse agonists.

### Increased Affinity of Agonists for Mutant Receptors—We next evaluated the competitive binding affinities of structurally diverse ligands for the wild-type, high constitutive activity Y7.53C and “locked-on” Y7.53N receptors (Table II and Fig. 5). Most inverse agonists showed similar affinities for the wild-type, Y7.53C, and Y7.53N receptors. In contrast, the affinities of agonists for the two Y7.53 mutant receptors assayed were higher than those observed for the wild-type receptor. Moreover, the two mutant receptors differed in the magnitude of the changes observed. The affinities of agonists for the Y7.53C mutant receptor showed a 2- to 6-fold increase in comparison to wild-type receptor. The agonist affinities for the Y7.53N mutant receptor were dramatically increased from 40- to 115-fold (Table II).

### Computational Modeling and Double Mutants Support Functional Helix 7-Helix 8 Interaction—The inactive Y7.53F mutant provides a unique substrate for computational and experimental investigation of the microenvironment of Y7.53. This mutation results in a receptor with no basal or inducible signaling, high agonist affinity, and decreased mesulergine affinity (50). Thus, only the phenylalanine side chain, among all naturally occurring amino acids, leads to the receptor being trapped in a conformation that has high affinity binding but not active coupling. These observations suggest that the stabilization of the phenylalanine mutant in an inactive conformation may be dependent on a unique set of side chain interactions with the phenylalanine side chain. Therefore, we attempted to identify the predominant local interaction of this side chain. Evaluation of a computational model based on the rhodopsin crystal structure suggests that the aromatic ring of Y7.53 interacts with the conserved aromatic side chain of the helix 8 residue 7.60 (Fig. 6). Function-restoring double mutants provide strong evidence that the side chains involved contribute to a common network of interactions (5, 17, 55). Therefore, we investigated the additive effects of mutations at position 7.53 and 7.60. Notably, although the single mutation in the wild-type receptor of Y7.60A had no effect on receptor function, this mutation was able to restore wild-type function to the inactive Y7.53F mutant, both in terms of stimulation by 5-HT and inhibition of basal signaling by SB206553 (Fig. 7). We also determined the affinity of \[^{3}H\]mesulergine for 7.53/7.60 double-mutant receptors. As previously reported, the Y7.53F mutant showed a decrease in mesulergine affinity (50) (Table III). The introduction of the second mutation at position 7.60, which restored wild-type coupling, also led to the restoration of wild-type affinity for mesulergine.

Our mutation series at position 7.53 suggests that the side chain present at this position, which causes an uncoupled re-

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ceptor conformation, has a highly restricted structural space: out of all 19 substitutions, only the Y7.53F mutant showed this phenotype (Fig. 1). The double mutants studied show a similar structural constraint at position 7.60. The inactive receptor phenotype was observed only with Y7.53F and the native Y7.60. Second mutations of Y7.60 to either Ala (Fig. 7), Phe, Leu, or Trp all restored the Y7.53F mutant receptor to a functional state. This high degree of restriction in the mutational space at both loci for this phenotype and the identification of function-restoring double mutants supports the hypothesis that the two side chains share a common microenvironment.

**DISCUSSION**

A study of all 19 possible substitutions for Y7.53 in the 5HT2C receptor revealed a variety of receptor phenotypes. As previously reported, Y7.53F had high agonist affinity, no detectable basal signaling, and no activation of [3H]IP accumulation in response to 5-HT (50). The 18 remaining mutants and the wild-type receptor all showed detectable constitutive activity. Three of the mutants, K, D, and E, had a more than 5-fold reduction in their level of expression in comparison with the wild-type receptor, making it difficult to assess their relative degree of constitutive activity. Among the remaining mutants, three classes of constitutive activity can be distinguished, which correlate with the side-chain properties of the substitutions introduced as follows.

**Class I: Moderate Constitutive Activity (Wild-type (Tyr), Val, Pro, Leu, Ile, Ala, and Arg)***—These receptors, representing the non-polar substitutions and the basic Arg show an elevated level of basal signaling that is well below that attained with 5-HT (Figs. 1 and 2). The constitutive activity of the wild-type receptor is consistent with previous reports (56–58).

