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Review

The DRY motif and the four corners of the cubic ternary complex model

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ABSTRACT

Recent structural data on GPCRs using a variety of spectroscopic approaches suggest that GPCRs adopt a dynamic conformational landscape, with ligands stabilizing subsets of these states to activate one or more downstream signaling effectors. A key outstanding question posed by this emerging dynamic structural model of GPCRs is what states, active, inactive, or intermediate are captured by the numerous crystal structures of GPCRs complexed with a variety of agonists, partial agonists, and antagonists. In the early nineties the discovery of inverse agonists and constitutive activity led to the idea that the active receptor state (R*) is an intrinsic property of the receptor itself rather than of the RG complex, eventually leading to the formulation of the cubic ternary complex model (CTC). Here, by a careful analysis of a series of data obtained with a number of mutants of the highly conserved E/DRY motif, we show evidences for the existence of all the receptor states theorized by the CTC, four 'uncoupled (R, R* and HR and HR*), and, consequently four 'coupled' (RG, R*G, HRG and HR*G). The E/DRY motif located at the cytosolic end of transmembrane helix III of Class A GPCRs has been widely studied and analyzed because it forms a network of interactions believed to lock receptors in the inactive conformation (R), and, thus, to play a key role in receptor activation. Our conclusions are supported by recent crystal and NMR spectra, as well as by results obtained with two prototypical GPCRs using a new FRET technology that de-couples G protein binding to the receptor from signal transduction. Thus, despite its complexity and limitations, we propose that the CTC is a useful framework to reconcile pharmacological, biochemical and structural data.

1. Introduction

Heptahelical G Protein Coupled Receptors (GPCRs) represent the most versatile form of transmembrane signaling protein and one of the largest families of potential targets for pharmacological interventions [1]. Therefore, it is not surprising that there has been widespread interest in the mechanism(s) by which GPCRs mediate their effects. In the late 80's, the concept of drug-receptor interactions underwent a profound change with: 1) the realization that receptors exhibit constitutive activity (CA) in the absence of agonist, 2) the parallel discovery of inverse agonists, and 3) analysis of pharmacological data obtained with genetically modified receptors. This led to a number of receptor theories of increasing complexity with the intent to reconcile experimental data to mathematical models. Despite the recent explosion of GPCR crystal structures of both active (R*) and inactive (R) receptor states (more than 30 structures from Class A, B, C and Frizzled), a lot of work will be still necessary to fully unravel the mechanism(s) through which they become active as well as in providing a full account of the ensemble of basal and active states [2,3].

One of the most conserved motifs within the Class A GPCR sequence is the E/DRY located at the cytosolic end of transmembrane helix III (TM3) where the Arg ($R^{3.50}$) is conserved in 96% of the receptors of this class [4]. This stretch of residues has been widely studied since the recognition that its central $R^{3.50}$ forms a network of interactions that is thought to favor receptor inactive conformation. This include contacts with the adjacent $E/D^{3.49}$ (intrahelical salt bridge) and, frequently, with another residue in position 6.30 (interhelical hydrogen bonding). Analyzing the function of all the published mutants within the E/DRY

Abbreviations: GPRC, heptahelical G protein-coupled receptors; CA, constitutive activity; CAM, constitutively active mutant; CIM, constitutively inactive mutant; TCM, ternary complex model; ETC, extended ternary complex model; CTC, cubic ternary complex model; TM, transmembrane helix

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motif of Class A GPCRs, we previously classified receptors, based on the effects of mutations in position E/D$^{3.49}$, into those that become constitutively active (constitutively active mutants or CAMs, P1-type) or do not (constitutively inactive mutants or CIMs, P2-type) [5]. An intriguing correlation was found between the effect of mutations in E/D$^{3.49}$ and R$^{3.50}$. Mutations of the Arg in P1-type receptors generated receptors that retained the capacity to bind agonist with high affinity but they often lost basal and agonist-induced signaling, a behavior suggestive of conserved G protein coupling but loss of G protein activation. In contrast, Arg mutations in P2-type receptors invariably generated loss of high affinity agonist binding as well as loss of agonist-induced responses, suggesting loss of G protein coupling. Thus, it was concluded that the two classes of receptors utilize this highly conserved triplet in different ways. For the P1-type receptor group, E/DRY appears to be the key motif in constraining the receptor in the ground state, while in the P2-type group it seems more directly implicated in G protein coupling/recognition [5].

