α_1-Adrenergic Receptor Subtype Determinants for 4-Piperidyl Oxazole Antagonists†

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ABSTRACT: Mutational studies in conjunction with ligand binding assays were used to examine the basis of α_1-adrenergic receptor subtype selectivity for a series of 4-piperidyloxazole antagonists. A set of chimeric α_{1A} receptors were created by systematically substituting individual transmembrane domains from α_{1D} adrenergic receptors. The oxazole antagonists exhibited significant reductions in affinity against the receptor construct $\alpha_{1A/D}$(TM2), and moderate reductions in affinity versus constructs $\alpha_{1A/D}$(TM5), $\alpha_{1A/B}$(TM5), and $\alpha_{1A/D}$(TM6). Antagonist affinities for these chimeras exceeded those found for wild type α_{1D} and α_{1B}. Site-directed mutagenesis methods were then used to explore the role that individual residues in TM2 and TM5 play in ligand binding affinity and selectivity. These studies revealed that mutations at position 86 in the second transmembrane domain and position 185 in the fifth transmembrane domain of the α_{1A} receptor have a major impact on receptor subtype selectivity.

α_1-adrenergic receptors (α_1-ARs)[1] are a family of G protein-coupled seven transmembrane biogenic amine receptors, which are involved in signaling processes that make them relevant as pharmacological targets for many diseases associated with the cardiovascular and nervous systems. Three known α_1-receptor subtypes, α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR, are expressed in a wide variety of tissues. An α_1-AR antagonist, terazosin, has been used for symptomatic relief of begnign prostatic hypertrophy. However, terazosin is nonselective among α_1-ARs and this has led to a number of undesirable side effects. Therefore, the development of α_{1A}-AR selective antagonists has received a great deal of attention.

The primary ligand binding site for the natural ligands in the biogenic amine subclass of seven transmembrane receptors is putatively located within the transmembrane region. Supporting evidence includes a variety of mutagenesis and ligand binding studies that implicate various residues of the transmembrane domain interior in direct ligand interactions (*1*−*3*). Energy transfer experiments with a fluorescent ligand indicate that the binding site is ∼12 Å below the extracellular surface (*4*). These experiments provide a general picture for the adrenergic ligand binding site. However, more detailed studies are necessary to understand how minor sequence differences control receptor subtype ligand selectivity. A mutational study of the β_2-adrenergic receptor suggested that conserved serine residues in the fifth transmembrane domain form hydrogen bonds with catecholamine ligands (*5*). A subsequent study in α_1 receptors (*6*) suggested the fifth transmembrane domain was involved in agonist selectivity for different α_1 receptor subtypes. Niguldipine, a dihydropyridine analogue, is reported to be an α_{1A} subtype-selective antagonist (*7*,*8*), and we have shown previously that the subtype selectivity of dihydropyridines is determined by a single residue at position 86 in the second transmembrane domain of α_{1A} (*9*). In the work reported here, we have explored the correlations between α_1 receptor subtype sequence variations and antagonist selectivity. A detailed understanding of these correlations would be quite useful for the rational design of subtype-selective α_1 antagonists.

An exhaustive study involving the synthesis and testing of several different classes of α_1 antagonists yielded a novel series of 4-piperidyloxazole analogues (Chart 1) that are highly selective for α_{1A}- versus α_{1B}- and α_{1D}-adrenergic receptors (*10*). Extensive study of the oxazole series analogues indicates that modification at the 4-position of the oxazole ring and at the 3- and 4-positions in the phenyl ring of a phenylethyl-substituted piperidine affect the subtype selectivity by several hundred-fold.

Previously, chimeric receptors and mutant receptors with specific amino acid substitutions have been used to identify residues responsible for ligand selectivity among different G-protein coupled receptor classes and subtypes (*11*−*18*). We have adopted this approach in an attempt to understand how substituents in the 4-piperidyloxazole series influence

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[1] Abbreviations: AR, adrenergic receptor; α_{1A}, α_{1A}-adrenergic receptor; α_{1B}, α_{1B}-adrenergic receptor; α_{1D}, α_{1D}-adrenergic receptor; ss-α_{1D}-AR, α_{1D}-AR containing a signal sequence; 4-piperidyloxazole, 2-methoxy-5-[2-[4-[5-phenyl-4-[(2,2,2-trifluoroethoxy)methyl]-2-oxazolyl]-1-piperidinyl]ethyl]benzenesulfonamide(9CI); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [^{125}I]HEAT, ([^{125}I]-2-β-(4-hydroxyphenyl))ethylaminomethyltetralone; TM, transmembrane; COS-7, monkey kidney epithelial cells, GPCR, G-protein-coupled receptor.

