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New insights into the human 5-HT_4 receptor binding site: exploration of a hydrophobic pocket

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1 A body of evidences suggests that a hydrophobic pocket of the human 5-HT_4 receptor contributes to the high affinity of some bulky 5-HT_4 ligands. A thorough study of this pocket was performed using mutagenesis and molecular modeling.

2 Ligand binding or competition studies with selected bulky ligands (RS39604, RS100235, [^{3}H]GR113808 and ML11411) and small ligands (5-HT and ML10375) were carried out on wild-type and mutant receptors (W7.40A/F, Y7.43F, R3.28L) transiently transfected in COS-7 cells. The functional activity of the mutated receptors was evaluated by measuring the ability of 5-HT to stimulate adenylyl cyclase.

3 For W7.40F mutation, no changes in the affinity of studied ligands and in the functional activity of the mutant receptor were observed, in contrary to W7.40A mutation, which abolished both binding of ligands and 5-HT-induced cAMP production.

4 Mutation R3.28L revealed a totally silent receptor with a basal level of cAMP production similar to the mock control despite its ability to product cAMP in the presence of 5-HT. Moreover, a one order loss of affinity of RS39604 and a 45-fold increase of ML11411 affinity were observed.

5 Mutation Y7.43F modified the affinity of GR113808, which displays a 13-fold lower affinity for the mutant than for the wild-type receptor.

6 In conclusion, in the hydrophobic pocket, two polar amino acids are able to interact through hydrogen bonds with bulky ligands depending on their chemical properties. Moreover, these experimental data may validate the proposed new three-dimensional model of the human 5-HT_4 receptor.

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Keywords: Site-directed mutagenesis; molecular modelling; 5-HT_4 receptor; hydrophobic pocket; 5-HT_4 bulky ligands

Abbreviations: CPU, central processing unit; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GPCR, G protein-coupled receptor; GR113808, 1-methyl-1*H*-indole-3-carboxylic acid 1-(2-methanesulfonylamino-ethyl)-piperidin-4-ylmethyl ester; h5-HT_4 receptors, human 5-HT_4 receptors; ML10375, 4-amino-5-chloro-2-methoxy-benzoic acid 2-(*cis*-3,5-dimethyl-piperidin-1-yl)-ethyl ester; ML11411, 4-amino-5-chloro-2-methoxy-benzoic acid 2-{4-[4-(5-dimethylamino-naphthalene-1-sulfonylamino)-butyl]-piperidin-1-yl}-ethyl ester; PDB, Brookhaven Protein Data Bank; PEI, polyethyleneimine; RASSL, receptor activated solely by synthetic ligands; r.m.s.d., root mean square deviation; RS39604, *N*-[2-(4-{3-[4-amino-5-chloro-2-(3,5-dimethoxy-benzyloxy)-phenyl]-3-oxo-propyl}-piperidin-1-yl)-ethyl]-methanesulfonamide; RS100235, 1-(8-amino-7-chloro-2,3-dihydro-benzo[1,4]dioxin-5-yl)-3-{1-[3-(3,4-dimethoxy-phenyl)-propyl]-piperidin-4-yl}-propan-1-one; TM, transmembrane domain; WT, wild type

Introduction

Since their discovery in mouse colliculi neuronal cells and in the guinea pig ileum (Dumuis *et al*., 1989; Hoyer *et al*., 1994), considerable interest has been devoted to the characterization and the distribution of the human 5-HT_4 (h5-HT_4) receptor (Eglen *et al*., 1990; Hedge & Eglen, 1996; Blondel *et al*., 1998; Claeysen *et al*., 1999). It belongs to the superfamily of G protein-coupled receptors (GPCRs) and is involved in a variety of pathological disorders such as irritable bowel syndrome, gastroparesis (Bouras *et al*., 1999), cardiac atrial arrhythmia (Kaumann, 1994), dysfunction of the urinary tract (Hedge & Eglen, 1996) and memory deficits (Marchetti-Gauthier *et al*., 1997). Particularly, recent studies have underlined the potential role of the h5-HT_4 receptor in Alzheimer's disease (Robert *et al*., 2001; Maillet *et al*., 2004). Thus, this receptor constitutes a valuable target for therapeutic applications such as the cure of irritable bowel syndrome, the prevention of atrial fibrillations, the restriction of cognitive decline and mental retardation observed in cognitive disorders (Langlois & Fischmeister, 2003), and the synthesis of new selective agonists, antagonists or inverse agonists is still a challenge.

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In the course of the rational design of new drugs, the binding site and molecular interactions involved in h5-HT$_4$ ligand binding were particularly studied (Buchheit *et al*., 1995; Gaster & King, 1997; Lopez-Rodriguez *et al*., 1997; 2001; 2002; Iskander *et al*., 1999; Mialet *et al*., 2000b; Bureau *et al*., 2002). Our previous work revealed several fundamental interactions between residues located in transmembrane domain (TM) 3, D3.32, TM5, S5.43 and TM6, F6.51 (to facilitate comparison of aligned residues in related GPCRs, the most conserved residue in a given TM X was given the index number X.50, and residues within a given TM were then indexed relative to the '50' position (Ballesteros & Weinstein, 1995). Note that the '50' position does not necessitate that the residue is in the middle of the TM; for example, the highly conserved R3.50 is at the cytoplasmic end of TM3 in the conserved E/DRY sequence) of the receptor (Mialet *et al*., 2000b) and the structural features that characterize the h5-HT$_4$ ligands: an aromatic moiety, a coplanar carbonyl function and a basic nitrogen atom. For many ligands, the nitrogen atom is substituted by a voluminous group, which should take place in a hydrophobic pocket of the receptor. Earlier studies have underlined the importance of this pocket for the ligand–receptor recognition (Gaster & King, 1997; Lopez-Rodriguez *et al*., 1997; 2001; 2002; Iskander *et al*., 1999; Claeysen *et al*., 2003; Pauwels, 2003). Moreover, h5-HT$_4$ RASSL (receptor activated solely by synthetic ligands) studies have shown that ligands where the basic nitrogen was linked to a voluminous group were still able to interact with the mutated receptor in contrary to the endogenous 5-HT or to small synthetic ligands such as tryptamine derivates, which do not possess a substituted basic nitrogen (Claeysen *et al*., 2003; Pauwels, 2003). Thus, the hydrophobic pocket could contain specific anchor points contributing to the affinity of bulky ligands. Surprisingly, this region of the receptor accommodates a large set of substituents including hydrophobic groups as well as polar groups without affecting the affinity of the compounds (Langlois & Fischmeister, 2003).

