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Effect of mutation of Phe $243^{6.44}$ of the histamine H_2 receptor on cimetidine and ranitidine mechanism of action

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ABSTRACT

Despite the pivotal role GPCRs play in cellular signaling, it is only in the recent years that structural biology has begun to elucidate how GPCRs function and to provide a platform for structure-based drug design. It is postulated that GPCR activation involves the movement of transmembrane helices. The finding that many residues, which have been shown to be critical for receptor activation and are highly conserved among different GPCRs, are clustered in particular positions of transmembrane helices suggests that activation of GPCRs may involve common molecular mechanisms. In particular, phenylalanine 6.44, located in the upper half of TMVI, is highly conserved among almost all GPCRs. We generated Phe $243^{6.44}$ Ala/Ser mutants of histamine H_2 receptor and found that while the substitutions do not affect receptor expression or ligand signaling, are able to specifically alter cimetidine and ranitidine mechanisms of action from simply inactivating the receptor to produce a ligand-induced G-protein sequestering conformation, that interferes with the signaling of β2-adrenoceptor. Taking advantage of the cubic ternary complex model, and mathematically modeling our results, we hypothesize that this alteration in ligand mechanism of action is consequence of a change in ligand-induced conformational rearrangement of receptor and its effect on G-protein coupling. Our results show that receptor point mutations can not only alter receptor behavior, as shown for activating/inactivating mutations, but also can have more subtle effects changing ligand mechanism of action.

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1. Introduction

Early formulations of receptor theory present a parsimonious model consisting of a single quiescent receptor that changes its conformation when activated by agonists. Within this framework, receptors were thought as adopting only two states, active and inactive, with agonists producing a response through the selection and enrichment of the natural receptor active state. Substantial experimental evidence now exists that show that G protein-coupled receptors (GPCRs) can adopt a collection of multiple conformations, that could be responsible for multiple receptor behaviors [1]. According to this view, the whole concept of ligand efficacy must be readdressed, considering that there are several non-sequential effects triggered by the receptor that can be called "receptor response". Receptor activation or inactivation of various signaling pathways, receptor phosphorylation, recruitment of different intracellular partners, or receptor internalization can all be thought as responses that can be independently initiated or terminated by each ligand-receptor pair [2]. This phenomenon came to be referred to as ligand bias of the signal or ligand functional selectivity [3].

It is acknowledged that the multiple receptor conformations responsible for the different responses are spontaneously adopted and then selected by the ligand [4]. GPCR ligands are classified according to their ability to induce a response in agonists, when selecting the active receptor conformation, antagonists, if they have no preference for any specific conformation, and inverse agonists, when selecting the inactive receptor species inactivating spontaneous receptor activity.

We and others described that some but not all inverse agonists can exert their effects stabilizing a spontaneous species of the receptor that is able to bind to an inactive form of the G-protein,

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making it unavailable to other receptor molecules. In this way, the inactivated receptor interferes with the signaling of other unrelated GPCRs aiming to signal through the same G-protein [5–9]. Remarkably, this behavior proved to be specific for the ligand/receptor pair considered.

Despite the pivotal role GPCRs play in cellular signaling, it is only in the recent years that structural biology has begun to elucidate how GPCRs function and to provide a platform for structure-based drug design [10]. It is postulated that GPCR activation involves the movement of transmembrane helices. Particularly, a primary movement of TMVI was identified for rhodopsin and β2-adrenoceptor [11–13]. Indeed, the finding that many residues, which have been shown to be critical for receptor activation and are highly conserved among different GPCRs, are clustered in this transmembrane helix suggests that activation of GPCRs may involve common molecular mechanisms. In particular, phenylalanine 6.44, located in the upper half of TMVI, is highly conserved among almost all GPCRs (from aminergic to peptide or lipid binding receptors) and its interactions with the hydrophobic isoleucine residue 3.40 are present in almost all receptors studied, belonging to a described consensus scaffold of non-covalent contacts in GPCRs [10,12]. Previous site-directed mutagenesis studies indicate that this residue, also plays a very relevant role on GPCR activation [14,15]. Moreover, recent crystallographic studies point to Phe $282^{6.44}$ in the β2-Adrenergic receptor being a key residue whose interactions stabilize the relative position of TMVI, which in turn is rotated around this residue swinging the helix outward when the receptor adopts its agonist stabilized active conformation [8]. These observations suggest that this residue plays a critical role in defining ligand mechanism of receptor activation.

Histamine H_2 receptors have the regulatory functions of histamine during cell proliferation, gastric acid secretion, airway and vascular smooth muscle relaxation and immune responses [16]. Among years, histamine ligands prove to be of clinical utility and are among the top marketed drugs around the world. H2R inverse agonists cimetidine, ranitidine, and famotidine are classic treatments for patients presenting gastric or duodenal ulcers, dyspepsia, or gastroesophageal reflux disease [17,18]. For this work, we generated Phe $243^{6.44}$ Ala/Ser mutant of histamine H_2 receptor in order to study the effects of the loss of its hydrophobic interaction in ligand efficacy and mechanism of action. We found that while the point mutation does not affect receptor expression or agonist signaling, is able to specifically alter the mechanisms of action of clinically relevant H_2 inverse agonists cimetidine and ranitidine, from simply inactivating the receptor to produce a cimetidine-induced G-protein sequestering conformation that interferes with the signaling of β2-adrenoceptor. Taking advantage of the cubic ternary complex model, and mathematically modeling our results, we can hypothesize that this alteration in ligand mechanism of action is a consequence of a change in the receptor conformational ensemble induced by the ligand and its effect on G-protein coupling and activation.

Our results show that receptor point mutations can not only alter receptor behavior, as shown for activating/inactivating mutations, but also can have more subtle effects changing ligand mechanism of action.

2. Materials and methods

2.1. Materials

DMEM medium, antibiotics, phosphate-buffered saline (PBS), amthamine and bovine serum albumin (BSA) were obtained from Sigma. Tiotidine, cimetidine, famotidine and ranitidine were from Tocris Bioscience (UK). Fetal bovine serum (FBS) was purchased from Natocor (Córdoba, Argentina). All other chemicals were of analytical grade and obtained from standard sources. [^{3}H]cAMP and [^{3}H]tiotidine were purchased from PerkinElmer Life Sciences (Boston, MA, USA).