One of the most intriguing and complex issues regarding receptor function and activation is the definition of a conformational landscape of different receptor states [6]. Strikingly, an in depth analysis of the growing number of the newly available crystal and NMR structures find evidences for such an ensemble of receptor states as envisioned by the Cubic Ternary Complex model (CTC). Thus, we propose to use this mathematical context as the minimum acceptable framework to contextualize most of the functional data published so far for wild type as well as for mutant GPCRs. This is suggested by a comparison of recent pharmacological data from E/DRY mutants of different Class A GPCRs with the FRET-based data from the two prototypical GPCRs, i.e. β_2-adrenergic (β_2-AR) and opsin.

2. From the conception of receptor theory to the cubic ternary complex model

The “occupancy theory” of Clark [7,8] along with modifications by Ariëns [9] and Stephenson [10], represent the simplest mechanistic models of ligand-receptor interaction. In 1980 DeLéan and colleagues introduced the next step of complication, the Ternary Complex Model (TCM), where agonists bind the receptor R with low affinity and the receptor-G protein complex (RG) with high affinity [11]. However, in 1989, the almost simultaneous discovery of inverse agonists [12,13] and Constitutively Active Mutant Receptors (CAMs) [14–16] led to the idea that the active state is an intrinsic property of the receptor itself rather than of the RG complex, leading Samama and colleagues to formulate the extended TCM (ETC) [17,18]. This model has several advantages, as it allows for selective affinity for different receptor species (as the TCM), but also allows for efficacy to be vectorial, i.e. positive ($\alpha > 1$) or negative ($\alpha < 1$) [19], providing an explanation for CA.

If we limit ourselves to the analysis of E/D$^{3.49}$ CAM receptors, then the six different receptor species predicted by the ETC model (R, HR inactive and R*, HR*, R*G and HR*G active) are sufficient to accommodate all of the experimental data published so far. However, studying R$^{3.50}$ mutants of the α_{1B} adrenergic receptor (α_{1B}-AR) within the highly conserved E/DRY motif, it was soon apparent that the high agonist affinity without CA observed with some of these mutants could not be explained within the context of the ETC [20]. At that time, Cotecchia and Costa speculated that these results could be interpreted by assuming that mutations at R$^{3.50}$ were able to isomerize the receptor to a state that favors high affinity for agonists, but is unable to either bind the G protein (R*, predicted by the ETC, but never demonstrated) or, despite binding the G protein, was unable to induce its activation (RG, not predicted by the ETC). However, they could not distinguish between them in the absence of direct measure of G protein binding.

The possible existence of two additional inactive species (RG and HRG) was, however, already anticipated by the CTC model of Weiss and colleagues [21–23]. Indeed, the ETC rapidly evolved in the CTC which

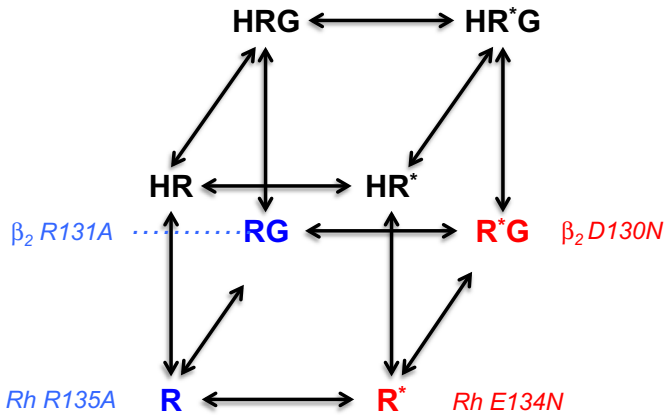


Fig. 1. The CTC model and the different receptor species as identified by DRY motif mutants. Blue for R$^{3.50}$ mutants and red for D/E$^{3.49}$ mutants.

is symmetric, comprehensive with respect to several other previous pharmacological models, and can be generalized to equilibrium involving multiple ligands (including biased agonists), G proteins (or other transducers) and receptors. Furthermore, because of the principle of free energy coupling [24], it is thermodynamically complete in the way that it describes all the possible three-ways interactions between receptor, ligand and G protein. In the CTC model each receptor is allowed to bind to only one G-protein and one ligand (no allosteric ligand) at a time, while G-proteins and ligands form a common pool accessible to each receptor. At variance with the ETC, each receptor can exist in an active (R*) or inactive (R) conformation, both of which are able to interact with ligand and G-protein (Fig. 1).