From all these observations, the need for a precise identification of the nature of the pocket and its potential interaction with bulky ligands emerged. In this work, we performed a thorough study of the hydrophobic pocket combining site-directed mutagenesis, molecular modeling and docking experiments. We selected several h5-HT$_4$ receptor ligands belonging to different structural classes but sharing a basic nitrogen included in different substituted piperidin rings (Figure 1): (i) two molecules, GR113808 and RS39604, which possess a sulfonamido group attached through a two carbon atom chain on the nitrogen atom of piperidin. GR113808 is an indole carboxylate generally used as reference in 5-HT$_4$ studies and RS39604 (Clark *et al*., 1994) is a phenylketone. Moreover, the phenyl moiety of RS39604 is substituted by a bulky (dimethoxyphenyl)methyl group; (ii) molecule ML11411 (Berque-Bestel *et al*., 2003), a benzoic ester, which possesses a sulfonamido group substituted by a voluminous dansylamino group and situated at a distance of more than two carbon atoms from the basic nitrogen atom; (iii) one molecule, RS100235 (Clark *et al*., 1995), a ketone, which does not possess a sulfonamido chain but a more hydrophobic (dimethoxyphenyl)propyl chain on the basic nitrogen atom of piperidin; and (iv) ML10375 (Yang *et al*., 1997), a small benzoic ester, which represents a reference for identifying some direct interactions between bulky ligands and residues of the hydrophobic pocket.

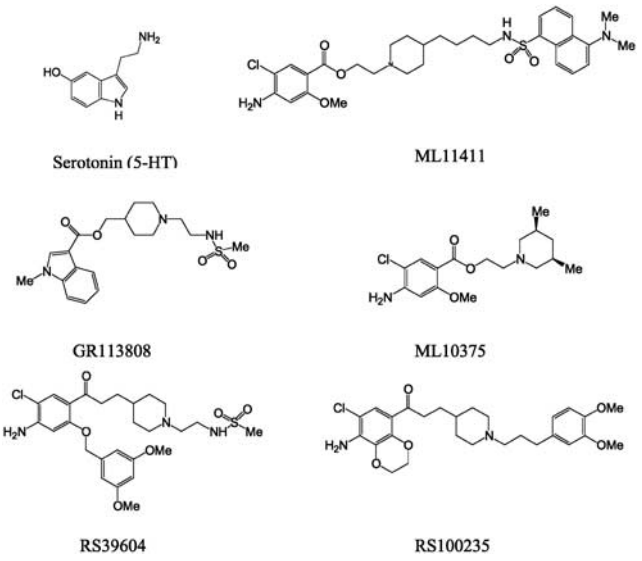


Figure 1 Chemical representation of the h5-HT$_4$ receptor ligands used in this study.

Methods

Materials

Complementary mutagenesis oligonucleotides and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco Life Technologies. 5-Hydroxytryptamine (5-HT) was from Aldrich (L'Isle d'Abeau Chesnes, France). 1-Methyl-1*H*-indole-3-carboxylic acid 1-(2-methanesulfonylamino-ethyl)-piperidin-4-ylmethyl ester (GR113808) was a gift from Glaxo Research Group (Ware, Hertfordshire, U.K.). 4-Amino-5-chloro-2-methoxy-benzoic acid 2-(*cis*-3,5-dimethyl-piperidin-1-yl)-ethyl ester (ML10375) and 4-amino-5-chloro-2-methoxy-benzoic acid 2-{4-[4-(5-dimethylamino-naphthalene-1-sulfonylamino)-butyl]-piperidin-1-yl}-ethyl ester (ML11411) were synthesized as previously described (Yang *et al*., 1997; Berque-Bestel *et al*., 2003), 1-(8-amino-7-chloro-2,3-dihydro-benzo[1,4]dioxin-5-yl)-3-{1-[3-(3,4-dimethoxy-phenyl)-propyl]-piperidin-4-yl}-propan-1-one (RS100235) was a gift from Roche (U.S.A.). *N*-[2-(4-{3-[4-amino-5-chloro-2-(3,5-dimethoxy-benzyloxy)-phenyl]-3-oxo-propyl}-piperidin-1-yl)-ethyl]-methanesulfonamide (RS39604) was purchased from Tocris (Fisher Bioblock Scientific, Illkirch, France).

Molecular modeling

The amino-acid sequence of the h5-HT$_4$ receptor was manually aligned with the sequence of other serotoninergic receptor subtypes and taking the bovine rhodopsin as the template (Palczewski *et al*., 2000).

The three-dimensional model of the seven h5-HT$_4$ receptor TM was built using the bovine rhodopsin crystallographic structure (A-1HZX) as template, in which the position of TM3 was modified (about 17° rotation around the helix axis) to take into account previous mutagenesis results (D3.32). The

ionizable residues (Asp, Arg, Glu, Lys) and the piperidin nitrogen atom were considered charged. During the first refinement steps of the model, the Cα backbone of each helix bundle was first constrained to its initial position and gradually relaxed. After obtaining an energy refined model of the seven TM of the h5-HT$_4$ receptor, the extra- and intracellular loops were then added. This was achieved using the 'homology' loops module, which uses a library of loop templates from the Brookhaven Protein Data Bank (PDB). Because no experimental information about the structure of these loops is yet available, extensive high-temperature molecular dynamics at 1000 K followed by annealing to 300 K were next carried out on these segments, with the seven TM being frozen in its most favorable position as obtained above. The final structure was energy minimized using 100,000 steps of a conjugated gradient procedure. During these calculations, the disulfide bridge (Beck-Sickinger, 1996) between C93 (extracellular loop 1) and C184 (extracellular loop 2) of the receptor was imposed as a constraint. Then, the whole system was relaxed taking into account possible translation and rotation movements of individual transmembrane helices.

Manual docking of studied ligands was performed so as to fit previous experimental data, which identified contact points with D3.32, S5.43 and F6.51. The resulting structure obtained for each ligand–receptor complex was further refined using molecular dynamics and energy minimizations. Then the docking was improved by intensive annealing calculations.

Models were built on an 'Octane' Silicon Graphics computer, using softwares from Molecular Simulation Inc. (InsightII, Biopolymer, Homology, Discover). Annealings were performed using the computing facilities of CINES (Centre Informatique National de l'Enseignement Supérieur, Montpellier, France). For all these calculations, a distance-dependent dielectric constant was used to avoid overevaluation of electrostatic interactions between charged groups. A cutoff of 2 nm was used to truncate the list of interactive atoms to a reasonable size in relation to the available central processing unit (CPU) time.