2.2. Plasmid constructions

pCEFL-H_2 receptor was previously generated in our laboratory [19]. Mutants were generated using the QuickChange XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions. In brief, mutations are generated by PCR using a pair of oligonucleotide primers designed with mismatching nucleotides at the center of the primers. The primers used were 5′-cgccgtcatgggggc**tag**catcatctgct ggtttcc-3′ for the replacement of phenylalanine 243 by serine and 5′-cgccgtcatgggggc**cg**ccatcatctgctggtttcc-3′ for the replacement of phenylalanine 243 by alanine. The mismatching nucleotides are underlined. All constructions were sequenced to confirm the expected base change.

2.3. Cell culture and transfection

HEK 293T (human embryonic kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 5Our results show that receptor point mutations μg/ml gentamicin and cells were incubated at 37 °C in humidified atmosphere containing 5% CO_2. HEK 293T cells seeded on 24-well plates were transfected using the K2 Transfection System (Biontex, Munich, Germany) with pcDNA3-GαS, pCEFL-H_2R wild type or derived mutant plasmids according to the manufacturer's instructions. After 4h, cells were re-seeded in accordance with the assay to be performed.

2.4. Binding experiments

Triplicate assays were performed in 50 mM Tris-HCl pH 7.4. For saturation studies, 10^4 HEK 293T transfected cells/well of a 48-well cluster plate were incubated for 40 min at 4 °C with increasing concentrations of [^{3}H]-tiotidine, ranging from 0.4 up to 240 nM in the absence or in the presence of 1 μM unlabeled tiotidine. For competition binding experiments, cells were incubated with [^{3}H]-Tiotidine (20 nM) in the presence of different concentrations of competitors in a final volume of 50 μl for 40 min at 4 °C. The incubation was stopped by dilution with 1 ml of ice-cold 50 mM Tris-HCl pH 7.4. After three washes with 1 ml ice-cold buffer the bound fraction was collected in 200 μl of ethanol. Experiments with intact cells were performed at 4 °C to avoid ligand internalization.

2.5. cAMP response experiments

For cAMP determination assays, cells were plated in 48-well cluster plates and incubated 3 min in basal culture medium supplemented with 1 mM IBMX at 37°C, followed by 9 min exposure to different ligand concentration. For concentration-response experiments, doses ranging between 10 pM and 10 μM were used. When interference on β2-Aderenrgic receptor signaling was studied, histaminergic ligands were co-incubated with isoproterenol 10 μM. The reaction was stopped by ethanol addition followed by centrifugation at 2000*g* for 5 min. The ethanol phase was then dried and the residue resuspended in 50 mM Tris-HCl pH 7.4, 0.1% BSA. cAMP content was determined by competition of [^{3}H]-cAMP for PKA, as previously described [20].

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2.6. Comparative homology modeling, molecular dynamics and residues interaction analysis

Human histamine H_2 receptor model was constructed using SWISS-MODEL, an automated protein structure homology-modeling server [21]. The model was built by contrasting the target sequence with the server template library. After selecting the most suitable templates, the corresponding all-atom models for the target sequence were generated using ProMod-II. Finally, the best quality model was selected by the scoring function QMEAN. Molecular dynamic simulations were carried out based on the homology model using CHARMM 22 force field executed on NAMD 2.6 software. Receptor structure was initially optimized through 5000 steps restraining the atoms of the backbone and subsequent additional 5000 steps unrestricted. This final structure was employed to prepare the mutant structures keeping the atom coordinates of the last minimization step. Then, the three structures were thermalized to 300 K escalating 10 K/100 ps using 1 fs steps. Once the temperature was reached, a harmonic potential was applied to the backbone with a 100 force constant that was gradually diminished at 10 /ns rate until completely removal. Finally, 30 ns molecular dynamics were run employing implicit solvent and Langevin thermostat. Data analysis were performed with Visual Molecular Dynamics (VMD) 1.9.1. Structure editing and non-bond interactions were performed using Discovery Studio Visualizer (Version 4.5.0) (Table 1). Molecular graphics were carried out with the UCSF Chimera package, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) [22].

2.7. Statistical analysis

Binding data and sigmoidal dose–response fittings were performed with GraphPad Prism 6.00 for Windows, GraphPad Software (San Diego, CA, USA). Specific binding was calculated by subtraction of nonspecific binding from total binding. One-way analysis of variance with Dunnett's post test was performed using InStat version 3.01 (GraphPad Software, San Diego, CA). One-way analysis of variance with Tukey's post test was performed using InStat version 3.01 (GraphPad Software, San Diego, CA).

3. Results

3.1. Homology modeling and molecular dynamics of H_2 receptor wild type and F243 mutants

G-protein coupled receptors share common structural patterns. Besides their familiar seven-helix transmembrane bundle, there are several highly-conserved motifs or even individual residues. Position 6.44 is occupied by phenylalanine in more than 80% of the overall human Class A Rhodopsin like receptors including more than 97% of amine receptors, and about 81% and 84% of peptide and nucleotide receptors according to GMOS web interface, specifically corresponding to Phe 243 in the histamine H_2 receptor (Fig. 1A and B). The fact that a residue is highly conserved among different GPCR families or species orthologs indicates that a different aminoacid would be not well tolerated by the protein or its function, highlighting the importance of the nature of the residue that occupies that position in the structure or the functionality of the protein. Of the possible templates available in the SWISS-MODEL library, β2-adrenoceptor (PDB 2RH1) predicted the best quality model for the histamine H_2 receptor (QMEAN-3,27). The homology molecular modeling and subsequent molecular dynamics show that F243$^{6.44}$ is deeply buried in TMVI, pointing to the inner portion of the seven-helix bundle and facing TM2 and 3 (Fig. 1C and D). Due to its hydrophobic nature, F243 establishes several interactions with various residues, mostly of TM3, playing a role in the stabilization of these two helices (Leu 60$^{2.46}$; Ile 106$^{3.40}$; Leu 109$^{3.43}$; Trp 247$^{6.48}$). Molecular dynamics show that when F243 is mutated, there is a rearrangement of the nearby residues, but some positions are conserved, indicating that general structure is not massively disturbed (Fig. 2A and B). As shown in Fig. 2C and D, the average positions and the distances between Leu 60$^{2.46}$, Leu 109$^{3.43}$ and Leu 283$^{7.48}$ are conserved. In contrast, the position and interaction of specific residues are largely affected. While in mutated variants Val 239$^{6.40}$ points to the center of the seven-helix bundle, in the wild type receptor its position results favorable when rotates to face the cell membrane. This is reflected in a change in the average distance to Leu 60$^{2.46}$ (Fig. 2E), which is decreased when F243 is mutated.

