Phenylalanine in the Second Membrane-Spanning Domain of α_{1A}-Adrenergic Receptor Determines Subtype Selectivity of Dihydropyridine Antagonists

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Received April 30, 1996; Revised Manuscript Received August 28, 1996[⊗]

ABSTRACT: The α_1-adrenergic receptors (α_1-AR) belong to the G-protein coupled seven-transmembrane biogenic amine receptor family. Three subtypes have been successfully cloned in the α_1-adrenergic receptor family, and they share 50% identical amino acid sequences and 70% similarity. We have constructed seven chimeric receptors of the α_{1A}-AR. Each of the chimeras contains α_{1D}-subtype amino acid sequences within the membrane-spanning domains. Comparisons of ligand affinities with these chimeras has provided information on the importance of certain amino acid residues in determining receptor subtype specificity in the α_{1A}- and α_{1D}-ARs. With ligands in the dihydropyridine series, the niguldipine analog **1** was found to have respective pK_i's of 9.32 ± 0.17 for α_{1A}-AR; 6.84 ± 0.24 for α_{1D}-AR; and 6.76 ± 0.28 for $\alpha_{1A/D}$(TM2), respectively. This trend was also exhibited by two other niguldipine analogs, **2** and **3**, which had similar pK_i's toward α_{1D}-AR and $\alpha_{1A/D}$(TM2). This subtype selectivity was also maintained in the piperdine derivative, **4**, an α_{1A}-AR selective ligand, which showed the same parallel trends in binding affinities with α_{1A}-AR and the six chimeras as the niguldipine analogs. Since in considering the second membrane-spanning domain, the α_{1A}- and α_{1D}-ARs only differ at positions 76, 77, 85, and 86, we were able to show through mutational studies that phenylalanine 86 is solely responsible for the selectivity found in the chimeric receptor $\alpha_{1A/D}$(TM2) exhibited against the ligands **1**−**4** used in this study. A model based on the rhodopsin structure places the amino acid at position 86 in the final turn toward the extracellular region. This is four helical turns above aspartic acid-79, a conserved amino acid in the second membrane-spanning domain. This is the first report that suggests a significant involvement of the second membrane-spanning domain in antagonist binding in the biogenic amines class of the superfamily of seven-transmembrane receptors.

The α_1-adrenergic receptors (α_1-AR)[1] are a family of G-protein coupled seven-transmembrane receptors, which are involved in the regulation of the cardiovascular and central nervous systems. The major three subtypes, α_{1A}-, α_{1B}-, and α_{1D}-AR, are expressed in a wide variety of tissues with different tissue specificity (Hwa et al., 1994). A strategy designed to discover highly selective compounds as agonists and antagonists against these targets provides an attractive approach to develop effective therapeutic agents to treat a range of diseases.

The α_1-ARs, like other G-protein coupled biogenic amine receptors, have a conserved aspartic acid residue (Asp-113) in the third membrane-spanning domain, which is essential for activity and is presumed to bind a quarternary amine counterion present in the ligands of these receptors. Mutational studies with various members of the family of biogenic amine G-protein coupled receptors have been undertaken in attempts to probe ligand interactions at other sites in neighboring membrane-spanning domains. In one of the most comprehensive studies it was suggested that conserved serines in the fifth membrane-spanning domain might act as hydrogen bonding partners for the catechol ring found in most β_2-AR agonists (Strader et al., 1989). The involvement of the fifth, sixth, and seventh membrane-spanning domains in influencing ligand selectivity has also been suggested from several studies (Link et al., 1992; Guan et al., 1992; Bluml et al., 1994; Adham et al., 1994; Wess et al., 1991). Recently, agonist selectivity among α_1-ARs was reported to be determined by a residue (Val-185) in the fifth membrane-spanning domain (which is valine in α_{1A}-AR and alanine in α_{1B}-AR and α_{1D}-AR) with an additional contribution from a residue (Met-293) within the sixth membrane-spanning region (Hwa et al., 1995). This is consistent with studies that suggest involvement of the fifth membrane-spanning domain in ligand binding to the α_2-AR (Link et al., 1992) and β_2-AR (Strader et al., 1989).