Site-directed mutagenesis

The full coding region of the 5-HT$_{4(g)}$ cDNA (previously named 5-HT$_{4(e)}$ and later renamed 5-HT$_{4(g)}$; Langlois & Fischmeister, 2003) was subcloned in the mammalian expression vector pRC/CMV (Invitrogen, Carlsbad, CA, U.S.A.) as previously described (Blondel *et al*., 1998). Mutations in the human 5-HT$_{4(g)}$ receptor were introduced using QuickChange Site-directed Mutagenesis kit (Stratagene, Montigny-le-Bretonneux, France). Mutants were analyzed by restriction enzymes and the authenticity of each mutation was confirmed by DNA sequencing (Genome Express, Montreuil, France).

Measurement of cAMP production

Transiently transfected COS-7 cells (Mialet *et al*., 2000b) were preincubated for 24 h with dialyzed fetal calf serum (FCS). The cells were then incubated for 15 min in a serum-free medium supplemented with 5 mM theophylline and 10 μM pargyline at 37°C in 5% CO_2. 5-HT was added (1 μM) and incubated for an additional 15 min at 37°C in 5% CO_2. The reaction was stopped by aspiration of the medium and addition of 100 μl ice-cold perchloric acid (20%). After a 30 min period at 4°C, neutralization buffer was added (HEPES 25 mM, KOH 2 N) and cAMP was quantified using a radioimmunoassay kit (cAMP competitive radioimmunoassay, Beckman, France).

Statistical analysis

One-way ANOVA, followed by Newman–Keuls *post hoc* test was used to assess the differences between the experimental groups. Values of $P<0.05$ were considered to be significant.

Ligand binding assays

Transfected COS-7 cells were washed twice with phosphate-buffered saline, scraped, collected and centrifuged at $3000\times g$ for 5 min. The pellet was resuspended in 10 volumes of ice-cold HEPES buffer (50 mM, pH 7.4) and homogenized by an Ultraturax tissue grinder. The lysate was subsequently centrifuged at $40{,}000\times g$ for 15 min at 4°C. The resulting pellet was resuspended in 10 volumes of HEPES buffer (50 mM, pH 7.4). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Radioligand binding assays were performed in 500 μl buffer (50 mM HEPES, pH 7.4) containing 20 μl of [^{3}H]GR113808, 50 μl (~50 μg) of membrane preparation and 20 μl of displacing drug or assay buffer. Nonspecific binding was measured in the presence of 20 μM of GR113808.

Saturation experiments were performed using [^{3}H]GR113808 at nine concentrations ranging from 0.01 mM to 4 nM. Competition assays were performed in the presence of nine concentrations of the displacing ligands (10^{-12}–10^{-4} M) and a concentration of [^{3}H]GR113808 corresponding to its K_D value over the wild-type (WT) receptor.

Incubation was performed at 25°C for 30 min and terminated by rapid filtration through Whatman GF/B filter paper using the Brandel model 48R cell harvester. Filters were presoaked in a solution of polyethyleneimine (PEI) (0.1%) to reduce the nonspecific binding. After filtration, they were subsequently washed twice with ice-cold buffer (50 mM Tris-HCl, pH 7.4) and placed overnight in 4 ml of Ready-Safe scintillation cocktail (Beckman, Fullerton, CA, U.S.A.). Radioactivity was measured using a Beckman model LS 6500C liquid scintillation counter. Binding data were analyzed by computer-assisted nonlinear regression analysis (Prism, Graph Pad Software, San Diego, CA, U.S.A.). IC_{50} values correspond to a displacement of 50% of specific radioligand binding and were converted to K_i values according to the equation: $K_i = IC_{50}/(1+(\text{concentration of } [^3\text{H}]\text{GR113808}/K_D))$.

Immunoprecipitation and immunoblotting

Membrane preparation was performed as follows. Briefly, cells were washed twice with ice-cold PBS and were then disrupted by homogenization with a syringe in 500 μl of ice-cold buffer A (5 mM Tris-HCl pH 7.4, 2 mM EDTA and protease inhibitors). Lysates were centrifuged at $500\times g$ for 5 min at 4°C. The supernatant was then centrifuged at $40{,}000\times g$ for 20 min, and the pellet was resuspended in the same above buffer.

Membrane proteins (100 μg) were precleared by incubation with a mixture of protein A and G-Sepharose beads (Sigma, L'Isle d'Abeau Chesnes, France). After centrifugation, a

monoclonal anti-Flag antibody (M2; Sigma) was added to the supernatant for 1 h at 4°C. Immunocomplexes were then isolated by incubation with 10% vol vol^{-1} protein G–Sepharose (Sigma) overnight at 4°C. The protein G–Sepharose beads were washed three times with buffer A and resuspended in sample buffer at 60°C for 20 min. The dissolved immunocomplexes were resolved on a 6% SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and subjected to immunoblotting using the anti-Flag antibody. Immunoreactive bands were visualized by light emitting nonradioactive method (ECL, Amersham, Orsay, France).

Results

The human 5-HT$_4$ receptor: three-dimensional model

In order to identify the amino acids of the hydrophobic pocket of the h5-HT$_4$ receptor potentially involved in interactions with bulky ligands, we first built a three-dimensional model of the h5-HT$_4$ receptor. Based on the crystallographic bovine rhodopsin structure of TM (Palczewski *et al*., 2000) and after inclusion of extra- and intracellular loops the model was refined by molecular dynamics and energy minimization. Comparison with the crystallographic bovine rhodopsin structure showed that the global arrangement of the TM was roughly maintained with a root mean square deviation (r.m.s.d.) of 0.26 nm between the TM backbones. As in bovine rhodopsin, an α-helical structure comprising residues S318–C328 was found in the C-terminal tail. However, differences between the primary sequences of the h5-HT$_4$ receptor and bovine rhodopsin led to some differences between the two three-dimensional structures. For instance, TM1 of the h5-HT$_4$ receptor was found oriented as a regular α-helix due to the absence of P residue in position 1.53. In contrast, P1.53 in rhodopsin bends this helix more inwards, and TM6 of the h5-HT$_4$ receptor was found more bent at P6.50 toward TM5 than in rhodopsin. As described for various GPCRs, an intramolecular network of hydrogen bonds was found between the well-conserved N1.50, D2.50 and N7.49 residues (Konvicka *et al*., 1998). We report for the first time a salt bridge between the carboxylate side chain of E2.64 and the guanidinium side chain of R3.28. After relaxing the system, the well-known disulfide bond between C93 and C184, located respectively in the first and the second extracellular loops, was conserved (Beck-Sickinger, 1996). Finally, a large hydrophobic pocket, delimited by residues V2.57, F2.60, V3.27, R3.28, L3.31, W7.40 and Y7.43, was observed on the model. This pocket occupied a volume of 15 nm^3 for a total binding site volume of 31 nm^3, estimated by calculations with the Surfnet software (Laskowski, 1995). This observation could be a first explanation of the fact that ligands containing bulky groups bound on their basic nitrogen atom possess high affinities for the h5-HT$_4$ receptor (Lopez-Rodriguez *et al*., 1997).