When mutated by alanine (ΔF243A), molecular dynamics show, as expected, that all its interactions are lost, and when replaced with serine (ΔF243S) the hydrophobic interactions disappear leading to the formation of a hydrogen bond between the OH group of the serine and the Val 239$^{6.40}$ but not with Trp 247$^{6.48}$ (Fig. 2F and G). The residues that interact with F243 establish new interactions when the aromatic residue is missing. The position of Trp 247$^{6.48}$ varies but is not largely affected, because it replaces the interactions that establishes in the wild type protein (hydrogen bond with Leu 276$^{7.41}$ and hydrophobic interaction with F243$^{6.44}$, as shown in Fig. 2H and I) making a new hydrogen bond with the oxygen of the backbone of the Ser 105$^{3.39}$ that is more stable in the ΔF243A mutant than in the ΔF243S (Fig. 2J). The criteria to establish the interactions are summarized in Table 1, and all the interactions described above are listed in Table 2. While the accurate conformation and the functional consequences of mutating F243 cannot be explicitly predicted, the results obtained from the molecular dynamics assay indicate that while the protein conformation is widely conserved, there are some specific significant changes that can account for differences in receptor behavior. In the following sections we attempt to experimentally determine the functional consequences of F243 substitution on H_2 receptor pharmacology.

Table 1
Residues interaction criteria.

Interaction type	Maximum distance (Å)	Min. Angle Criteria (°)	Max. Angle Criteria (°)
Hydrogen Bond	3.4	90	180
Pi-Alkyl	4.2	–	45
Pi-Pi	6.0 (centroid); 4.5 (closest atom)	–	50 (θ); 35 (γ)
Pi-Sigma	7.0	–	45

3.2. Ligand binding and signaling profiles of H_2 receptor wild type and F243 mutants

When ΔF243A or ΔF243S mutant receptors are expressed in HEK 293T cells, displayed a [^{3}H]-tiotidine binding profile similar to wild type protein. Saturation assays were performed in intact cells, showing that wild type as well as mutant constructions are correctly expressed in the cell membrane and keep the expected binding profile (Fig. 3 and Table 3). Binding competition experiments show that except for ranitidine, the affinity of histaminergic ligands was unchanged when F243 was mutated (Table 3). Ranitidine significantly diminishes its affinity for mutant receptor variants, although this has no impact in ligand's potency (Fig. 4D).

Similarly, when expressed at comparable levels, the signaling properties of the three constructions are essentially equal. Regarding cAMP production, amthamine and all four inverse agonists tested conserved their ligand profiles (as agonist and inverse

(A)

Receptor		TMVI													
H2 histamine	237	A	A	V	M	G	A	**F**	I	I	C	W	F	P	249
H1 histamine	418	G	F	I	M	A	A	**F**	I	L	C	W	I	P	430
5-HT4 serotonine	262	C	I	I	M	G	C	**F**	C	L	C	W	A	P	274
β2 adrenoceptor	275	G	I	I	M	G	T	**F**	T	L	C	W	L	P	287
M1 muscarinic	369	S	A	I	L	L	A	**F**	I	L	T	W	T	P	381
D1 dopaminergic	275	S	V	I	M	G	V	**F**	V	C	C	W	L	P	287
α1b adrenergic	297	G	I	V	V	G	M	**F**	I	L	C	W	L	P	309
A1 adenosine	237	A	L	I	L	F	L	**F**	A	L	S	W	L	P	249
MT1 melatonin	241	F	V	V	F	V	L	**F**	A	I	C	W	A	P	253
Rhodopsin	254	I	I	M	V	I	A	**F**	L	I	C	W	V	P	266
H3 histamine	281	A	V	I	V	S	I	**F**	G	L	C	W	A	P	293
H4 histamine	273	A	S	K	M	G	S	**F**	A	V	C	W	A	P	285
P2Y purinergic	248	A	V	V	L	A	V	**F**	A	L	C	F	L	P	260

(B)

Organism		TMVI													
Homo sapiens	237	A	A	V	M	G	A	**F**	I	I	C	W	F	P	249
Gorilla gorilla	237	A	A	V	M	G	A	**F**	I	I	C	W	F	P	249
Macaca mulatta	237	A	A	V	M	G	A	**F**	I	I	C	W	F	P	249
Pan troglodytes	237	A	A	V	M	G	A	**F**	I	I	C	W	F	P	249
Heterocephalus glaber	237	A	A	V	M	G	A	**F**	I	I	C	W	F	P	249
Pongo pygmaeus	237	A	A	V	M	G	A	**F**	I	I	C	W	F	P	249
Cavia porcellus	237	A	A	V	M	G	A	**F**	I	I	C	W	F	P	249
Mus musculus	236	A	A	V	M	G	A	**F**	I	V	C	W	F	P	248
Rattus norvegicus	236	A	A	V	M	G	A	**F**	I	I	C	W	F	P	248
Bos Taurus	237	A	A	V	M	G	A	**F**	I	V	C	W	L	P	249
Cricetulus griseus	236	A	A	V	M	G	A	**F**	V	I	C	W	F	P	248
Danio rerio	274	A	V	V	L	G	A	**F**	V	V	C	W	F	P	286