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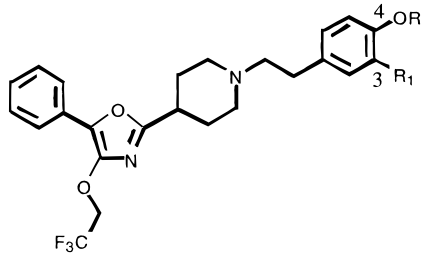
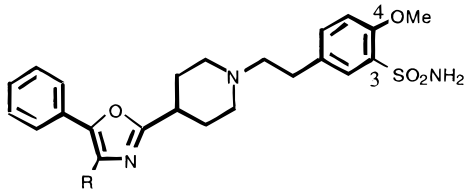
Chart 1



1: R=OH, R1=H
2: R=Et, R1=$SO_2NH(CH_2)_2NHCOMe$
3: R= $(CH_2)_3Me$, R1=H
4: R=Me, R1=$S0_2NH(CH_2)_2NHCOMe$
5: R=Me, R1=H
6: R=Me, R1=SO_2NH_2
12: R=Me, R1=$SO_2NHCH_2CONH_2$



7: R-CH_2OMe
8: R=CH_2OEt
9: R=H
10: R=Me
11: R=CH_2OBu

the observed ligand binding selectivities for α_1-receptor subtypes. We have measured binding affinities for these ligands with various α_{1A}/α_{1D} chimeric receptors and α_{1A} receptor constructs with specific point mutations. The results are discussed in the context of a model of the binding site that provides a consistent explanation for the α_1 receptor subtype selectivity and SAR for these compounds (*19*).

METHODS AND MATERIALS

Materials. 4-Piperidyloxazoles were synthesized in-house and the synthesis of these compounds is described elsewhere (*10*). The structures of the compounds tested in this study are shown in Chart 1. These compounds were dissolved in 100% DMSO prior to appropriate dilution for the binding assays.

Site-Directed Mutagenesis, Transient Expression, and Ligand Binding Assay. The mutant receptors studied are illustrated schematically in Figure 1 and the number and location of mutations together with the K_d values of [^{125}I]-HEAT for each receptor is provided in Table 1. The construction of mutant receptor cDNAs and transient expression in COS-7 cells were described previously (*9*).

Three new receptors with a single point mutation (α_{1A}-185A, $\alpha_{1A/D}$(TM5-185V), and $\alpha_{1A/D}$(TM5-189A)) were created by using the oligonucleotides AGCGCTGAGAATAAT-GCATAGCCCGGCAG (containing a new restriction site AvaIII), GAAGGAGCACACGGCCGAGAAGACAGC (containing a new restriction site Alw21I), GAAGGAGCA-CACGGACGAGAAGACAACGTAGCCCGCCTC (containing a new restriction site Alw21I) for α_{1A}-185A, $\alpha_{1A/D}$(TM5-185V), and $\alpha_{1A/D}$(TM5-189A), respectively. The $\alpha_{1A/D}$(TM5-185V) and $\alpha_{1A/D}$(TM5-189A) mutants were created by in vitro mutagenesis using the Sculptor system (Amersham, IL) with $\alpha_{1A/D}$(TM5) as a template. The α_{1A}-185A mutant was created by using PCR and the mutation site was inserted into α_{1A} cDNA by using Eco47III and KpnI. All the constructs were sequenced. The preparation of crude membrane and the ligand binding assay were described previously (*9*). The binding assay for [^{125}I]HEAT was performed with a various concentrations of membrane protein and 70 000 cpm/100 μL of [^{125}I]HEAT. The displacement assay against [^{125}I]HEAT was performed with 5−10 μg of membrane protein at various concentrations of antagonists. Crude membranes from Rat-1 fibroblast cells stably expressing human ss-α_{1A}-AR and α_{1B}-AR were also used to determine pK_is (*20*). Statistical analyses were employed with Instat 2.0 (Texas A&M University) to derive the standard deviation and p values for the pK_is of the ligands **1**−**12** against each of the constructs. The number of experiments used to compute the standard deviation for the pK_is of the ligands for the various receptors is listed in Table 2.

