Amino acids of the α_{1B}-adrenergic receptor involved in agonist binding: differences in docking catecholamines to receptor subtypes

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Abstract Site-directed mutagenesis and molecular dynamics analysis of the 3-D model of the α_{1B}-adrenergic receptor (AR) were combined to identify the molecular determinants of the receptor involved in catecholamine binding. Our results indicate that the three conserved serines in the fifth transmembrane domain (TMD) of the α_{1B}-AR play a distinct role in catecholamine binding versus receptor activation. In addition to the amino acids D125 in TMDIII and S207 in TMDV directly involved in ligand binding, our findings identify a large number of polar residues playing an important role in the activation process of the α_{1B}-AR thus providing new insights into the structure/function relationship of G protein-coupled receptors.

Key words: G protein-coupled receptor; Adrenergic receptor; Catecholamine; Molecular dynamics

1. Introduction

The adrenergic receptors (AR) mediate the functional effects of catecholamines like epinephrine and norepinephrine by coupling to several of the major signalling pathways modulated by G proteins. 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_B, α_{1D}) receptor subtypes.

Like almost all G protein-coupled receptors (GPCR), the AR family shares the presence of seven hydrophobic regions that are believed to form a bundle of α-helical transmembrane domains (TMD), connected by alternating intra- and extracellular hydrophilic loops. Mutational analysis of the ARs has revealed that the TMD contribute to the formation of the ligand binding pocket, whereas amino acid sequences of the intracellular loops appear to mediate receptor–G protein coupling [1].

The pharmacological heterogeneity of various AR subtypes must result from differences of their ligand binding pockets. However, identification of the interaction sites on the receptors for a wide range of synthetic agonists and antagonists remains a complex task.

Several studies have focussed 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 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 [2]. Mutagenesis studies of the β_2- [3] and α_{2A}-AR [4] suggested that the amino group of the catecholamines makes an electrostatic interaction with the carboxylate side chain of an aspartate on TMDIII which is highly conserved in all GPCR-binding amine ligands. This aspartate is involved in high-affinity binding of agonists as well as antagonists at both the β_2- and α_{2A}-AR. On the other hand, there is evidence that the catechol *meta*- and *para*-hydroxyl groups interact with serine residues present in TMDV of all GPCR which bind catecholamines with high affinity.

The serines of TMDV range from two to three in different receptors (Fig. 2) and the individual role of each has been assessed by site-directed mutagenesis only for few GPCR including the β_2- [5], α_{2A}- [4] and α_{1A}-AR [6] subtypes as well as dopamine D1 [7] and D2 [8] 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. Thus, the results obtained on catecholamine interaction with one receptor cannot be directly extrapolated to another receptor subtype.

In this study, we have investigated the catecholamine binding site of the α_{1B}-AR subtype [9] combining site-directed mutagenesis of a large number of amino acids with molecular dynamics analysis of the 3-D model of the α_{1B}-AR bound to (−)-epinephrine. Our results indicate a distinct role of the three conserved serines in TMDV of the α_{1B}-AR in catecholamine binding versus receptor activation, providing striking evidence that the docking sites for catecholamines can differ even among closely related AR subtypes.

2. Materials and methods

2.1. Construction of mutated α_{1B}-ARs

The cDNA encoding the hamster α_{1B}-AR [9] was mutated by polymerase chain reaction-mediated mutagenesis technique using Taq DNA polymerase (Boehringer). The mutated DNA fragments obtained were digested with the appropriate enzymes and cloned into the expression vector pRK_5 containing the α_{1B}-AR cDNA. Recombinant clones were isolated and sequenced by cycle sequencing using Exo^- *Pfu* polymerase (Stratagene). For permanent expression in Rat-1 cells, the receptor cDNAs were subcloned into the pZip-Neo containing the neomycin resistance gene as previously described [10].