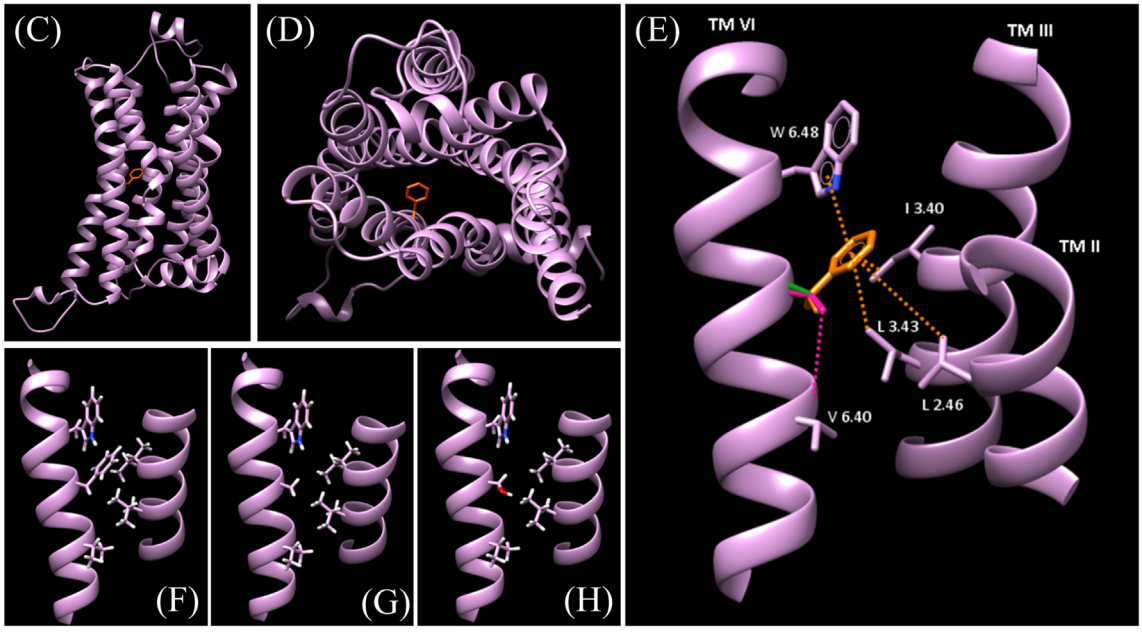

Fig. 1. Sequence alignment of the histamine H_2 receptor against the sequences of several GPCRs of human origin (A) or against receptor orthologs from different species (B). A fragment of the predicted transmembrane helix VI is shown where Phe 6.44 is bolded. Homology modeling of the histamine H_2 receptor. Lateral (C) and upper (D) views of the structure obtained, highlighting the phenylalanine 243 residue. Panel (E) shows the interactions of the wild type residue (orange) and the interactions when position 6.44 is occupied by an alanine (green) or a serine (pink), as summarized in Table 2. A detail of the positions of the lateral chains of phenylalanine, alanine, and serine are shown in panels (F) to (H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

agonists) when acting on receptor mutants, although for the case of amthamine there is a loss in its potency when phenylalanine is mutated (Fig. 4A-E and Table 4).

As a conclusion, at least concerning ligand binding and second messenger production, ΔF243A and ΔF243S mutations do not entail any substantial differences despite the observed changes in aminoacid interactions predicted with the homology modeling and molecular dynamic studies. These observations suggest that the molecular rearrangements induced by phenylalanine mutation are not crucial neither for the binding of the ligands studied nor for receptor signaling. However, since the efficacy of a ligand transcends second messenger production, we aimed to study alternative receptor behaviors.

3.3. Interference efficacy of H_2 receptor inverse agonists

As stated above, we and others observed that certain ligands stabilize a receptor conformation able to couple to the G-protein in an inactive conformation [5–9]. Consequently, signaling of other unrelated receptors that couple to the same G-protein is dampened due to a G-protein kidnapping. This interference in receptor signaling can be envisaged as a ligand-promoted alteration in receptor behavior and then as ligand efficacy, which becomes evident when measuring the ability of other Gαs coupled receptor to signal in presence of the H2 ligand [5,7]. We tested this phenomenon stimulating the cells with isoproterenol in the absence or presence of different concentrations of H_2 receptor inverse agonists. When acting on wild type H2 receptor tiotidine and famotidine, interferes with isoproterenol-induced β2-adrenoceptor cAMP response diminishing it about a 30% from 82 ± 9 to 53 ± 6 and to 60 ± 7 pmol respectively (Fig. 5B and D). On the other hand, cimetidine and ranitidine increase isoproterenol response, to 147 ± 7 and to 126 ± 4 pmol respectively, suggesting that the ligand augments G protein availability (Fig. 5A and C). While tiotidine and famotidine behavior is preserved when acting on the mutated receptors, cimetidine and famotidine effect on isoproterenol-induced cAMP response is strikingly affected. The efficacy of the last two ligands to modulate the β2-adrenoceptor response is altered in such a way that, the potentiation can be completely abolished, or even though, when cimetidine acts on ΔF243A mutant, behaves as tiotidine or famotidine, dampening β2-adrenoceptor cAMP response (Fig. 5A and B). In our hands, F243 seems to be crucial for cimetidine and ranitidine mechanism of action.

It is worth noting that the interference on β2-adrenoceptor cAMP response by H_2 inverse agonists can be due to the differential interaction with G-proteins or β-arrestin, or even alterations in downstream signaling that interact with β2-adrenoceptor. However, the fact that the dampening on isoproterenol-induced cAMP

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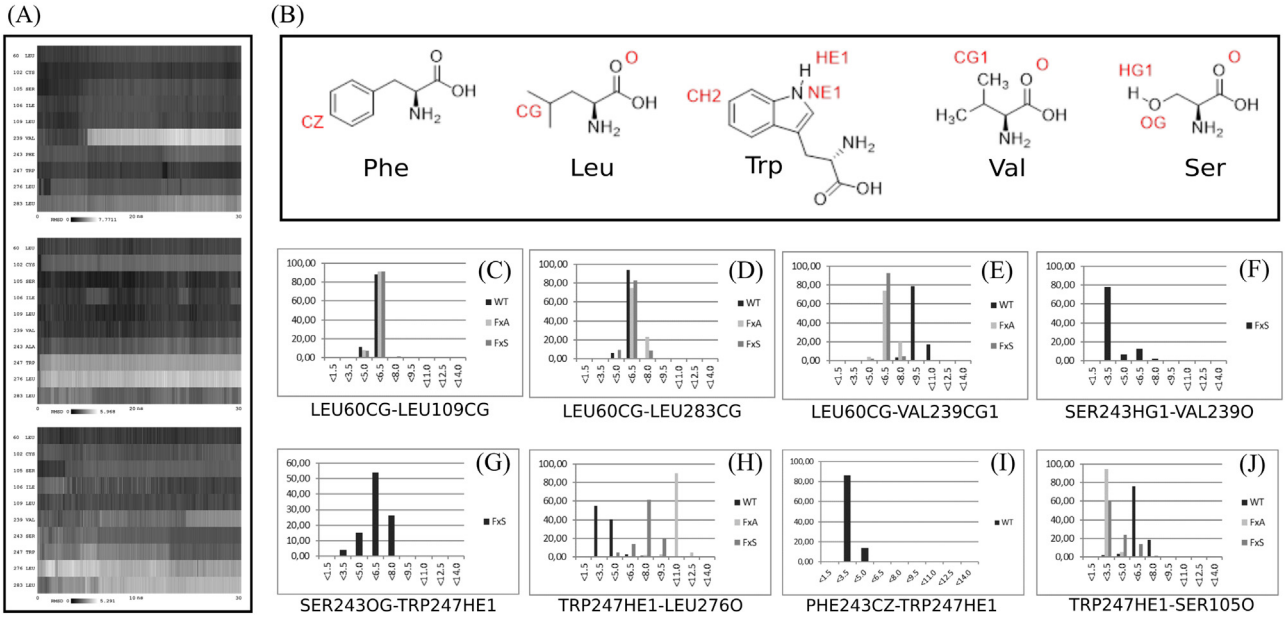

Fig. 2. Results obtained in the 30-ns molecular dynamic assay. (A) Representation of the root mean square deviation (RMSD) of the atomic positions of the specified residues. The scale indicates the deviation from the initial position determined in the number 1 frame. The upper panel represents data for wild type protein, middle panel for ΔF243A and bottom panel for ΔF243S. (B) Schematic representation of aminoacids to indicate the nomenclature used for the different atoms. (C)-(J) Frequency histograms of the measured distances between the specified atoms during the 30 ns.

