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Molecular mechanisms involved in the activation and regulation of the α_1-adrenergic receptor subtypes [1]

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Abstract

The adrenergic receptors (ARs) belong to the superfamily of membrane-bound G protein coupled receptors (GPCRs). Our investigation has focused on the structure–function relationship of the α_{1b}-AR subtype used as the model system for other GPCRs. Site-directed mutagenesis studies have elucidated the structural domains of the α_{1b}-AR involved in ligand binding, G protein coupling or desensitization. In addition, a combined approach using site-directed mutagenesis and molecular dynamics analysis of the α_{1b}-AR has provided information about the potential mechanisms underlying the activation process of the receptor, i.e. its transition from the 'inactive' to the 'active' conformation.
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1. The α_1-adrenergic receptor subtypes

The receptors for a large number of hormones and neurotransmitters regulate cellular activity via the intermediary role of guanine nucleotide regulatory proteins (G proteins) [1]. Among these receptors, the adrenergic receptors (ARs) mediate the effects of epinephrine and norepinephrine by coupling to several of the major signalling pathways modulated by G protein. The adrenergic receptor family now includes nine different gene products, three β (β_1, β_2, β_3), three α_2 ($\alpha_{2\text{-C10}}$, $\alpha_{2\text{-C4}}$, $\alpha_{2\text{-C2}}$) and three α_1 (α_{1a}, α_{1b}, α_{1d}) receptor subtypes.

The α_1-AR is present in many tissues including brain, heart, blood vessels, liver, kidney, prostate and spleen. In these tissues the α_1-ARs mediate a variety of physiological effects such as neurotransmission, vasoconstriction, cardiac inotropy and chronotropy, and glycogenolysis [2]. Radioligand binding studies in different rat tissues have demonstrated two classes of α_1-AR binding sites, 'A' and 'B', with high and low affinity for the α-AR antagonists WB4101 and phentolamine, respectively [2]. After large-scale purification of the α_1-AR from smooth muscle cells DDT1 MF-2, a first receptor was cloned, unequivocally assigned to the pharmacological α_{1B} subtype and hence named α_{1b}-AR [3]. On the other hand, the pharmacological α_{1A} subtype is mainly coded by the α_{1a}-AR initially cloned from a bovine brain library and inappropriately named α_{1C}-AR [4]. Finally, the cloned α_{1d}-AR (initially named α_{1A}-AR or $\alpha_{1A/D}$-AR) represents a receptor subtype not clearly identified by previous pharmacological studies [5].

Functional differences in α_1-AR-mediated responses have been described in various tissues. Activation of the α_1-ARs causes polyphosphoinositide (PI) hydrolysis catalysed by phospholipase C (PLC) via pertussis toxin-insensitive G proteins in almost all tissues where this effect has been examined. Recent studies have shown that other signalling pathways can be activated upon α_1-AR stimulation such as phosphatidylcholine hydrolysis and phospholipase A2 (for review, see Ref. [2]). However, the comparison among different α_1-AR-mediated responses in various tissues has not allowed the assessment of any clear signalling differences among distinct α_1-AR subtypes.

In vivo studies aiming to assess a specificity of the functional responses mediated by distinct α_1-AR subtypes have been hampered by the fact that the subtype-selective drugs are only moderately selective and might interact with both other adrenergic and non-adrenergic receptors. Thus, the functional implications of α_1-AR heterogeneity and their

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physiological relevance remain largely unknown. Recently, targeted gene disruption has been increasingly used to elucidate the in vivo functions of several receptor subtypes. Thus, to contribute to the elucidation of the physiological role of the α_1-AR subtypes in vivo we have used gene targeting to create knockout mice lacking the α_{1b}-AR [6]. Our findings provide strong evidence that the α_{1b}-AR can be a mediator of the blood pressure response as well as of the aorta contractility induced by α_1-agonists. This was demonstrated by the finding that the mean arterial blood pressure response to phenylephrine was decreased by 45% in $\alpha_{1b}-/-$ as compared to $+/+$ mice. In addition, phenylephrine-induced contractions of aortic rings were also decreased by 25% in $\alpha_{1b}-/-$ mice. The α_{1b}-AR knockout mouse model provides a useful tool to elucidate the functional specificity of different α_1-AR subtypes and to better understand the effects of adrenergic drugs. A full understanding of the functional implications of adrenergic receptor heterogeneity awaits the knockout of all AR subtypes as well as the intercross among different knockout models.

