A 5-HT4 RECEPTOR TRANSMEMBRANE NETWORK IMPLICATED IN
THE ACTIVITY OF INVERSE AGONISTS BUT NOT AGONISTS

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RUNNING TITLE: Molecular events in 5-HT4 R inverse agonist action
SUMMARY

Activation of G protein-coupled receptors is thought to involve disruption of intramolecular interactions that stabilize their inactive conformation. Such disruptions are induced by agonists or by constitutively active mutations. In the present study, novel potent inverse agonists are described to inhibit the constitutive activity of 5-HT$_4$ receptors. Using these compounds and specific receptor mutations, we investigated the mechanisms by which inverse agonists may reverse the disruption of intramolecular interactions that causes constitutive activation.

Two mutations (D100$^{3.32}$A in TMD-III and F275$^{6.51}$A in TMD-VI) were found to completely block inverse agonist effects without impairing their binding properties, nor the molecular activation switches induced by agonists. Based on the rhodopsin model, we propose that these mutated receptors are in equilibrium between two states R and R* but are unable to reach a third “silent” state stabilized by inverse agonists. We also found another mutation in TMD-VI (W272$^{6.48}$A) that stabilized this silent state. This mutant remained fully activated by agonists. Molecular modeling indicated that Asp100, Phe275 and Trp272 might constitute a network required for stabilization of the silent state by the described inverse agonists. However, this network is not necessary for agonist activity.
INTRODUCTION

G protein-coupled receptors (GPCRs) are the most numerous and versatile family of proteins that control cell-cell communications (1-3). Their endogenous ligands include hormones, neurotransmitters, growth and survival factors, smells, tastes and photons.

Both wild type (WT) and mutant GPCRs have been discovered to be spontaneously active and to adopt an active ($R^*$) conformation in absence of agonists (4,5,) (6). In the simplest model, GPCRs exist in a dynamic equilibrium between active ($R^*$) and inactive (R) states governed by an allosteric constant ($J = [R]/[R^*]$). Different models have been proposed to simulate the transition of receptor between ligand or G protein-bound states. In the extended ternary complex, $R^*$ interacts with the G-protein either spontaneously ($R^*G$) or through agonist binding ($AR^*G$). In the cubic ternary complex model, both the R and $R^*$ states interact with the G-protein (for a review see (7)). The efficacy of a ligand is thought to be a function of its relative affinity for R and $R^*$. Therefore, neutral antagonists have equal affinity for both receptor states, agonists preferentially bind to $R^*$, thereby shifting the position of equilibrium towards active conformation, and inverse agonists preferentially bind to R, shifting the position of equilibrium towards the inactive state. A growing body of evidence suggests the existence of multiple active and inactive conformations. A model of these multiple conformations has been proposed (2,7) which accurately simulates the biochemical results of rhodopsin studies (8,9).

Understanding the function of GPCRs at a molecular level requires better understanding of the mechanisms by which the binding of agonists and inverse agonists preferentially stabilizes the $R^*$ and R conformations respectively. An important contribution to this understanding was the identification of key residues which, when altered by mutation, led to increased constitutive activity of GPCRs (i.e. accumulation of $R^*$ in the absence of agonists). These residues have been identified
not only in the intracellular domains known to be involved in G protein activation, but also in transmembrane domains (TMDs) and even in some cases in extracellular domains.

Most of these residues have been proposed to participate in networks of intramolecular interactions, which stabilize the receptors in inactive conformations R. Disruption of these networks results in a conformational shift from R to R* (2,10-15).

In the case of rhodopsin, a salt-bridge constraint between E113 in TMD-III and K296 in TMD-VII stabilizes the receptor in the R state (10). Similar intramolecular interactions have been demonstrated for α_{1A}-adrenoceptors between D125 of TMD-III and K331 in TMD-VII. Activation of this receptor is believed to result from the disruption of the D125 - K331 salt-bridge by the neurotransmitter biogenic amine that acts as counter-ion of the D125 (11). However, this mechanism of receptor activation is likely to be specific for α_{1B}-adrenergic receptor since the K in TMD-VII is not conserved within related GPCRs sequences. β_{2}-adrenergic receptors are kept under an inactive state by an ionic lock between the adjacent Arg and Asp of the DRY (D^{3.49}R^{3.50}Y^{3.51}) sequence (cytoplasmic end of TMD-III) and a conserved Glu in the cytoplasmic end of TMD-VI (E268 in β2-adrenergic receptor)(13). For several GPCRs, charge-neutralizing mutations of these residues increase constitutive activity by disrupting this lock (15-17). In rhodopsin, similar disruption is observed following protonation of the Glu in the ERY sequence (equivalent to the DRY sequence) and another acidic residue that could be the conserved Glu of TMD-VI (18). Taken together, these findings support the idea that the disruption of intramolecular interactions constitutes the receptor activation switch, although the precise residues involved in these interactions may vary between different receptors. Such an hypothesis is also supported by the observation that constitutively active receptors are characterized by structural instability (16,19,20).

In contrast to the rapid progress in understanding molecular mechanisms underlying the transition from R to R*, mechanisms by which inverse agonists favor the conversion from R* to R (inactive conformation) remain unclear. An important observation was that mutations of residues that
constrain the receptor in the inactive state lead to constitutively activated receptors that can be switched back to an inactive state by inverse agonists. This suggests to us that inverse agonist action may implicate different constraining networks than those disrupted by agonists.