Table 2
Interactions of 6.44 residue with neighboring aminoacids.

WT H_2 receptor		
Aminoacid	Type of interaction	
L $60^{2.46}$	Hydrophobic	Pi-sigma
I $106^{3.40}$	Hydrophobic	Pi-alkyl
L $109^{3.43}$	Hydrophobic	Pi-sigma
W $247^{6.48}$	Hydrophobic	Pi-Pi stacked
ΔF243A H_2 receptor		
Aminoacid	Type of interaction	
None	–	–
ΔF243S H_2 receptor		
Aminoacid	Type of interaction	
V $239^{6.40}$	Hydrophilic	Hydrogen bond

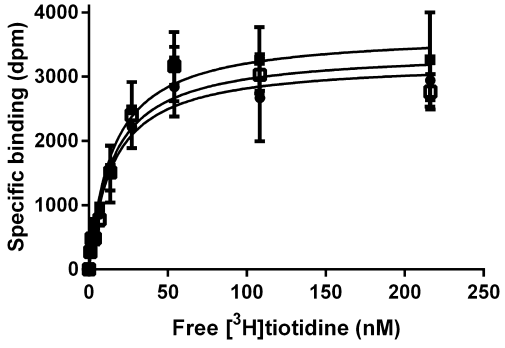

Fig. 3. [^{3}H]-tiotidine saturation binding assay in HEK 293T cells transfected with pCEFL-H_2R wild type (□); pCEFL-H_2R ΔF243A (■); or pCEFL-H_2R ΔF243S (●). Results are expressed as the mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. Results of nonlinear curve fitting are detailed in Table 3.

levels is reduced or abolished when Gαs is overexpressed, strengthen the hypothesis of G-protein kidnapping as the main cause of the observed effect (Fig. 5E).

Considering the G-protein kidnapping effect and employing the CTC model, we were able to simulate dose-response curves using

Table 3
Binding parameters for the wild type H_2 receptor and its variants. Values are the best fitted parameters and expressed as the means ± SD of at least three independent experiments performed in triplicate. Significant differences between treatments were taken at $p < 0.05$ (* corresponds to $p < 0.05$).

	WT H_2 receptor	ΔF243A	ΔF243S
Kd (nM)	14.6 ± 1.6	15.2 ± 1.6	14.8 ± 1.6
Bmax (%)	100 ± 5.7	109 ± 5.2	95 ± 5.4
Amthamine pKi	5.1 ± 0.4	6.0 ± 0.2	5.7 ± 0.4
Cimetidine pKi	6.5 ± 0.1	5.9 ± 0.3	6.5 ± 0.3
Famotidine pKi	6.8 ± 0.1	6.3 ± 0.3	6.8 ± 0.4
Ranitidine pKi	6.9 ± 0.2	5.8 ± 0.3*	5.8 ± 0.4*
Tiotidine pKi	7.3 ± 0.1	6.9 ± 0.2	6.7 ± 0.2

different sets of parameters in order to conclude about ligand behavior. The utility of this model was proved in previous works, where we could successfully predict the mechanism of action of histamine inverse agonists and its significance on receptor signaling.

In this case, we focused on the change in the mechanism of action of cimetidine due to H_2 receptor mutation.

3.4. The cubic ternary complex receptor-occupancy model can explain the inverse agonist behavior

In previous works, we used the CTC model to explain some otherwise unexplainable ligand features. This model is unique since it permits the receptor to bind to G-protein in an inactive form, allowing the formation of a ternary complex (ligand-receptor-G protein). This ternary complex can sequester the G-protein, making it unavailable for other GPCRs and thus interfering in cell signaling. Briefly, the CTC model depicted in Fig. 6A is described by three equilibrium constants (the equilibrium constant between free ligand and ligand bound to receptor represented by *Ka*, the equilibrium constant between free inactive receptor and coupled to G-protein designated as *Kg*, and the equilibrium constant for the transition between the active and inactive

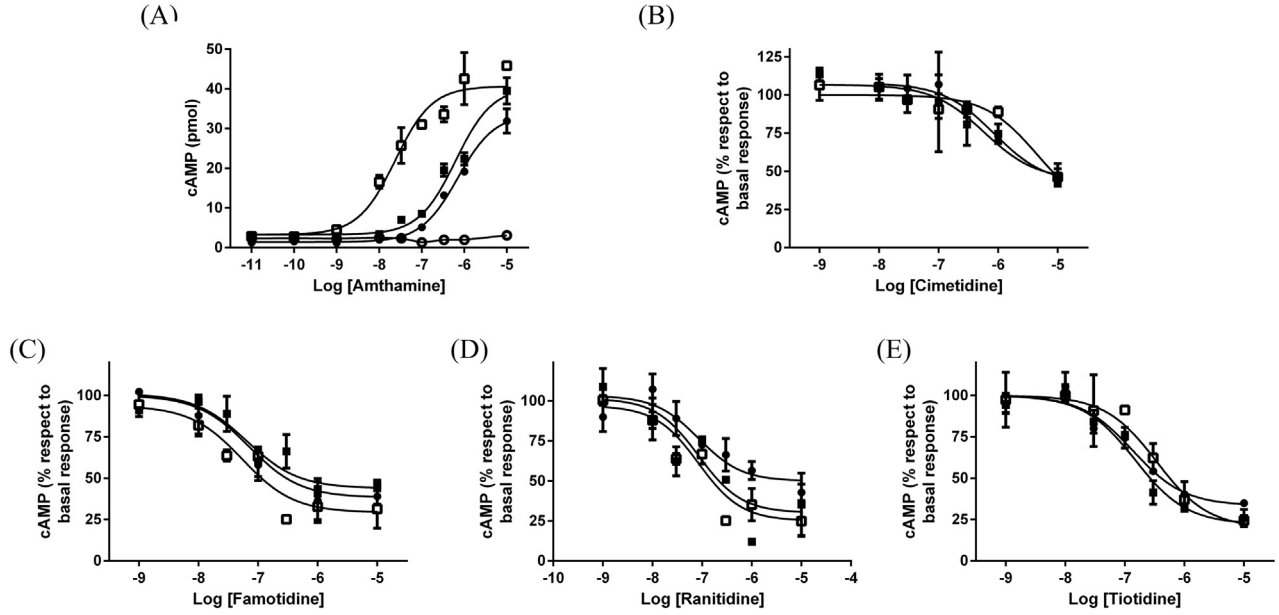

Fig. 4. Concentration-response curves in HEK 293T cells transfected with pCEFL-H_2R wild type (□); pCEFL-H_2R ΔF243A (■); pCEFL-H_2R ΔF243S (●) or mock (○). Cells were treated with increasing concentrations of amthamine (A), cimetidine (B), famotidine (C), ranitidine (D) or tiotidine (E). Results are expressed as the mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. Results of nonlinear curve fitting are detailed in Table 4.

