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Constitutive activity and inverse agonism at the $\alpha_1$adrenoceptors

Susanna Cotecchia *

Department of Pharmacology and Toxicology, University of Lausanne, Rue du Bugnon 27, 1005 Lausanne, Switzerland

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ABSTRACT

Mutations of G protein-coupled receptors (GPCR) can increase their constitutive (agonist-independent) activity. Some of these mutations have been artificially introduced by site-directed mutagenesis, others occur spontaneously in human diseases. The α_{1B}adrenoceptor was the first GPCR in which point mutations were shown to trigger receptor activation. This article briefly summarizes some of the findings reported in the last several years on constitutive activity of the $\alpha_1$adrenoceptor subtypes, the location where mutations have been found in the receptors, the spontaneous activity of native receptors in recombinant as well as physiological systems. In addition, it will highlight how the analysis of the pharmacological and molecular properties of the constitutively active adrenoceptor mutants provided an important contribution to our understanding of the molecular mechanisms underlying the mechanism of receptor activation and inverse agonism.

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1. The $\alpha_1$adrenoceptors: structure–functional relationships

The mammalian G protein-coupled receptors (GPCRs) can be divided in three main classes according to sequence homology: class I or rhodopsin-like (which is the largest subfamily), class II or secretin-like, and class III or glutamate-metabotropic-like [1]. Within class I, the adrenoceptors (AR) mediate the functional effects of epinephrine and norepinephrine. The AR family includes nine different gene products: three β (β_1, β_2, β_3), three α_2 (α_{2A}, α_{2B}, α_{2C}) and three α_1 (α_{1A}, α_{1B}, α_{1D}) receptor subtypes.

Within the subfamily of the α_1AR subtypes [2], extensive mutational analysis performed by various investigators helped to identify the structural determinants involved in each of the three main ''classical'' functional properties of GPCRs: (1) ligand-binding; (2) coupling to G protein-effector systems; (3) desensitization. Beyond these classical features, a number of additional functional paradigms of GPCRs have recently emerged including constitutive activity, oligomerization and their interaction with a variety of signaling proteins.

The molecular interactions of the endogenous catecholamines, epinephrine and norepinephrine, with different AR subtypes has been explored in different studies. Epinephrine and norepinephrine contain a protonated amino group separated from the aromatic catechol ring by a β-hydroxylethyl chain. Mutagenesis studies of the α_{1B}AR [3,4] suggested that the amino group of the catecholamines makes an electrostatic interaction with the carboxylate side chain of an aspartate on helix 3, $Asp^{125(3.32)}$, which is highly conserved in all GPCR binding biogenic amines (Fig. 1) (the amino acid numbering in parentheses, used only for the amino acids in the helical bundle, is that proposed in Ref. [5]). Similar findings were obtained mutating the homologous aspartate in the α_{1A}AR subtype (S.C., unpublished results). For both the α_{1A} and α_{1B}AR, the catechol *meta*- and *para*-hydroxyl groups of epinephrine and norepinephrine make weak hydrogen

* Tel.: +41 21 6925400; fax: +41 21 6925355.
E-mail address: Susanna.Cotecchia@unil.ch.

Abbreviations: GPCR, G protein-coupled receptor; AR, adrenoceptor; CAM, constitutively active mutant; MD, molecular dynamics

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bonding interactions with serine residues in helix 5 which are conserved in all catecholamine-binding GPCRs (Fig. 1) [3,4].

The roles of a cluster of aromatic amino acids in helices 6 and 7 in ligand binding of the α_{1B}AR were also investigated [6]. The results indicated that, among these aromatic residues, Phe$^{310(6.51)}$ is the only one involved in binding epinephrine probably interacting with the catechol ring of the ligand.

In contrast, very little is known so far about the amino acids of the α_{1B}AR which interact with different antagonists as well as about the structural basis underlying receptor selectivity for different ligands. Mutation of Asp$^{125(3.32)}$ (Fig. 1) to alanine profoundly impaired the ability of the hamster α_{1B}AR to bind antagonists [3]. Experimental mutagenesis studies suggested that a conserved tyrosine in the extracellular half of helix 7 should interact with prazosin [3].