**Class II: High Constitutive Activity (Gly, Gin, Met, Thr, Ser, Cys, and His)***—These receptors, representing the polar substitutions as well as Met and Gly, show a nearly maximal level of basal signaling (Figs. 1 and 2). The level of basal signaling is suppressible by the inverse agonist SB206553. 5-HT causes little or no additional [3H]IP accumulation. Notably, in addition to its unusual flexibility, the carbonyl of Gly is more reactive than that of other non-polar amino acids, because it is not shielded by the side chain. Thus, with the exception of Met, all substitutions in this group introduce additional hydrogen-bonding capacity, albeit at different positions in space.

**Class III: Locked-on Constitutive Activity***—The Y7.53N mutation manifested a unique constitutively active phenotype

![Graph](http://www.jbc.org/)

**FIG. 1. Activity of WT and Y7.53 mutant 5-HT2C receptors.** Accumulation of [3H]IP in the presence of vehicle (basal), 10 μM 5-HT, or 10 μM SB206553 was normalized by dividing by protein content/well and subtracting the dpm/μg of protein for accumulation in the presence of SB206553 for the WT from all other constructs. The data were obtained from 3–25 independent experiments performed in triplicate.

![Graph](http://www.jbc.org/)

**FIG. 2. Concentration dependence of the effects of 5-HT and SB206553 on [3H]IP accumulation in 5-HT2C receptors.** [3H]IP accumulation in cells expressing WT (A), Y7.53Q (B), and Y7.53N (C) mutant 5-HT2C receptors incubated with the agonist 5-HT (closed circles) and the inverse agonist SB206553 (open circles) as described under “Experimental Procedures.” Data were corrected for protein content and are shown as mean ± S.E. of triplicate determinations. Data shown are representative of results obtained in 25 (WT), 3 (Glu), and 6 (Asp) independent experiments.
that, to our knowledge, has never been reported for any GPCR. Similar to several of the class II mutants, the N mutant showed a high level of basal signaling that was not significantly augmented in the presence of 5-HT (Figs. 1–3). However, in contrast to the class II mutants, the N mutant showed no significant suppression of signaling in the presence of the inverse agonist SB206553 (Figs. 1–3). The signaling of this receptor was unaffected by exposure to three additional agonists and to eight additional inverse agonists (Fig. 3). Consistent with phospholipase C-mediated signaling, this mutant showed an elevated rate of [3H]IP accumulation over time (Fig. 5) and an inhibition by the phospholipase C inhibitor U73122.

The results of competition binding suggest that the predominant conformation of this locked-on constitutively active receptor may be distinct from that of the wild-type and the Y7.53C class II receptor. The Y7.53C mutant showed a modest increase in affinity for agonists in comparison to wild-type receptor, varying from quipazine (1.7-fold increase) to 5-methoxytryptamine (5.8-fold increase). In contrast, the Y7.53N mutant showed a markedly elevated affinity for agonists, with the affinity for 5-methoxytryptamine increased 115-fold over that of the wild-type receptor (see Table II).

Our mutation series results differ from a previous complete mutation series at a locus associated with constitutive activity. Lefkowitz and coworkers (59) studied A6.34 in the /H9251-H/9281 adrenergic receptor and found that all 19 natural amino acid substitutions resulted in receptors with increased constitutive activity. Their results suggest that these mutations all interfered with a mechanism responsible for stabilizing the receptor in an inactive conformation, possibly the ionic lock between R3.50 and E6.30 (24, 34, 35). In our mutation series of a different locus, Y7.53, we identify an uncoupled receptor mutant, constitutively active receptors, and a locked-on receptor mutant. This variety of active and inactive phenotypes suggests that Y7.53 may be involved in the transitions among several conformers. Specific mutations have the effect of trapping the receptor in a particular region of the conformational landscape. The effects of these mutations may be analogous to the cold-temperature trapping of rhodopsin photostates (60).