The present study, combining mutagenesis, pharmacology and molecular modeling of 5-HT$_4$ receptor, sheds new light on this issue. We previously reported that 5-HT$_4$ receptors display high constitutive activity under wild type and mutated forms (20,21). In this study, three mutations are reported (D100$^{3.32}$A in TMD-III; F275$^{6.51}$A and W272$^{6.48}$A in TMD-VI) that completely blocked the effects of inverse agonists while keeping their specific binding on the receptor. Besides, when combined with other potent constitutively activating mutations, these three mutations were discovered to be “dominant” as inverse agonist effects were still blocked. Surprisingly, they also left unchanged molecular activation switches induced by agonists. Molecular modeling revealed a network of interactions between these three residues.
EXPERIMENTAL PROCEDURES

Construction of Mutated m5-HT₄ (a) Receptor cDNA.

The mutants were generated by exchanging the endogenous residues to Alanine in the m5-HT₄ (a) R cDNA sequence with the QuikChange Site-Directed Mutagenesis Kit from Stratagene. The sense primers used were respectively:

F275: 5'-CTG TTT CTG CTG GGC CCC CGC CTT TG -3'
D100: 5'- ACC TCT CTG GCT GTC CTA CTC ACC- 3'
W272: 5'- CTG TTT CTG CGC GGC CCC CTT CTT-3'
S197: 5'- TAT GCT ATC ACC TGC GCT GTG GT-3'
R118: 5'- TTC CCT GGA CGC TTA TTA CGC CAT-3'
N308: 5'- GGT TGG ACC CTT TTC TCT ATG CCT -3'
T134: 5'- TGC GTA GAG GGG CCA TCT TGT TCC -3'

Cell Culture and Transfection.

The cDNAs, subcloned into pRK5, were introduced into COS-7 cells by electroporation. Briefly, cells were trypsinized, centrifuged, and resuspended in EP Buffer (50 mM K₂HPO₄, 20 mM CH₃CO₂ K, 20 mM KOH, 26.7 mM MgSO₄; pH 7.4) with 25-2000 ng of receptor cDNA. The total amount of cDNA was kept constant at 15 µg per transfection with wild-type pRK5 vector. After 15 min at room temperature, 300 ml of cell suspension (10⁷ cells) were transferred to a 0.4-cm electroporation cuvette (Bio-Rad Ivry sur Seine, France) and pulsed with a gene pulser apparatus (setting 1000 mF, 270 V). Cells were diluted in Dulbecco’s modified Eagle’s medium (DMEM; 10⁶ cells/ml) containing 10% dialyzed and decomplemented fetal bovine serum (dFBS) and plated on 10cm Falcon Petri dishes or into 12 wells clusters at the desired density.

Determination of cyclic AMP (cAMP) Production in Intact Cells.

Six hours after transfection, the surrounding cell media was exchanged for DMEM without dFBS with 2 mCi [³H]adenine/ml to label the ATP pool, and incubated overnight. cAMP accumulation
was measured as described previously (22).

**Membrane Preparation and Radioligand Binding Assay.**

Membranes were prepared from transiently transfected cells plated on 15-cm dishes and grown in DMEM with 10% dFBS for 6 h, followed by incubation for 20 h in DMEM without dFBS. The cells were washed twice in PBS, scraped with a rubber policeman, harvested in PBS, and centrifuged at 4°C (200g for 4 min). The pellet was resuspended in buffer containing 10 mM HEPES (pH 7.4), 5 mM EGTA, 1 mM EDTA, and 0.32 M sucrose, and homogenized 10 times with a glass-Teflon potter at 4°C. The homogenate was centrifuged at 20,000g for 20 min, and the membrane pellet was resuspended in 50 mM HEPES (pH 7.4; 5 mg of protein in 1 ml of solution) and stored at -80°C until use. 5-HT<sub>4</sub> receptor densities were estimated with the specific radioligand [³H]GR 113808 at saturating concentration (0.4-0.6 nM, K<sub>d</sub> = 0.12 nM) as described previously (23). 5-HT (50 µM) or RS 100325 (10 µM) was used to determine non-specific binding. Protein concentration in the samples was determined with the Bio-Rad protein assay (24).

**Data Analysis.**

Using Kaleidagraph software, the dose-response curves were fitted according to the equation

\[ Y = \left( (y_{max} - y_{min})/(1+x/EC_{50})^{nH} \right) + y_{min} \]

where EC<sub>50</sub> (or EC<sub>50inv</sub>) is the concentration of agonist (or inverse agonist) that evokes a half-maximal response; \( y_{max} \) and \( y_{min} \) correspond to the maximal and minimal responses, respectively; and \( nH \) is the Hill coefficient. Statistical differences were examined with the Stat-View Student program (Abacus Concepts, Berkeley, CA) with t tests.