forms of the receptor denoted by *Kact*). These three basic equilibriums are modified by four different factors. α, indicating the effect of ligand binding on receptor activation; β, accounting for the effect of G-protein binding on receptor activation; γ, quantifying the effect of ligand binding on G-protein coupling; and δ, describing the extent to which the joint effect of any two of receptor activation, G-protein coupling, or ligand binding, varies conditional of the level of the third. A full description of the model and its parameters can be found in [23–25].

According to the CTC model, if we consider that the species able to generate physiological responses are R*G and LR*G, we can define a normalized factor called f^* as the addition of those species over the total receptor amount:

$$f^* = \frac{[R^*G] + [LR^*G]}{[R] + [R^*] + [RG] + [R^*G] + [LR] + [LR^*] + [LRG] + [LR^*G]} \quad (1)$$

Assuming a proportionality between f^* and cell response, a graph depicting f^* as function of ligand concentration represents a concentration–response curve.

Table 4
Ligand potency parameters for the wild type H_2 receptor and its variants. Values are the best fitted parameters of the curves represented in Fig. 4 and expressed as the means ± SD of at least four independent experiments performed in triplicate. Significant differences between treatments were taken at $p < .05$ (* corresponds to $p < .05$).

pEC50	WT H_2 receptor	ΔF243A	ΔF243S
Amthamine	7.6 ± 0.1	6.2 ± 0.1*	6.1 ± 0.1*
Cimetidine	5.4 ± 0.4	6.2 ± 0.3	6.1 ± 0.2
Famotidine	7.3 ± 0.2	7.2 ± 0.2	7.2 ± 0.3
Ranitidine	7.1 ± 0.2	7.1 ± 0.2	6.9 ± 0.3
Tiotidine	6.4 ± 0.2	6.8 ± 0.1	6.9 ± 0.2

The representation of the amount of these sequestering species as function of ligand concentration shows the ability of a ligand to interfere in the signaling of other GPCRs attempting to signal through the same pathway.

$$f^* = \frac{\beta \cdot Kact \cdot Kg[G] + \alpha\beta\gamma\delta \cdot Ka \cdot Kact \cdot Kg[G][L]}{1 + Kact + Kg[G] + \beta \cdot Kg \cdot Kact[G] + (Ka + \alpha \cdot Ka \cdot Kact + \gamma \cdot Ka \cdot Kg[G] + \alpha\beta\gamma\delta \cdot Ka \cdot Kact \cdot Kg[G]) \cdot [L]} \quad (2)$$

$$f = \frac{Kg[G] + \gamma \cdot Ka \cdot Kg[G][L]}{1 + Kact + Kg[G] + \beta \cdot Kg \cdot Kact[G] + (Ka + \alpha \cdot Ka \cdot Kact + \gamma \cdot Ka \cdot Kg[G] + \alpha\beta\gamma\delta \cdot Ka \cdot Kact \cdot Kg[G]) \cdot [L]} \quad (4)$$

On the other hand, the addition of RG and LRG over total receptor amount represents the fractional quantity of receptor species able to sequester G-protein:

$$f = \frac{[RG] + [LRG]}{[R] + [R^*] + [RG] + [R^*G] + [LR] + [LR^*] + [LRG] + [LR^*G]} \quad (3)$$

And the availability of G-protein for other GPCRs can be obtained as

$$G_{available} = G_{total} - f \quad (5)$$

Fig. 5 shows that, for the set of parameters chosen, while a ligand still behaves as an inverse agonist diminishing the amounts

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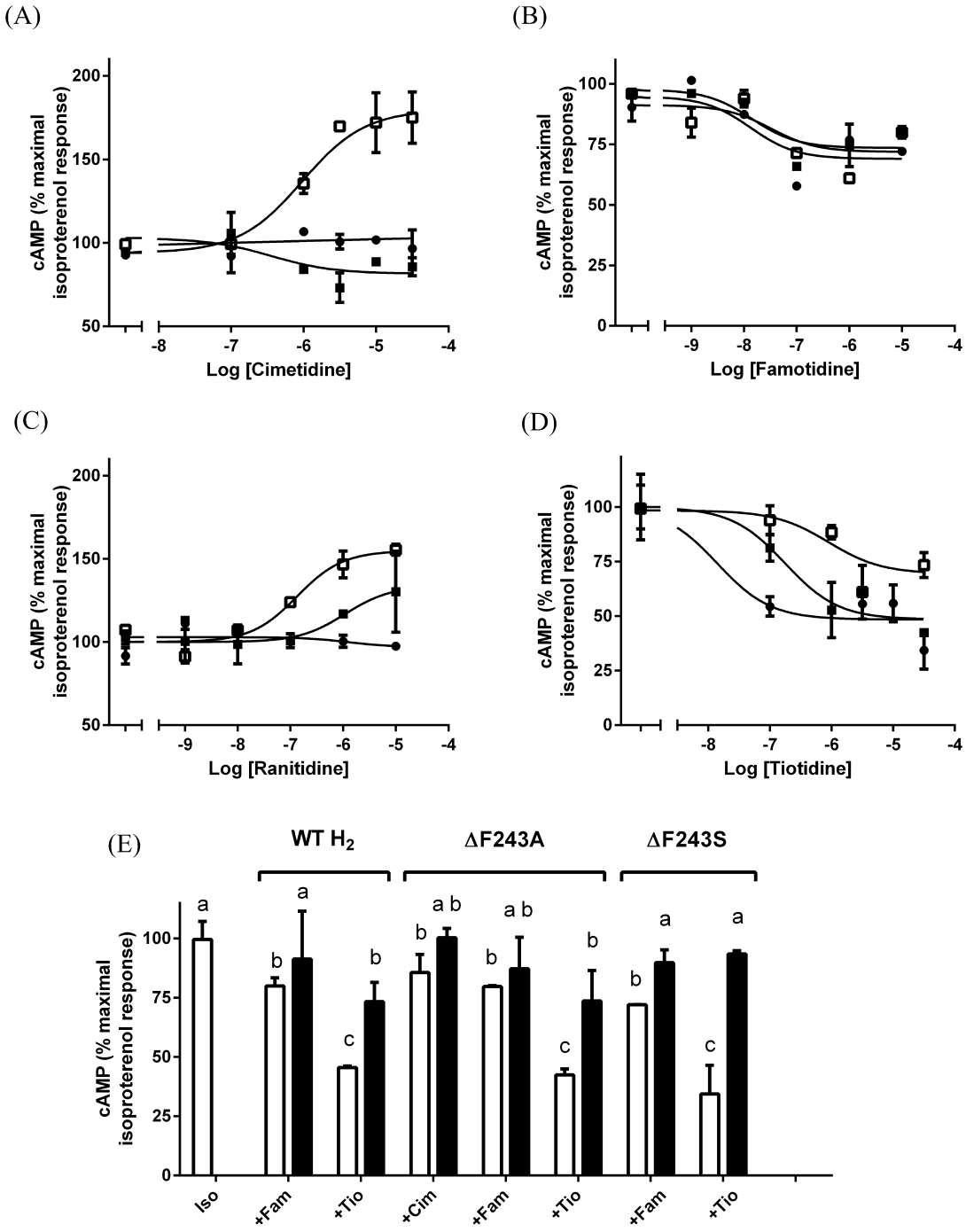