The CTC has been described by its authors to be “heuristic” in nature, in the sense that it is complex, encompassing eight different receptor species (four without a ligand bound: R and RG inactive, R* and R*G active; four bound to a ligand: HR and HRG inactive, HR* and HR*G active) and seven different parameters that often cannot be experimentally demonstrated or estimated and, therefore, frequently neglected. As we will discuss here, a careful analysis of crystal and NMR structures, as well as pharmacological, biochemical, and FRET data obtained with a number of mutants of the highly conserved E/DRY motif reveals evidence of the existence of all the GPCR states theorized by the CTC model.

3. What do the crystal structures tell us?

3.1. Crystal structures and E/DRY motif

While we should always take into consideration that many GPCR crystals do not mimic native structures due to unphysiological crystallization conditions or extensive engineering [25], their crystallization has provided a powerful tool to understand their structure and functioning. Generally speaking, the presence of the so called ‘ionic lock’ has been considered as indication of an inactive receptor state [26–28]. However, while the intrahelical salt bridge (E/D$^{3.49}$ - R$^{3.50}$) has been found in the vast majority of the crystal structures resolved so far, (including CXCR1, CXCR4 and AT$_1$ receptors; Table 1, reporting measurements of the distances between the intra-helical residues in TM3 not discussed in the original crystal structure papers), with some notable exceptions (H$_1$ [29], P2Y$_1$ [30], M$_1$, M$_3$, M$_4$ and LPA$_1$ (Table 1)), surprisingly, the interhelical hydrogen bonding (R$^{3.50}$ - E$^{6.30}$) has been found only in rhodopsin [31], D$_3$ [32], β_1-AR [33] and in few antagonist- or inverse agonist-bound structures (A$_{2A}$ [34], β_1-AR [35] as well as in M$_4$ [36]). Of notice, in the crystal structure of CXCR4 [37] R$^{3.50}$ is bound to helix VI through a water-mediated hydrogen bond which could perform a role similar to that of the Glu residue in rhodopsin, despite the presence of a Lys in position 6.30 [38]. CXCR1

Table 1
Measured distances between the residues forming the intrahelical salt bridge in TM3 (D/E$^{3.49}$ - R$^{3.50}$) in antagonist-bound structures.

Receptor	PDB	PubMed ID	Residue 1	Residue 2	Dist. (Å)
M1	5CXV (human)	26958838	D$^{3.49}$ (D122)	R$^{3.50}$ (R123)	9.6
M3	4U15 (rat)	25450769	D$^{3.49}$ (D164)	R$^{3.50}$ (R165)	4.5
M4	5DSG (human)	26958838	D$^{3.49}$ (D129)	R$^{3.50}$ (R130)	6.9
CXCR1	2LNL (human)	23086146	D$^{3.49}$ (D134)	R$^{3.50}$ (R135)	2.4
CXCR4	3ODU (human)	20929726	D$^{3.49}$ (D133)	R$^{3.50}$ (R134)	3.5
AT1	4YAY (human)	25913193	D$^{3.49}$ (D125)	R$^{3.50}$ (R126)	3.6
LPA1	4Z34 (human)	26091040	E$^{3.49}$ (E145)	R$^{3.50}$ (R146)	8.7

also has a Lys in position 6.30 [39] analogously to CXCR4, and could behave similarly.

Strikingly, rhodopsin, D_3, CXCR4 and CXCR1 mutated in position E/D$^{3.49}$ where unable to induce CA [40–43], and for this reason we previous classified them as belonging to the CIM category (P2-type) [5]. In addition, M_4 [44], A_{2A} [45] and β_1-AR [46] have all been demonstrated to possess little basal activity compared, for example, to β_2-AR [46].

On the other hand, the crystal structure of receptors known to possess a fair amount of basal activity such as β_2-AR [47] μ-OR [48], M_3 [49] and AT_1 [50] do not show the presence of the interhelical ionic lock in their reported crystal structures even bound to an antagonist (μ-OR, [51] and AT_1 [52]) or inverse agonist (β_2-AR, [53] and M_3 (see Fig. 2, not discussed in the original crystal structure paper)), and, accordingly, may fall into the CAM category (P1-type).