Docking results: identification of the candidate amino-acid residues of the h5-HT$_4$ receptor in potential interaction with bulky ligands

Whatever the synthetic ligand, the pharmacophoric elements (the aromatic moiety, the coplanar carbonyl function and the basic nitrogen atom included in a piperidin ring and substituted by bulky substituents) were found to take place between TM3, TM4, TM5 and TM6. A hydrogen bond (around 0.17 nm) was observed between the hydroxyl group of S5.43 and the carboxylic oxygen (ester or ketone) of ligands. Finally, the proximity of the piperidin ring of the different ligands and F6.51 allowed hydrophobic interactions.

In addition to these fundamental interactions, other interactions appeared to take place depending on the structure of the ligands: (i) a hydrogen bond was found to link one oxygen atom of the sulfonamido functional group of GR113808 with the phenolic hydrogen of Y7.43 (Figure 2a) and a similar interaction was observed with ML11411; (ii) in contrary, sulfonamido functional group of RS39604 was not found to interact with the phenolic hydrogen of Y7.43 but with a hydrogen of the guanidinium group of R3.28, which was located just below the extracellular disulfide bridge described above (Figure 2b). In fact, although RS39604 possesses a sulfonamido ethyl chain, analogous or smaller than that of other tested molecules, the presence of the (dimethoxyphenyl)methyl group on the aromatic moiety was observed to induce a different positioning of this ligand in the receptor.

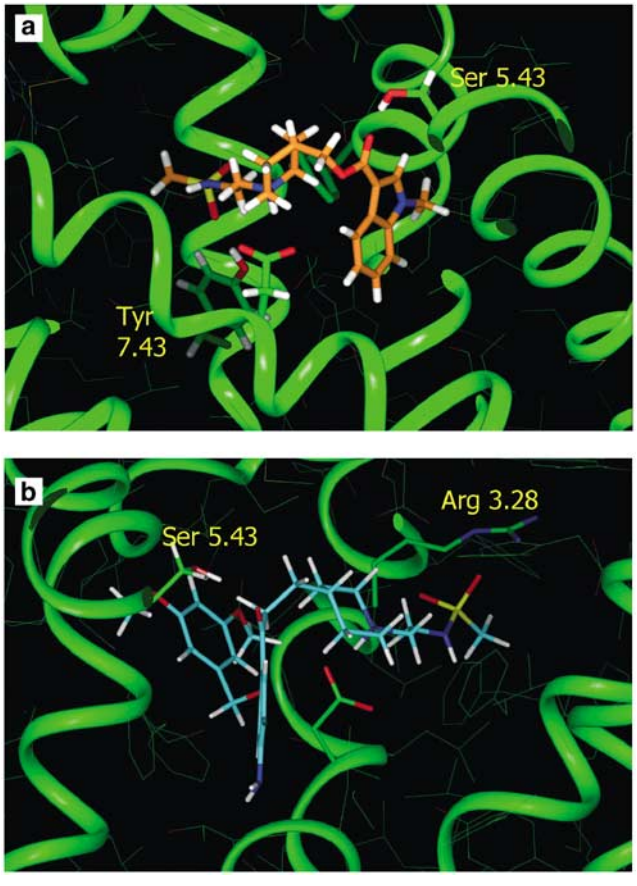
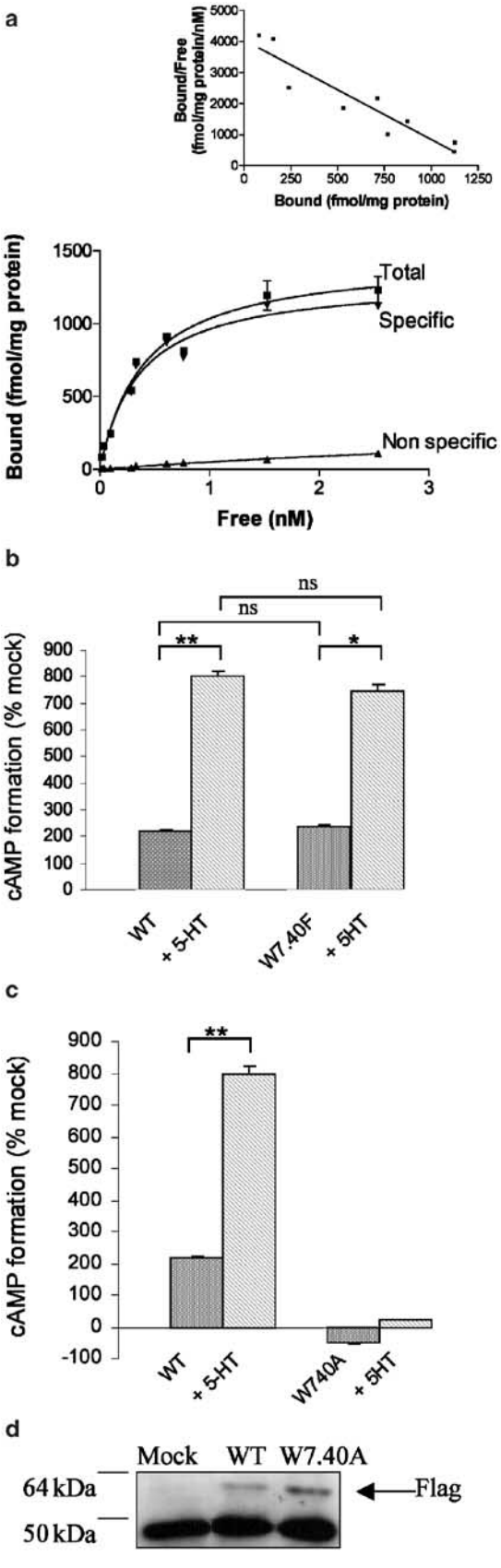


Figure 2 Molecular model for the interactions involved in the binding site of the h5-HT$_4$ receptor. (a) Molecular model of a region of the GR113808 binding site of the h5-HT$_4$ receptor. This model predicted a possible hydrogen bond interaction between the hydroxyl group of the residue Y7.43 of the h5-HT$_4$ receptor and the sulfonamido group of the antagonist GR113808. (b) Molecular model of a region of the RS39604 binding site of the h5-HT$_4$ receptor. The molecular model shows a potential hydrogen bond between the sulfonamido group of RS39604 and the guanidinium extremity of R3.28 of the h5-HT$_4$ receptor.