In terms of the ligands and their selectivity, the dihydropyridine, niguldipine, was first reported to be a several hundred-fold selective α_{1A}-AR antagonist (Boer et al., 1989). Another dihydropyridine analog similar to niguldipine (SNAP 5089) was reported to be greater than a thousand-fold selective in binding the α_{1A}-AR (Wetzel et al., 1995). It was reported that this latter compound was not affected by the various mutations within the fifth membrane-spanning domain while several other compounds showed decreased affinity induced by these mutations (Salon et al., 1995). If it is accepted that the Asp-113 in the third membrane-spanning domain anchors the quartenary amine in these ligands, then

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[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: AR, adrenergic receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction; [^{125}I]HEAT, ([^{125}I]-2-β-(4-hydroxyphenyl))ethylaminomethyltetralone; TM, transmembrane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); COS-7, monkey kidney epithelial cells.

S0006-2960(96)01024-0 CCC: $12.00

Table 1: Sequence Difference in the Membrane-Spanning Domains between α_{1A}-AR and α_{1D}-AR[a]

	TM1			TM2			TM3			TM4			TM5			TM6			TM7	
res no.	α_{1A}	α_{1D}	res no.	α_{1A}	α_{1D}	res no.	α_{1A}	α_{1D}	res no.	α_{1A}	α_{1D}	res no.	α_{1A}	α_{1D}	res no.	α_{1A}	α_{1D}	res no.	α_{1A}	α_{1D}
29	L	V	88	V		94	F		167	Q	E	182	P	A	278	F		283	P	
30	G		87	E		95	G		166	R	K	183	G		277	F	L	284	S	
31	V		86	F	M	96	R		165	W		184	Y		276	S		285	E	
32	I	F	85	I	T	97	V	A	164	G		185	V	A	275	G		286	T	G
33	L		84	A		98	F		163	F	L	186	L	V	274	I	L	287	V	
34	G	A	83	S		99	C		162	L		187	F		273	P		288	F	
35	G	A	82	F		100	N	D	161	P		188	S		272	M		289	K	
36	L	F	81	P		101	I	V	160	G		189	A	S	271	V		290	I	V
37	I		80	L		102	W		159	I	V	190	L	V	270	L	F	291	V	I
38	L		79	V		103	A		158	S		191	G	C	269	F		292	F	
39	F	M	78	T		104	A		157	I	V	192	S		268	F		293	W	
40	G	A	77	S	A	105	V		156	V		193	F		267	P		294	L	
41	V		76	T	S	106	D		155	L		194	Y		266	L	F	295	G	
42	L	A	75	L		107	V		154	S	A	195	L		265	W		296	Y	
43	C	G	74	L		108	L		153	L	V	196	P		264	C		297	L	F
44	N		73	L		109	C		152	A	V	197	L	M	263	L		298	N	
45	I		72	D		110	C		151	W		198	A		262	V		299	S	
46	L		71	A		111	T		150	V	L	199	I	V	261	F		300	C	
47	V		70	V		112	A		149	C	L	200	I		260	CV	V	301	I	V
48	I		69	A		113	S		148	L	A	201	L	V	259	G		302	N	
49	L		68	L		114	I		147	L		202	V		258	V		303	P	
50	S		67	N		115	M	L				203	M		257	V				
51	V		66	V		116	G	S				204	Y		256	I				

Summary of Mutated Receptors

mutated receptors	no. of residues mutated	location
$\alpha_{1A/D}$(TM1)	8	TM1
$\alpha_{1A/D}$(TM2)	4	TM2
$\alpha_{1A/D}$(TM3)	5	TM3
$\alpha_{1A/D}$(TM4)	11	TM4
$\alpha_{1A/D}$(TM5)	9	TM5
$\alpha_{1A/D}$(TM6)	6	TM6
$\alpha_{1A/D}$(TM7)	6	TM7
α_{1A}85T	1	TM2
α_{1A}86M	1	TM2
α_{1A}85T86M	2	TM2
$\alpha_{1A/D}$(TM23)	9	TM2 and 3
$\alpha_{1A/D}$(TM2367)	21	TM2, 3, 6, and 7
α_{1D}156F	1	TM2

[a] The residues closer to the extracellular region in each membrane-spanning domain appear on the top of the table. The residue numbers are based on wild type human α_{1A}-AR. [b] The chimeric receptors between α_{1A}- and α_{1D}-AR were named $\alpha_{1A/D}$ followed by the position of the membrane-spanning domain(s) containing sequences from α_{1D} in a parenthesis. The positions and changes in the sequence in each membrane-spanning domain are shown in the top seciton of this table. The receptors with point mutations were named with the position(s) of the mutation(s) and the amino acid(s) to which they are mutated.

the results suggest that different ligands can occupy different sites on the other domains.