Table 1: Summary of Mutant Receptors in This Study and K_ds for [^{125}I]HEAT[a]

constructs	location of mutation(s)	no. of residues mutated	K_d for [^{125}I]HEAT (pM)
α_{1A}-AR	NA	0	66 ± 15
$\alpha_{1A/D}$(TM2)	TM2	4	71.3
$\alpha_{1A/D}$(TM3)	TM3	5	95
$\alpha_{1A/D}$(TM4)	TM4	11	115
$\alpha_{1A/D}$(TM5)	TM5	9	235 ± 64
$\alpha_{1A/D}$(TM6)	TM6	6	30
$\alpha_{1A/D}$(TM7)	TM7	6	42 ± 40
$\alpha_{1A/B}$(TM5)	TM5	4	54
α_{1A}85T	TM2	1	21
α_{1A}86M	TM2	1	30
α_{1A}85T86M	TM2	2	272
α_{1A}185A	TM5	1	13
α_{1A}85T85M$_{/D}$(TM5)	TM2&5	11	362
$\alpha_{1A/D}$(TM5,6)	TM5&6	15	150
$\alpha_{1A/D}$(TM5-185V)	TM5	10	78 ± 15
$\alpha_{1A/D}$(TM5-189A)	TM5	10	390 ± 16
ss-α_{1D}-AR	NA	0	63 ± 47

[a] The first row and the last row are wild-type α_1-ARs. The exact locations and amino acids for the chimeric receptors were previously shown (*9*). The K_ds were measured for each membrane preparation used. When more than two separate membrane preparations were used, the average of the K_d and standard deviations are shown.

RESULTS AND DISCUSSION

Transmembrane Chimeras. We reported previously that each of five α_{1A}-AR chimeric receptors containing sequences from the α_{1D}-AR at the membrane spanning domains had, with one exception, K_ds for [^{125}I]HEAT comparable to those of wild-type α_{1A}-AR (Table 1). The only exception was the $\alpha_{1A/D}$(TM5) which had a similar K_d to ss-α_{1D}-AR (*9*). Unfortunately, the chimeric receptor containing the first membrane spanning domain, $\alpha_{1A/D}$(TM1), was poorly expressed in the membrane fraction of COS cells and could not be used for further studies. The binding characteristics of each of the other chimeras were first explored with the