More precisely, this group pushed the sulfonamido functional group of the bulky side chain close to the R3.28 residue; (iii) the salt bridge observed in our h5-HT$_4$ receptor model between the guanidinium group of R3.28 and the carboxylate group of E2.64 might form a steric hindrance preventing a convenient positioning of ML11411; (iv) the dimethoxy phenyl group of RS100235 was found to take place between TM2, TM3 and TM7 developing hydrophobic interactions with those helixes *via* residues V2.57, L3.31, V3.27, W7.40 and Y7.43. Although W7.40 was positioned at a very large distance from ligands to interact through a hydrogen bond, its location between Y7.43 and R3.28 in the binding pocket combined with its high conservation degree within the 5-HT receptor family makes it a good candidate to study its interactions with ligands.

Thus, besides the interactions already described (Mialet *et al*., 2000b), this three-dimensional model enabled us to identify additional potential interactions with residues of the hydrophobic pocket, which might contribute to the high affinity of bulky ligands, especially those possessing a sulfonamido moiety. These candidate residues were W7.40, Y7.43 located on TM7 and R3.28 located on TM3.

Experimental data of mutations: W7.40F. and W7.40A

First, the mutation W7.40F was performed. This mutation maintains the aromatic nature of the amino acid but suppresses the possibility of a hydrogen bond involving the indolic amino group of W. Scatchard analysis (Figure 3a) using the specific antagonist [^{3}H]GR113808 was performed on COS-7 cell membranes transfected with W7.40F. This mutant bound [^{3}H]GR113808 with the same affinity as the WT receptor (K_D W7.40F $= 0.46 \pm 0.14$ nM, K_D WT $= 0.23 \pm 0.06$ nM) although its expression was higher (B_{max} W7.40F $= 1306 \pm 85$ fmol mg^{-1} proteins, B_{max} WT $= 850 \pm 47$ fmol mg^{-1} proteins). Furthermore, no difference in competition binding on the W7.40F mutant using [^{3}H]GR113808 was observed between 5-HT and the synthetic ligands RS39604, ML11411, RS100235 and ML10375 (Table 1). The biological function of the mutated receptor was evaluated by determining cAMP accumulation in transfected COS-7 cells. 5-HT induced cAMP production similar to the value observed for the WT receptor (Figure 3b). These results clearly show that mutation W7.40F did not

Figure 3 Mutants W7.40F and W7.40A. (a) Scatchard analysis of saturation experiments of [^{3}H]GR113808 binding to the W7.40F h5-HT$_{4(g)}$ receptor mutant. Membranes were prepared from COS-7 cells transiently transfected with the cDNA encoding the mutated receptors. (b, c) Functional response with or without 5-HT of the WT and mutant receptors. Experiments were performed in COS-7 cells transiently expressing the WT, W7.40F (b) and W7.40A (c) receptors. Data are expressed as a percentage of cAMP production in mock-transfected cells taken as control. Values are the mean $\pm$ s.e.m. values determined from three experiments performed in triplicate. $^{**}P<0.01$; $^{*}P<0.05$ *versus* indicated values; ns, non-significant. (d) Protein immunoblotting of the W7.40A mutant expressed in COS-7 cells. COS-7 cells were transiently transfected with the WT Flag-h5-HT$_{4(g)}$ receptor, W7.40A Flag-h5-HT$_{4(g)}$ receptor mutant or control empty vector (mock). After immunoprecipitation with an anti-Flag antibody, the immunocomplexes were resolved on a 6% polyacrylamide gel and revealed by a monoclonal anti-Flag antibody. A specific band migrating to the level of 60 kDa was detected whereas in mock-transfected cells no labeling was detected at this position.

modify the binding of the studied ligands and cAMP production in response to 5-HT.

To gain further insights into the role of this highly conserved residue W7.40, we performed a second mutation W7.40A in order to evaluate the contribution of the aromatic moiety to binding and efficacy. In fact, this mutation abolished totally the binding of [^{3}H]GR113808 and the 5-HT-induced cAMP accumulation (Table 1 and Figure 3c). Compared to other mutants where the binding and the activation properties were conserved, immunoprecipitation and immunoblotting experiments of the W7.40A receptor were performed in order to verify its expression in transfected cells (Figure 3d).

Experimental data of mutations: R3.28L

To get experimental support to the molecular modeling predictions, which identify R3.28 as an important amino acid of the hydrophobic pocket of the h5-HT$_4$ receptor, this residue was mutated for L. This mutation should not modify the hydrophobic nature of the pocket but should reveal involvement of a charged environment in this position. Scatchard analyses with [^{3}H]GR113808 demonstrated that mutation R3.28L did not induce any significant change in [^{3}H]GR113808 binding properties of the receptor mutant (K_D R3.28L $= 0.55 \pm 0.25$ nM, K_D WT $= 0.23 \pm 0.06$ nM) (Figure 4a). Analysis of the maximal binding capacity of this mutant demonstrated that it was expressed at a level similar to the WT receptor (B_{max} R3.28L $= 908 \pm 36$ fmol mg^{-1} proteins, B_{max} WT $= 850 \pm 47$ fmol mg^{-1} proteins). From competition experiments, we observed that this mutation disturbed neither the binding of the two small ligands, 5-HT and ML10375, nor the binding of RS100235. On the other hand, the affinity of RS39604 for the mutated receptor was 17-fold lower than the WT receptor (K_i R3.28L $= 55.8 \pm 29.6$ nM, K_i WT $= 2.15 \pm 1.92$ nM) (Figure 4c). Binding of ML11411 to the R3.28L mutant occurred with an apparent 45-fold higher affinity than the WT receptor (K_i R3.28L $= 1.16 \pm 0.44$ nM, K_i WT $= 53.5 \pm 24.6$ nM) (Figure 4d). Then, cAMP accumulation was determined (Figure 4b) and compared to the WT receptor. The R3.28L mutant displayed a basal level of cAMP production similar to the mock control indicating the abolition of the constitutive activity. This abolition was confirmed by experiments with GR113808, which has been shown to behave as an inverse agonist of the WT 5-HT$_{4(g)}$ receptor isoform (Mialet *et al*., 2000a). However, in the presence of 5-HT, the mutated receptor induced a cAMP production similar to the WT receptor.