The major three subtypes of α_1-AR share 50% identical amino acid sequences and 70% similarity while the membrane spanning regions share even higher homology. Since ligands for these receptors presumably have a common binding site in the third membrane-spanning domain at Asp-113, the subtype selectivity may be found in slight sequence differences within the other membrane-spanning domains. Using α_{1A}-AR as a template we constructed a series of chimeric receptors between α_{1A}-AR and α_{1D}-AR using α_{1A}-AR as template and determined the residue(s) involved in the antagonist subtype selectivity.

MATERIALS AND METHODS

Site-Directed Mutagenesis. All constructs including the wild type human α_{1A}-AR contained shortened EE-epitopes, EYMPME (Grussenmeyer et al., 1985), at the N- and C-termini of the receptors, which permitted the detection of receptor expression by immunological methods. First, several unique restriction sites were introduced and one restriction site was eliminated by silent mutations to create an α_{1A}-AR cDNA containing unique restriction sites between the coding regions corresponding to the membrane-spanning domains (Figure 1). This cDNA is available upon request. Each of the cDNA constructs which comprised the individual membrane-spanning domains from α_{1D}-AR was synthesized by PCR with primers to include the unique restriction sites. These cDNAs were sequenced and then exchanged to the equivalent position in the α_{1A}-AR cDNA using the appropriate restriction sites. The mutated cDNAs were inserted into an expression vector pMT4 (Kaufman et al., 1989). The changes in each membrane-spanning domain that resulted are shown in Table 1.

A point mutation from methionine to phenylalanine in the second membrane-spanning domain in α_{1D}-AR cDNA was created using Sculptor in vitro mutagenesis system (Amersham, IL). The mutated α_{1D}-AR cDNA (α_{1D}156F) was then introduced into an expression vector, pMT4ss-α_{1D}-AR (Buckholz et al., 1994), by exchange to the equivalent position using *Apa*I sites.

Transient Expression and Membrane Preparation. The pMT4 plasmids containing mutated α_1-AR cDNAs were introduced into COS-7 cells using Lipofectamine (GIBCO

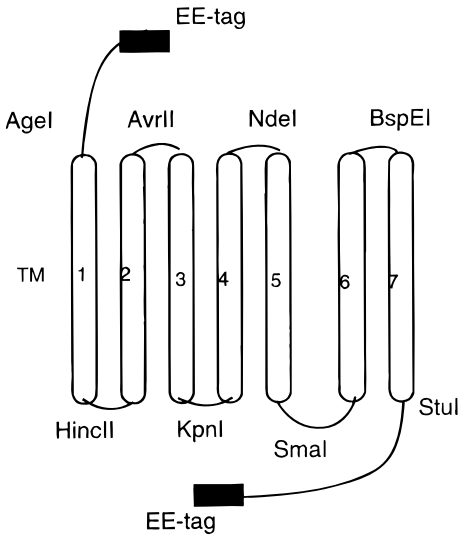


FIGURE 1: Unique restriction sites in α_{1A}-adrenergic receptor cDNA. Sequences coding shortened EE-epitope were added 3′ and 5′ of α_{1A}-AR cDNA. *Kpn*I, *Nde*I, and *Bsp*EI were newly introduced into the cDNA, and a *Sma*I site at the 3′ end of the cDNA was deleted. *Age*I, *Hinc*II, *Avr*II, and *Stu*I existed in the original cDNA.

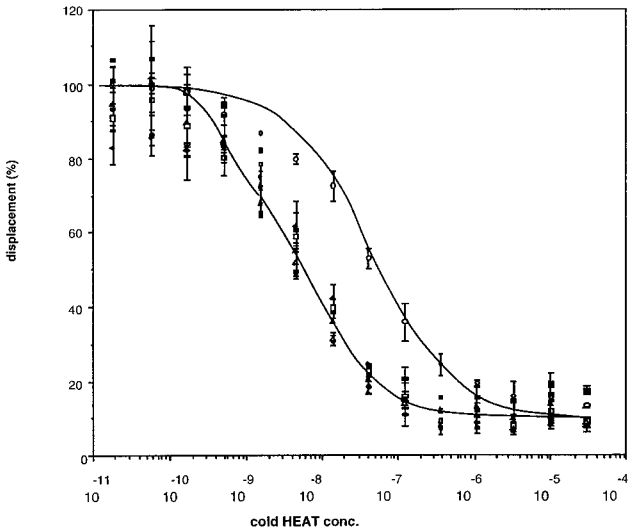


FIGURE 2: Displacement of [I^{125}]HEAT with cold HEAT against α_{1A}-AR (●) and six chimeric receptors; $\alpha_{1A/D}$(TM2) (◆), $\alpha_{1A/D}$(TM3) (■), $\alpha_{1A/D}$(TM4) (◇), $\alpha_{1A/D}$(TM5) (○), $\alpha_{1A/D}$(TM6) (□), and $\alpha_{1A/D}$(TM7) (△). The chimeric receptors with the exception of $\alpha_{1A/D}$(TM5) showed indistinguishable displacement curves with α_{1A}-AR($K_i \approx 0.2$ nM) on the left side. $\alpha_{1A/D}$(TM5) showed a similar displacement curve with α_{1D}-AR ($K_i \approx 1.0$ nM) on the right side.