2.2. Cell culture and transfections

COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium

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(DMEM) supplement with 10% fetal calf serum (FCS) and transfected with different DNAs following the DEAE-dextran method, as previously described [9]. COS-7 cell (0.5×10^6) grown in 35-mm dishes were transfected with the DNA (2 μg/million of cells) encoding the various receptors and harvested 48 h after their transfection. For permanent expression, Rat cells were transfected with the pZip-Neo containing the neomycin resistance gene and the cDNA of the receptors using Ca^{2+}-phosphate precipitation. Clones resistant to the antibiotic G418 (300 μg/ml) were isolated and tested for their ability to bind the α_1-AR antagonist [^{125}I]HEAT [10].

2.3. Ligand binding

Membrane preparations derived from cells expressing the different α_{1B}-ARs and ligand-binding experiments using [^{125}I]HEAT (Dupont, New England Nuclear) were performed as previously described [9,10]. Prazosin (10^{-6} M) was used to determine non-specific binding. For competition curves, a final [^{125}I]HEAT concentration of 80 pM was used. For saturation curve analysis, [^{125}I]HEAT concentrations ranged from 10 to 500 pM. Data were analysed by computer, using an interactive non-linear regression program (LIGAND).

2.4. Inositol phosphate determination

For the determination of labelled inositol phosphates, COS-7 or Rat cells expressing the different α_{1B}-ARs were grown in 35-mm dishes and labelled with [^{3}H]inositol (Anawa) at 3–5 μCi/ml for 15–18 h in inositol-free DMEM supplemented with 1% FCS. After labelling, cells were stimulated for 45 min with (−)-epinephrine in the presence of 20 mM LiCl. Total inositol phosphates were extracted and separated as described in our previous studies [9,10].

2.5. 3-D model building of the α_{1B}-AR model and computational procedure

The 3-D building of the receptor model and molecular dynamics (MD) analysis were performed as extensively described in our previous work [11]. The input structure of the (−)epinephrine–α_{1B}-AR complex has been obtained by docking the agonist into the binding site of the minimized average structure of the wild-type α_{1B}-AR carrying the aspartate D142 in its protonated form [11]. The input structure was energy minimized and subjected to 150 ps of MD simulations. The structures averaged over the last 100 ps of the equilibrated time period of each MD simulation were then minimized. Modelling studies were performed with the molecular graphics package QUANTA (version 4.0; Molecular Simulations, 1990). Energy minimizations and MD simulations of the receptors were achieved on a HP-720 workstation by means of the program CHARMM (version 22) [12].

3. Results and discussion

3.1. Mutagenesis of the aspartate D125 in TMDIII

D125 of the α_{1B}-AR is homologous to the aspartate on TMDIII of the β_2- and α_{2A}-AR as well as D1-R involved in the interaction with the amino group of catecholamines. Mutation of D125 to alanine totally impaired the ability of the α_{1B}-AR to bind both agonists and antagonists. This is shown by the fact that COS-7 cells expressing the D125A receptor mutant displayed no specific [^{125}I]HEAT binding and no epinephrine-induced stimulation of inositol phosphate (IP) production. On the other hand, the expression of the D125A receptor was similar to that of the wild-type α_{1B}AR as shown by the immunoprecipitation of the phosphorylated receptors following $^{32}P_i$ labelling of transfected COS-7 cells (Fig. 1). These results strongly suggest that, similarly to the β_2- and α_{2A}-AR [3,4], also in the α_{1B}-AR the conserved aspartate D125 on TMDIII interacts with the functional amino group of both agonists and antagonists.

3.2. Mutagenesis of the serines in TMDV

S207, 208 and 211 of the α_{1B}-AR are homologous to the cluster of two to three serines conserved in TMDV of GPCR which bind catecholamines with high affinity (Fig. 2). To assess the interaction of these serines with the catechol *meta*- and *para*-hydroxyl groups, they were mutated into alanine and the receptor mutants were tested for their ability to interact with ligands carrying both hydroxyl groups (epinephrine and norepinephrine), the *meta*-hydroxyl (phenylephrine) or the *para*-hydroxyl group (synephrine). All the receptors mutated at the serines of TMDV displayed expression levels similar to that of the wild-type α_{1B}-AR. Mutations of S208 or S211 into alanine either individually or in combination did not result in any important effect on the receptor's ability to bind ligands (Table 1). On the other hand, mutation of S207 to alanine decreased the affinity of (−)- and (+)-epinephrine as well as that of (−)-norepinephrine of about 250-, 150- and 350-fold, respectively, without changing that of the antagonist prazosin (Table 1). The affinity of phenylephrine and synephrine were decreased of 10- and 2-fold, respectively.