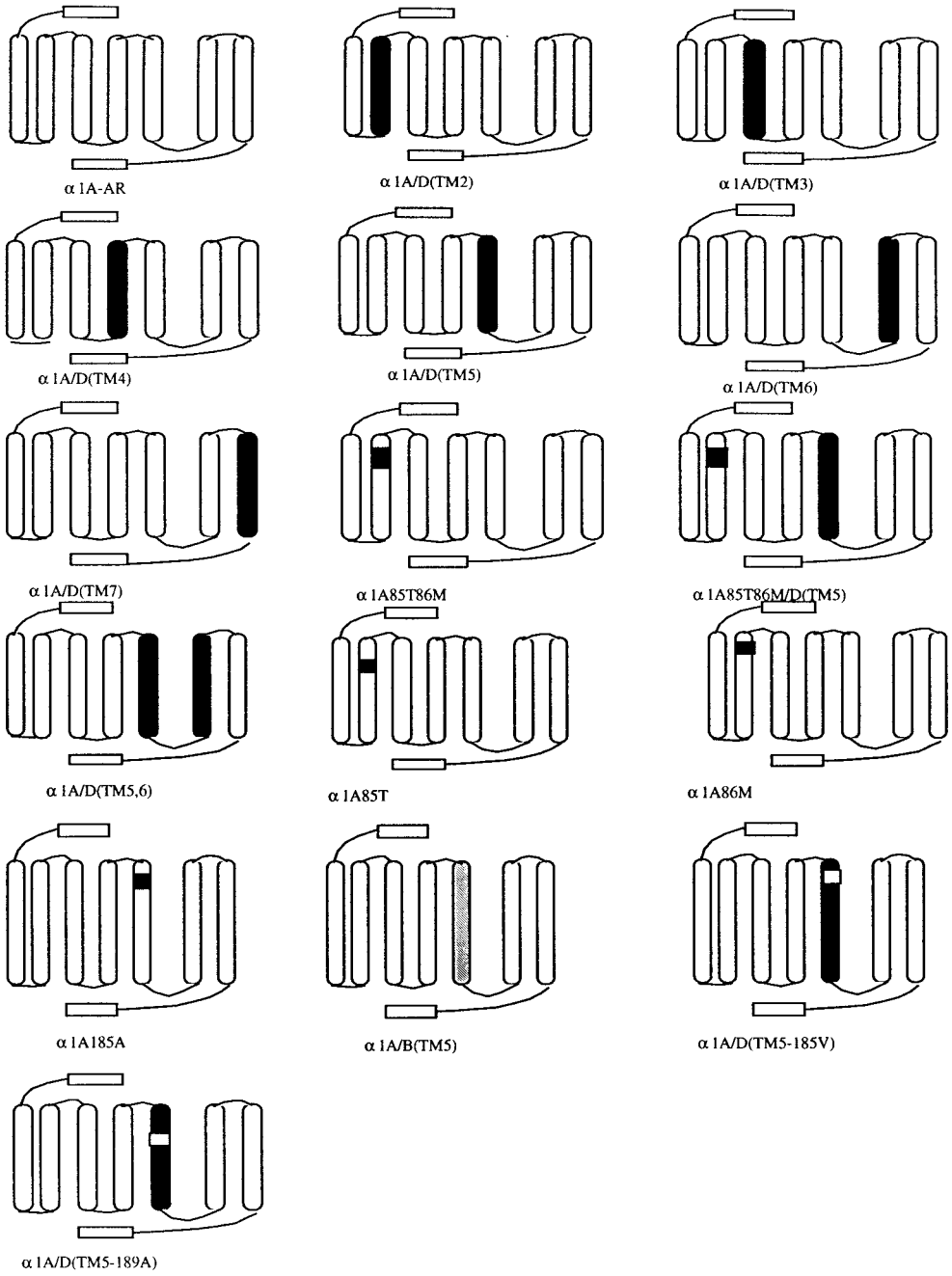


FIGURE 1: Schematic presentation of chimeric receptors studied. The N-terminus is at the top of each figure and the C-terminus is at the bottom. The boxes at the N- and C-terminus represent epitope tags (*34*) that were added for immunological detection of the recombinant proteins. The open ovals represent transmembrane domains with an α1A sequence; the heavily shaded ovals represent transmembrane domains with α1D sequence, and the lightly shaded oval indicates transmembrane domains with α1B sequence. Site specific mutations are represented by boxes within the ovals. The lines connecting the ovals represent the loops with an α1A sequence.

nonselective antagonist prazosin (Table 2). The pK_is of prazosin for $\alpha_{1A/D}$(TM2), $\alpha_{1A/D}$(TM4), $\alpha_{1A/D}$(TM5), and $\alpha_{1A/D}$(TM7) were similar to the pK_i of 9.70 for α_{1A}-AR, while the pK_is for $\alpha_{1A/D}$(TM3) and $\alpha_{1A/D}$(TM6) were slightly higher than those for α_{1A}-AR.

Two α_{1A}-selective 4-piperidyloxazole analogues, **2** and **3**, were tested against the six chimeric receptors. The chimeras, $\alpha_{1A/D}$(TM3), $\alpha_{1A/D}$(TM4), and $\alpha_{1A/D}$(TM7), exhibited binding affinities for both compounds comparable to that of the parent α_{1A} receptor (Table 2). In contrast, $\alpha_{1A/D}$(TM2) showed significantly reduced affinity for both selective antagonists while $\alpha_{1A/D}$(TM5) showed moderately reduced affinity for these antagonists (Table 2). The α_{1A} and ss-α_{1D} receptors differ by only four residues (α_{1A} positions 75, 76, 85, and 86) in the second transmembrane domain (Table 3). We recently reported that α_{1A} residue 86 is the key determinant for the α_1-subtype selectivity of dihydropyridine antagonists (*9*). This is of interest since the primary alkylation site of the antagonist *p*-(bromoacetamido)benzyl-1-iodocarazolol in the β_2-adrenergic receptor was localized to a peptide fragment from the second transmembrane domain (*21*), and the only nucleophiles present in this peptide segment are Ser92 and His93, which correspond to α_{1A} residues Ile85 and Phe86. The analogous residues in α_{1D} are Thr155 and Met156. To define more precisely the role of TM2 residues in subtype selectivity for the 4-piperidyloxazole compounds, we con-