Activation of the α_1AR subtypes causes polyphosphoinositide hydrolysis catalyzed by phospholipase C *via* pertussis toxin-insensitive G proteins of the $G_{q/11}$ family in almost all tissues where this effect has been examined. Polyphosphoinositide hydrolysis results in the increase of intracellular inositol phosphate production. Several lines of evidence demonstrated that the i3 loop contains the main structural determinants involved in α_{1B}AR coupling to G proteins of the $G_{q/11}$ family. A detailed analysis of the molecular basis of the receptor–G_q coupling was carried on by combining computational modeling and experimental mutagenesis of α_{1B}AR [7]. The functional analysis of a large number of receptor mutants in conjunction with the predictions of molecular modelling support the hypothesis that Arg^{254} and Lys^{258} in the i3 loop as well as Leu^{151} in the i2 loop (Fig. 1) are directly involved in receptor-G protein interaction and/or receptor mediated activation of the G protein [8]. It is important to highlight that mutations of the homologous leucine or hydrophobic residue in the i2 loop resulted in receptor–G protein uncoupling for other GPCRs as well [1].

The α_{1B}AR expressed in various cell types can undergo phosphorylation and desensitization upon exposure to agonists as well as to the protein kinase-C (PKC) activator phorbol-myristate-acetate [9,10]. In particular, we demonstrated that a stretch of serines in the C-tail of the receptor represents the main phosphorylation sites (Fig. 1) [11]. Three of them (Ser^{404}, Ser^{408} and Ser^{410}) are involved in agonist-induced phosphorylation whereas two others (Ser^{394} and Ser^{400}) represent the sites for PKC-mediated phosphorylation of the α_{1B}AR. Agonist-induced regulation of the receptor seems to be mainly mediated by members of the G protein-coupled receptor kinase (GRK) family. In fact, GRK_2-mediated phosphorylation of Ser^{404}, Ser^{408} and Ser^{410} is crucially involved in the desensitization of the α_{1B}AR.

2. The discovery of constitutively active receptor mutants and its implications

The α_{1B}AR was the first GPCR in which point mutations were shown to trigger receptor activation [12]. A conservative substitution (Ala^{293}Leu) in the cytosolic extension of helix 6 (Fig. 1) of the α_{1B}AR resulted in its constitutive (agonist-independent) activity. In the absence of agonist, cells expressing the mutated receptor exhibited higher basal levels of inositol phosphates as compared to cells expressing the wild type α_{1B}AR. To further assess the role of this amino acid, Ala$^{293(6.34)}$ was systematically mutated by substituting each of the other 19 amino acids [13]. Remarkably, all possible amino acid substitutions of Ala^{293} in the α_{1B}AR induced variable levels of constitutive activity which was the highest for the Ala^{293}Glu mutant.

To extend the generality of this finding within the AR family, similar mutations were performed in the β_2 and α_{2A}AR which are coupled to G_s-mediated stimulation or G_i-mediated inhibition of adenylyl cyclase, respectively [14,15]. Both β_2 and α_{2A}AR mutants exhibited increased constitutive activity leading to increased or decreased agonist-independent adenylyl cyclase activity, respectively.

The discovery of the constitutively active mutants (CAMs) in the AR family catalyzed the interest of a large number of groups towards the elucidation of two main aspects of GPCR function and drug action: (a) the activation process of GPCRs and (b) the identification of ligands with negative efficacy. In addition, the discovery of the CAM GPCRs encouraged the search for spontaneously occurring activating mutations of different receptors which are responsible for a number of human diseases [16].

This article will briefly review some of the findings obtained in the last several years on constitutive activity of the $\alpha_1$adrenoceptor subtypes and its implications on our understanding of inverse agonism.

3. Constitutively activating mutations in the $\alpha_1$adrenoceptor subtypes

Among the three α_1AR subtypes, the largest number of activating mutations have been described in the α_{1B} (Fig. 1), few in the α_{1A} and none in the α_{1D}AR subtype (Table 1). For all the CAMs their constitutive activity was mainly assessed measuring inositol phosphate accumulation in whole cells whereas for few of them other biochemical pathways, like phospholipase A_2 or D, have also been explored.

As mentioned above, in the α_{1B}AR all possible amino acid substitutions of Ala$^{293(6.34)}$ in the cytosolic extension of helix 6 induced variable levels of constitutive activity [13]. No quantitative relationship was found between the physicochemical properties of the substituting amino acids and the levels of agonist-independent activity of the CAMs. The greatest increase in constitutive activity was observed for the mutation of Ala$^{293(6.34)}$ into glutamate. All CAMs displayed increased affinity for the full agonist epinephrine which was, at least to some extent, correlated with their degree of constitutive activity.