Experimental data of mutations: Y7.43F

As the Y7.43A mutation has been already performed in a previous work (Mialet *et al*., 2000b), Y7.43 was mutated into F maintaining the aromatic nature but lacking the phenolic hydroxyl group found in the model to be responsible for a hydrogen bond with the sulfonamido moiety of voluminous ligands.

Because of the low level of reproducibility of Scatchard analyses (Figure 5a and Table 1), experiments were repeated 10 times and the most representative graph is presented in Figure 5a. In all cases, the expression level of the Y7.43F mutant was low as compared to the WT receptor (B_{max} Y7.43F $= 390 \pm 54$ fmol mg^{-1} proteins, B_{max} WT $= 850 \pm 47$ fmol mg^{-1} proteins). The nonspecific binding was high compared to total binding (35% for [^{3}H]GR113808 concentration 2.5 nM). Nevertheless, we found for GR113808 a 13-fold lower affinity ($K_D = 3.06 \pm 0.65$ nM) for the mutant receptor than for the WT receptor ($K_D = 0.23 \pm 0.06$ nM). Furthermore, the determination of the affinity of RS39604, ML11411 and RS100235 for the Y7.43F mutant revealed no differences in affinity for ML11411 and RS100235 and a small not significant decrease for RS39604 (K_i Y7.43F $= 15 \pm 9.5$ nM, K_i WT $= 2.15 \pm 1.92$ nM) (Table 1). Competition binding of 5-HT was not performed, considering that the position Y7.43, very far from the binding site of 5-HT, should not induce any perturbation. This is confirmed by the evaluation of the biological activity of the mutated receptor (Figure 5b). The maximal response of 5-HT obtained with the mutant was quite similar to that obtained with the WT receptor.

Discussion

In this work, we focused our interest on a specific hydrophobic pocket of the h5-HT$_4$ receptor binding site able to interact with voluminous ligands, and investigated potential important residues around this specific pocket. For that purpose, we first proposed a new molecular model of the h5-HT$_4$ receptor based on the crystallographic structure of bovine rhodopsin and including for the first time the extra- and intracellular loops to the TM. The modeling of this entire receptor (helices and loops) appeared necessary to respect the packing arrangement of the TM of the receptor, which is important due to the localization of the studied hydrophobic pocket, close to the membrane surface. This model was structurally characterized by the location of the well-documented residues D3.32, S5.43

Table 1 Summary of the binding properties of the WT and mutated h5-HT$_4$ receptors

Ligands	*WT* K_i/K_D (nM)	*Y7.43F* K_i/K_D (nM)	*W7.40F* K_i/K_D (nM)	*W7.40A* K_i/K_D (nM)	*R3.28L* K_i/K_D (nM)
GR113808	0.23±0.06	3.06±0.65	0.46±0.14	No binding	0.55±0.25
5-HT	653±223	ND	627±270	—	402±180
ML10375	0.57±0.38	ND	0.64±0.38	—	0.85±0.43
ML11411	53.5±24.6	79.7±42.9	33.9±14.8	—	1.16±0.44
RS100235	2.11±1.12	1.1±0.66	2.12±1.18	—	3.84±2.22
RS39604	2.15±1.92	15±9.5	3.27±0.93	—	55.8±29.6
B_{max} (fmol mg^{-1} protein)	850±47	390±54	1306±85	—	908±36

Binding experiments were performed on COS-7 cells transiently transfected with cDNA encoding the WT and mutated receptors. Results are expressed as means±s.e.m. of three independent experiments performed in triplicate. ND: not determined.

and F6.51, which allow fundamental interactions with classical pharmacophore elements of ligands. Secondly, (voluminous ligands/h5-HT$_4$ receptor) complexes docking experiments were performed, underlining three particular amino acids, W7.40, R3.28 and Y7.43, which represented possible points of interaction with bulky ligands.

The first amino acid, W7.40, widely conserved within the 5-HT receptors family was particularly interesting to examine. Indeed, a W residue can participate to direct hydrophobic interactions or to hydrogen bonds. For example, in bovine rhodopsin, bulky aromatic groups and notably W residues are essential for chromophore stabilization and photoisomerization (Kristiansen & Dahl, 1996). In 5-HT$_2$ receptors, substantial contributions of W7.40 for both ligand binding and second messenger production were reported (Roth *et al*., 1997). In the D$_2$ dopamine receptor, this residue forms part of the binding crevice (Fu *et al*., 1996). Yet we found that mutation W7.40F in the h5-HT$_4$ receptor caused no change in binding, indicating that W7.40 is not involved in a hydrogen bond interaction with ligands. This observation was consistent with the large distance deduced from our molecular model between the NH of W7.40 and the polar groups of the tested ligands. On the other hand, when W7.40 was mutated into A, both binding and activity of the h5-HT$_4$ receptor were lost, most likely due to nonspecific conformational change in the receptor. Indeed, a single mutation could cause improper protein folding leading to loss of binding and activation despite the expression of this mutant in cell membranes. Taken together, these results indicated that in this position the aromatic nature of the residue is necessary for maintaining the binding and functional properties of the h5-HT$_4$ receptor.