**Drugs.**

GR 113808 ([1-[2(methylsulfonyl-amino)ethyl]4-piperidyl-nyl] methyl-1-methyl-indole-3-carboxylate, maleate) and GR 125487 ([1-[2-(methylsulfonyl-amino)ethyl]4-piperidinyl]methyl-5-fluoro-2-methoxy-1H-indole-3-carboxylate, hydrochloride) were generously donated by Glaxo (Ware, Herts, U.K.); [³H]GR 113808 was purchased from Amersham Pharmacia Biotech; 5-HT
(serotonin) was purchased from Sigma Chemical Co. (St. Louis, MO). BIMU8 [endo-N-8-methyl-8-azabicyclo(3.2.1)oct-3-yl]-2,3-dihydro-3-isopropyl-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride was obtained from Boehringer Ingelheim, Milan, Italy.

RS100235 (1-(8-Amino-7-chloro-2,3-dihydrobenzo[1,4]dioxin-5-yl)-3-[1-[3-(3,4-dimethoxyphenyl)propyl]-piperidin-4-yl]-propan-1-one hydrochloride salt); RO116-0086 (2,3-Dihydrobenzo[1,4]dioxine-5-carboxylic acid 1-butyl-piperidin-4-ylmethyl ester hydrochloride salt); RO116-1148 (2,3-Dihydrobenzo[1,4]dioxine-5-carboxylic acid 1-butyl-piperidin-4-ylmethyl amide hydrochloride salt) were generously donated by Roche Bioscience (Palo Alto, CA).

**Molecular modeling of the 5-HT₄ receptor**

A model of the transmembrane domain in the mouse 5-HT₄ receptor was constructed using the bovine rhodopsin structure (PDB code: 1F88 :A) as a template. Receptor sequence alignment was determined using GRAP Mutant database ([http://tGRAP.uit.no/fam1asel.html](http://tGRAP.uit.no/fam1asel.html)). All 5-HT₄ receptor modeling was conducted following virtual truncation of certain amino acid residues: 1 to 10 of the N-terminal region, 163 to 183 of the e2 loop, 219 to 250 of the i3 loop and 332 to 387 of the C-terminal region. Five different models were developed using the program Modeller in the Insight-II environment (Molecular Simulation Inc.; San Diego, CA) on a Silicon Graphics R10000 O2 workstation. Although these models generated very similar molecular structures, the findings reported here are based on the model that returned the best 3-dimensional likeness of rhodopsin. This model was submitted to energy minimization using the program Discover 3 and the CFF force field. Cα-trace was tethered by applying a force constant value of the quadratic potential of 100. A steepest descent followed by a conjugate gradient method then was applied until convergence with 0.5 kcal mol⁻¹ Å⁻¹ root mean square energy gradient difference between successive minimization steps.
RESULTS

1. Novel and highly potent 5-HT₄ receptor specific inverse agonists

GR 125487 and SB 207266 have previously been described to be inverse agonists at the 5-HT₄ receptor (21). In order to find new 5-HT₄-receptor inverse agonists we synthesized numerous compounds having common pharmacophoric features with the SB 204070, a specific benzoate dioxane 5-HT₄ receptor antagonist, (25). Among them, two highly potent inverse agonists were identified, RO116-0086, a benzoate dioxane which is the des-amino, des-chloro version of SB 204070 and RO 116-1148 which is the corresponding amide.

By measurement of cAMP accumulation in cell lines that transiently express wild-type 5-HT₄ receptor, both RO116-0086 and RO116-1148 were found to have high potencies (EC₅₀inv = 0.3 ± 0.1nM) and high efficacies. Indeed, they inhibited 80% of basal constitutive activity i.e. cAMP accumulation above mock cAMP accumulation, in the absence of agonist (Figure 1B). Both are much more potent and efficacious than previously described 5-HT₄ inverse agonists (21).

2. Effects of Asp100 (D100³.32A) mutation on responsivity to agonists and inverse agonists

Screening for mutations that would impair the efficacy of inverse agonists without modifying the agonist-induced activation, we analyzed the mutation of Asp100. This highly conserved residue within the biogenic amine receptor family (26) is believed to make an ionic interaction with neurotransmitters protonated amine. As expected, 5-HT was completely unable to stimulate the D100A mutated receptor (Fig. 2A), due to its loss of binding (Fig. 2B). Interestingly, the binding of the labelled antagonist [³H]GR 113808 was only slightly affected by the mutation (Kᵰ = 0.12 ± 0.05 nM and 0.26 ± 0.04 nM at WT and D100A receptors, respectively). In contrast to 5-HT, the benzimidazolone derivative BIMU 8 remained fully and even showed a higher potency on the D100A mutant than on the WT 5-HT₄ receptor. The EC₅₀ values for cAMP stimulation were...
equal to 4 ±1.5 and 1 ± 0.5nM for WT and D100A respectively (Fig.2A). Affinity of BIMU-8 for 5-HT$_4$ receptor was also slightly better on the D100A mutant than on the WT. $K_D$ values for BIMU 8 measured by competition with the [³H]GR 113808 radioligand were 30±11nM and 6.5 ± 3nM for WT and D100A respectively (Fig.2B).

These data suggest that, the D100$^{3,32}$A mutant has not lost the ability to be activated as long as the agonist is able to bind. When expressed at densities ranging from 200 up to 10000 fmol/mg protein, the basal level of cAMP synthesis of the D100A mutant was 1.5 – 2-fold higher than that of WT. Receptor expression levels of WT or D100A were also not significantly different following transfection of 300 ng cDNA (1350± 60 and 1580±60 pmol/mg protein for WT and D100A, respectively).