Studies from our laboratory combining site-directed mutagenesis of the α_{1B}AR and molecular dynamics (MD) simulations on computational models of the receptor highlighted the potential role played in receptor activation by the Glu/AspArgTyr (E/DRY) motif at the cytosolic end of helix 3 (Fig. 1), which is highly conserved in GPCRs of the rhodpsin-like class. Mutations of the aspartate (Asp$^{142(3.49)}$) of the E/DRY motif resulted in high constitutive activity (Fig. 2). Similarly to the Ala$^{293(6.34)}$ replacements, all the 19 possible natural amino acid substitutions of the aspartate resulted in variable levels of

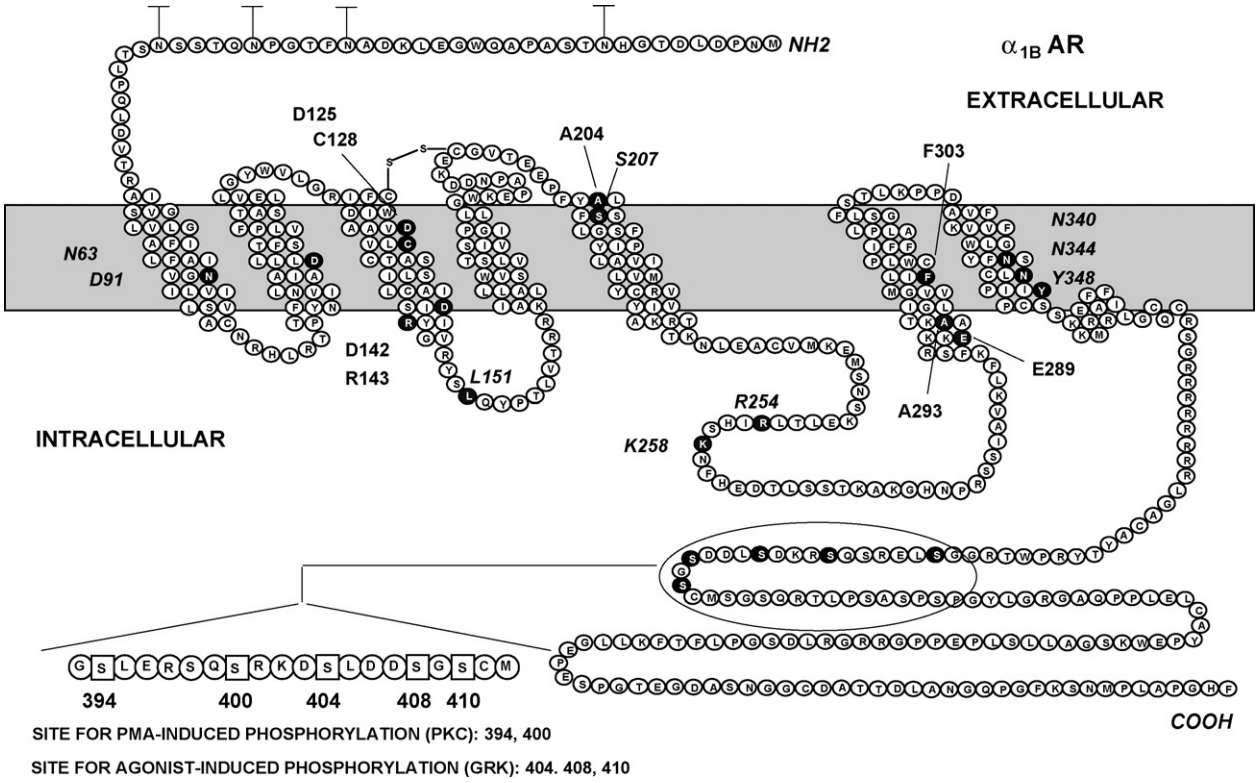

Fig. 1 – Topographical model of the α_{1B}AR displaying key amino acids involved in receptor function. The sequence of the hamster α_{1B}AR is topographically arranged according to its alignment with the crystal structure of bovine rhodopsin. The four N-linked glycosylation sites of the receptor are indicated with crosses. The black circles indicate most of the amino acids mentioned in the text which can be divided in four groups. (i) in bold, main sites where constitutively activating mutations have been described: Asp^{125}, Cys^{128}, Asp^{142}, Arg^{143}, Ala^{204}, Glu^{289}, Ala^{293}, Phe^{303}; (ii) in italic, key amino acids playing a role in catecholamine binding (Asp^{125}, Ser^{207}), coupling to G_q (Leu^{151}, Arg^{254}, Lys^{258}); (iii) in italic, highly conserved polar residues within the helical bundle: Asn^{63}, Asp^{91}, Asn^{340}, Asn^{344}, Tyr^{348}; (iv) in the C-tail, phosphorylation sites (Ser^{394}, Ser^{400}, Ser^{404}, Ser^{408} and Ser^{410}) for protein kinase C (PKC) and G protein-coupled receptor kinases (GRK).

agonist independent activity [17]. The greatest constitutive activity was observed for the mutation of Asp$^{142(3.49)}$ into Thr. All CAMs carrying mutations of Asp$^{142(3.49)}$ displayed increased affinity for the full agonist epinephrine which was, at least to some extent, correlated with their degree of constitutive activity. Increased constitutive activity was also found after mutating the acidic residue of the E/DRY motif in other receptors including rhodopsin [18], the β_2AR [19], the histamine H_2 [20], the vasopressin V_2 [21] and muscarinic M_1 [22] receptors.