2. Structure–function relationship of the α_1-AR subtypes

The three cloned α_1-AR subtypes share similar structural features characterized by the seventh transmembrane domain (TMD) motif common to other G protein coupled receptors (Fig. 1). The TMDs of the three α_1-ARs show 65–75% of amino acid identity when compared among each other. Potential sites of phosphorylation by protein kinase C and A are present in the intracellular domains of all three receptor subtypes, suggesting that protein phosphorylation might play a role in receptor regulation.

Like most G protein coupled receptors (GPCRs), the α_1-AR subtypes share three fundamental functional features: (1) they discriminate and bind the appropriate ligands; (2) they activate specific G protein-effector systems; (3) their functional response can be dynamically regulated, often resulting in the attenuation of receptor-mediated effects (desensitization). The focus of much investigation has centred on understanding the structural basis for each of these functional properties.

2.1. Ligand binding

Several studies have focused on the molecular interactions of the endogenous catecholamines, epinephrine and norepinephrine, with different AR subtypes. Epinephrine and norepinephrine contain a protonated amino group separated from the aromatic catechol ring by a β-hydroxylethyl chain. The molecular requirement for catecholamine binding to the AR should include the electrostatic interaction between the receptor and the amino group of the ligand, hydrogen bonds

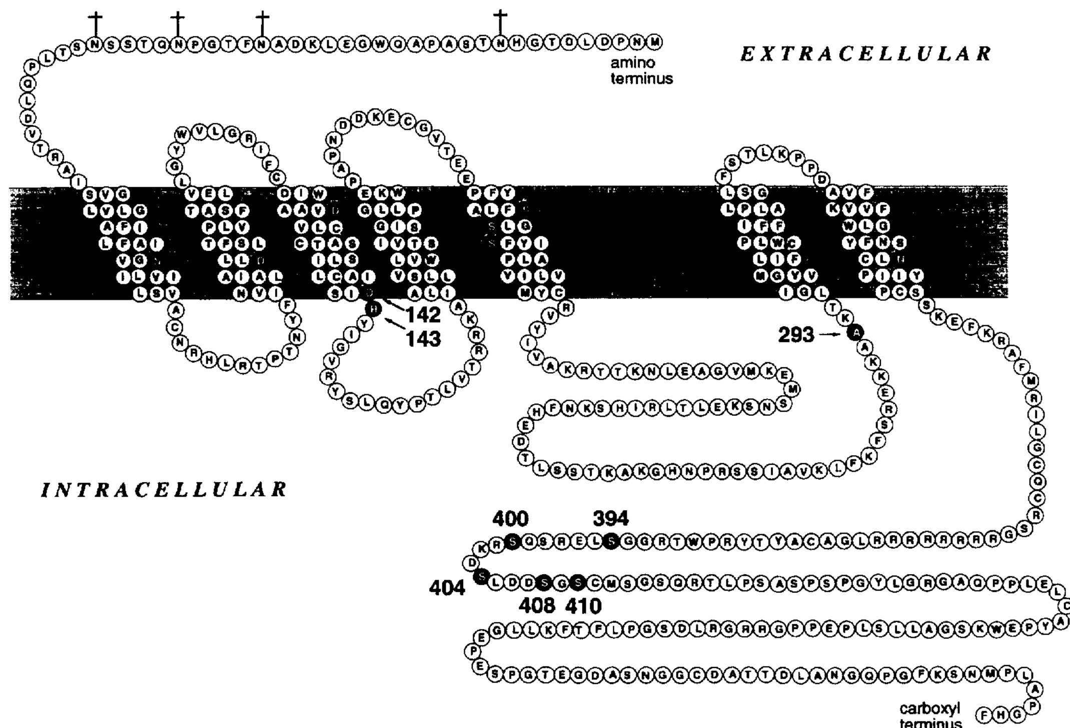

Fig. 1. Topographical model of the α_{1b}-AR. The black circles indicate the amino acids mentioned in the text. The aspartate (D125) in the third transmembrane domain and the three serines (S207, S208 and S211) in the fifth transmembrane domain participate to the binding of catecholamines in different α_1-AR subtypes [13]. N63, D91, R143 and N344 contribute to form a 'polar pocket' within the transmembrane domains [14]. D142 and A293 are the positions at which constitutively activating mutations have been described [17,14]. S394 and S400 in the C-tail are the phosphorylation sites for protein kinase C, whereas S404, S 408 and S410 are the phosphorylation sites for G protein coupled receptor kinases [28].

