Mutational Analysis of the α_{1a} -Adrenergic Receptor Binding Pocket of Antagonists by Radioligand Binding Assay

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Computer simulations of the human α_{1a} -adrenergic receptor (α_{1a} -AR) based on the crystal structure of rhodopsin have been combined with experimental site-directed mutagenesis to investigate the role of residues in the transmembrane domains in antagonist binding. Previous molecular dynamics studies from our laboratory indicated that the amino acids Asp106 in the third transmembrane domain (TMD), Gln167 in TMD IV of α_{1a} -AR were directly involved in prazosin, tamsulosin and KMD-3213 binding. The Asp106Ala mutant did not exhibit any affinity for [³H]prazosin. On the other hand, the Gln167Phe mutant α_{1a} -AR showed reduced binding affinity for [³H]prazosin. In competition binding experiment the binding affinities of prazosin and tamsulosin were increased 11-fold and 33-fold respectively to Gln167Phe mutant in comparison with wild type receptor. It seems that mutation of this residue by phenylalanine has offered more interaction for the ligands with its aromatic ring. The results provide direct evidence that these amino acid residues are responsible for the interactions between α_{1a} -AR and radioligand [³H]prazosin as well as tamsulosin and KMD-3213.

Key words α_{1a} -adrenergic receptor; site-directed mutagenesis; binding pocket; binding assay

The α_1 -adrenergic receptors (α_1 -AR) belong to Class A of the super family of G protein coupled receptors (GPCRs). They comprise at least three cloned subtypes, α_{1a} -, α_{1b} - and α_{1d} -ARs.^{1,2)} α_1 -ARs are of particular therapeutic interest due to their important roles in the control of blood pressure, in the contraction and growth of smooth muscle and in renal and hepatic metabolism. The α_{1a} -AR located in large abundance in the prostate is thought to be influential in the condition of benign prostatic hyperplasia (BPH), a considerable health problem for aging man.³⁾ The development of high affinity α_{1a} -AR antagonists is of paramount importance for the treatment of BPH.

To rationally design selective drugs, an understanding of subtype differences in the ligand-binding pockets would be invaluable. In this paper the attention is focused on α_{1a} -AR due to its role in neurotransmission processes, in pharmacological treatment of BPH.^{4,5)} Indeed, many researchers are involved in mutagenesis studies on the α_{1a} -AR, which revealed the key residues of both agonist and antagonist binding sites.⁶⁾

In contrast to the wealth of information about the agonist binding pocket of adrenergic receptors, the knowledge about the binding mode of antagonists is much more limited. Three consecutive residues set on second extracellular loop (Gln177, Ile178 and Asn179) are involved in interaction with some α_{1a} -AR selective antagonists.⁷⁾ Two conserved phenylalanine residues on transmembrane domain (TMD) VII (Phe308 and Phe312) are involved in π – π stacking with almost all α_{1a} -AR antagonists.⁸⁾

Previous molecular modeling studies of α_{1a} -AR in our laboratory predicted that the protonated amino group of prazosin, tamsulosin and KMD-3213 make ionic interactions with the carboxylate side chain of Asp106 in TMD III, which is highly conserved in all GPCR-binding amine ligands.⁹⁾

Docking study and subsequent molecular simulation studies with α_{1a} -AR antagonist/receptor complex by other investigators have also revealed the same aspartate (Asp106) to be interacting with doxazosin (a quinazoline based antagonist, with a similar basic structure of prazosin) and tamsulosin.¹⁰⁾ Pedretti and colleagues published a homology model of α_{1a} -AR where both the agonist and antagonist interacted with Asp106.¹¹⁾

KMD-3213 is a selective α_{1a} -AR antagonist.^{12,13)} α_{1a} -AR selective antagonist would be very useful for the treatment of BPH without any untoward effect on the blood pressure. By homology modeling of α_{1a} -AR with KMD-3213, we identified a key amino acid residue responsible for higher binding affinity to α_{1a} -AR. The study revealed that carboxamide nitrogen of KMD-3213 forms an ionic bond with the carboxylate side chain of Gln167 in TMD IV.⁹⁾ Prazosin and tamsulosin are proposed to interact with the side chain of Asp106 in TMD III.

The objectives of this study are both the construction of mutant α_{1a} -ARs such as Asp106Ala, double mutants Asp106Ala-Gln167Phe and Gln167Phe by site-directed mutagenesis and the analysis of their interactions with prazosin, tamsulosin and KMD-3213 by radioligand binding assay. We also confirm the modeling data that Gln167 of α_{1a} -AR is a novel residue to report for antagonist binding.