An interesting pattern of functional responses was also obtained by mutating the arginine of the E/DRY sequence of the hamster α_{1B}AR into Lys, His, Asp, Glu, Ala, Iso, Asn [23]. The charge-conserving mutation of Arg$^{143(3.50)}$ into lysine and histidine conferred, respectively, high and low degree of constitutive activity to the receptor. In contrast, all the other replacements of Arg$^{143(3.50)}$ were not constitutively active and were dramatically impaired in their ability to mediate agonist-induced inositol phosphate response.

Altogether, these findings on the E/DRY sequence led to the hypothesis that the aspartate might constitute a fundamental switch of the α_{1B}AR activation through the protonation/deprotonation of its side chain, whereas the main role of the conserved arginine is to mediate receptor activation.

Activating mutations of the α_{1B}AR were also found in the extracellular half of the seven-helix bundle. In particular, mutation of Cys$^{128(3.35)}$ in helix 3 into different amino acids led

Table 1 – Constitutive activity at the $\alpha_1$adrenoceptor subtypes

Experimental conditions	α_{1A}AR	α_{1B}AR	α_{1D}AR
Mutation-induced	Refs. [25,28]	Refs. [12,13,17,24,25,26]	n.d
Overexpression of native receptor	Ref. [28]	Refs. [17,28]	Refs. [36,37]
Physiological system	n.d.	n.d.	Ref. [38]

n.d.: not determined.

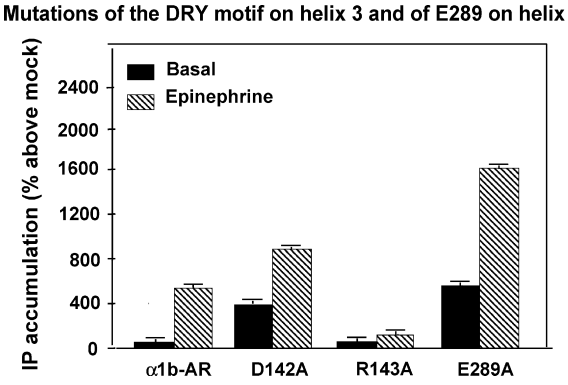

Fig. 2 – Effect of mutations of the E/DRY motif (helix 3) and of E289 (helix 6) in the α_{1B}AR. Total inositol phosphates (IP) were measured in COS-7 cells expressing the wild type α_{1B}AR and its mutants D142A, R143A and E289A in the absence (Basal) or in the presence of 10^{-4} M epinephrine for 45 min, as described in Ref. [25]. IP accumulation is expressed as % above mock which represents the IP levels measured in cells transfected with empty DNA vector.

to constitutively active forms of the receptor, with large, bulky or hydrophobic residues inducing greater constitutive activity [24]. The increase in constitutive activity correlated with increased affinity of the receptor mutants for epinephrine. Increased constitutive activity was interpreted as the result of enhanced translational motion of helix 3 induced by the mutations.