BRL, Gaithersburg, MD) according to the manufacturer's instructions. The cells were incubated with Opti-MEM (GIBCO BRL), containing Lipofectamine and DNA for 3 h. The cells were harvested 48 h after the transfection. The cells were homogenized with a micro-homogenizer for 10−15 s in phosphate saline buffer (pH 7.5) containing protease inhibitors; 1 μg of leupeptin/mL, 25 μg of Pefablock SC/mL, 1 μg of aprotin/mL, and 1 μg of pepstatin A/mL. Crude membrane fractions were collected using a benchtop ultracentrifugation TL-100 (436 000*g*, 15 min). Membranes were washed with 5 mM HEPES buffer (pH 7.4). The protein concentration was determined using the Bio-Rad Protein Assay Reagent.

Ligand Binding Assay. α_{1A-}AR selective antagonists were synthesized in house using published procedures (Wetzel et al., 1995) and used in a displacement assay against [^{125}I]HEAT. 5−10 μg of membrane protein in 100 μL of 5 mM HEPES buffer (pH 7.4), 22 μL of 25 mM acetic acid containing 0.3% DMSO with a various concentration of antagonists, and 100 μL of [^{125}I]HEAT (2200 Ci/mmol, Dupont-New England Nuclear, Boston, MA) (70 000−100 000 cpm/100 μL) in assay buffer (25 mM PIPES, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5) were mixed and incubated in a 96-well plate for 90 min at room temp. The membrane samples were then filtered over a GF/B filter and washed with 4 mL of ice-cold 25 mM Tris buffer (pH 7.5). The membrane-bound radioactivity was determined by a γ scintillation top-counter. Nonspecific binding was determined in the presence of 100 μM phentolamine. All compounds were tested at least twice for each receptor. Crude membranes from Rat-1 fibroblast cell stably expressing human α_{1D}-AR with mellitin signal peptide (ss-α_{1D}-AR) were used to determine pK_i for α_{1D}-AR (Goetz et al., 1994).

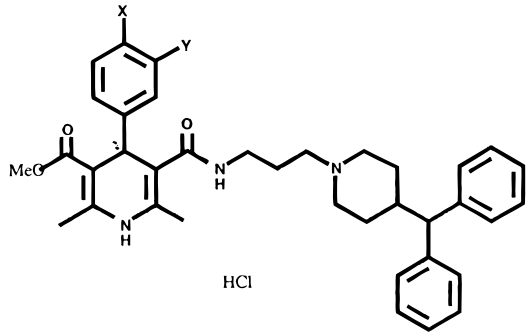
RESULTS AND DISCUSSION

The seven chimeric receptor cDNAs were transfected into COS-7 cells using the pMT4 expression vector. In the case of the $\alpha 1_{A/D}$(TM1) receptor, although expression was detected by western blotting, no significant binding to HEAT was observed. Furthermore the staining of the cells with an immunofluorescence probe indicated that the expressed receptor only existed in the cytoplasmic region. In contrast, the membrane from COS-7 cells expressing $\alpha_{1A/D}$(TM5) bound HEAT with a comparable affinity to that found for wild type α_{1D}-AR (pK_i 9.0). Similarly membranes from COS-7 cells expressing the other five chimeric receptors also displayed comparable affinities (pK_i of 9.5−9.7) for HEAT as that observed with the wild type α_{1A}-AR (pK_i 9.7) (Figure 2).