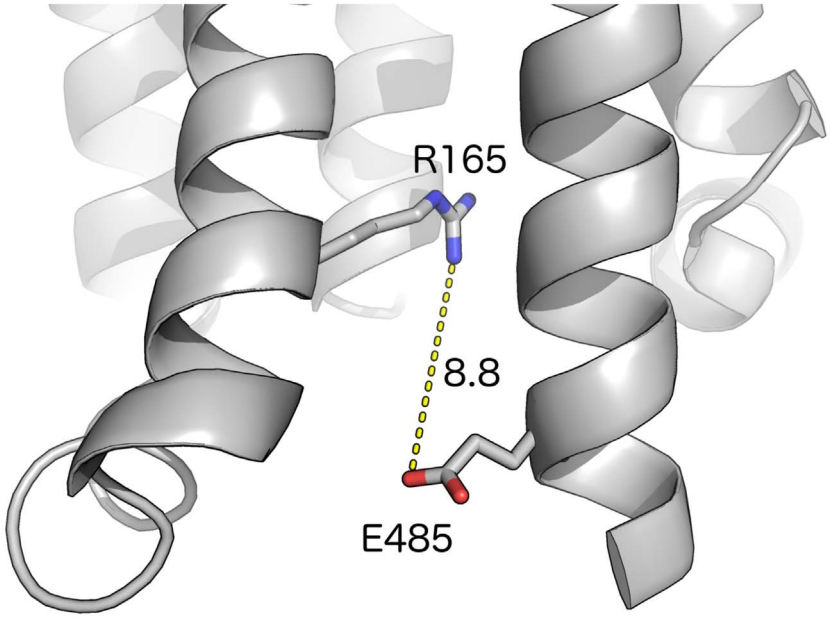
Thus, despite crystals are only a "snapshot" of one structure of that specific receptor that is favored in the crystal environment [54], the presence or absence of the ionic lock seems to represent a different propensity to form this intra-molecular network, indicating that some receptors have a lower energy barrier between the R and R* states leading to a relevant basal activity that might reflect different needs in a physiological context [55].

In addition, any mutation at R$^{3.50}$ causes loss of agonist-induced activity for rhodopsin [26,40,56,57], as well as disrupting high affinity agonist binding for D_3 [41] or CXCR4 [42], and for a number of other CIM (P2-type) receptors, including α_{2A} [58] and TPα [59]. On the other hand, high affinity agonist binding is preserved in β_2-AR R$^{3.50}$ mutants [57,60], as well as in all the other CAM (P1-type) receptors [5], indicating that for this sub-class of receptors R$^{3.50}$ is not necessary for G protein coupling. Accordingly, no direct interaction between R$^{3.50}$ and the C-terminus of Gαs has been appreciated in two active x-ray crystal structure of the β_2-AR [61,62].

Therefore, despite the majority of these structures, with the notable exception of rhodopsin and CXCR1 (the last structure was determined, indeed, by NMR spectroscopy), have been heavily engineered (T4 lysozyme or apocytocrome b_{562} RIL (BRIL) insertion and/or thermostabilizing mutations) to allow crystallization and, thus, information from these crystals should be taken with caution, these structural data corroborate our previous suggestions of a dual role for the E/DRY motif for CAMs, representing the main switch to the active receptor state(s), as opposed to CIMs, where it is directly involved in G protein coupling/recognition [5].

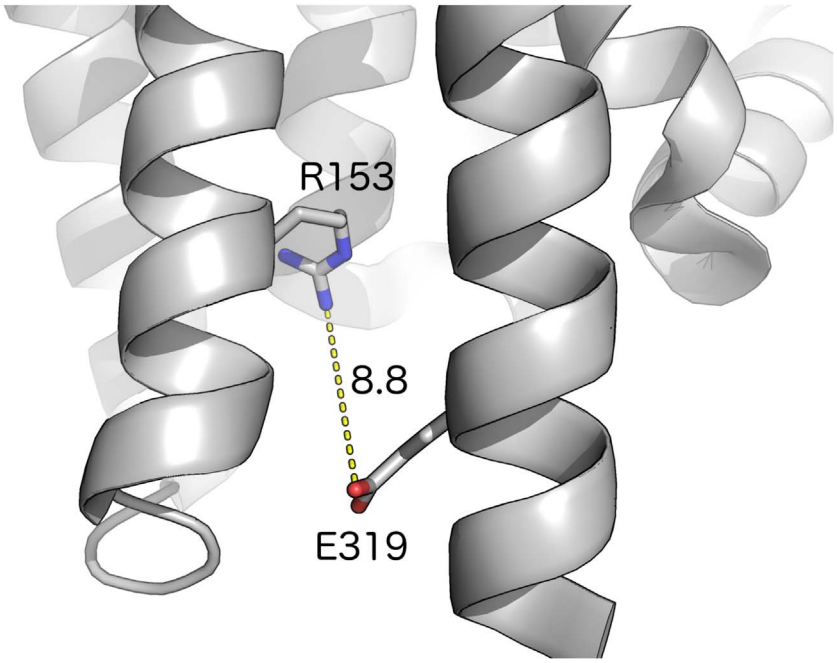
3.2. Crystal structures and the ensemble of states