MATERIALS AND METHODS

Drugs Prazosin hydrochloride was purchased from RBI (Research Biochemical Incorporated, Natick, MA, U.S.A.), tamsulosin was donated by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan) and KMD-3213 was gifted from Kissei Pharmaceutical Co., Ltd. (Matsumoto, Japan).

DNA Constructs A cDNA clone encoding human α_{1a} -

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AR receptor in the plasmid vector (pCR3) was introduced into the DH5 α strain of *Escherichia coli* by the electroporation method. The culture of *E. coli* was used to prepare plasmid DNA by plasmid purification midi kit (Qiagen, Germany). The mutant human α_{1a} -AR, Asp106Ala, Asp106Ala-Gln167Phe and Gln167Phe were created by a QuikChange[®] site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) using the forward primer 5'-ATCTGGGCGGCA-GTGGCTGTGCTGTGCTGCACC-3' and reverse primer 5'-GGTGCAGCACAGCACAGCCACTGCCGCACCGAT-3' for Asp106Ala and the forward primer 5'-CTGTTCCGGCT-GGAGGTTCCCGCCCCCGAGGAC-3' and reverse primer 5'-GTCCTCGGGGGCCGGGAACCTCCAGCCGAAC-AG-3' for Gln167Phe, according to the manufacturer's protocol. The mutant receptors were confirmed by DNA sequencing analysis (Beckman Coulter CEQ2000XL, Beckman/Coulter, Fullerton, CA, U.S.A.).

Cell Culture and Transfection HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. HEK293 cells were transfected in 10-cm dishes at about 80% confluence with Lipofectamine[™]2000 (Invitrogen, Carlsbad, CA, U.S.A.)—a lipofection transfection kit, according to the manufacturer's protocol. Twenty four hours after transfection, the selection for stably expressed cells was done for two weeks in the medium containing 10% fetal bovine serum and 500 μ g/ml G418. A stably expressing cell lines were subsequently cultured in 200 μ g/ml G418-containing growth medium.

Membrane Preparations from HEK293 Cells For the preparation of membranes, cell pellets were homogenized with 1 ml of buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA-Na and 10 mM NaCl with a Polytron homogenizer (setting 7 for 8 s). The homogenates were centrifuged at 800 $\times$ *g* for 5 min. The supernatant was further centrifuged at 100000 $\times$ *g* for 30 min. The precipitate was resuspended into binding buffer. Protein content was measured by the method of Bradford¹⁴⁾ and kept at -80 °C until used.

Ligand Binding Assays Each binding incubation tube contained 10–20 μ g of membrane protein, [³H]prazosin (Amersham Biosciences, U.K.), unlabeled drug as required, and binding buffer in a final volume of 1 ml. For saturation binding studies, six concentrations (30–2000 pM for wild type and Gln167Phe mutants, 30–3000 pM for Asp106Ala, Asp106Ala-Gln167Phe mutants) of [³H]prazosin were tested in duplicate. For competition binding studies, the concentration of [³H] prazosin with wild-type receptors was 200 pM and 8 concentrations of competing ligands were used in duplicate. Nonspecific binding was defined with the use of 10 μ M phentolamine. For both the radioligand binding assays, incubations were carried out for 45 mins at 30 °C and were terminated by rapid filtration through Whatman GF/C filters that had been presoaked in 0.3% polyethyleneimine followed by washing with 10 ml ice-cold wash buffer. The radioactivity retained on the filters was quantitated by liquid scintillation spectrophotometry.

Data Analysis Nonlinear regression analysis of saturation and competition binding assay were performed using GraphPad Prism software (San Diego, CA, U.S.A.). The results of experiments are expressed as means $\pm$ S.E.M. The inhibition concentration (IC₅₀) in displacement analysis were

determined as the concentrations of ligands that inhibited [³H]prazosin binding by 50%; the values of inhibition constants (*K_i*) were calculated by the equation of Cheng and Prussoff¹⁵⁾ and expressed as p*K_i* (-log *K_i*). Student's unpaired *t*-test was performed to assess the significance of the difference. A *p* value of less than 0.05 was taken as significant.

RESULTS

Mutational analysis of α_{1a} -AR has resulted in identification of several amino acids that are involved in the binding of agonists^{16–18)} and antagonists.^{8,9,19)} To verify the modeling data and the involvement of corresponding amino acids (Asp106 and Gln167) in the human α_{1a} -AR, these amino acids were mutated to alanine and phenylalanine. Mutated receptors were initially characterized by [³H]prazosin that was used to determine the dissociation constant (*K_d*) and maximum bound (*B_{max}*) values for each of the mutant and wild type receptors. The wild-type receptor bound the ligand with high affinity (Table 1), whereas significantly reduced affinity (2-fold in comparison with wild-type respectively) was found with Gln167Phe mutant receptor. Single mutation of Asp106 to alanine or double mutants Asp106Ala and Gln167 to phenylalanine resulted in a loss of [³H]prazosin binding. So, no *B_{max}* values were obtained with the Asp106Ala and Asp106Ala-Gln167Phe mutants. The expression level for a given construct was found to be consistent in separate preparations.