Whereas, as already discussed, highly potent inverse agonists (RO116-0086, RO116-1148) blocked the WT constitutive activity, they were absolutely unable to inhibit basal constitutive activity of the D100A mutant (Fig.3A). This effect is not unique to these two newly highly potent inverse agonists, similar results were obtained with the previously described 5-HT$_4$ receptor inverse agonists, GR 125487 and SB 207266 (21) (data not shown). This effect can therefore not be attributed to a loss of binding to the D100A mutant receptor, as shown in Fig.4 A and B. RO116-0086 competition binding experiments showed a very small decrease in affinity for D100A (Kd = 3 ±1.4 x 10$^{-10}$ M) relative to WT (Kd= 8.4±2.6 x 10$^{-11}$ M). RO116-1148 showed a 10-fold decrease in affinity for D100A (Kd = 5.1 ±1.8 x 10$^{-9}$ M) as compared to the WT (Kd = 4.7 ± 1.5 x 10$^{-10}$ M)

The influence of receptor expression on inverse agonist activity was then analyzed. While the receptor constitutive activity increased as expected as a function of the receptor concentration, RO116-1148 remained totally inactive as inverse agonist on the D100A mutant (Fig.5B). In comparison, its effect on WT receptor was constant whatever the receptor concentration (Fig.5A).
Thus, a mutant was generated that was fully activated by but insensitive to inverse agonists in spite of the ability of these later to bind to the receptor.

3. D100$^{3-32}$A mutation is a dominant mutation, which prevents the effect of inverse agonists whatever the level or nature of the constitutive activation of the 5-HT$_4$ receptor:

In a previous study we showed that either truncation of the C-terminal domain of 5-HT$_4$ receptor ($\Delta$327$^{7-70}$) or the A258$^{6-34}$L mutation in the third intracellular loop elevated the constitutive activity relative to the WT 5-HT$_4$ receptor (21) (Fig. 6A). At both of these mutations, 5-HT and BIMU 8 increased cAMP synthesis over basal (Figure 6B) and RO116-1148 decreased cAMP synthesis relative to basal (Figure 6C and D). Following dual mutation of D100A+$\Delta$327 or D100A+A258L, the inverse agonist property of RO 116-1148 was lost (Figure 6 C and D). Furthermore, as expected 5-HT was inactive on these double mutants whereas, as on D100A, BIMU8 remained fully efficient (Fig.6 B). Taken together, these results shown that the D100A+$\Delta$327 and D100A+A258L dual mutants were qualitatively similar in their pharmacological behaviour to the D100A mutant on its own. Thus, the D100A mutant is a “dominant” mutant. It blocked the effects of inverse agonists on constitutively activated 5-HT$_4$ receptors, whatever the nature or intensity of the constitutive activation.

4. Among other mutations leading to different constitutively active 5-HT$_4$ receptors, mutations of two aromatic residues, F275A and W272A block RO116-1148 inverse agonist activity:

We investigated the effects of mutation of other residues known to be involved in the binding of 5-HT or the constitutive activation of the receptor (Figure 7A). The T134$^{4-38}$A mutation is located in the second intracellular loop (T134$^{4-38}$ is a conserved threonine in many 5-HT receptors (27)). The
A mutation is located at the putative interface between TMD-III and the i2 loop (R118 residue belongs to the DRY sequence). In addition to the D100A mutant, four mutants were generated in the membrane-spanning regions. The S197 of TMD-V, which likely interacts via a hydrogen bond with the OH of 5-HT, was mutated into Ala (S197A). Two highly conserved aromatic residues of TMD-VI (F275A and W272A) were mutated into Ala. The Asn residue in of TMD-VII (N308), which is conserved across the family 1 GPCRs, was mutated to Asp.

The S197A, T134A and F275A mutants were similar to the WT receptor in their levels of constitutive activity. The N308D and R118A mutants had levels of constitutive activity, similar to that of D100A (Fig. 7B). Of all the mutants investigated, we found only F275A to be similar to D100A in its insensitivity to the potent inverse agonist, RO116-1148 (Figs 7B and 7C). As with the D100A mutation, the absence of effect of RO116-1148 cannot be attributed to an absence of binding. Competition-binding experiments with RO116-1148 demonstrated a very small decrease in the affinity for F275A (9±2.3 x 10⁻¹⁰ M) relative to WT (4.7±1.5 x10⁻¹⁰ M) (Figure 8A). In addition, the 5-HT-mediated cAMP accumulation at this F275A mutant was antagonized by RO116-1148 (Fig. 8B).

The W272A mutant expressed at a standard level of 450 fmol/mg, was totally silent, with a level of basal cAMP accumulation similar to that of mock-transfected controls (Figure 7B). However, as shown in Fig 8B, the 5-HT efficiency to stimulate cAMP accumulation was the same on the W272A mutant as on WT 5-HT₄ receptor. Since it displayed no constitutive activity at all, this silent mutant was, by definition, insensitive to the inverse agonist activity of RO116-1148. In order to check if this mutation was also a “dominant” mutation that prevents the effect of inverse agonists, we performed dual mutation studies with the constitutively active mutant, A258L. The W272A+A258L mutant was found to have a modest level of constitutive activity (Figure 7B). Note that this modest constitutive activity could not be further increased, due to a low expression level of
W272A mutant. The same phenomenon was already observed with a mutation of the 5-HT\textsubscript{2A} receptor when the analogous tryptophan was mutated into alanine at W336\textsuperscript{6.48}A (29). However, if modest this basal level of cAMP accumulation was sufficient to reveal that RO116-1148 did not induce detectable decreases in basal cAMP accumulation (Figure 7C).