So, the question now is 'which state, active or inactive, these structures really represent'? As expected, the ionic lock is broken in both opsin/metarhodopsin and β_2-AR active structures [61,63], as well as in A_{2A} [64], NTS_1 [65] M_2 [66] and 5-HT_{2B} (Fig. 3, not discussed in the original crystal structure paper) agonist-bound structures. However, most of the agonist-bound structures resolved so far exhibit only small



Receptor	PDB	PubMed ID	Residue 1	Residue 2	Dist. (Å)
M3	4U15 (rat)	25450769	R$^{3.50}$ (R165)	E$^{6.30}$ (E485)	8.8

Fig. 2. Interhelical ionic lock distances between R165 (Blue) and E485 (red) in M_3 muscarinic receptor bound to an inverse agonist. Each GPCR residue was matched to a numerical amino acid location using the GPCRdb protein selection tool (http://tools.gpcr.org/visualise/proteinselection). ICM Browser Pro's Distance Between Two Atoms tool was used to measure distance between residues. Distance measurements represent the shortest distance between a terminal nitrogen atom on the indicated R residue and a terminal oxygen or nitrogen atom on the indicated E residue.



Receptor	PDB	PubMed ID	Residue 1	Residue 2	Dist. (Å)
5HT2B	4IB4 (human)	23519215	$R^{3.50}$ (R153)	$E^{6.30}$ (E319)	8.8

Fig. 3. Interhelical ionic lock distances between R153 (Blue) and E319 (red) in 5-HT_{2B} receptor bound to an agonist. Residue matching and distance measurements have been performed as described in Fig. 2.

scale changes compared with their antagonist-bound counterparts, and only the crystallographic structures of opsin/metarhodopsin [67–70] as well as β_2-AR [62] in their G protein-interacting conformation show significant rearrangement of the transmembrane regions (particularly TM3 and TM6) beyond the disruption of the E/DRY network [38,71]. This demonstrates that more profound rearrangements than the mere breakage of the ionic lock are associated with full receptor activation [72], and that only these G protein interacting structures represent the real active receptor state (R*G) [73].

4. MD simulation and NMR studies

Proteins, however, are not static as displayed in models derived from crystallization data, but rather dynamic structures that adopt different states over the time. Indeed, MD simulations performed on the β_2-AR [74–76] and β_1-AR [77] show that the interhelical hydrogen bond can form and break, suggesting the existence of an equilibrium between different receptor states.

More recently, two very elegant ^{19}F NMR studies of the A_{2A} [78] and of β_2-AR [73] demonstrate the existence of two states, named S1 and S2, described as 'inactive' (because not coupled to the G protein) representing the ionic lock formed and broken conformation of the receptor, respectively. It is quite possible that these two states, that we will call 'uncoupled', represent the R (inactive, ionic lock formed, S1) and R* (active-like, ionic lock broken, S2) states captured in different crystal structures in the absence of G protein/G protein mimetic and predicted by the CTC. The same studies also identified two additional states, S3 and S3′ (also called S4), induced by the addition of a peptide derived from the carboxyl (C)-terminal domain of the G-protein Gαs in the case of A_{2A} receptor [78], or a G protein mimetic antibody Nb80 in the case of β_2-AR [73]. These two states, described as 'active' by their ability to interact with the Gαs-derived peptide or the G protein mimetic, have, however, different conformations, and have been postulated to possess a different ability to activate the G proteins. These conformations that we will call 'coupled' may well correspond to the RG (unable to activate the G protein, S3) and R*G (able to activate the G protein, S3′/S4) states predicted by the CTC (Fig. 1).

5. The signature DRY motif in the context of the CTC model

Recently, one of us developed a technique termed Systematic Protein Affinity Strength Modulation (SPASM) to dissect G protein coupling from conformational change (Fig. 4). It is based on a novel FRET-based sensor that uses a peptide comprising the last 27 residues of a Gα C-terminus tethered to the receptor C terminal domain to detect the ligand/mutagenic stabilization of GPCR conformations that promote interactions with the cognate G protein in living cells and, thus, allowing us to distinguish between coupled (RG and R*G) and uncoupled (R and R*) forms of the receptor, independently from their ability to induce a signal [57].