To assess their activation properties, the receptor mutants were expressed transiently in COS-7 cells as well as permanently in Rat-1 cells. Dose–response curves of (−)-epinephrine in Rat cells expressing the S207A receptor indicated that the maximal levels of IP accumulation (R_{max}) were comparable to that of cells expressing the wild-type α_{1B}-AR, whereas its potency (EC_{50}) was about 100-fold lower in agreement with its reduced binding affinity (Fig. 2). Mutations of either S208 or S211 did not impair the receptor's ability to mediate epinephrine-induced IP accumulation. However, surprisingly the double mutant S208-S211 was completely impaired in its ability to be activated by epinephrine (Table 1 and Fig. 2).

These results indicate that the individual serines on TMDV play a different role in agonist binding versus receptor activation. Whereas S207 is primarily involved in catecholamine binding, the integrity of at least one of S208 and S211 is a crucial requirement for receptor activation despite having a marginal role on ligand binding. Thus, the role of the serines in TMDV of the α_{1B}-AR is strikingly different not only when compared to other catecholamine receptors, but also to the closely related α_{1A}-AR subtype. In this receptor, only two of

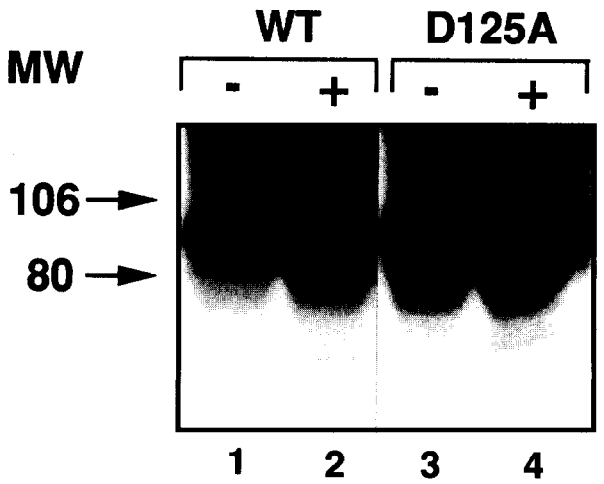

Fig. 1. Phosphorylation of the α_{1B}AR and its mutant D125A. COS-7 cells transfected with the DNA encoding the wild-type (WT, lanes 1 and 2) or mutated receptor (D125A, lanes 3 and 4) were labeled with $^{32}P_i$ and the phosphorylated receptors were immunopreciptated as described in [10]. Receptor phosphorylation was assessed in the absence (−) or presence (+) of PMA (4-phorbol 12-myristate 13-acetate). Position of the prestained molecular mass markers are indicated in kDa.

the three serines of the α_{1B}-AR are conserved (Fig. 2). Recent studies [6] have shown that in the α_{1A}-AR the serine homologous to S207 of the α_{1B}-AR is important for receptor activation, *but not* for catecholamine binding. On the other hand, both serines present in the α_{1A}-AR might contribute to form one, *but not* two hydrogen bondings with the catechol ring.

3.3. Mutagenesis of several other amino acids in the TMD

To investigate the role of other amino acids in catecholamine binding as well as agonist-induced receptor activation, we mutated several polar residues in different TMD of the α_{1B}-AR. For most receptor mutants the expression levels were not dramatically different than that of the wild-type α_{1B}-AR with the exception of S135A which displayed very low levels of expression (Table 1). None of the mutations induced any great change in the stereospecificity of (−)- versus (+)-epinephrine binding to the receptor. Many of them did not have any important effect either on ligand binding or on the receptor's ability to mediate catecholamine-induced IP accumulation. However, few mutations were identified having an effect on ligand binding and/or the activation process of the α_{1B}-AR. In particular, as shown in Table 1, mutation of Y203 in TMDV into alanine induced about 30- and 90-fold decrease in the receptor's binding affinity for (−)-epinephrine and (−)-norepinephrine, respectively, as well as a profound (≈80%) impairment of the receptor-mediated IP response. On the other hand, mutation of Y338 in TMDVII into alanine resulted in about 10-, 30- and 70-fold decrease of the binding affinity of (−)-epinephrine, (−)-norepinephrine and prazosin, respectively. The IP response mediated by the Y338A mutant was similar, if not greater, than that of the wild-type α_{1B}-AR.