It is not surprising that a helix 7 locus has been found to play a critical role in the control of the activation state of the 5HT2C receptor. The hydrogen bonding network identified in the rhodopsin structure suggests that helix 7 is likely to be a principal player in the helical movement underlying activation. The predominant changes in the relative configurations of the helixes during receptor activation is a movement of helix 6 away from helix 3 (8) and closer, at its cytoplasmic side, to helix 5 (12, 13). A model of the hydrogen bonding network connecting the rho-
Dopamin helices in the ground state suggests that the only helix with which helix 6 is connected is helix 7 (7). Furthermore, helix 7 is predicted to maintain ground state hydrogen bonds with all other helices except helices 4 and 5 (16). Thus helix 7 is uniquely situated to transmit changes induced either by ligand binding or by mutagenesis to changes in the relative positioning of helix 6, which is a main determinant of the conformational state of the protein.

If the highly conserved residues have similar functions in the various GPCRs, why are the effects of mutations at these loci so varied? In the 5HT2C receptor we find that many of the mutations at position 7.53 result in constitutive activity. However, this locus has been mutated in several other GPCRs, and a variety of receptor phenotypes have resulted, none of which include constitutive activity. The previously reported effects of mutations at position 7.53 include changes in affinity, coupling, and sequestration (21, 61–73). One explanation for the variety of effects seen with mutation of the same conserved side chain in different GPCRs is that the functional effects of a mutation depend not merely on the common role of the side chain but, more importantly, on the local microenvironment that is unique to the particular receptor studied. Evidence for the critical role of the local microenvironment in the phenotype of

| TABLE II |
| 3H]Mesulergine competition binding for WT, Y7.53C and Y7.53N mutant 5-HT2C receptors |

|                | WT Ki | Y7.53C Ki | Ratio* | Y7.53N Ki | Ratio* |
|----------------|-------|-----------|--------|-----------|--------|
| Inverse agonists |       |           |        |           |        |
| SB206553       | 5.7 ± 1.0 | 14 ± 1 | 0.4    | 25 ± 7 | 0.2    |
| Mianserin      | 1.1 ± 0.3 | 1.3 ± 0.2 | 1.2    | 1.1 ± 0.2 | 1.0    |
| Pirenzipine    | 10 ± 1 | 9 ± 1 | 1.1    | 13 ± 3 | 0.8    |
| Metergoline    | 1.1 ± 0.4 | 0.6 ± 0.1 | 1.8    | 1.4 ± 0.8 | 0.8    |
| Methiothepin   | 0.5 ± 0.1 | 0.2 ± 0.1 | 2.5    | 0.8 ± 0.1 | 0.6    |
| LY53,857       | 3.6 ± 0.5 | 3 ± 1 | 1.2    | 4.8 ± 1.3 | 0.8    |
| Cinanserin     | 49 ± 2 | 78 ± 7 | 0.6    | 89 ± 14 | 0.6    |
| Amitriptyline  | 7.4 ± 0.2 | 9 ± 1 | 0.8    | 16 ± 0.5 | 0.5    |
| Agonists       |       |           |        |           |        |
| 5-HT           | 101 ± 10 | 25 ± 3 | 4.0    | 1.0 ± 0.1 | 101    |
| 5-MeO-N,N dimethyl-tryptamine | 313 ± 61 | 73 ± 8 | 4.3    | 7.4 ± 1.1 | 42     |
| 5-MeO-tryptamine | 81 ± 6 | 14 ± 2 | 5.8    | 0.7 ± 0.1 | 115    |
| a-Me-5-HT      | 136 ± 23 | 28 ± 7 | 4.9    | 1.5 ± 0.1 | 90     |
| (±)-2,5-Dimethoxy-4-iodophentamine | 50 ± 8 | 15 ± 2 | 3.3    | 0.6 ± 0.1 | 83     |
| Quipazine      | 383 ± 21 | 222 ± 11 | 1.7 | 9 ± 1 | 42     |

* Ratio, Ki, WT/Ki, mutant.