5.1. The β_2-AR case

We have analyzed by means of this technique, two mutants of the β_2-AR that have been shown to posses most, if not all, the traits of CAMs, the D130N [27,79,80] and R131A [76]. As it appears from the SPASM data showing an increase of the FRET signal in basal condition (i.e. increase G protein interaction) and the increase in agonist binding affinity, both mutants are stabilized in a G_S interacting conformation (Fig. 5). However, only D130N showed enhanced CA, agonist-induced signaling and G protein activation [57]. These data, therefore, indicate that D130N displays an active R*G conformation (S3′/S4) in its basal state, whereas R131A results in a coupled but activation-deficient RG conformation (S3) (Fig. 1). Interestingly, both species can bind agonists with high affinity becoming HR*G and HRG, respectively. It is therefore conceivable that the $R^{3.50}$ mutation induces the opening of the G

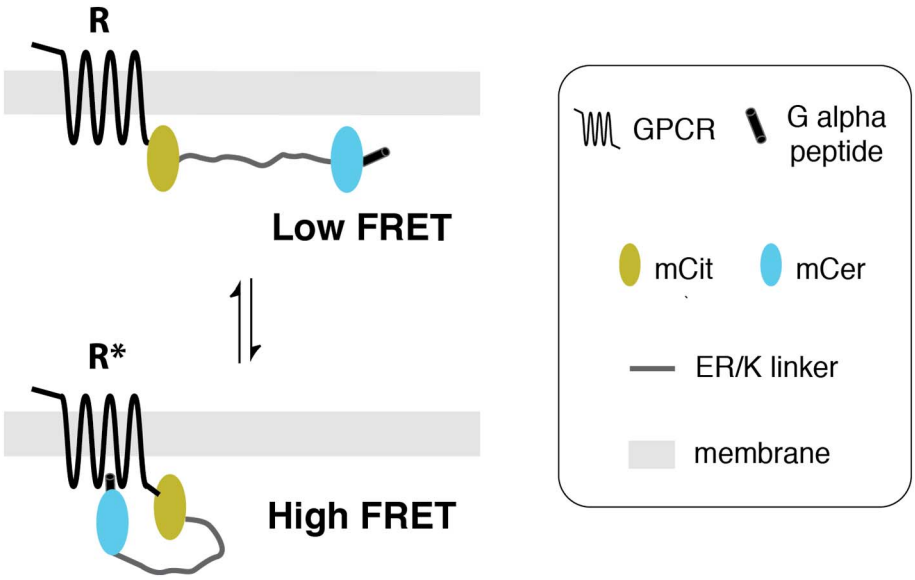
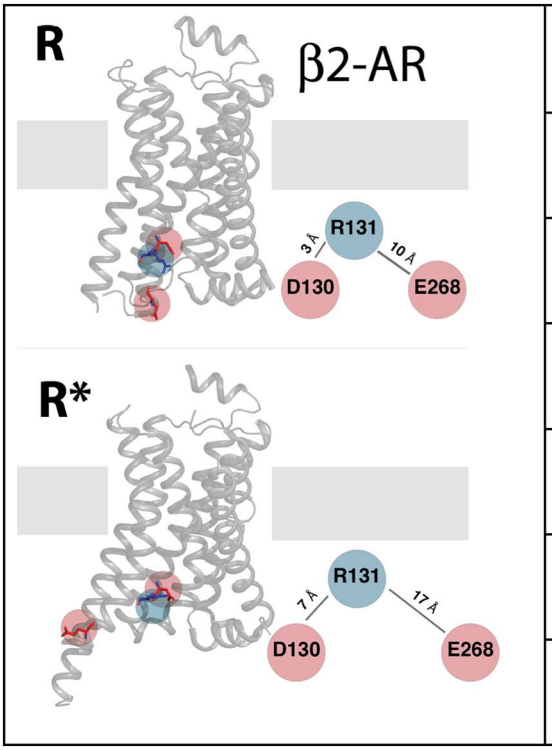


Fig. 4. Schematic of SPASM FRET sensors that report the active state of the GPCR. The sensor consists of a single polypeptide comprising the GPCR and a peptide derived from the C-terminus of a G alpha subunit, separated by an ER/K α-helical linker flanked by a FRET donor (mCerulean) and acceptor (mCitrine) pair. Activation of the receptor (R to R*) leads to interaction with the Galpha C-terminus, which brings the FRET donor and acceptor in proximity resulting in higher FRET [57].

protein binding site (ionic lock broken) to allow Gα docking without being able to induce its activation. To our knowledge, this was the first experimental evidence of the existence of a high affinity, G protein coupled but activation-deficient RG conformation as envisioned by the CTC.