Competition binding studies with [\textsuperscript{3}H] GR113808 demonstrated that RO116-1148 binds to the W272A mutant (1.14 ±0.3 \times 10^{-9} \text{ M}) with a very small decrease in affinity relative to WT (Figure 8A). In addition, the 5-HT-mediated cAMP accumulation at this W272A mutant was antagonized by RO116-1148 (Figure 8B).

Taken together, these results revealed that, two mutations (W272\textsuperscript{6.48}A and F275\textsuperscript{6.51}A), in addition to the D100\textsuperscript{3.32}A mutation, inhibit the effects of inverse agonists on the 5-HT\textsubscript{4} receptor constitutive activity, but not the ability of these compounds to bind to the receptor.

5. Molecular modeling of the 5-HT\textsubscript{4} receptor

A model of the molecular structure of the 5-HT\textsubscript{4} receptor was developed, using a template based on the corresponding region of high structural resolution recently determined for rhodopsin (Figure 9A). With this model we aimed to investigate the relationships between the three residues (D100\textsuperscript{3.32}, F275\textsuperscript{6.51}, W272\textsuperscript{6.48}) that were experimentally determined to contribute to the effects of inverse agonists. With this model we hoped to elucidate the relative positions of these three residues in three-dimensional space.

Using this model, the distances between D100\textsuperscript{3.32}, W272\textsuperscript{6.48} and F275\textsuperscript{6.51} side chains were determined to range from 3 to 4 Angstroms (Figure 9B). In addition the side chains of F275 and W272 contribute to an aromatic cluster of π-interactions, similar to those described previously for catecholamine receptors (30). The acidic side chain of D100 was found to be oriented towards the amine of W272 indole group, and might participate in a polar interaction that would be stabilized by the aromatic ring of the F275 residue that is predicted to reside immediately above it. The intramolecular interactions predicted by this model would be expected to be disrupted by the
D100A, W272A and F275A mutations, which were found to inhibit the activity (but not significantly affinity) of inverse agonists.
DISCUSSION

One of the key findings of this study was the observation that the potent inverse agonists, RO116-0086 and RO116-1148, reverse basal levels of cAMP accumulation in WT but not the D100A mutant. In addition, these agents were ineffective following dual mutation of D100<sup>3.32</sup>A with the constitutively active mutations, Δ327<sup>7.70</sup> or A258<sup>6.34</sup>L.

Highly conserved among biogenic amine receptors, the Asp residue D100<sup>3.32</sup> is known to make an ionic bond with the protonated amine of the ligand. Mutation of this Asp to Ala generally leads to a loss of binding for the neurotransmitter structurally related to amine biogene (28). This was also the case for the D100<sup>3.32</sup>A mutant that proved unable to bind 5-HT. However, in contrast to what has been observed for other biogenic amine receptors, this mutation did not significantly affect the binding of the radioligand antagonist [³H]GR 113808, or the binding of the synthetic agonist BIMU 8. Indeed, this mutant might be considered to be a RASSL (receptor activated solely by synthetic ligand (31)). Since BIMU 8 was at least equi-potent and equi-effective at the D100A and WT receptor, we conclude that D100<sup>3.32</sup> is not involved in the conformational shifts that result in receptor activation, at least for this synthetic ligand. In contrast, D100 is essential for the shifts in receptor conformation that result in receptor inactivation by the studied inverse agonists.

The two-state model is now believed to be a simplification of a more general concept of allosteric equilibrium (7,32,33). According to this concept, the receptor undergoes transitions between different allosteric states. Agonists preferentially stabilize different R* states, and consequently can stimulate different signal transduction pathways by “receptor trafficking” (7). Our group previously showed that constitutively active 5-HT₄ receptor mutants (Δ327 and A258L) have different R* conformations (20).

A recent study on agonist-mediated changes in conformation of the G protein-coupling domain of β2-adrenoceptors found that conformations stabilized by full or partial agonists were
different (14). Therefore, it seems likely that the inactive R state is a heterogeneous population of different inactive conformations. Using this logic, the rhodopsin receptor, proposed to exist in at least three different states, might be expected to exist instead in at least three different populations of states. The “ground” silent state (Rg) of rhodopsin is stabilized by the inverse agonist, cis-retinal. It is distinct from another low activity state, the ligand-free opsin state, R. The third state, metarhodopsin II, is characterized by being bound to all-trans-retinal, and is fully active (R*) (8,34).

The activity of the R state was determined to be 10⁻⁶ times lower than that of the R* state (34). Stabilization of the Rg state by cis-retinal, the inverse agonist, is necessary for the physiological activity of rhodopsin as it enables the rod cell to operate as a single photon detector even in the presence of high levels of activated rhodopsin.