Fig. 5. [3H]Mesulergine competition binding curves for wild-type, Y7.53F, and Y7.53N mutant 5-HT2C receptors. [3H]Mesulergine competition binding assays carried out with membranes prepared from cells expressing WT (closed circles), Y7.53C (closed triangles), and Y7.53N (open circles) receptors are shown for agonists (A, B) and inverse agonists (C, D). Data are mean ± S.E. of triplicate determinations and are representative of at least three independent experiments. For each ligand shown, the data were from the same experiment.

Fig. 6. Computational model of the 5HT2C receptor based on the rhodopsin crystal structure showing proximity of the Y7.53 and Y7.60 side chains. The helix 7 backbone is indicated by purple ribbon. For clarity, only the position 7.53 and 7.60 side chains are shown.
a particular mutant is found in the function-rescuing double mutants we have identified involving the 7.53 and 7.60 locus. Generating an uncoupled receptor requires the presence of both a phenylalanine side chain at 7.53 and a tyrosine side chain at 7.60. Any other substitution examined at either position leads to an active receptor. These results indicate that the precise microenvironment found with the side-chain geometries present in the 5HT2C receptor influence the pattern of interactions resulting in the receptor phenotype observed. Furthermore, although all rhodopsin-like GPCRs may share a common activation mechanism, the structure of the energy landscape for transitions among conformers is likely to vary for specific receptors. The physiological role of rhodopsin, for example, demands an extremely low level of basal signaling. Therefore, rhodopsin is relatively tightly held in the inactive conformer, both by a series of interconversions in the vicinity of the helix 7 Schiff base linkage (6, 16) and by an inverse agonist that stabilizes the inactive conformer (75). The present data are consonant with this formulation. The different receptor phenotypes we identified are likely to represent different accessible conformations of the 5-HT2C receptor. The mutation of Y7.53, a locus that we propose is involved in the structural path that connects agonist binding to helix movement, leads to an alteration of the energy landscape. In this new landscape, the relative predominance of specific receptor conformers having distinct phenotypes is modified from the wild-type. Although some behavior of GPCRs can be explained by a ligand-dependent activation switch, an increasing body of evidence suggests that the activated form of the receptor can involve multiple conformational states. Our data in the 5HT2C receptor suggest that the conserved Y7.53 may be a common microdomain. Y7.53 forms part of the conserved helix 7 NPXXY domain, and Y7.60 is a component of the cytoplasmic helix 8 domain identified in the rhodopsin crystal structure (6). The helix 8 domain has been implicated in forming the interface with G-proteins (7). Thus the computational model and double revertant mutation results provide a potential structural link between the helix rearrangement and the G-protein interface. The lack of effect observed with the single Y7.60 mutations studied suggests that the 5HT2C receptor has redundancy in the connections between these two domains. The identification of three classes of constitutively active receptor and an inactive receptor with high agonist affinity supports the existence of multiple distinguishable receptor conformations and implicates the conserved Y7.53 in contributing to the transition among the conformations. The funnel shape energy landscape theory has been applied to binding behavior in proteins (78, 79). In this theory, proteins exist in a population of conformations that are attained in the valley of a folding funnel. Binding selects one population, with the effects of binding at a few residues in the binding site propagating via cooperative interactions that originate there to distant loci in the protein (79). The present data are consonant with this formulation. The different receptor phenotypes we identified are likely to represent different accessible conformations of the 5-HT2C receptor. The mutation of Y7.53, a locus that we propose is involved in the structural path that connects agonist binding to helix movement, leads to an alteration of the energy landscape. In this new landscape, the relative predominance of specific receptor conformers having distinct phenotypes is modified from the wild-type. Although some behavior of GPCRs can be explained by a ligand-dependent activation switch, an increasing body of evidence suggests that the activated form of the receptor can involve multiple conformational states. Our data in the 5HT2C receptor suggest that the conserved Y7.53 contributes to the switching among these multiple conformations and implicates, for the first time, the helix 8 segment.

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