The affinities of α_{1A}-AR selective antagonists **1**−**4** (Chart 1) were first tested with wild-type α_{1A}-AR and ss-α_{1D}-AR (Table 2). For compound **1** the value obtained was consistent with the reported K_ds of compound **1** (SNAP5089), 0.35 nM (pK_i 9.46) and 540 nM (pK_i 6.27) for α_{1A}- and α_{1D}-AR, respectively (Wetzel et al., 1995). It should be noted that the K_i's obtained for compound **1** with α_1-AR's expressed in Rat-1 fibroblast cells and COS-7 cells are also self consistent. Each of the compounds **1**−**4** was then tested with the six chimeric receptors. The results are shown in Figure 3 and Table 2. Only $\alpha_{1A/D}$(TM2) receptor had significantly reduced affinity to all four antagonists relative to α_{1A}-AR, whereas $\alpha_{1A/D}$(TM5) and $\alpha_{1A/D}$(TM6) receptors had slightly lower affinity (Figure 3a, Table 2) with compounds **2** and **3**. The other chimeric receptors $\alpha_{1A/D}$(TM3), $\alpha_{1A/D}$(TM4), and $\alpha_{1A/D}$(TM7) had similar or slightly elevated affinities toward these compounds relative to α_{1A}-AR.

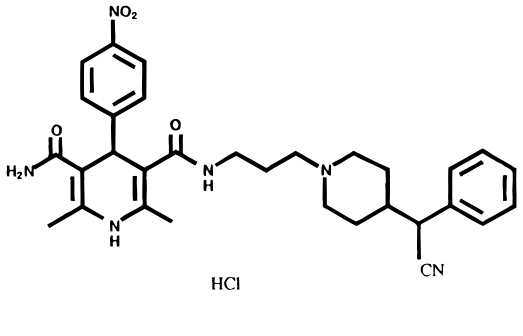
α_{1A}-AR and α_{1D}-AR only differ by four amino acids at positions 76, 77, 85, and 86 in the second membrane-spanning domain (α_{1A}-AR numbering) (Table 1). We constructed three mutant receptors, α_{1A}85T86M containing double point mutations at 85 (I to T) and 86 (F to M), α_{1A}85T containing a single point mutation at 86 (I to T), and α_{1A}86M containing a single point mutation at 86 (F to M). Both α_{1A}85T86M and α_{1A}86M showed reduced affinity against three of the compounds in a manner similar to $\alpha_{1A/D}$(TM2) (Figure 3b, Table 2), whereas α_{1A}85T bound compound **1** as well as α_{1A}-AR [pK_i 9.52(±0.05)] (Table 2). These results indicated that phenylalanine at position 86 is the principal determinant responsible for the subtype selectivity of α_{1A}-AR and α_{1A}-AR toward these α_{1A}-AR selective compounds.

Chart 1



1 X=NO_2, Y=H

5 X=H, Y=NO_2



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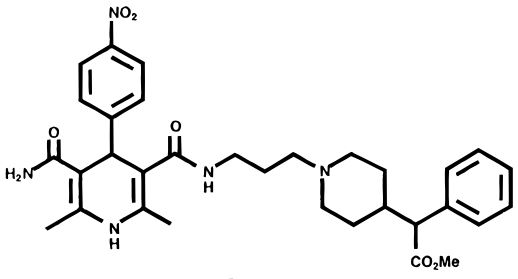

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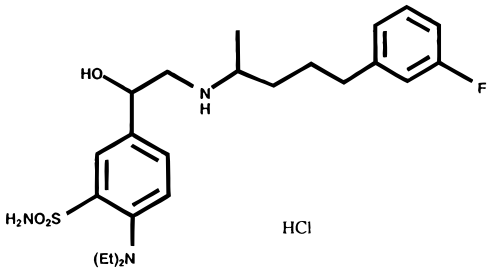

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Table 2: Summary of pK_i (±SE) Determined Using Recombinant α_1-ARs in This Study[a]

receptors	compound **1**	compound **2**	compound **3**	compound **4**
α_{1A}-AR	9.32 (±0.17) (+) 6.50 (±0.01) (−) 9.22 (±0.01)	8.54 (±0.03)	8.92 (±0.02)	7.50 (±0.06)
ss-α_{1D}-AR	6.36 (±0.07) (+) 5.83 (±0.10) (−) 6.45 (±0.14)	5.24 (±0.07)	5.70 (±0.29)	5.35 (±0.20)
$\alpha_{1A/D}$(TM2)	6.76 (±0.28)	5.80 (±0.01)	6.36 (±0.02)	5.71 (±0.01)
$\alpha_{1A/D}$(TM3)	10.40 (±0.42)	9.73 (±0.01)	9.35 (±0.00)	7.85 (±0.18)
$\alpha_{1A/D}$(TM4)	10.20 (±0.28)	8.47 (±0.005)	9.24 (±0.001)	7.67 (±0.10)
$\alpha_{1A/D}$(TM5)	9.14 (±0.01)	7.96 (±0.16)	8.42 (±0.07)	6.71 (±0.21)
$\alpha_{1A/D}$(TM6)	9.19 (±0.19)	8.31 (±0.17)	9.06 (±0.04)	6.96 (±0.06)
$\alpha_{1A/D}$(TM7)	9.82 (±0.48)	8.39 (±0.13)	9.08 (±0.13)	7.74 (±0.03)
α_{1A}85T	9.52 (±0.05)	ND	ND	ND
α_{1A}86M	6.62 (±0.06) (+) 5.54 (±0.08) (−) 6.45 (±0.20)	6.71 (±0.42)	6.35 (±0.08)	ND
α_{1A}85T86M	6.42 (±0.07)	5.54 (±0.10)	6.22 (±0.08)	ND
$\alpha_{1A/D}$(TM23)	6.61 (±0.15) (+) 5.64 (±0.12) (−) 6.53 (±0.01)	ND	ND	ND
$\alpha_{1A/D}$(TM2367)	6.95 (±0.08) (+) 5.91 (±0.06) (−) 6.66 (±0.09)	5.58 (±0.22)	6.06 (±0.16)	ND
α_{1D}156F	9.28 (±0.30)	7.14 (±0.03)	ND	ND