The second mutation concerned the R3.28 residue. It is interesting to note that among the different 5-HT receptor subtypes, this residue is only found in the h5-HT$_4$ receptor. Its physico-chemical properties allowing ionic and/or hydrophobic bonds and its particular localization on TM3, which plays a crucial role in GPCR activation (Gether *et al*., 1997; Kobilka & Gether, 1998; Shapiro *et al*., 2002), might confer a particular interest with respect to this residue. Moreover, R3.28 is located one turn above D3.32, a key amino acid for receptor activation (Claeysen *et al*., 2003). In this study, the R3.28L mutant exhibited a very low level of constitutive activity and became insensitive to inverse agonists, indicating that this mutant receptor might exist mainly in the inactive state. However, the fact that it conserved the ability to activate cAMP production upon 5-HT stimulation suggests that this mutation did not perturb the conversion from inactive to active state. Conse-

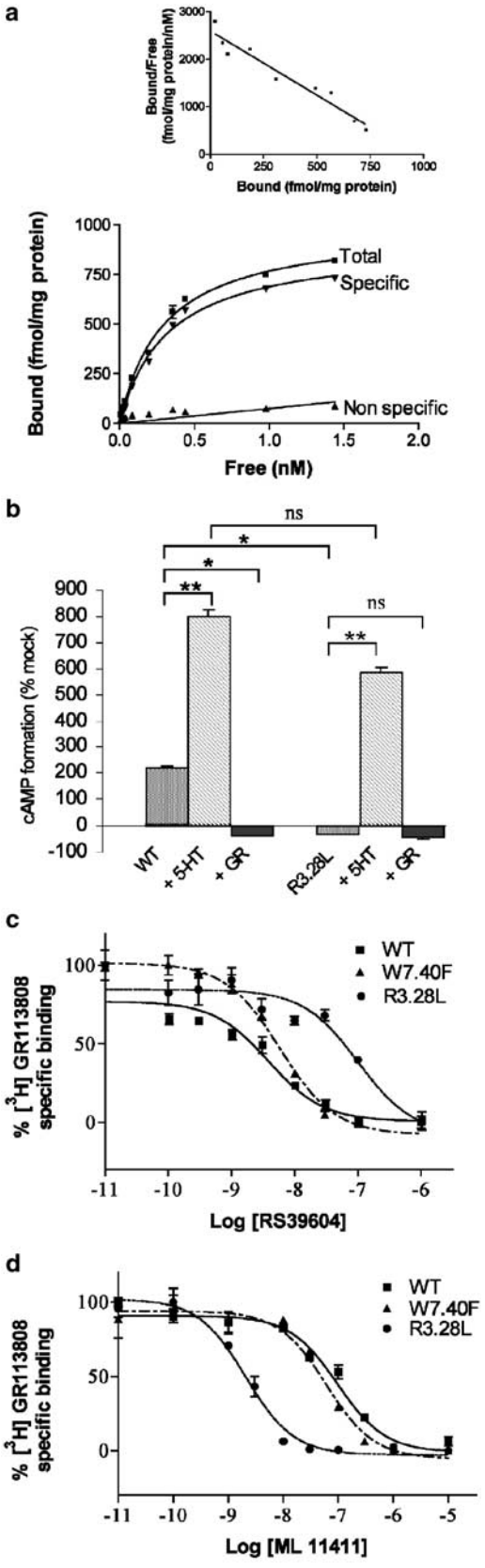


Figure 4 R3.28L mutation exhibited an abnormal constitutive activity and a modified binding for RS39604 and ML11411. (a) Saturation analysis of [^{3}H]GR113808 binding to COS-7 cells transfected with the R3.28L h5-HT$_{4(g)}$ receptor mutant. Nonspecific binding was determined with 10 μM cold GR113808. Experiments were performed in triplicate using a range of nine concentrations of radioligand. (b) cAMP accumulation in the absence or presence of 5-HT and in the presence or absence of cold GR113808 in COS-7 cells transiently expressing the WT and R3.28L human h5-HT$_{4(g)}$ receptors. Data are expressed as a percentage of cAMP production in mock-transfected cells taken as control. Data are the means$\pm$s.e.m. values of five independent experiments performed in triplicate. $^{**}P<0.01$; $^{*}P<0.05$ *versus* indicated values; ns, non-significant. (c, d) Competition of RS39604 (c) and ML11411 (d) for [^{3}H]GR113808 binding to the WT, R3.28L and W7.40F h5-HT$_{4(g)}$ receptors. The assays were carried out on membranes derived from COS-7 cells and in the presence of 0.2 nM of [^{3}H]GR113808. Results are expressed as a percentage of the specific binding in the absence of competing ligand. Data are the means$\pm$s.e.m. of three experiments performed in triplicate.

quently, the role of R3.28 residue, found only in the 5-HT$_4$ receptor, could be relevant to a participation in the constitutive activity of the receptor. In our model, R3.28 was supposed to interact with RS39604 through a hydrogen bond and with residue E2.64 through a salt bridge, thereby able to prevent the binding of ML11411. The R3.28L mutant did not change [^{3}H]GR113808 binding but revealed a 17-fold decrease in affinity of RS39604 compatible with the disruption of a hydrogen bond. Indeed, such a decrease in affinity represents a loss of about 2–3 kcal mol^{-1} of binding energy and seems to agree with the existence of a hydrogen bond between R3.28 and the sulfonamido group of RS39604 as anticipated by the molecular model.

In contrast, the R3.28L mutation induced a 45-fold increase in the affinity of ML11411, the biggest ligand of our study, which possesses a middle-range affinity for the WT receptor. Our model showed that the presence of a salt bridge between the guanidinium group of R3.28 and the carboxylate group of E2.64 could prevent a convenient positioning of ML11411. Moreover, the charged environment induced by the side chain of R3.28 might lead to a charge repulsion with the charged dansylamino group of this compound. When R3.28 was changed for L, the charged environment was lost and the salt bridge was disrupted, releasing the structural constraint, which could explain the increase of the affinity.

The third amino acid pointed out by our model, Y7.43, occupies a strategic position. In bovine rhodopsin receptor, a K is located in the 7.43 position and represents the covalent binding site of retinal (Ballesteros *et al*., 2001; Sakmar *et al*., 2002). In amine biogenic GPCRs (m3 muscarinic (Schwartz *et al*., 1995), β_2 adrenergic (Strader *et al*., 1989) or D$_2$ dopaminergic receptors (Fu *et al*., 1996)), Y in the 7.43 position contributes to agonist and/or antagonist binding affinities. In our model, a potential hydrogen bond between Y7.43 and the sulfonamido group of GR113808 and ML11411 was observed. Our previous mutation Y7.43A abolished binding of [^{3}H]GR113808 and 5-HT-induced cAMP accumulation (Mialet *et al*., 2000b) probably due to a nonspecific conformational change of the mutated receptor. Our present results showed that the Y7.43F mutation caused a 13-fold decrease of [^{3}H]GR113808 affinity for the mutant, without affecting the 5-HT-induced activity of the receptor. Thus, the aromatic nature of the amino acid in the 7.43 position of the h5-HT$_4$ receptor could be sufficient to maintain the correct folding of the receptor and its activation properties. Nevertheless, as for R3.28L mutation, a 13-fold decrease of affinity could be consistent with the disruption of a hydrogen bond. Consequently, the predicted hydrogen bond in our molecular model, between Y7.43 and GR113808, probably occurs and could confirm the orientation of the sulfonamido chain toward