Table 2: Summary of pK_is reported in this study[a]

receptor	prazosin	1	2	3	4	5	6	7	8	9	10	11	12
α_{1A}-AR	9.70(0.26,4)	9.43(0.05,4)	8.83(0.26,3)	8.13(0.31,3)	8.95(0.13,3)	8.59(0.14,4)	9.20(0.05,4)	8.31(0.04,3)	8.56(0.24,3)	9.47(0.32,3)	9.86(0.18,3)	8.15(0.23,4)	8.55(0.02,3)
$\alpha_{1A/D}$(TM2)	9.50(0.38,2)	ND	6.83(0.01,3)	6.97(0.01,3)	ND	ND	ND	ND	ND	ND	ND	ND	ND
$\alpha_{1A/D}$(TM3)	10.75(0.01,3)	ND	8.73(0.03,3)	8.03(0.01,3)	ND	ND	ND	ND	ND	ND	ND	ND	ND
$\alpha_{1A/D}$(TM4)	10.29(0.71,3)	ND	8.53(0.03,3)	7.85(0.01,3)	ND	ND	ND	ND	ND	ND	ND	ND	ND
$\alpha_{1A/D}$(TM5)	9.81(0.17,3)	8.46(0.08,6)	7.92(0.10,3)	7.18(0.22,3)	8.00(0.17,4)	7.71(0.15,6)	8.08(0.08,4)	8.15(0.03,3)	7.86(0.08,3)	8.45(0.10,3)	8.57(0.07,3)	7.81(0.06,3)	7.94(0.17,3)
$\alpha_{1A/D}$(TM6)	10.74(0.13,3)	ND	8.25(0.02,3)	7.47(0.01,3)	ND	ND	ND	ND	ND	ND	ND	ND	ND
$\alpha_{1A/D}$(TM7)	9.95(0.13,3)	ND	8.90(0.06,3)	8.11(0.02,3)	ND	ND	ND	ND	ND	ND	ND	ND	ND
α_{1A}85T86M	8.95(0.12,3)	8.75(0.18,3)	6.79(0.07,3)	6.68(0.01,3)	7.38(0.18,3)	7.20(0.08,3)	8.15(0.10,3)	7.96(0.17,3)	7.88(0.22,3)	9.28(0.07,3)	9.21(0.03,3)	7.68(0.11,3)	7.86(0.08,3)
α_{1A}85T86M$_{/D}$(TM5)	ND	8.10(0.10,3)	6.08(0.01,3)	6.20(0.17,3)	7.06(0.01,3)	6.31(0.15,3)	7.50(0.13,3)	7.71(0.16,3)	7.46(0.15,3)	8.94(0.02,3)	ND	7.29(0.14,3)	7.03(0.22,3)
$\alpha_{1A/D}$(TM5,6)	10.04(0.21,3)	ND	7.90(0.01,3)	7.14(0.01,3)	ND	ND	ND	ND	ND	ND	ND	ND	ND
α_{1A}85T	ND	9.56(0.04,3)	8.77(0.08,3)	8.08(0.01,3)	8.69(0.08,3)	8.59(0.12,3)	9.10(0.11,3)	ND	ND	ND	ND	ND	ND
α_{1A}86M	ND	9.28(0.16,3)	7.40(0.12,3)	7.37(0.03,3)	8.22(0.05,3)	7.79(0.15,3)	8.99(0.04,3)	8.32(0.22,3)	8.42(0.21,3)	9.13(0.18,3)	9.59(0.09,3)	8.00(0.34,3)	ND
α_{1A}185A	ND	8.45(0.07)	7.86(0.12,3)	7.28(0.04)	8.22(0.10,3)	7.91(0.06,3)	8.40(0.01,3)	8.62(0.08,3)	8.17(0.09,3)	9.44(0.29,3)	9.46(0.08,3)	7.99(0.24,3)	8.00(0.18,3)
$\alpha_{1A/B}$(TM5)	ND	8.46(0.08,)	ND	ND	8.04(0.02,3)	7.72(0.09,3)	8.04(0.06,3)	8.22(0.13,3)	7.56(0.10,3)	ND	ND	7.60(0.17,3)	ND
$\alpha_{1A/D}$(TM5-185V)	ND	9.44(0.36,3)	8.77(0.33,3)	7.90(0.26,3)	9.04(0.30,3)	ND	9.47(0.25,3)	9.78(0.01,3)	9.52(0.22,3)	ND	ND	9.07(0.19,3)	9.72(0.37,3)
$\alpha_{1A/D}$(TM5-189A)	ND	8.38(0.11,3)	ND	7.08(0.33,3)	7.92(0.10,3)	7.52(0.12,3)	8.09(0.05,3)	8.34(0.03,3)	7.99(0.04,3)	ND	ND	7.90(0.01,3)	8.00(0.21,3)
α_{1B}-AR	ND	ND	5.83(0.04,3)	6.58(0.01,3)	ND	ND	7.20(0.05,3)	7.82(0.09)	7.40(0.07)	ND	ND	7.25(0.02,3)	ND
ss-α_{1D}-AR	11.11(0.06,4)	7.71(0.01,3)	6.04(0.10,3)	6.28(0.01,3)	7.02(0.11,3)	6.54(0.04,4)	7.09(0.09,3)	7.59(0.06,3)	7.22(0.05,3)	7.91(0.05,3)	8.14(0.11,3)	7.19(0.09,3)	6.95(0.05,3)