For the receptors showing saturable [³H]prazosin binding (wild-type and Gln167Phe), affinities of prazosin, tamsulosin and KMD-3213 were determined by radioligand displacement studies (Table 2). Figure 1 shows that mutation of Gln167 to phenylalanine increased the affinities of prazosin and tamsulosin more than 30-fold (*p*<0.05). Furthermore, Table 2 shows that mutation of Gln167 into phenylalanine resulted in a 2-fold loss in affinity to KMD-3213, corresponding to a loss of an electrostatic bond. Because of the lack of saturable [³H]prazosin binding of two mutant receptors (Asp106Ala and Asp106Ala-Gln167Phe), antagonist binding could not be studied in these receptors (Table 2).

Table 1. Analysis of Saturation Binding Data for Both Wild-Type and Mutant α_{1a} -AR

	<i>K_d</i> (pM)	<i>B_{max}</i> (fmol/mg protein)
WT(3)	130 $\pm$ 5.22	5478 $\pm$ 126
Asp106Ala (3)	>30000	ND
Asp106Ala-Gln167Phe (3)	>30000	ND
Gln167Phe (3)	265.2 $\pm$ 7.2***	3575.3 $\pm$ 239.1**

WT, wild-type. ND, no detectable specific binding with [³H]prazosin. The data shown indicate mean $\pm$ S.E.M. and numbers in parentheses indicate number of experiments. ***p*<0.005 and ****p*<0.001, vs. wild-type.

Table 2. Analysis of Binding Affinities (p*K_i*) Data of Several α_{1a} -AR Antagonists for Wild-Type and Mutant α_{1a} -AR

p <i>K_i</i>	Prazosin	Tamsulosin	KMD-3213
WT(3)	10.76 $\pm$ 0.13	11.37 $\pm$ 0.38	11.14 $\pm$ 0.06
Gln167Phe (3)	11.8 $\pm$ 0.28*	12.9 $\pm$ 0.21*	10.79 $\pm$ 0.1

The data shown indicate mean $\pm$ S.E.M. and numbers in parentheses indicate number of experiments. p*K_i*=-log (*K_i*), where *K_i* is in nM. **p*<0.05, vs. wild-type.

Fig. 1. Competition Binding Studies of Prazosin (A), Tamsulosin (B), and KMD-3213 (C) for [^3H]Prazosin Binding to Wild-Type as Well as Gln167Phe Mutant of α_{1a} -AR

The data represented the mean $\pm$ S.E.M. of three to six experiments performed in duplicate.

DISCUSSION

α_{1a} -ARs seem to play key roles in the regulation of the vascular smooth muscle contraction.²⁰ There is evidence suggesting that they are regulators of hypertrophic growth responses primarily in neonatal cardiomyocytes. Since the α_{1a} -AR is known to play a major role in cardiac physiology, the antagonists of α_{1a} -AR are useful for the treatment of cardiovascular diseases occurring due to α_1 -ARs.

Recently, with the development of computer-based homology modeling, the three dimensional structure of receptors of cardiac muscles and blood vessels and its binding with a ligand could be anticipated. The binding sites of the receptor complex and the analysis of the binding profile were possible.

As for the binding sites in α_{1a} -AR of prazosin, tamsulosin and KMD-3213, it is evident that amino acids on 3rd, 4th, 5th and 6th TMDs of α_{1a} -AR are important according to the report which analyzed the binding sites with the receptor by the computer-based homology modeling.⁹ Among the other amino acid residues, Asp106 and Gln167 on α_{1a} -AR are sup-

posed to interact with α_1 -AR antagonists. Discrete residues for antagonist binding have been well-established.²¹ However, Gln167 on α_{1a} -AR is another residue to report for the first time for antagonist binding. Therefore, in this study, we constructed and characterized the single as well as double mutations of these amino acid residues by site-directed mutagenesis. These amino acids, aspartate and glutamine were substituted for alanine and phenylalanine respectively. Since alanine substitutions introduce changes in both charge and H-bonding capabilities and in size, potentially leaving a cavity in comparison to other side chains, aspartate was substituted by alanine. Phenylalanine substitution will impart aromatic ring for binding with ligands. We analyzed the mutant receptors by ligand binding assays. The effects of the mutations studied on the affinity of prazosin, tamsulosin and KMD-3213 correlated fully with the expectations from the computational modeling of these antagonists with the α_{1a} -AR.