Fig. 5. HEK 293T cells transfected with pCEFL-H_2R wild type (□); pCEFL-H_2R ΔF243A (■); or pCEFL-H_2R ΔF243S (●) were treated with isoproterenol 10 μM and increasing concentrations of cimetidine (A), famotidine (B), ranitidine (C) or tiotidine (D). (E), HEK 293T cells transfected with indicated receptors were co-transfected (filled bars) or not (empty bars) with pcDNA3-GαS and treated with isoproterenol and indicated ligands in a concentration of 10 μM. Results are expressed as the mean ± S.D. of assay triplicates obtained in at least three independent experiments. Different letters indicate statistically significant differences ($p < 0.05$).

of R∗G+LR∗G in a concentration dependent manner (Fig. 6B, C and D), varying γ factor allows to change ligand behavior towards RG+LRG. For γ values < 0.5, G-protein sequestering receptor species RG + LRG dose dependently decrease, augmenting the availability of the G-protein, while for γ > 0.5 RG + LRG increases when ligand concentration is augmented decreasing G-protein availability (Fig. 6E, F and G). This change from ligand-dependent decrease to increase in G-protein sequestering receptor species, augmenting the value of γ factor, can account for the change in cimetidine and ranitidine effects on isoproterenol response when acting on mutant H2 receptor. γ factor, which quantifies the effect of ligand binding on G-protein coupling, depends on the ligand, the receptor and the G-protein. Fig. 6B and E are derived from Fig. 6C and F respectively, simulating a behavior similar to those of tiotidine and famotidine with γ varying from 10 to 1000. On the other hand, Fig. 6D and G simulates the behavior of cimetidine with γ varying from 0.1 to 1. While for tiotidine and famotidine, the change in γ values induces no qualitative changes in efficacy or in the amounts of G-protein availability, the values chosen for cimetidine and ranitidine ($0.1 < \gamma < 1$), cause a change in the

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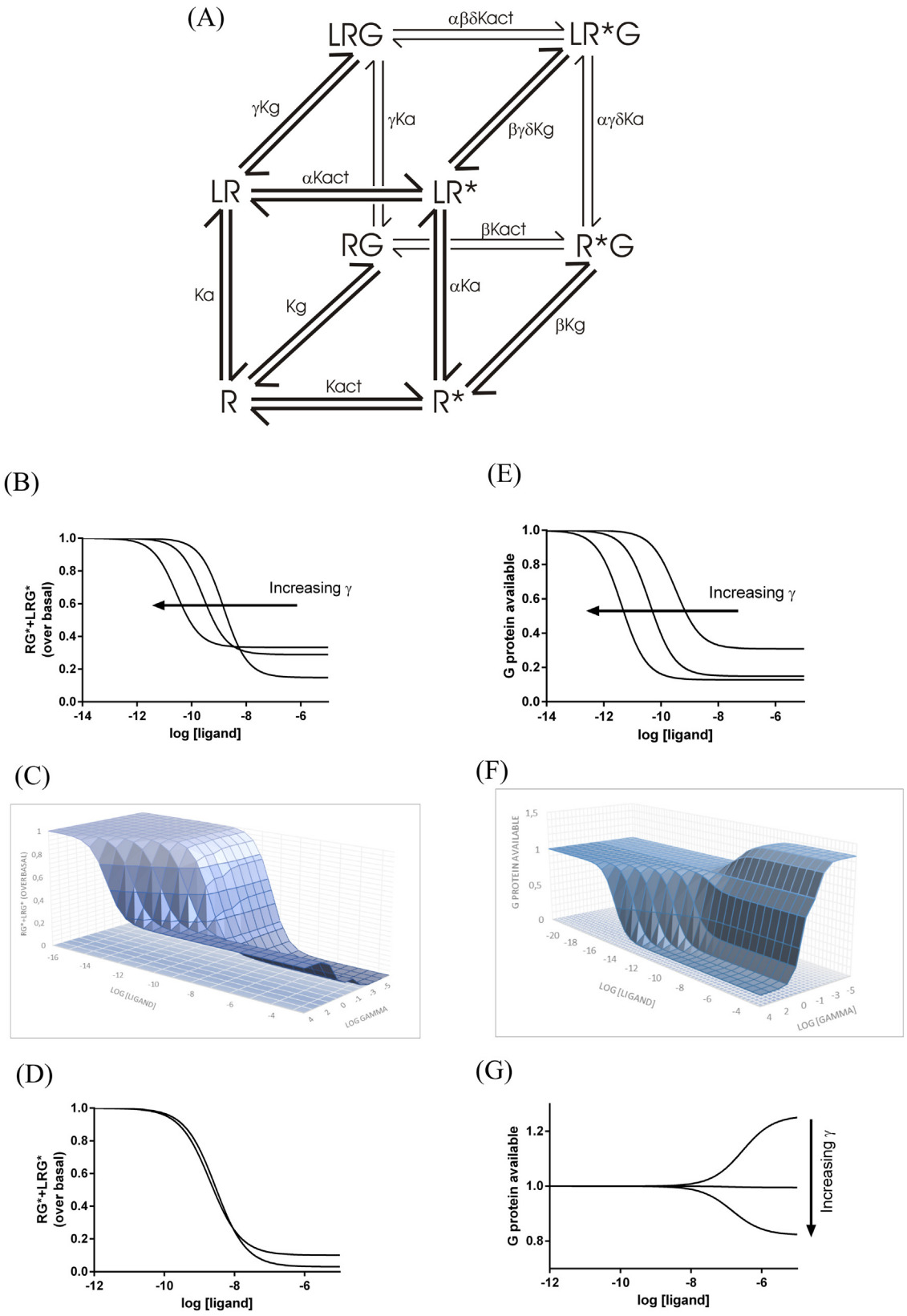