[a] The statistical deviation (SD) and number of experiments are shown in parentheses.

Table 3: Sequence Data for Regions of the Second and Fifth Transmembrane Domains of the Human α_1-Adrenergic Receptors

	TM2																						
	69																					**86**	
α_{1A}	A	V	A	D		L	L	T	S		T	V	L	P	F	S	A	I	F	E	V
α_{1D}	-	-	-	-		-	-	S	A		-	-	-	-	-	-	-	T	M	-	-
	TM5																				
				185				**189**													
α_{1A}	P	G	Y	V	L	F	S	A	L	G	S	F	Y	L	A	I	I	L			
α_{1D}	A	-	-	A	V	-	-	S	V	C	-	-	-	M	-	V	-	V			
α_{1B}	-	F	-	A	-	-	-	S	-	-	-	-	-	-	-	V	-	-			

structed the double mutant, α_{1A}85T86M. As shown in Table 2, this double mutant exhibits low affinities for both α_{1A} selective compounds, with pK_is comparable to those observed for $\alpha_{1A/D}$(TM2).

Double Domain Chimeras. Previous studies have shown that Val185 in the fifth transmembrane domain and Met292 in the sixth transmembrane domain of the α_{1A} receptor are critical for subtype selectivity of agonists and, in some cases, have additive or cooperative effects on binding affinity (*6*). In this study we observed that the chimeric receptor $\alpha_{1A/D}$(TM5) showed an 8- to 11-fold reduction in affinity versus α_{1A} for compounds **2** and **3**, while $\alpha_{1A/D}$(TM6) exhibited a 3- to 5-fold reduction in affinity for these selective antagonists (Table 2). To determine if the effects of TM5 and TM6 on binding affinity were independent and/or additive, we constructed a chimeric receptor, $\alpha_{1A/D}$(TM5, 6), which contained the sequences of both the fifth and sixth transmembrane domains from ss-α_{1D}. The chimeric receptor $\alpha_{1A/D}$(TM5, 6) exhibited only a 6- to 7-fold reduction in affinity for the selective compounds **2** and **3** (Table 2). Thus, we cannot conclude from these data alone that the effects of TM5 and TM6 are independent or additive for this series of 4-piperidyloxazole compounds. The binding affinities observed for $\alpha_{1A/D}$(TM5, 6) may merely reflect conformational changes in the receptor due to altered packing between the fifth and sixth transmembrane domains.