The findings of this study might be explained by applying the rhodopsin three-states model to the 5-HT₄ receptor. In this model, the 5-HT₄ receptor exists in an equilibrium between three states (or populations of state), Rg, R* and R, stabilized by inverse agonist, agonist, ligand-free, respectively. The Rg state would be preferred in the presence of inverse agonists. Indeed, in the presence of inverse agonists, the levels of cAMP accumulation were close to those observed in the absence of functional receptor (the mock transfected controls, see Figures 1 and 5A).

Inversely, the absence of constitutive activity and insensitivity to inverse agonists observed with the W272A mutant might indicate that this receptor exist predominantly in the Rg state. The observed agonist-mediated increases in cAMP accumulation demonstrate that this mutation does not interfere with agonist-mediated conversion from Rg to R* (Figure 8). The lack of effect of inverse agonists in the W272A mutant is possibly due to the very low levels of constitutive activity. However, from separate studies in which basal cAMP accumulation is increased by the additional A258L mutation, the persistent insensitivity to inverse agonists supports the idea that the W272₆.₄₈ residue (TMD-VI) is highly conserved in Family I GPCRs. In the crystal structure of rhodopsin, an analogous tryptophan residue, W265₆.₄₈, points downwards to the retinylidene group, near the B-ionone ring of cis-retinal and close to its C13-
methyl group (3.8Å)(34,35). Since rhodopsin was crystallized in the presence of the inverse agonist cis-retinal (34), this orientation of W265 is likely to correspond to the Rg state. Conversely, rhodopsin adopts a conformation in the absence of activation by light (R), in which the cross-linking group located on retinal ionone ring is directly bound to W265 (36). Finally, deletion of the C-13 methyl group on the retinal results in an increase constitutive activity that might indicate a stabilization of the R* state (37).

We can apply the three states model to our observations with the D100A, D100A+Δ327 and D100A+A258L mutants. Although these mutants had different levels of constitutive activity, they retained sensitivity to the agonist. This indicates that the D100A mutation does not impair the agonist-mediated transition to the R* conformation. Conversely, in these mutants the transition to the Rg state is no longer favored by inverse agonists. Similar logic might be applied to the influence of the F275A mutant on intramolecular interactions.

In dual mutation studies, the observed failure of the constitutively activating mutations, Δ327 or A258L, to overcome the insensitivity of D100A to inverse agonists suggests that the intramolecular interactions associated with activation of cAMP synthesis, and hence R*, are different from those associated with inactivation of cAMP synthesis and Rg.

It is now well established that TMD-III and TMD-VI play a crucial role in GPCR activation (2). For many GPCRs, activation involves disruption of a network of interactions consisting of the Arg and the Asp/Glu residues of the conserved DRY sequence of the cytoplasmic side of TMD-III, and a conserved Glu residue at the cytoplasmic end of TMD-VI. It has been proposed that this disruption, possibly following protonation of the Asp/Glu or Glu residues, is critical to receptor activation (15-17). Moreover, in studies of rhodopsin or β2-adrenoceptors, activation by site-directed labeling of residues on the cytoplasmic side of the TMDs III and VI strongly suggests that activation involves clockwise rotation and separation of TMD-III from TMD-VI (38,39).
The data presented in this study suggest that a different network of intramolecular interactions exists in the 5-HT$_4$ receptor between the D100$^{3.32}$ residue of TMD-III and the W272$^{6.48}$ and F275$^{6.50}$ residues of TMD-VI. This network underlies the actions of inverse agonists but not agonists. The resulting structural model of the 5-HT$_4$ receptor, presented in Fig 9A was based on the structure of the corresponding sequence of rhodopsin which has previously been elucidated (35). In this model, the side chains of residues D100$^{3.32}$, W272$^{6.48}$ and F275$^{6.51}$ are 3 - 4 Angstroms apart. In addition, the aromatic cluster formed between the side chains of F275 and W272 has previously been described for other catecholamine GPCRs (40). Finally, we propose that a polar interaction between the acidic side chain of D100 and the amine of W272 is stabilized if the aromatic ring of F275 is localized immediately above it.

We hypothesize that the electrostatic influence of the aromatic W272 residue on its surroundings must be neutralized in order for the receptor to adopt the Rg conformation. This occurs when the W272 residue is engaged in the W272-D100-F275 network. Mutations of D100 and F275 to alanine explain the absence of effect of inverse agonists, and hence the failure to stabilize the W272-D100-F275 network that defines the Rg conformation, because under the alanine substitutions, neutralization of the electrostatic influence of W272 is no longer possible. Using the same logic, we predict that the W272A mutation permanently neutralizes the electrostatic influence of the W272 thereby stabilizing the Rg state.

We propose that 5-HT$_4$ receptor inverse agonists described here, favor a D100$^{3.32}$-F275$^{6.51}$-W272$^{6.48}$ constraining network, which is not required for agonist activation. How this occur is a matter of speculation. One hypothesis is that the aromatic moiety of inverse agonists interact with hydrophobic residues of the network to favor the receptor constrain. Indeed, a shift in the position of the aromatic moiety of RO 116-0086 by adding a chloro and an amino substituents to give the SB 204070 compound, suppress the inverse agonist property of the drug.