Interestingly both these two states showed high affinity for agonist, in agreement with the 'closed' high affinity receptor conformations induced by a nucleotide-free G protein recently identified for a number of GPCRs [81]. In addition, these data nicely fit with our hypothesis on the role of the E/DRY motif in P1 type of GPCRs (CAM), i.e. its role as the main restraint to the active receptor conformations, and not in G protein binding/recognition, as all the $R^{3.50}$ mutants have increased or unaltered agonist affinity [5] (see also crystal structure paragraph above). These results, of course, may also provide the framework as to interpreting the α_{1B}-AR data [20], as well as data from other P1-type receptors [5].



E/DRY mutant	SPASM FRET (Malik et al., 2013)
β2-AR – D130N	++
β2-AR – R131A	++
β2-AR – E268N	+
Opsin – E134N	No change
Opsin – E247N	No change
Opsin – R135A	No change

Fig. 5. Summary of data on E/DRY motif obtained using SPASM GPCR sensors. (Left) Schematic of β2-AR E/DRY residue contacts in the inactive (R) and active (R*) states. For β2-AR, mutagenesis of either E268, D130, or R131 residues results in receptor transition to an active conformation as witnessed by an increase in measured FRET. In contrast, mutagenesis of either the E134, E247, or R135 residues within opsin that constitute its E/DRY motif does not activates the receptor given the dominance of another set of ionic interactions centered on K296 in the retinal binding site (not shown).

5.2. The rhodopsin case

Numerous rhodopsin mutants have been described to differently affect receptor activity [26,40,56], which have lead to misleading classification of this prototype GPCR. We analyzed two rhodopsin mutants, namely E134N and R135A, and ultimately demonstrated that non-conservative mutagenesis of either E/R residue did not increase basal G protein coupling (Fig. 5) (and, likely, basal activity [82]) compared to WT receptor (P2 type, CIM). In addition, we, provided evidence that R135A is an inactive receptor conformation unable to couple G protein (R, S1) and to signal even in the presence of agonist (HR), while E134N can ultimately bind and activate G protein upon agonist stimulation (HR*G) (Fig. 1). These data are in strong agreement with the proposed direct role for $R^{3.50}$ in G protein binding for the P2 type of receptors [5]. Interestingly, spectroscopic data have demonstrated that several mutations of E134 cause an increase in the amount of metarhodopsin II, the active form of rhodopsin, at equilibrium [83]. Thus, considering the spectrophotometric and the SPASM data, we can, for the first time, realistically postulate here that E134N mutant represents a conformation of rhodopsin uncoupled from G protein in its basal condition, but in a 'active-like' conformation (R*, S2).

5.3. The α_{2A}-AR and TPα receptor cases

We have previously identified $R^{3.50}$ mutants of both α_{2A} adrenergic receptor (α_{2A}-AR) [58] and TPα [59] receptors closely matching the rhodopsin R135A phenotype being characterized by absence of CA, impairment of agonist-induced activity and lack of high affinity agonist binding, suggestive of lack of G protein coupling (R, S1). However, we also identified α_{2A}-AR and TPα $E/D^{3.49}$ mutants that, despite lacking any augmented CA, retained agonist-induced signaling (indicating the capacity to couple to G proteins) and showed enhanced agonist affinity in binding studies [58,59,84]. Intriguingly, some $E^{3.49}$ mutants of the TPα produced receptors with even more efficient signaling properties, i.e. increased agonist potency and efficacy as well as increased efficiency in G protein activation, and after that named Super Active Mutants (SAMs) in alternative to CAMs [84,85]. Thus, these data suggest that in some receptors $E/D^{3.49}$ mutations induce a transition from the ground state (R) toward a different conformation characterize by a lack of increase in CA. It is therefore possible that, in the absence of agonist, these α_{2A}-AR and TPα $E/D^{3.49}$ mutants could assume either an uncoupled active-like state (R*, S2) as the rhodopsin E134N mutant, or, more likely a G proteins coupled but activation deficient conformation (RG, S3), as the β_2-AR R131A mutant. This is something we are currently investigating by means of the SPASM technique in our labs. Upon agonist addition, both receptors become fully active, i.e. HR*G. In fact, while it is now clear that agonists will bind receptor with high affinity only in the presence of G protein coupling (closed conformation) [81], and that agonist alone are not sufficient to induce a full active receptor state [73], agonist are, however, essential in altering the distribution of the different conformations (conformational selection) [78,86]