Residues in the fifth transmembrane domain are important for ligand binding in some biogenic amine GPCRs (*5*, *6*, *22*−*28*). Since the $\alpha_{1A/D}$(TM5) chimera showed reduced affinities for the oxazole antagonists (Table 2) compared to $\alpha_{1A/D}$(TM6), we decided to examine in more detail the effects of the fifth transmembrane domain on receptor subtype selectivity. We constructed a chimeric receptor, $\alpha_{1A/D}$(TM5), that also contains two mutations in TM2 at positions 85 and 86. This chimeric receptor, α_{1A}85T86M$_{/D}$(TM5), was examined for additive or cooperative effects of both TM2 and TM5 on antagonist binding. Ten of the 4-piperidyloxazole antagonists were tested against this chimera and the results are shown in Figure 2, together with pK_is for α_{1A} and ss-α_{1D} for comparison. The pK_i for each antagonist was lower for α_{1A}85T86M and $\alpha_{1A/D}$(TM5) relative to α_{1A} and higher compared to ss-α_{1D}. The double domain chimera, α_{1A}85T86M$_{/D}$(TM5), exhibited binding affinities for all antagonists comparable to ss-α_{1D}. These values were significantly lower than the observed binding affinities for α_{1A}-85T86M, $\alpha_{1A/D}$(TM5), or α_{1A}. These results suggest that selectivity for this series of antagonists is determined by residues in both the second and fifth transmembrane domains and that the contributions of TM2 and TM5 are independent and additive.

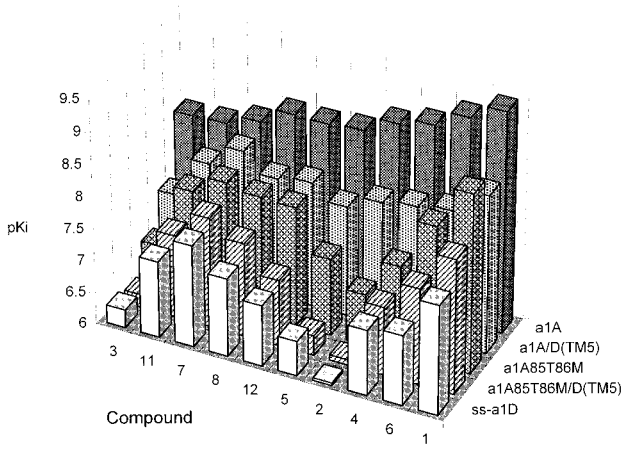


FIGURE 2: Representation of the average pK_is of 10 4-piperidyloxazole analogues against three chimeras, α_{1A}85T86M, $\alpha_{1A/D}$(TM5), and α_{1A}85T86M$_{/D}$(TM5), compared against α_{1A} and ss-α_{1D}. The compounds are ordered based on increasing pK_is for α_{1A}. The standard deviations (SD) and the number of experiments used to determine the values of the pK_i for each ligand are provided in Table 2.

3- and 4-Phenyl Substituent Effects on Selectivity. Six compounds, **1**-**6,** with various modifications at the 3- and 4-position of the phenethyl ring were tested against α_{1A}, ss-α_{1D}, and the two chimeras, α_{1A}85T86M and $\alpha_{1A/D}$(TM5). These initial results revealed no clear relation between the substitution patterns and the selectivity. To facilitate a discussion of the results, the antagonists are shown ordered by increasing pK_i for α_{1A} in Figure 3A. Both α_{1A}85T86M and $\alpha_{1A/D}$(TM5) had significantly reduced affinity toward the six ligands (Figure 3A), in comparison to that observed for α_{1A}. However, each receptor construct exhibited different responses to the substituent variations at the 3- or 4-position of the phenethyl ring. Like α_{1A}, the chimera $\alpha_{1A/D}$(TM5) was relatively insensitive to the presence of bulky groups, e.g., the sulfonamide on the phenylethyl ring in compound **2.** In contrast, α_{1A}85T86M and ss-α_{1D} were much more sensitive to modifications of these substituents. For example, the change from an ethoxy to a methoxy at the 4-position (**2** versus **4**) resulted in increases in affinity that were significant for ss-α_{1D} and α_{1A}85T86M, but not for α_{1A} or $\alpha_{1A/D}$(TM5). These results suggest that the determinant(s) for selectivity relative to modifications at the 3- or 4-position of the phenyl ring reside within the second transmembrane domain.