[a] The pK_i for isomers for compound **1** are also shown. ND, not determined.

We did not mutate the residues at 75 and 76. However, given that the results that α_{1A}85T86M and α_{1A}86M showed essentially the same reduced affinities as $\alpha_{1A/D}$(TM2) regardless of the difference in amino acids at 75 and 76 strongly suggest that amino acids 75 and 76 make no significant contribution to the subtype selectivity found in compound **1**.

The enantiomers of niguldipine **5** have been previously shown to be stereoselective antagonists for α_{1A}-AR with the *S*-isomer exhibiting the higher affinity (Boer et al., 1989). The enantiomers of compound **1** were also shown to be stereoselective antagonists for α_{1A}-AR with the (−)-isomer exhibiting the higher affinity whereas with the α_{1D}-AR there is less descrimination (Wetzel et al., 1995). The mutant receptor α_{1A}86M, like α_{1D}-AR, also exhibited less stereoselective descrimination with the enantiomers of compound **1** (Figure 4, Table 2). We observed that α_{1A}86M had slightly lower affinity for the S-isomer[2] of **1** than either α_{1A}-AR or α_{1D}-AR; however, with $\alpha_{1A/D}$(TM2367), which contains the second, third, sixth, and seventh membrane-spanning domains from α_{1D}-AR, the binding was restored to the same level as that of α_{1D}-AR (Figure 4, Table 2).

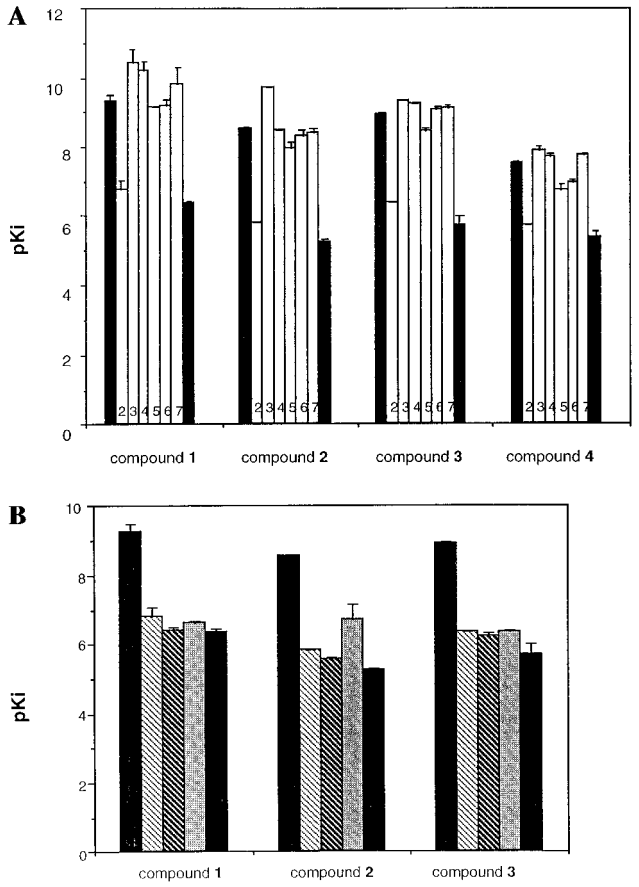

FIGURE 3: Binding of α_{1A} selective antagonists to mutated receptors. The pK_i's were measured against [I^{125}]HEAT. For each compound, the first solid column shows pK_i for α_{1A}-AR and the last solid column shows pK_i for α_{1D}-AR. (A) The middle six columns are pK_i's for the chimeric receptors $\alpha_{1A/D}$(TM2), $\alpha_{1A/D}$(TM3), $\alpha_{1A/D}$(TM4), $\alpha_{1A/D}$(TM5), $\alpha_{1A/D}$(TM6), and $\alpha_{1A/D}$(TM7). The numbers in the columns indicate the position of the transmembrane-spanning domain mutated. (B) The middle three columns represent pK_i's for $\alpha_{1A/D}$(TM2) (light hatch marks), α_{1A}85T86M (heavy hatch marks), and α_{1A}86M (gray bars).