It has been reported that constitutive activation of the α_{1B}AR could result from mutations of the $Asp^{125(3.32)}$ (i.e. the helix 3 aspartate of the putative ligand binding site) into alanine or lysine and those of $Lys^{331(7.36)}$ in helix 7 into different residues [24]. The $Asp^{125(3.32)}Lys/Lys^{331(7.36)}Asp$ switch mutant displayed similar basal signaling activity as the wild type receptor. These results together with those from pH dependent binding studies suggested a potential mechanism of α_{1B}AR activation. The hypothesis was that, when the α_{1B}AR ligand binding pocket is empty, a salt bridge between $Lys^{331(7.36)}$ (helix 7) and $Asp^{125(3.32)}$ (helix 3) constrains the receptor in its ground state. Destruction of the $Asp^{125(3.32)}$/$Lys^{331(7.36)}$ salt bridge might release a structural constraint thus triggering the α_{1B}AR activation [24]. However, our group could not confirm that mutations of $Asp^{125(3.32)}$ increase the constitutive activity of the α_{1B}AR [3]. The reasons for this discrepancy should be further investigated.

Constitutive activation could also be induced by mutating $Ala^{204(5.39)}$ into valine in the hamster α_{1B}AR and Met^{292} into leucine in the rat α_{1A}AR [25].

Constitutively activating mutations of the α_{1B}AR have been described also at two residues in the cytosolic half of helix 6, $Glu^{289(6.30)}$ and $Phe^{303(6.44)}$ [26]. The mutations of $Glu^{289(6.30)}$, at the cytosolic end of helix 6, into Ala, Asp, Phe, Lys and Arg resulted in a marked increase in the constitutive activity of the receptor as well as in its affinity for epinephrine. It is worth noting that, in one of the three crystal structures of dark rhodopsin released so far, $Glu^{289(6.30)}$ is involved in a salt bridge with the arginine of E/DRY motif [27]. MD simulations on the α_{1B}AR models based upon rhodopsin structure suggest that the salt bridge between positions 3.50 and 6.30 might be a feature of the ground state [26].

Replacement of the highly conserved $Phe^{303(6.44)}$ in helix 6 with leucine also resulted in increased constitutive activity of the α_{1B}AR [26]. In contrast, mutations of this residue into Ala, Gly, Asn and Tyr impaired the receptor-mediated inositol phosphate response. It was suggested that the replacement of $Phe^{303(6.44)}$ with leucine perturbs the helix 3-helix 6 packing interactions thus resulting in constitutive activation of the α_{1B}AR.

Finally, it was recently found that also in the human α_{1A}AR mutations of $Asp^{123(3.49)}$, the aspartate of the E/DRY sequence, into isoleucine and of $Ala^{271(6.34)}$ into glutamate or lysine increase the constitutive activity of the receptor, similarly to the α_{1B}AR [28].

Altogether these findings support the hypothesis that the process of agonist-independent activation for both the α_{1A} and α_{1B}AR subtypes involves, at least in part, structural determinants in the cytosolic halves of helices 3 and 6.

4. Constitutively active mutants as a tool to investigate receptor activation

The analysis of the pharmacological and molecular properties of CAMs (Fig. 3) has been instrumental to generate hypothesis on the molecular basis of receptor activation. In particular, a detailed analysis of the properties of the adrenergic CAMs resulted in the elaboration of the ''allosteric ternary complex model'' to describe receptor activation [15]. The results of this study suggested that in the absence of agonist a structural constraint keeps the wild type receptor inactive (*R*) preventing sequences of the intracellular loops to interact with the G proteins. Activating mutations might release such constraint triggering the conversion into the active state (*R**), which couples to G proteins. One hypothesis is that activating mutations mimic, at least to some extent, the conformational change triggered by agonist binding to GPCR. This extended version of the ternary complex model introduced for the first time an explicit isomerization constant regulating the equilibrium of GPCR between at least two interconvertible allosteric states, *R* (inactive or ground state) and *R** (active) (Fig. 3).

However, a precise structural description of the molecular changes underlying the conversion from the inactive states (*R*) to the active states (*R**) of the receptor is still lacking. Which is the nature of the ''constraint''? Is it similar for GPCRs of different classes? How can mutations in apparently unrelated regions of a GPCR release this constraint? Are CAMs representative of the agonist-bound wild type receptor?