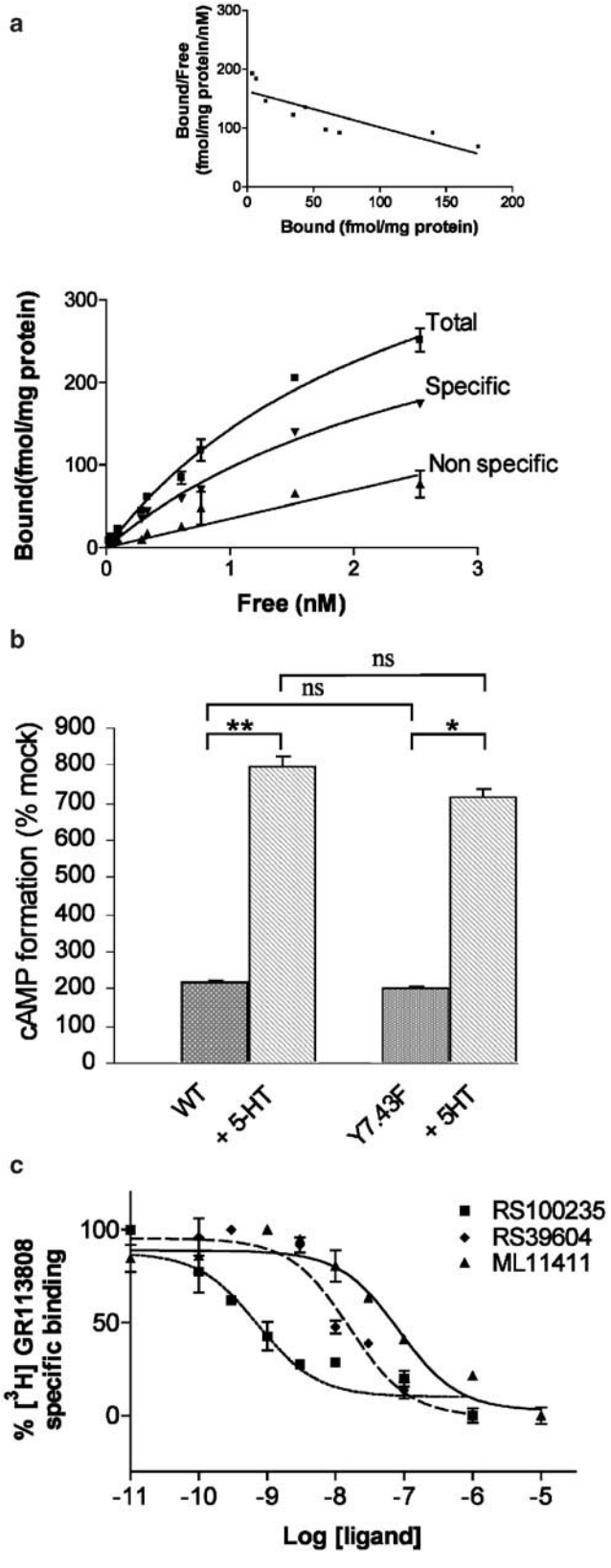


Figure 5 Y7.43F mutation involved a lowest binding of the h5-HT$_4$ receptor antagonist GR113808 but a normal activity. (a) Saturation analysis of [^{3}H]GR113808 binding to COS-7 cells transfected with the Y7.43F h5-HT$_{4(g)}$ receptor mutant. Data are the means ± s.e.m. of six experiments performed in triplicate. (b) cAMP accumulation in the absence or presence of 5-HT in COS-7 cells expressing the WT or mutant receptors. Levels of cAMP accumulation are expressed as a percentage of cAMP production measured in mock-transfected COS-7 cells. Each value represents the mean ± s.e.m. of three independent experiments performed in triplicate. $^{**}P<0.01$; $^{*}P<0.05$ *versus* indicated values; ns, nonsignificant. (c) Competition of RS100235, RS39604 and ML11411 for [^{3}H]GR113808 binding to the Y7.43F h5-HT$_{4(g)}$ receptors. The assays were carried out on membranes derived from COS-7 cells and in the presence of 0.2 nM of [^{3}H]GR113808. Results are expressed as a percentage of the specific binding in the absence of competing ligand. Data are the means ± s.e.m. of three experiments performed in triplicate.

Y7.43 rather than N7.45 more embedded in the receptor, as suggested by a molecular model previously described in literature (Lopez-Rodriguez *et al*., 2001).

Although ML11411 affinity was not affected by Y7.43F mutation, it is difficult to conclude to te absence of the hydrogen bond predicted by the model. Indeed, the middle-range affinity of this compound has been shown to result probably from steric and/or charge interactions with R3.28, which could mask the positive contribution of a hydrogen bond to the affinity of ML11411. Taken together, these results showed that Y7.43, pointed out by our model, should represent a putative anchor point in the hydrophobic pocket for bulky ligands possessing a polar sulfonamido chain.

In conclusion, this exploration of the hydrophobic pocket of the h5-HT$_4$ receptor by molecular modeling and site-directed mutagenesis confirms the large volume of the pocket and the main location of hydrophobic residues such as L and V. These residues create a hydrophobic environment strengthening the interactions with ligands possessing voluminous hydrophobic substituents on their basic amino part. Moreover, the proposed theoretical molecular model allows us to suggest new hypotheses on the origin of the high affinity of h5-HT$_4$ receptor ligands possessing a polar substituent on their basic amino part. The model points out two polar amino acids, R3.28 and Y7.43, for their potential interaction with voluminous ligands and for their localization in the binding crevice. Thus, residue R3.28, found only in the h5-HT$_4$ receptor, seems necessary for the constitutive activity of the receptor but not essential for its activation. Moreover, this residue could create a strain at one end of the binding crevice. Finally, R3.28 and Y7.43 could both be involved in hydrogen bonds with ligands possessing a polar extremity and more particularly a sulfonamido chain on the basic amino part.

Taken together, the obtained results provide a new important step toward the complete delineation of the h5-HT$_4$ receptor binding site identifying amino acids of the large hydrophobic pocket as putative specific anchor points of several bulky ligands depending on their structural properties.

Furthermore, our experimental results obtained with mutated receptors may validate the proposed new three-dimensional model of the h5-HT$_4$ receptor. Further calculations in lipidic bilayer are in progress in order to study the behavior of the receptor model in a more realistic environment.

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