Fig. 6. Schematic representation of the cubic ternary complex model (A). CTC model whereby both inactive and active states of the receptor are allowed to interact with the G-protein, but only R*G γand LR*G mediates response. L represents the ligand; *Ka* and *kg* represent the association constants to ligand and G-protein respectively; *Kact* is the allosteric constant; and α, β, γ the modifiers of the equilibriums once the receptor is active, or G-protein or ligand bound respectively. δ represents the joint effect of any two of receptor activation, G-protein coupling, or the binding of ligand varies conditional on the level of the third. (C) Simulation of dose-response experiments and (F) ligand concentration dependent variation of G-protein availability according to the CTC model. (B) and (D) depict three specific curves taken from (C) for γ values of 10, 100 and 1000; and 0.1, 0.5 and 1 respectively. (E) and (G) depict three curves taken from (F) for the same values of γ. Simulations were based on equations 2 and 5, using values of: α = 0.1; β = 1; δ = 1; Ka = kg = 10^9; Kact = 1; [G]total = 0.5; [R]total = 1.

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behavior towards G-protein, sequestering it for γ values higher than 0.5. It is worth noting that the change in the behaviors of tiotidine and famotidine is not exactly the same. While for tiotidine there is a potentiation in the β2 signaling interference effect, with pIC_{50} varying from 6.0 ± 0.5 for the WT receptor to 6.8 ± 0.4 for the ΔF243A and to 7.9 ± 0.7 for ΔF243S mutants (Fig. 5D); there is no statistically significant change in famotidine pIC50 (7.5 ± 0.7; 7.8 ± 0.7 and 7.8 ± 0.8, respectively; Fig. 5B). Likewise, while cimetidine behavior changes from potentiating β2 signaling when acting on WT receptor, to have a neutral effect or to interfere when binds to ΔF243S or to ΔF243A respectively (Fig. 5A), ranitidine decrease its potentiating effect or changes it to neutral when acting on ΔF243A or on ΔF243S, respectively (Fig. 5C). Since γ factor its both ligand- and receptor-dependent, this differential behavior can be well accommodated choosing a suitable set of γ values for each ligand-receptor pair.

In any case, it can be concluded that F243 receptor mutants have higher γ values, favoring ligand-induced G-protein receptor coupling in an inactive conformation.

4. Discussion

If it is considered that the receptor molecule possesses a network of intramolecular interactions whose modifications can propagate through the macromolecule, a minor modification in the extracellular part of the receptor protein could have a major impact on the intracellular domains and the coupling to G-proteins. The information about GPCRs functionality based on point mutations indicates that what can be considered minor changes in protein structure could have vast consequences on its function. This conclusion cannot be easily explained considering that receptors undergo large conformational changes when switch between states. On the contrary, if receptor states are considered as collections of microconformations (or protein ensembles) that can be rapidly switched and that are responsible for diverse receptor behaviors, it can be simply thought that a slight modification in a protein domain could be conveyed to a distant portion of the macromolecule having functional consequences [26].

In this context, the main finding of the present work is to describe a point mutation able to alter in a subtle manner protein conformation and to modify specifically the mechanism of action of cimetidine and ranitidine without changing their negative efficacy as inverse agonists.

When protein mutation technology became readily accessible for pharmacologists, there was a burst in the research of the effects of point mutations on GPCRs functions. There were found several specific residues responsible for receptor activation or inactivation, mostly conserved among different GPCRs belonging to several families. The finding of constitutively activated mutant receptors (CAM receptors) was the first experimental proof that receptors spontaneously alternate between active and inactive states, giving support to the so-called *two sate models of receptor activation* [27]. Later, there were published several reports showing that receptor mutants were accountable for several pathologies [28].

In the cases described above, receptor mutation implies a major change on ligand efficacy. On the contrary, the results presented herein show that a change in a few intramolecular interactions of the upper portion of the receptor protein can have subtler functional consequences that are not evident when ligand efficacy is evaluated in classical terms.

According to current parsimonious pharmacological ligand-receptor functional models, an inverse agonist can diminish receptor activity favoring its inactive state, uncoupling the receptor from G-protein, or promoting receptor coupling to G-protein but in its inactive form [29,30]. Distinct models account for these potential mechanisms of inverse agonist action from the simple two state model, to the extended ternary complex model and the CTC model.

Our results show that cimetidine and ranitidine act as inverse agonists by one of the two first mechanisms described (favoring receptor inactive state or uncoupling it from the G-protein) when binds to wild type H_2 receptor. As seen in Figs. 4B and D and 5A and C, when cimetidine and ranitidine act on H_2 receptor mutants, although they keep their negative efficacy, their behavior towards β2-adrenoceptor signaling is strikingly altered, indicating a change in the mechanism of action that can be explained by the formation of an inactive receptor-G-protein complex.

The CTC model, as the only model that contemplates this last mechanism of action, was used to simulate the results. Fig. 5 shows that a change in the parameter γ, which describes the modulation of G-protein coupling by ligand binding, does not affect whether a ligand possess negative efficacy or not (always inactivating receptor function behaving as an inverse agonist), but alters the balance between the different inactive receptor species (G-protein bound and unbound). This difference implies a modification in the mechanisms of action of cimetidine and ranitidine, modifying their efficacy regarding β2-adrenoceptor signaling, in such a degree that turns cimetidine into a ligand able to interfere in the isoproterenol-induced signaling of the β2-adrenoceptor.

Our results show that the mutation in position 6.44 affects whether ligand binding promotes the receptor interaction with the G-protein. We conclude that changes in the network of the receptor intramolecular interactions can induce changes in ligand mechanism of action, emphasizing that the efficacy of the ligand does not rely only on its chemical properties but depends on the pair ligand-receptor.

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Competing interests

The authors declare that no competing interests exist.

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