To address some of these questions, we combined site-directed mutagenesis of the α_{1B}AR and molecular dynamics simulations of receptor models [17,23,26]. The results of these studies highlighted the important role played in receptor activation by the E/DRY motif at the cytosolic end of helix 3 (Fig. 1), which is highly conserved in GPCRs of the rhodopsin-like class. As mentioned above, mutations of the aspartate of the E/DRY increased the constitutive activity of the α_{1B}AR [17]. Increased constitutive activity was also found after mutating the acidic residue of the E/DRY motif in other receptors

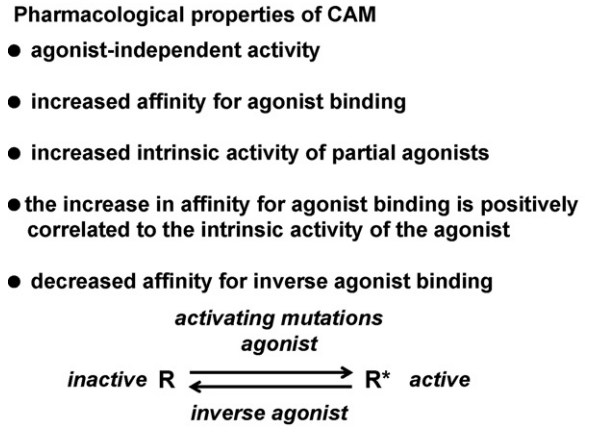

Fig. 3 – Pharmacological properties of constitutively active adrenoceptor mutants. These properties, measured for different constitutively active adrenoceptor mutants [15,17], can be interpreted in the context of the "allosteric ternary complex model" [15]. However, these pharmacological features have not been systematically tested for CAMs of other GPCRs. According to the "allosteric ternary complex model", GPCRs exist in equilibrium between at least two interconvertible allosteric states, *R* (inactive or ground state) and *R (active). Activating mutations might mimic, at least to some extent, the conformational change triggered by agonist binding to GPCR.**

including rhodopsin [18] and the β_2-AR [19]. In contrast, mutations of the conserved arginine in a number of GPCRs can profoundly impair receptor function [1,23].

In the latest computational model of the α_{1B}AR, shown in Fig. 4, which is based upon the first crystal structure of rhodopsin [27], the double salt bridge between the arginine of the E/DRY motif and both the adjacent aspartate and $Glu^{289(6.30)}$, as inherited from rhodopsin structure, constitutes a feature of the ground state. At the experimental level, similarly to the effect induced by mutating the aspartate of the E/DRY motif, mutations of the $Glu^{289(6.30)}$ markedly increased the constitutive activity of the α_{1B}AR [26].

In contrast, the structures of the active mutants, despite the different locations of the mutations, tend to share the release or weakening of one or both the charge reinforced H-bonding interactions involving $Arg^{143(3.50)}$ in the ground state. However, the lower conservation of the anionic amino acid at 6.30 as compared to that at 3.49 supports the hypothesis that the latter plays a more important role in stabilizing the ground state of GPCRs. Along the same line, in the latest structures of rhodopsin $Arg^{3.50}$ retains its interaction with the adjacent glutamate while loosing the charge-reinforced H-bond with $Glu^{6.30}$ [29,30].

Altogether these findings on the α_{1B}AR support the hypothesis that receptor activation involves the weakening or breaking of the interhelical interactions between the cytosolic halves of helices 3 and 6 (Fig. 4). This putative model of receptor activation seems to be shared by other GPCRs belonging to the rhodopsin-like class (reviewed in [31]). However, other potentially constraining interactions have been described in GPCRs beyond those involving helices 3 and 6 [32]. A particularly important challenge for the future will be to explain how agonists can activate GPCRs and to what extent a common mechanism of activation is shared by GPCRs of different classes.

5. Constitutive activity of the wild type $\alpha_1$adrenoceptors

The "allosteric ternary complex model" predicts that the constitutive activity measured in cells depends on the amount of expressed receptor in its active state R^* [15]. This prediction is clearly supported by findings demonstrating that increasing density of GPCRs results in the progressive elevation of basal (agonist independent) receptor-mediated production of second messengers [15,28]. This implies also that native GPCRs might display spontaneous activity in physiological systems which has been demonstrated only for few receptors [33]. There are documented differences in the extent of constitutive activity among even highly related GPCRs (reviewed in [34]) and this might depend on differences in their intrinsic activation properties, on the cellular environment in which signaling is measured as well as on experimental factors. Some discrepancies can also be observed comparing results on the same receptor from different groups, as it will be illustrated here below. Such discrepancies highlight the general difficulty of measuring constitutive activity which is often quite small for native receptors.