6. The DRY motif in the "real world"

To date, four class A GPCRs have been reported to have different natural occurring mutations in the central $R^{3.50}$ of the DRY motif that are linked to clinical identified syndromes, the vasopressin V_2 [87,88], the gonadotropin-releasing hormone GnRH [89,90], GPR40 (NCBI http://www.ncbi.nlm.nih.gov, accessed 21 June, 2015) and the $P2Y_{12}$ receptors [91]. Both mutations of the GnRH receptor (R139H/C), resulting in the clinical manifestation of hypogonadotropic hypogonadism, are characterized by lack of their plasma membrane expression precluding analysis of their functionality. Other mutations in position $D^{3.49}$ or $R^{3.50}$ [92,93], however, define this receptor as a CIM [5]. The situation is more complicated for the V_2 receptor which has variants leading to two different diseases with opposite clinical manifestation, nephrogenic diabetes insipidus (NDI) for R137H [87], and nephrogenic syndrome of inappropriate antidiuresis (NSIAD) for R137C/L [88]. Of note, while R137C/L variants are characterized by elevated basal agonist-independent signal [94], R137H is likely characterized by an elevated endocytosis, possibly due to an increase in CA [95]. Indeed, the 'artificial' D136A mutant has been shown to be a CAM [96]. R104P mutation of GPR40, an attractive drug target for the treatment of type 2 diabetes, has been recently identified as a 'loos of function' variant [97]. Despite this Single Nucleotide Polymorphism is characterized not only by a reduced receptor expression at the plasma membrane, but also by a strong impairment in agonist-induced intracellular Ca^{2+} mobilization, no information about its CA has been reported [97]. Finally, the $P2Y_{12}$ R122C variant, clinically resulting in a deficiency of ADP-induced aggregation responses and in a chronic bleeding disorder, is characterized by a lack of agonist-induced response and reduced agonist affinity in vitro [91]. These features point to a "loss of function" receptor variant likely due to an impairment in G protein coupling. However, the very recent resolution of highly engineered $P2Y_{12}$ crystal structure shows a peculiar rearrangement of TM6 which together with a reported significant basal activity of the WT receptor [98] seems to exclude the formation of the inter-helical ionic lock [99], making it necessary to analyze the signaling effects of mutations in position $D^{3.49}$ to definitively classifying this receptor as belonging to the P1- or P2-type.

7. Conclusions

Clearly more structural work will be necessary to fully understand the highly regulated process of GPCR activation, but the analysis we present here supports the CTC as the minimum model to explain the complex pharmacological behavior of GPCRs and their mutants. Furthermore, it provides evidence that the intra/interhelical network involving the highly conserved E/DRY motif plays a more complex role than previously hypothesized. Giving rhodopsin unique role and segregation, it might not be a surprise that a second set of opsin-specific ionic interactions are important for retinal binding [100,101]. Crystal structure of a growing number of GPCRs, however, suggest that these multiple controls that must be "unleashed" to achieve full receptor activation are more common that previously appreciated [38,102].

Will The CTC be the 'ultimate model'? Likely not. While is now becoming evident that receptors possess at least two inactive and two active conformations [73,78], it is also evident that more active conformations exit [103,104]. In fact, an individual receptor might have basal activity or might be inactive depending on the particular signal output measured (biased signaling). Therefore, the single cube of the CTC is certainly an oversimplification of the family of 'cubes' that would be necessary to describe the full ensemble of receptor states induced by different (biased) agonists. In addition, the recent finding of a 'closed' active receptor conformation induced by a nucleotide-free G protein suggest that also the CTC should be refined to include the role of nucleotides in stabilizing the active receptor complexes [81]. These limitations notwithstanding, the analysis presented here provides evidence for the GPCR species envisioned by the CTC model, and suggests that, regardless of its limitations, it is a useful paradigm to reconcile pharmacological, biochemical and structural data. The ever growing progress in experimental techniques will allow us to reveal more details on the complexity of receptor function and regulation in the very near future.

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