Further studies may reveal the existence of analogous networks in other Family I GPCRs which would help to define the molecular determinants of inverse agonists. Exploration of such
networks would provide a better understanding of the influences that govern conversion between different receptor conformations, and the effects of agonists, inverse agonists and antagonists in terms of their impact on the GPCR structure.
REFERENCES


FOOTNOTES

Abbreviations:

GPCRs, G protein-coupled receptors; 5-HT, 5-hydroxytryptamine; 5-HT$_4$ receptor, 5-hydroxytryptamine receptor of class 4, Gs, G protein that stimulates adenylate cyclase; TDM, transmembrane domain; WT, wild-type; R, inactive receptor conformation; R*, active receptor conformation, Rg, "ground state" receptor conformation;

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LEGENDS TO FIGURES

Figure 1. New highly potent 5-HT$_4$ receptor inverse agonists


B) Concentration-effect curves of the new potent 5-HT$_4$ receptor inverse agonists on the agonist independent cAMP production in COS-7 cells transiently expressing m5-HT$_4$(a) receptors at a level of 1500 ±130 fmol/mg proteins.

cAMP production was measured for 15 min and expressed as a percentage of basal cAMP formation. Basal cAMP basal production, defined as 100%, was equal to 720 ± 50% of the mock-transfected controls. In mock transfected cells, the percentage conversions of [³H]ATP to [³H]cAMP was equal to 0.12 ± 0.04 %.

The results are representative of three independent experiments performed in triplicate.

Figure 2. Mutation of the conserved D100$^{332}$ in TM III impedes effect and binding of 5-HT but not of the synthetic agonist, BIMU 8.

A) The effects of a range on concentrations of 5-HT or BIMU 8 on intracellular cAMP levels were measured in COS-7 cells in which 5-HT$_4$(a) or D100A were expressed at equivalent levels (1350 ± 60 and 1580 ±110 fmol/mg protein, respectively). Results are expressed as a percentage of the cAMP production in mock transfected cells. In mock transfected cells, 0.10 ± 0.08 % of [³H]ATP was converted to [³H]cAMP.

B) Competition of agonists for [³H]GR 113808 binding ( 0.38nM) at membranes derived from the same batches of transfected COS-7 cells as in A. The results are expressed as a percent of the mean specific binding in the absence of competing ligand. The results are the mean of four independent determinations.
Figure 3. Lack of effect of inverse agonists following mutation of the conserved D100<sup>3.32</sup> in TM III.

A) COS-7 cells transiently expressing 2500 ± 220 fmol/mg of D100A mutant 5-HT<sub>4</sub> receptor were incubated with the potent and selective 5-HT<sub>4</sub> inverse agonists (R0116-0086, R0116-1148).

B) and C) Dose–response curves of the same inverse agonists on BIMU 8-mediated cAMP accumulation in COS-7 cells expressing 1900 ±150 fmol/mg protein WT 5-HT<sub>4</sub> receptors (B) or 2500 ±220 fmol/mg of D100A (C). cAMP production was measured for 15 min and expressed as a percentage of cAMP formation in mock-transfected control cells. In mock transfected cells, the percentage conversions of [<sup>3</sup>H]ATP to [<sup>3</sup>H]cAMP was equal to 0.11 ± 0.02 %.

BIMU 8 concentration was 3x10<sup>-8</sup> M and cAMP production was measured as a percent of mock. The results are representative of three independent experiments performed in triplicate.

Figure 4. The binding of the 5-HT<sub>4</sub> receptor inverse agonists was conserved in the mutated D100<sup>3.32</sup>A receptor.

Competition binding was performed for the inverse agonists (A: RO116-0086; B: RO116-1148), in presence of 0.24nM of [<sup>3</sup>H]GR113808. The essays were carried out on membranes derived from COS-7 cells and expressing similar levels of WT or D100A receptor (2500 ±180 and 2650 ± 210 fmol/mg protein, respectively). Results are expressed as a percent of the specific binding in the absence of a competing ligand. Representative data are presented from one of six replicate experiments.
Figure 5. Effects of receptor density on the effects of agonists and inverse agonists on constitutively active WT and D100A receptors

Levels of cAMP accumulation in COS-7 cells expressing WT and D100A over a range of receptor densities (from 200 to 9500 fmol/mg proteins) were determined in the absence (basal) or presence of inverse agonist or agonists.

A) and B) cAMP accumulation in WT or D100A transfected cells in the absence or presence of the inverse agonist (RO116-1148 at 10^{-7} M) is presented as a function of receptor expression levels.

C) and D) cAMP accumulation was also determined in the absence or presence of agonists (5-HT or BIMU 8 at 10^{-6} M).

cAMP accumulation was measured for 15 min and expressed as a percentage of cAMP production in mock transfected cells. In mock transfected cells, the percentage conversions of \[^3\text{H}\]ATP to \[^3\text{H}\]cAMP was equal to 0.12 ± 0.04 %. Representative data are presented from one of three replicate experiments.