For the α_1AR subtypes, we reported constitutive activity of the wild type α_{1A} and α_{1B}AR when the receptors were overexpressed in COS-7 cells [28]. The spontaneous activity of the α_{1B} was greater than that of the α_{1A}AR expressed at similar levels (3–4 pmol/mg of protein). However, in another study basal activity of wild type α_1AR subtypes overexpressed in HEK cells was not observed [35]. A possible explanation of this discrepancy is that the maximal expression level of receptors (1 pmol/mg of protein) at which basal activity was measured in this study [35] was lower than that in Ref. [28] (3–4 pmol/mg of protein).

For the wild type α_{1D}AR, constitutive activity and internalization was reported for the receptor expressed in Rat fibroblasts [36,37]. The constitutive activity of the α_{1D}AR was also observed in physiological systems like in aorta and mesenteric arteries where it could inhibited by inverse agonists [38]. In contrast, for the α_{1A} or α_{1B}AR the constitutive activity in physiological systems has not been investigated (Table 1).

Altogether, these findings indicate that there might be subtle differences in the constitutive activity of native α_1AR subtypes which might be fine-tuned by nature to fulfill specific roles in signaling. Such differences should be further explored and the elucidation of their physiological implications might represent an important area of investigation.

6. Inverse agonism at the $\alpha_1$adrenoceptors

Negative efficacy, i.e. the capacity of an antagonist binding to its receptor to repress its spontaneous activity, has been a

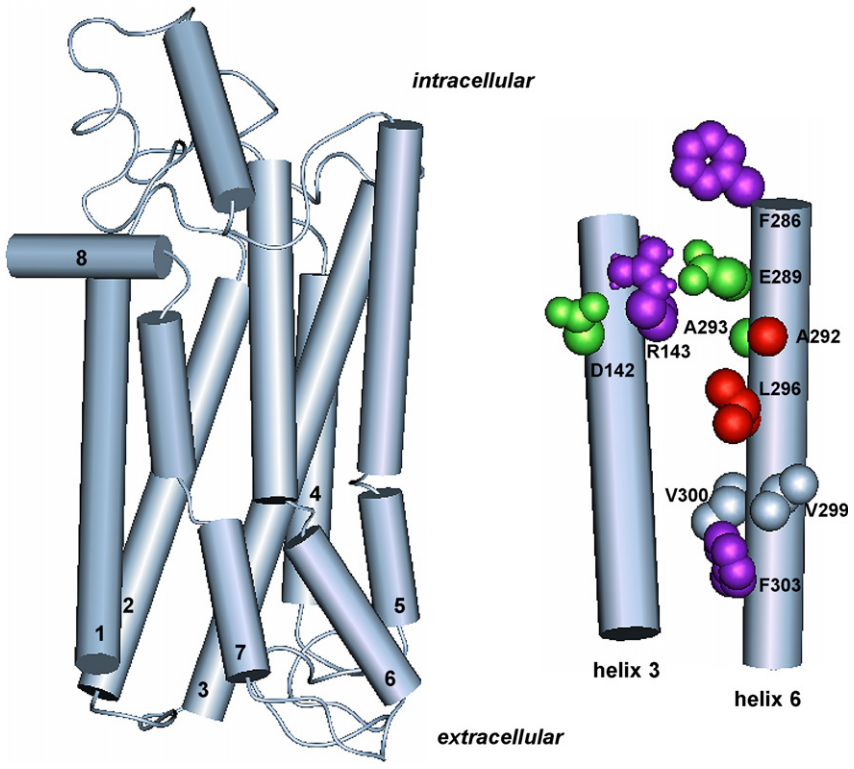

Fig. 4 – Relative position of helices 3 and 6 in the homology model of the wild type α_{1B}AR. Comparative modeling and molecular dynamic simulations were performed as described in Ref. [26]. The receptor is seen from a direction parallel to the membrane surface with the extracellular side on the bottom and the intracellular one on the top. The right view displays the amino acids of helices 3 and 6 involved in receptor activation. Van der Waals spheres whose radius has been reduced by 40% depict each side chain. The effect of mutations at each residue is depicted by their colour, with white representing no effect, green being constitutively active, red impaired receptor mediated signalling and violet being either impairing or constitutively activating depending upon the substituent amino acid.