between donor/acceptor sites of the receptor and the β-hydroxyl as well as the catechol *meta*- and *para*-hydroxyl groups of the ligand, and finally van der Waals attractive interactions. Mutagenesis studies of the β_2- [7] and α_{2A}-AR [8] suggested that the amino group of the catecholamines makes an electrostatic interaction with the carboxylate side chain of an aspartate on TMD III which is highly conserved in all GPCR binding amine ligands. On the other hand, there is evidence that the catechol *meta*- and *para*-hydroxyl groups interact with serine residues present in TMD V of all GPCRs which bind catecholamines with high affinity.

The serines of TMD V (Fig. 1) range from two to three in different receptors and the individual role of each of them has been assessed by site-directed mutagenesis only for a few GPCRs, including the β_2- [9], α_{2A}- [8] and α_{1a}-AR [10] subtypes as well as dopamine D1 [11] and D2 [12] receptors. These studies have clearly shown that, despite their conservation, the role of individual serines in ligand binding and/or receptor activation can vary among different catecholamine receptors. Our recent work on the α_{1b}-AR [13] provided solid evidence that D125 in TMD III interacts with the amino group of both agonists and antagonists, whereas S207 in TMD V interacts with both catecholic hydroxy groups of(−)-epinephrine. On the other hand, our findings indicated that both serines 208 and 211 in TMD III are crucially involved in receptor activation. This was shown by the fact that the mutation of both S208 and S211 completely impaired epinephrine-induced activation of phospholipase C, despite the fact that epinephrine binding was only modestly changed by the mutations.

The results of site-directed mutagenesis have been interpreted by molecular dynamics (MD) analysis of the three-dimensional model of the α_{1b}-AR previously described [14]. We have performed MD simulations of the (−)-epinephrine–α_{1b}-AR complex, testing different combinations of distance constraints between S207 and the catecholic oxygens of the ligand. Finally, we selected the minimized average structure resulting from the simulations in which S207 has been constrained to act as an H-bonding donor and acceptor for the *meta*- and *para*-hydroxyl groups, respectively, of the ligand. In fact, this interaction pattern involving S207 allows the cationic nitrogen atom of the ligand to perform a strong charge-reinforced H-bonding interaction with D125 in TMD III. This is in agreement with the experimental findings showing that D125 in TMD III is essential for both agonist and antagonist binding. Moreover, the constrained interaction with S207 allows the *meta*-and *para*-hydroxyl groups of epinephrine to form additional hydrogen bonds with S208 and S211, respectively. We propose that S207 makes a strong interaction with both the catecholic hydroxy groups of (−)-epinephrine. Such an interaction seems to be the necessary step for promoting other stabilizing or functionally important intermolecular interactions. This is in agreement with our experimental findings showing that the extent of the decrease of binding affinity induced by mutation of S207 into alanine accounts for the breakage of more than one hydrogen bonding interaction (the disruption of a strong hydrogen bond is expected to result in about a 20-fold reduction of binding affinity).

Very little is known so far about the receptor amino acids which interact with different antagonists as well as about the structural basis underlying receptor selectivity for different ligands. Ongoing studies in our laboratory are investigating the interaction pattern of different antagonists at both the α_{1a}- and α_{1b}-AR subtypes.

2.2. G protein coupling

Several studies have provided information about the domains of the α_{1b}-AR involved in receptor–G protein coupling. A chimeric β_2/α_{1b}-AR in which the third intracellular loop of the β_2-AR was replaced with the corresponding region of the α_{1b}-AR was able to activate phospholipase C [15]. This result clearly indicated that the third intracellular loop of the α_{1b}-AR was able to confer to the β_2-AR the ability to couple to phospholipase C. We next investigated which amino acid sequences in the third intracellular loop of the α_{1b}-AR determine the selectivity of receptor–G protein coupling. We determined that 27 residues of the α_{1b}-AR (residues 233–259) derived from the N-terminal portion of its third intracellular loop were sufficient to confer to the β_2-AR the ability to activate PI hydrolysis [15].