Figure 6. Lack of effect of inverse agonists in D100^322A mutation is dominant over constitutively activating mutations

A) Diagram of the double mutant 5-HT\(_{d(a)}\) receptors used in this study: D100A+\(\Delta\)327 and D100A+A258L

B) cAMP accumulation in the absence or presence of (5-HT and BIMU 8) in COS-7 cells expressing WT or mutant 5-HT\(_{d(a)}\) receptors D100A, \(\Delta\)327, D100A+ \(\Delta\)327 (truncation of C-terminal domain) or A258L and D100A+A258L (mutation in the I3 loop). The expression levels expressed in fmol/mg proteins, were for WT (3500±420), D100A(3850±350), \(\Delta\)327(3470±250), D100A+ \(\Delta\)27(3620±530), A258L(3750±450) and D100A+A258L(3250±410)
C) and D). Efficacy of the inverse agonist, RO116-1148, on basal constitutive activities of the WT and of these three mutated 5-HT$_4$ receptors expressed as the same level as in B. Levels of cAMP accumulation were measured after 15 min incubation and expressed as a percentage of basal cAMP production measured in mock-transfected COS-7 cells. The conversion percentage of [$^3$H]ATP to [$^3$H]cAMP in mock-transfected cells was 0.150±0.05. Each value represents the mean ± SEM determined from three independent determinations.

**Figure 7. Among the highly conserved residues, two aromatic residues are necessary for the inverse agonist activity.**

**A)** Diagram of all the mutations performed on the 5-HT$_{4a}$ receptor used in this study.

**B)** Native and mutant receptors were transiently expressed in COS-7 cells at 2500 - 3000 fmol/ mg proteins, WT (2560±120), D100A (2720±250), F275A (2520±240), S197A (2900±340), N308D (2570±280), R118A (2950±380), T134A (2800±430). W272A and W272A+A258L mutants were expressed only at 500±44 and 570± 65 fmol/mg proteins respectively.

Basal cAMP production was determined by measuring the accumulation of cAMP over 15 min, and expressed as a percent of cAMP accumulation in mock-transfected cells. The basal cAMP levels calculated for all mutated 5-HT$_4$ receptors ranged from 100 to 1250% of the cAMP levels measured in mock-transfected cells. In mock transfected cells, the percentage conversions of [$^3$H]ATP to [$^3$H]cAMP was equal to 0.13 ± 0.05 %.

**C)** Effects of 10$^{-7}$ M of RO116-1148 in cells transfected as described in B. cAMP accumulation over 15 min was determined in the absence or presence RO116-1148. Data are expressed as a percent of basal cAMP accumulation. The percentage conversion of [$^3$H]ATP to [$^3$H]cAMP in mock transfected cells was 0.12±0.04.
Figure 8. Responses to 5-HT$_4$ receptor agonist and antagonist were conserved in F275A and W272A mutant receptors

A) Competition of the inverse agonist RO116-1148 for $[^3]$HGR113808 binding (0.28nM) in membranes derived from COS-7 cells expressing mutated F275A and W272A receptors.

B) cAMP accumulation was measured in COS-7 cells transiently expressing WT, F275A or W272A receptors expressed at similar densities (620 ± 40, 750 ± 50, 650 ± 35fmol/mg protein respectively) in the absence and presence of 10$^{-5}$M 5-HT. The efficacy of RO116-1148 to inhibit 5-HT-mediated cAMP accumulation was tested in the same COS-7 cells. Levels of cAMP accumulation were measured after 15min incubation and expressed as a percentage of basal cAMP production measured in mock-transfected COS-7 cells. The conversion percentage of $[^3]$HATP to $[^3]$HcAMP in mock-transfected cells was 0.150±0.05. Each value represents the mean ± SEM determined from three independent determinations.

Figure 9. Molecular modeling of the 5-HT$_4$ receptor

A) A model of the 5-HT$_4$ receptor, based on the high-resolution structure elucidated for bovine rhodopsin. Three-dimensional side view of the Calpha trace with transmembrane helix (represented as ribbons) colored as followed: TMD-I in dark blue, TMD-II in light blue, TMD-III in violet, TMD-IV in pink, TMD-V in orange, TMD-VI in red and TMD-VII in green. The side chains of D100 (TMD-III), W272 and F275 (TMD-VI), located within the binding pocket of the 5-HT$_4$ receptor, are shown in black.

B) Side view of the binding pocket of the m5-HT$_4$ receptor. Calpha trace are represented as ribbons, residues shown are known to be involved in 5-HT binding or surrounding D100, W272 and F275. Molecular modeling reveal the tight network of these three side chains distant from 3.5 to 4.6 Angstroms. Color coding of D100, W272 and F275 atoms: O, red; N, cyan; C, green; H, white.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Additions and Corrections


A 5-HT₄ receptor transmembrane network implicated in the activity of inverse agonists but not agonists.

Lara Joubert, Sylvie Claeysen, Michèle Sebben, Anne-Sophie Bessis, Robin D. Clark, Renee S. Martin, Joël Bockaert, and Aline Dumuis

Page 25504, fifth line from the bottom of the first column and page 25506, right-hand column, line 6: Insert the following reference:


Influences of base excision repair defects on the lethality and mutagenicity induced by Me-lex, a sequence selective N3 adenine methylating agent.

Paola Monti, Paola Campomenosi, Yari Ciribilli, Raffaella Iannone, Alberto Inga, Dharini Shah, Gina Scott, Philip A. Burns, Paola Menichini, Angelo Abbondandolo, Barry Gold, and Gilberto Fronza

The affiliation for Paola Campomenosi was partially omitted. The additional affiliation should read:

“Human Genetics Laboratory, Department of Structural and Functional Biology (DBSF), University of Insubria, Varese, Italy

Also, Paola Monti was supported by a “Fondazione Italiana per la Ricerca sul Cancro” (FIRC) fellowship.


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