Bovine	**α1A**	**193**	**G Y V L F S A L G S F Y V P L**
Hamster	**α1B**	**202**	**F Y A L F S S L G S F Y I P L**
Rat	**α1D**	**182**	**G Y A I F S S V G S F Y L P M**
Hamster	**β2**	**198**	**A Y A I A S S I V S F Y V P L**

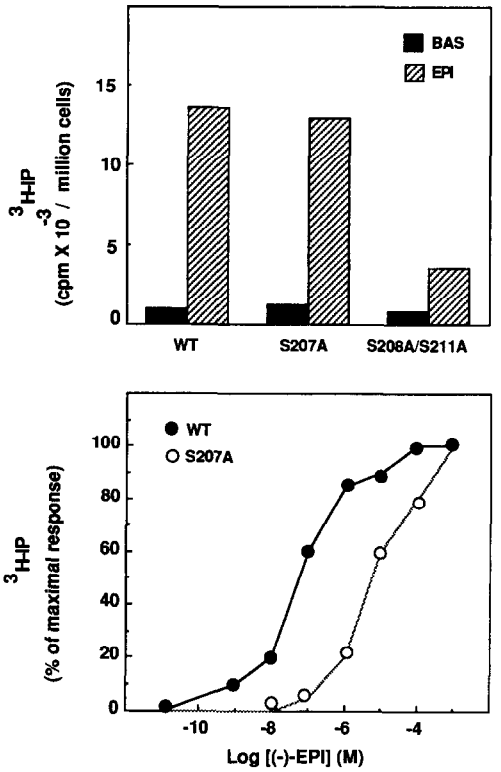

Fig. 2. Top panel: Alignment of the conserved serines in TMD V of the bovine α_{1A}AR [13], hamster α_{1B}AR [9], rat α_{1D}AR [14] and hamster β_2AR [3]. Middle panel: Total labeled inositol phosphates (^{3}H-IP) were measured in Rat-1 fibroblasts permanently expressing the wild-type α_{1B}AR (WT), its single mutant S207A or its double mutant S208A/S211A in the absence (BAS) or presence of 10^{-4} M (−)-epinephrine (EPI) for 45 min. Receptor expression ranged from 1 to 2 pmol/mg protein for all recepors. Bottom panel: Dose response of EPI to stimulate ^{3}H-IP accumulation in Rat-1 cells expressing the wild-type α_{1B}AR (WT) or its mutant S207A.