concept implicit in receptor theory from the start (reviewed in [39]). However, it remained an undeveloped idea for many years largely because of the difficulty to find adequate experimental systems to test it. Both the availability of CAMs and of cell systems overexpressing wild type GPCRs raised widespread interest in drugs with negative efficacy commonly measured as the ability of inhibiting the agonist-independent activity of receptors [33,34]. In most studies, ligands displaying negative efficacy have been indicated with the term of inverse agonists or negative antagonists without any specific reference to the mechanistic basis of their effect which might differ among ligands [39].

A large number of findings on inverse agonism concern ligands binding to β-AR, dopamine and histamine receptors (reviewed in [33,34]) whereas very little is known on antagonists of the α1AR subtypes. In one study, 24 alpha-antagonists differing in their chemical structures were tested for their effect on the basal activity of both the wild type α_{1A} and α_{1B}AR subtypes and on their CAMs [28]. The vast majority of alpha-antagonists displayed inverse agonism. However, the various alpha-antagonists differed in their negative efficacy and some of these differences depended on the α_1AR subtype. In fact, a large number of structurally different alpha-antagonists including all the tested quinazolines were inverse agonists at both the α_{1A} and α_{1B}AR subtypes. In contrast, several *N*-arylpiperazines displayed different properties at the two α_1AR subtypes being inverse agonists with profound negative efficacy at the α_{1B}AR, but not at the α_{1A}AR.

Inverse agonism has also been observed at the recombinant α_{1D}AR subtype as well as on the receptor expressed in arteries [36,38].

Many clinically effective medicines can be shown to be inverse agonists [34]. The therapeutic benefit of inverse agonists in diseases related to spontaneous activating mutations of GPCRs is quite obvious. However, a question which remains to be answered is whether therapeutic differences and benefits exist in the clinical use of drugs having negative efficacy *versus* those that behave as neutral blockers at native receptors. With respect to this question it is important to highlight that drugs with different degrees of negative efficacy might differ in their ability to induce upregulation of GPCRs upon chronic treatment.

In fact, beyond their ability to decrease constitutive activity, it has been reported that inverse agonists are also able to induce the ''upregulation'' of several CAMs. For the α_1AR subtypes, it has been reported that a number of inverse agonists increase the receptor number measured by ligand binding of a CAM, but not that of the wild type α_{1B}AR [40].

The effect of inverse agonists on receptor ''upregulation'' has been reported for different GPCRs and gave origin to a

variety of interpretations. One hypothesis is that the ability of inverse agonists to increase receptor number is linked to the instability of several CAMs. For example, CAMs of the histamine H_2 and of the α_{1B}AR carrying mutations of the aspartate of the E/DRY motif, displayed an increased instability compared to the wild type receptors as demonstrated by the time-dependent loss of binding activity at 37 °C which could be prevented by the incubation with inverse agonists [20]. However, as demonstrated for the H_2 histamine receptor, receptor instability has been reported for mutants which are constitutively active as well as inactive [20]. Finally, the stabilization of receptor function is not a unique property of inverse agonists since it can be induced by various ligands behaving as agonists or antagonists depending on the receptor system.

In conclusion, inverse agonism has emerged as a new dimension to the study of functional chemistry of receptors. Answering to many open questions about the therapeutic implications of inverse agonism will remain an interesting and important area of investigation in the next years.

7. Conclusions

The studies on CAMs of the α_1AR subtypes and of other GPCRs have had an important impact on our understanding of the molecular mechanisms underlying GPCR activation and inverse agonism. They provided further insight into several basic aspects of molecular pharmacology and drug action, a field in which the contribution of Arthur Hancock will be remembered forever. Despite the large number of studies on GPCRs, several important questions remain to be answered. Structural information at high resolution on other GPCRs than rhodopsin will be necessary to improve our understanding of GPCR activation and drug action at a molecular level. In addition, it will be important to understand the implications of drugs with negative efficacy *in vivo*, evaluate their benefits and improve future therapeutic strategies.

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