Another finding of our mutagenesis studies indicated that in the α_{1b}-AR the C-terminal portion of the third intracellular loop plays a crucial role in receptor–G protein coupling [16,17]. Mutations of A293 in this region of the loop resulted in the constitutive activation of the receptor (Figs. 1 and 2). In the absence of agonist, cells expressing the mutated receptor exhibited higher basal levels of inositol phosphates than cells expressing the wild type α_{1b}-AR (Fig. 2). Remarkably, all 19 substitutions of A293 conferred constitutive activity to various extents [17]. All these mutated receptors demonstrated higher affinity for agonist binding and higher potency of agonists to activate phospholipase C than the wild type. G protein coupled receptors exist in equilibrium between the 'active' and 'inactive' state. The 'active' state of the receptor, resulting from both G protein and agonist binding, is char-

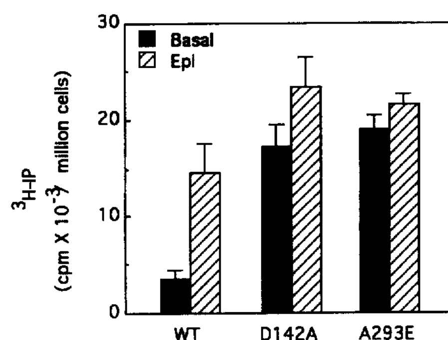

Fig. 2. Constitutively active α_{1b}-AR mutants. Total inositol phosphates (IPs) were measured in COS-7 cells expressing the wild type α_{1b}-AR (WT) and its constitutively active mutants D142A and A293E in the absence (basal) or presence of 10^{-4} M epinephrine (epi).

acterized by high affinity for the agonist and leads to G protein-mediated activation of the effector system [18]. The properties of the α_{1b}-AR constitutively active mutants (high affinity for agonists and activation of the G protein) are similar to those of the 'active' state of the receptor. This suggests that these mutated receptors mimic the 'active' conformation of the wild type α_{1b}-AR to various degrees, perhaps due to conformational changes similar to those induced by agonist when it binds to the wild type receptor.

Interestingly, the increased basal activity of the constitutively active α_{1b}-AR mutants was inhibited by some classical α-receptor antagonists. 'Competitive antagonists' are thought to act by blocking the access of the agonist to the binding site by steric hindrance. On the other hand, 'negative antagonists' or 'inverse agonists' have the defining property of inhibiting the agonist-independent activity of the receptor. The existence of antagonists with negative intrinsic activity [20] has been previously documented for some G protein coupled receptors [19,20]. Negative antagonism at the α_{1b}-AR had not been previously described. Thus, the constitutively active α_{1b}-ARs seem to provide a useful tool to further characterize different α_1-specific ligands with respect to their intrinsic activities and pharmacological properties. These studies might contribute to better understanding of the effects of drugs in vivo and to assess novel pharmacological parameters.

The discovery of the constitutively active adrenergic receptors [21] has also encouraged the search for spontaneously occurring activating mutations of other G protein coupled receptors which might be responsible for certain pathologies [22,23].

2.3. Receptor desensitization

Agonist-induced desensitization has been described for a variety of G protein coupled receptors [24]. We have provided evidence that the response mediated by the α_{1b}-AR expressed in various cell types could undergo desensitization upon exposure to agonists as well as to the phorbol ester [25]. In addition, a correlation between agonist-induced phosphorylation and desensitization could be demonstrated. Our results indicated that the biochemical mechanisms underlying epinephrine versus phorbol ester-induced phosphorylation of the α_{1b}-AR are different. This was demonstrated by the fact that the protein kinase C (PKC) inhibitor RO-318220 could abolish the effect of phorbol ester on phosphorylation of the α_{1b}-AR without altering that of epinephrine. On the other hand, rapid agonist-dependent regulation of the α_{1b}-AR seems to be mediated by G protein coupled receptor kinases (GRKs) [26]. GRKs have the unique property of phosphorylating G protein coupled receptors once they are occupied by agonists [27]. We demonstrated that overexpression of GRK2 or GRK3 could increase epinephrine-induced phosphorylation of the wild type α_{1b}-AR above basal as compared to that of the receptor expressed alone. On the other hand, overexpression of the dominant negative GRK2 (K220R) mutant impaired agonist-induced phosphorylation of the receptor. Recently, we have assessed that a stretch of serines in the C-tail of the receptor represents the main sites of phosphorylation (Fig. 1) [28]. Following extensive mutagenesis studies, we have been able to identify the three serines (S404, S408 and S410) involved in agonist-induced phosphorylation from the two serines (S394 and S400) involved in PKC-mediated phosphorylation of the α_{1b}-AR [28].