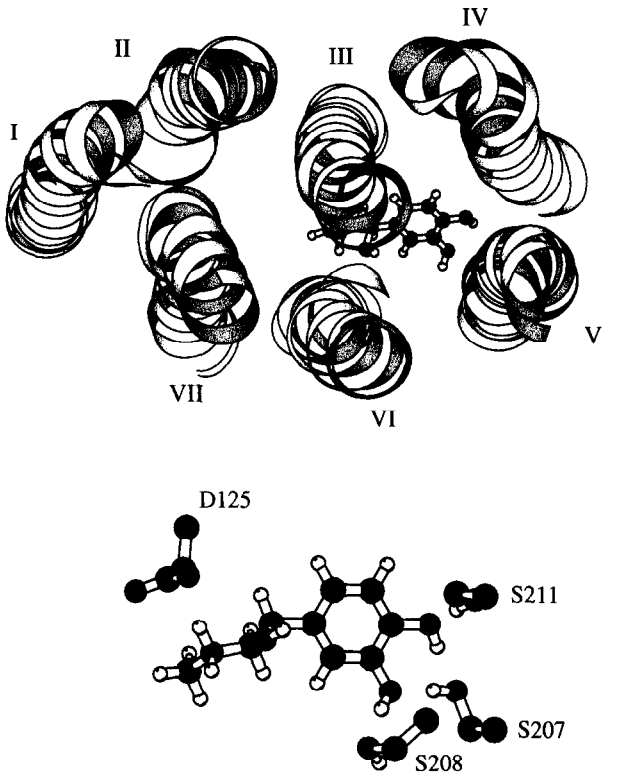

Fig. 3. Top panel: View of the minimized average structure of the (−)-epinephrine-α_{1B}AR complex in a direction parallel to the helix main axes from the intracellular side. Bottom panel: Details of the interaction of (−)-epinephrine with residues D125 in TMD III and serines 207, 208 and 211 in TMD V. Drawings were made with MOLSCRIPT (Kraulis, 1991).

Two other mutants, T130A and S132A, carrying mutations of T130 and S132 in TMDIII, were also impaired (≈50%) in their IP response without any important change in their ligand binding properties. The S135A mutant displayed an IP response similar to that of the wild-type α_{1B}-AR despite the fact that its expression levels were much lower. Thus, this mutant seems to be 'hyperactive' when compared to the wild-type α_{1B}-AR expressed at similarly low levels (results not shown).

3.4. Molecular dynamics analysis of the 3-D (−)-epinephrine–α_{1B}-AR complex

The results of site-directed mutagenesis have been inter-

preted by analysing the inter- and intramolecular interaction patterns involving the mutated amino acids in the 3-D model of the α_{1B}-AR previously described [11]. 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 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 TMDIII (Fig. 3). This interpretation is in agreement with the experimental findings showing that D125 in TMDIII is essential for both agonist and antagonist binding. Moreover, the constrained interaction with S207 allows the *meta*- and *para*-hydroxyl groups of epinephrine to perform additional hydrogen bonds with S208 and S211, respectively. The (−)-epinephrine–α_{1B}AR complex is further stabilized by the intermolecular van der Waals attractive interactions between the ligand and several amino acids, i.e., W121, L181, S211, F311 and L314.

In conclusion, on the basis of this analysis, we propose that S207 makes a strong interaction with both the catecholic hydroxy-groups of (−)-epinephrine. Such interaction seems to be the necessary step for promoting other stabilizing or functionally important intermolecular interactions. This interpretation is in agreement with our experimental findings showing that the extent of 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 boind is expected to result in about 20-fold reduction of binding affinity).

The results of the MD analysis also indicate that S208 and S211 in TMDV are mainly directed towards the TMDVI and TMDIV, respectively, and may exert a structural/functional role thus stabilizing the active conformation of the α_{1B}-AR. This agrees with the experimental findings showing that the integrity of at least one of S208 and S211 is crucial for receptor activation (Table 1 and Fig. 2).

Our analysis highlights several other amino acids including T130, S132, S135, Y203 and Y338, which might play a role in preserving the receptor structure and function rather than directly interacting with the agonist. In the theoretical model, these residues mainly occupy interhelical positions away from the putative agonist binding site, thus performing intramolecular interactions. In particular, T130 in TMDIII interacts with T174 on TMDIV whereas the conserved S132 in TMDIII makes H-bonding interactions with both the highly conserved W307 in TMDVI and N340 in TMDVII. This is consistent with the fact that both T130A and S132A mutants are partially impaired in their activation process.

Y203 in TMDV is rather directed towards the TMDIV, but it is close to the putative agonist binding site of the α_{1B}-AR. This might explain the fact that the mutation of Y203 into alanine results in a decrease of receptor activation as well as in a modest decrease of the affinity for agonist binding.

Concerning S135 in TMDIII, our MD analysis indicates that its role is to stabilize the interactions of the residues forming the 'polar pocket' of the receptor which plays a fundamental role in the activation process of the α_{1B}-AR [11]. Thus, the disruption of the 'polar pocket' might underlie the complex effects resulting from the mutation of S135 into alanine, i.e., on one hand, a profound impairment of receptor expression and, on the other, the 'hyperactivity' of the S135A mutant which seems fully activated despite its low expression levels.