Finally ss-α_{1D}156F was expressed transiently in COS-7 cells and tested against compounds **1** and **2** (Table 2). This point mutation from Met to Phe in the second membrane-spanning domain increased the affinity of compound **1** for α_{1D}-AR 1000-fold (pK_i from 6.36 to 9.28) bringing it to the same level of affinity found in α_{1A}-AR (pK_i 9.32). Together with the fact that α_{1A}86M and α_{1D}-AR had similar affinities to compound **1**, it is now clear that the α_{1A}-AR selectivity found in compound **1** solely depends on the type of amino acid at the position 86 in α_{1A}-AR. The affinity of compound **2** for ss-α_{1D}156F was also increased by 100 fold (pK_i 7.14) relative to that of α_{1D}-AR (pK_i 5.24), but lower than α_{1A}-AR (pK_i 8.54). This may suggest a minor contribution of other residues, most likely residue 85 (in the α_{1A}-AR numbering), in the subtype selectivity of compound **2**. Compound **2** had a higher affinity for α_{1A}86M (pK_i 6.71) than α_{1D}-AR (pK_i 5.24), wheras the affinity for α_{1A}85T86M was comparative to that of α_{1D}-AR (Table 2), supporting the possibility of the minor contribution of residue 85 to the selectivity. Nonetheless, the significantly increased affinity

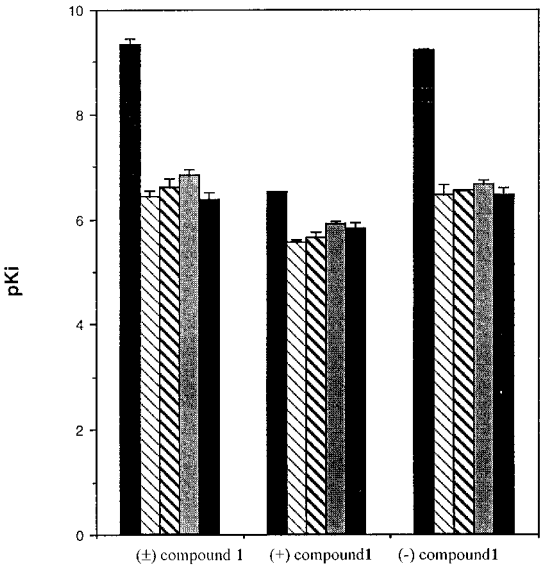

FIGURE 4: Binding of enantiomers of compound **1**. The first solid column represents pK_i's of α_{1A}-AR. The second, third, and fourth columns represent α_{1A}86M (light hatch marks), $\alpha_{1A/D}$(TM23) (heavy hatch marks) containing the second and third transmembrane-spanning domain from α_{1D}-AR, and $\alpha_{1A/D}$(TM2367)(gray bars) containing the second, third, sixth, and seventh transmembrane-spanning domain from α_{1D}-AR, respectively. The last solid column represents pK_i's of α_{1D}-AR.

for both compounds **1** and **2** by the single mutation from Met to Phe at 156 in α_{1D}-AR confirmed that Phe at 86 in α_{1A}-AR is indeed the major determinant for the subtype selectivity of dihydropyridine antagonists.