Effects of Asp106Ala and Asp106Ala-Gln167Phe Mutant on Antagonist Binding to α_{1a} -AR Early studies with the β -AR suggested that the agonist binding pocket was constituted by the interaction of the protonated amine of the catecholamine that hydrogen bonds to an aspartate residue in TMD III. Both agonist and antagonist binding are more severely affected by mutation of the aspartate in TMD III of the β -AR.²² It can be argued that mutants of this residue are highly unstable and very little surface expression occurs, making binding analysis difficult. So, the mutation of Asp106 to alanine or double mutants Asp106Ala and Gln167 to phenylalanine resulted in a loss of [^3H]prazosin binding and as a result no B_{max} values were obtained with the Asp106Ala and Asp106Ala-Gln167Phe mutants.

In previous mutagenesis studies of the β_2 -²³ and α_{2A} -AR²⁴ the authors 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 amino ligand. Furthermore, Asp125 of the α_{1b} -AR is the homologous to the aspartate on TMD III of β_2 - and α_{2A} -AR and dopamine1-receptor involved in the interaction with the amino group of catecholamines.²⁵ Cavalli and co-workers reported that mutation of Asp125 to alanine totally impaired the ability of the α_{1b} -AR to bind both agonists and antagonists. This was shown by the fact that Asp125Ala displayed no specific [^{125}I]HEAT binding and no epinephrine-induced stimulation of IP production.²⁵ Another report mentioned that a negative charge at position 125 on TMD III is thought to be important for agonist as well as antagonist affinity at β - and α_2 -ARs.²⁶ Recently, we obtained similar results for α_{1b} -AR in our laboratory.²⁷ Mutation of Asp107 in histamine H_1 -receptor which is thought to be the main interaction point of the protonated amine function of aminergic agonists and antagonists with their receptors, also led to total loss of specific [^3H]mepyramine binding.²⁸ These results may be extrapolated to conserved aspartate at position 106 in α_{1a} -AR. In the present study, we also report that single mutation of Asp106Ala and double mutation of Asp106Ala-Gln167Phe of α_{1a} -AR eliminated [^3H]prazosin as well as tamsulosin and KMD-3213 binding. These results strongly suggest that the same conserved aspartate Asp106 on TMD III of α_{1a} -AR interacts with the functional amino group of prazosin, tamsulosin and

KMD-3213.

Effects of Gln167Phe Mutant on Antagonist Binding to α_{1a} -AR Mutation of Gln167 in TMD IV showed increased binding affinity to prazosin and tamsulosin in [3 H]prazosin displacement in contrast with that of KMD-3213 where the mutation resulted in little and insignificant loss of affinity (Fig. 1). As proposed in the literature by Ishiguro *et al.*, Gln167 in TMD IV of α_{1a} -AR being uncharged could form an ionic bond with carboxamide side chain but does not form any interaction with prazosin or tamsulosin.⁹⁾ As a result, the increased pK_i values of prazosin and tamsulosin might be the result of the mutation to phenylalanine which offered aromatic interaction with these ligands. Certainly, the decreased affinity of the mutant for KMD-3213 proves the importance of Gln167 for interaction with the ligand. However, mutation by phenylalanine might have served binding of the residue with the aromatic ring of KMD-3213 (Table 2). It is noteworthy that, despite a favorable phenylalanine residue, KMD-3213 lost affinity for which it can be assumed that glutamine at position 167 could be necessary for binding KMD-3213.

Studies suggested that in contrast to agonist binding, which is localized to the interior core of the receptor, antagonists interact with residues closer to the extracellular surface of adrenergic receptors, above the plane of agonist binding pocket.^{7,29,30)} In this study, we also confirm that antagonist binding pocket is near the surface of the receptor and not down into the ligand-binding pocket as agonists (Fig. 2). α_1 -AR antagonists tend to be much larger than agonists (as much as two to four times larger), and comparable in size to peptide ligands. The size difference may be the reason for the antagonists' tendency to bind to the surface of the receptor and antagonize by blocking the agonist-binding pocket.³¹⁾

In conclusion, our studies showed that a combination of computer modeling, site-directed mutagenesis and pharmacological analysis was helpful to explore that Asp106, Gln167 and Asp106-Gln167 in TMD III, IV and TMD III & IV respectively in α_{1a} -AR are involved in prazosin, tamsulosin and KMD-3213 binding.

Fig. 2. Schematic Representation of TMD III and IV of α_{1a} -AR Highlighting in 'Gray' the Two Amino Acid Residues Selected for Single and Double Mutagenesis and Their Transmembrane Locations

The mutations were as follows: TMD III, Asp106→Ala; TMD IV, Gln167→Phe and TMD III & IV Asp106→Ala-Gln167→Phe. Amino acids are represented by the one-letter code.

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