Furthermore, C306 in TMDVI is directed towards TMDVII acting as a 'ridge' filling the 'groove' formed by F339 and N340 thus stabilizing the TMDVI/TMDVII pack-

Table 1
Ligand binding properties and IP accumulation in COS-7 cells expressing the wild-type α_{1B}-AR (WT) or mutant receptors

Receptor	Expression	IP levels	IC_{50} of ligands					
	B_{max} (pmol/mg)	R_{max} (% over basal)	(−)-EPI (μM)	(+)-EPI (μM)	(−)-NE (μM)	PHE (μM)	SYN (μM)	PRA (nM)
WT	1.2 ± 0.1	454 ± 55	8.8	64	9.7	8.4	223	0.71
S207A	1.4 ± 0.2	353 ± 39	2200	> 10 000	3300	64	476	0.62
S208A	0.9 ± 0.05	375 ± 16	6.1	59	5.5	11	173	0.59
S211A	1.2 ± 0.2	306 ± 32	5.1	25	2.7	9.8	249	0.52
S208A/S211A	0.7 ± 0.1	112 ± 13	12	101	17	3.7	100	0.66
C129A	0.9 ± 0.1	472 ± 31	9.8	59	8	–	–	2
T130A	0.9 ± 0.1	158 ± 29	3.3	35	7.3	–	–	4
S132A	1.1 ± 0.1	171 ± 52	3.2	45	4	–	–	1.1
S135A	0.1 ± 0.01	312 ± 37	–	–	–	–	–	–
S140A	1.1 ± 0.3	520 ± 63	8.7	48	10	–	–	3
S173A	1.4 ± 0.04	568 ± 100	7.3	93	26	–	–	1.1
S177A	1.4 ± 0.2	306 ± 22	3.1	18	15	–	–	1.6
Y203A	0.6 ± 0.1	98 ± 1	240	900	903	–	–	0.82
Y213A	0.5 ± 0.1	540 ± 41	19	53	25	–	–	1.1
C306A	0.3 ± 0.04	366 ± 90	1.7	16	3.8	–	–	0.78
S318A	1.8 ± 0.3	390 ± 24	6.2	43	–	–	–	1.2
Y338A	0.5 ± 0.1	600 ± 42	69	336	320	–	–	49

Receptor expression was assessed measuring the binding of 250 pM [^{125}I]HEAT on membranes from cells expressing the various receptors.
The IC_{50} of (−)-epinephrine ((−)-EPI), (+)-epinephrine ((+)-EPI), (−)-norepinephrine ((−)-NE), phenylephrine (PHE), synephrine (SYN) and prazosin (PRA) were assessed in competition binding experiments using 80 pM [^{125}I]HEAT.
R_{max} indicates the EPI-induced accumulation of total inositol phosphates (IP) expressed as % increase above basal levels in the absence of EPI. Results concerning the expression and IP mesurements are the mean ± SE of independent experiments for the WT ($n = 9$) and mutant ($n = 3$) receptors. The IC_{50} values were from 2–3 experiments which agreed within 20%. The ligand binding properties of the S135A mutant were not assessed because of its low expression level.

ing. Thus, C306 seems to play a structural role in the α_{1B}-AR, consistently with the finding that the C306A mutant displys lower expression levels as compared to the wild-type receptor.

Finally, an important structural role is played by the highly conserved Y338 in TMDVII. This residue is not directly involved in agonist binding despite the fact that its mutation has profound effects on ligand binding as in the Y338A receptor mutant. In particular, Y338 is directed towards TMDII interacting with S102. Thus, the integrity of Y338 is important to maintain the receptor sructure and thus, indirectly, preserving the ligand binding properties of the α_{1B}-AR.

4. Conclusions

The binding site of catecholamines in the α_{1B}-AR had not been previously defined in great detail [15]. In our study, the combination of mutational and structural/dynamical analysis of the α_{1B}-AR provides new informations about both the inter- and intramolecular interactions characterizing the (−)-epinephrine–α_{1B}AR complex. Our analysis provides solid evidence that D125 in TMDIII interacts with the amino group of both agonists and antagonists, whereas S207 in TMDV interacts with both catecholic hydroxy-groups of (−)-epinephrine. In addition, our results identify a large number of polar residues playing an important role in the activation process of the α_{1B}-AR, thus providing new insights into the structure/function relationship of GPCR.

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