3. The activation process of the α_{1b}-AR

The observation that mutations of GPCR can activate the receptor suggests that in the absence of agonist a structural constraint keeps the wild type receptor inactive (**R**), preventing sequences of the intracellular loops from interacting with the G proteins. Activating mutations might release such a constraint, triggering 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 GPCRs.

A structural description of the molecular changes underlying the conversion from **R** to **R*** is still lacking. One obvious problem is our limited knowledge of the three-dimensional structure of GPCRs, due to difficulties linked to their non-degenerative purification and crystallization. However, this information, once available, will not entirely reveal how the receptors function. Thus, the elucidation of the process of receptor activation relies on the combination of different biochemical, biophysical, pharmacological and modelling approaches.

In a recent study [14,29], we have proposed a novel strategy to explore the structural and dynamic properties of the active state of a GPCR and the potential molecular changes correlated with the transition from **R** to **R***. Combining site-directed mutagenesis of the α_{1b}-AR and comparative molecular dynamics simulations of the wild type and several constitutively active receptor mutants we have built a theoretical model which defines the essential features of the pattern of conformers associated with **R** and **R***. In particular, molecular dynamics analysis was used to compare the structural/dynamic features of constitutively active mutants with those of the wild type α_{1b}-AR and to predict key residues, the mutations of which would either constitutively activate or inactivate the receptor.

We proposed that the equilibrium between the inactive (**R**) and active (**R***) states of the α_{1b}-AR depends, at least in part, on the prototropic equilibrium between the deprotonated (anionic) and protonated (neutral) forms, respectively, of D142 of the DRY motif located at the end of TMD III (Fig. 1). This is supported by the observation that replacement of D142 with the hydrophobic amino acids confers constitutive activity to the α_{1b}-AR, as shown in Fig. 2 [29].

Our analysis highlighted a series of intramolecular interactions that might be of fundamental importance for the process of receptor activation.

(1) The structural constraint stabilizing the α_{1b}-AR in its inactive state (**R**) is a network of H-bonding interactions among N63, D91, N344 and Y348 forming a conserved 'polar pocket' near the cytosol and R143 of the DRY sequence. Disruption of these intramolecular interactions induced by replacing N63 with alanine constitutively activates the α_{1b}-AR [14].

(2) The receptor 'active states' induced either by protonation of D142 (**R***) or by constitutively activating mutations, despite being not structurally identical, all show two features in common: the shift of R143 out of the polar pocket and the cytosolic exposure of several residues of the intracellular loops. Therefore, we suggest that the main role of R143 is to mediate receptor activation, allowing several amino acids in the second intracellular loop and both N-terminal and C-terminal portions of the third intracellular loop to attain the right configuration for the formation of a site with docking complementarity with the G protein. This is in agreement with the experimental finding that mutations targeting R143 totally inactivate the α_{1b}-AR [14].

Our molecular dynamics analysis has focused so far on the transition from **R** to **R*** in the absence of agonist. It will be our next goal to show that the postulated active conformers thus found are also shared within the conformational space of the α_{1b}-AR bound to agonists of varying efficacies. This will require the extensive characterization of the docking sites of the receptor for various ligands.

The findings obtained with the constitutively active α_{1b}-AR mutants might provide interesting generalities about the molecular mechanisms underlying the activation process of other α_1-AR subtypes. We believe that a careful interdisciplinary approach combining site-directed mutagenesis, molecular dynamics and thermodynamic analysis of receptor mutants is useful to elucidate the structural/dynamic properties of GPCRs.

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