A number of studies have demonstrated the involvement of various residues in the fifth, sixth, and seventh membrane-spanning domains in ligand binding to various G-protein coupled biogenic amine receptors (Gantz et al., 1992, Link et al., 1992, Guan et al., 1992; Bluml et al., 1994; Adham et al., 1994; Wess et al., 1991; Suryanarayana & Kobilka, 1993; Kobilka et al., 1988). However, no study indicates any significance of the second transmembrane-spanning domain for ligand binding among biogenic amine G-protein coupled receptors (Schwartz, 1994). Mutational studies in the second membrane-spanning domain have been concentrated on a conserved aspartic acid, and the studies performed with β_2-AR, and α_2-AR (Strader et al., 1988; Ceresa and Limbird, 1994) indicated the important role of the conserved aspartic acid in activation/signal transduction rather than ligand binding. This aspartic acid is located close to the cytoplasm while the phenylalanine mutated in this study is predicted to be located in the final helical turn closest to the extracellular region. These two positions are 14 residues apart, and they are predicted to be facing into the interior hydrophilic core on the basis of the hydropathy plot. While the above data provide the most direct evidence for ligands **1**−**4** interacting with Phe-86 in the α_{1A}-AR, in a study with β_2-AR, a radioligand, a derivative of *p*-aminobenzylcarazolol was found cross-linked to a proteolytic fragment of residues 83−96 from the second membrane-spanning domain (Dohlman et al., 1988). Ser-92 and His-93 of were suggested as possible alkylation sites. It should to be noted that His-93 of β_2-AR corresponds to Phe-86 in α_{1A}-AR and this is in accord with the results presented for the α_1-AR antagonist.

In relation to other membrane domains, there have been a number of studies which suggest a significant role for the fifth membrane-spanning domain in ligand binding to bio-

[2] The (−)-enantiomer of compound **1** gave a similar CD spectrum to *S*-(+)-niguldipine, each showing a negative Cotton effect at 380 nm, whereas the (+)-enantiomer of compound **1** had a positive Cotton effect at 380 nm. We are thus able to assign the (−)-**1** enantiomer with the *S*-configuration.

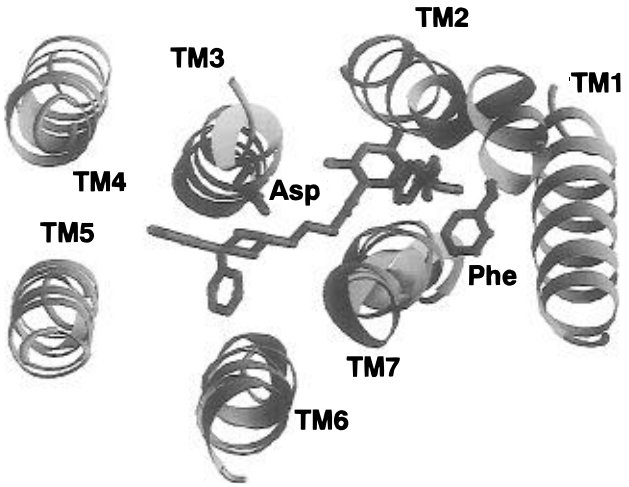

FIGURE 5: A possible binding mode of compound **1** in α_{1A}-AR. The model was built from the automated G-protein coupled receptor modeling in Swiss Model (http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html) using the β-adrenergic receptor model as a template. The ligand was manually docked into the binding crevice based on the mutagensis data and was minimized to reduce unfavorable steric overlaps.

genic amine receptors (Strader et al., 1989; Hwa et al., 1995). On the basis of the observation we have found that the fifth membrane-spanning region did not play significant roles in the subtype selectivity of these tested compounds between α_{1A}-AR and α_{1D}-AR. It was also reported that mutations at 192 and 193 in the fifth membrane-spanning domain of α_{1A}-AR decreased the affinity of 5-methyl Urapidil, WB4101, and HEAT, but no mutations within the fifth membrane-spanning domain (S188A, S192A, and F193L) affected the high affinity of compound **1** (Salon et al., 1995).

Using a model based on bovine rhodopsin, the binding mode of compound **1** to the receptor is shown (Figure 5) to portray the overall details of the putative binding mode of the antagonists in α_{1A}-AR deduced from the experiments described in this study. This is not intended to be construed as supporting a rhodopsin-like structure for α_1-AR's over alternative models (MaloneyHuss & Lybrand, 1992; Pardo et al, 1992): it is provided simply to visually illustrate the results in this study.

It is reasonable to assume the α_1-AR's and the other members of the larger subset of the catecholamine binding G-protein-coupled receptors to which they belong are likely to share a similar global structure. However, it is clear that their ligand binding sites may differ in each receptor and that the binding of different classes of ligands may involve different membrane-spanning domains. Therefore, if we are to gain a better understanding of ligand interactions in this superfamily it is important to focus on seeking a complete understanding of an individual receptor and its subtype with a variety of structurally diverse ligands. This would appear to be necessary from the largely empirical approaches that are replied upon today for the development of highly selective compounds.

ACKNOWLEDGMENT

We wish to thank Aaron Goetz for the assistance in the binding assay and John Ray and Istvan Kaldor for the synthesis of the antagonists.

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BI961024E