To further characterize the basis of this selectivity, two mutant receptors with single point mutations at α_{1A} positions 85 or 86 were constructed. The mutant receptor, α_{1A}86M, was found to have significantly lower affinities for four of the antagonists (compounds **2**−**5**) versus α_{1A}, whereas the same four compounds exhibited significantly higher affinities versus α_{1A}85T86M when compared to α_{1A}. The mutant α_{1A}-86M showed a similar trend as ss-α_{1D} for compounds **1**−**6**, but with overall higher affinities and the same relative increase in binding brought about by the substituent change from ethoxy (**2**) to methoxy (**4**) (Figure 3B). A parallel to the latter trend for compounds **1**−**6** was also observed for $\alpha_{1A/D}$(TM2). The mutant receptor had comparable affinities to α_{1A} (Figure 3B) against the antagonist set **1**−**6**, which are significantly higher than is observed versus α_{1A}85T86M.

Previously**,** we found the α_{1A}-subtype selectivity of dihydropyridines was determined solely by the residue in

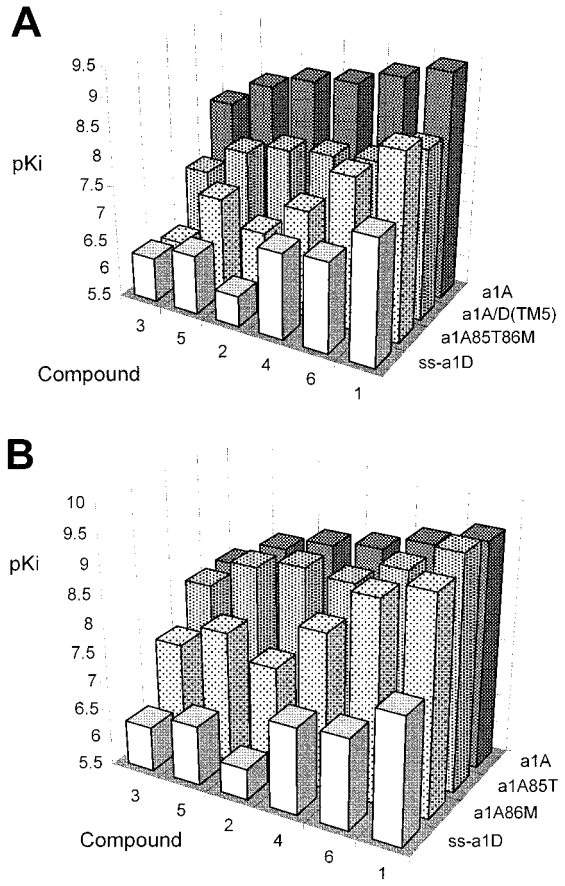


FIGURE 3: Representation of the average pK_is of 4-piperidyloxazole analogues **1**−**6** which differ only in the substituents at the 3- and/or 4-positions of the phenylethyl ring. The compounds are ordered based on increasing pK_is for α_{1A}. (A) The pK_is for **1**−**6** against α_{1A}, α_{1A}85T86M, $\alpha_{1A/D}$(TM5), and ss-α_{1D} are shown while in (B) the pK_is against α_{1A}, α_{1A}85T, α_{1A}86M, and ss-α_{1D} are portrayed.

α_{1A} at position 86 (*9*). In this current study, the selectivity found in the 4-piperidyloxazole antagonists also appears to be governed primarily by the residue at position 86. In fact, the mutant α_{1D}156F (*9*), which contains Phe at the position corresponding to 86 in α_{1A}, shows significantly increased affinity for compound **2**, pK_i 7.41 ± 0.03 (not in Table 2), and slightly increased affinity for compound **3**, pK_i 6.56 ± 0.04 (not in Table 2). Multiple sequence alignment data for adrenergic receptors show that the position analogous to position 86 in α_{1A} is different in each subtype (e.g., α_{1A}Phe, α_{1B}Leu, α_{1D}Met) and strictly conserved among species (*19*). By contrast, there is sequence variation among species for the position analogous to position 85 in α_{1A} (e.g., Thr in rat α_{1B} and Ala in human α_{1B}). Since subtype selectivity for the oxazole analogues is indeed conserved between human and rat (data not shown), it seems probable that position 86 in α_{1A} plays an important role in determining binding selectivity for these ligands, quite possibly via a direct ligand interaction. Furthermore, position 85 in α_{1A} clearly has a less significant effect on ligand binding selectivity, and its effect is likely an indirect one (e.g., local helix packing effects).

Bulkier substituents at the 4-position of the phenylethyl ring appear to result in decreased affinity for both α_{1A} and ss-α_{1D}, with ss-α_{1D} showing greater sensitivity (Figure 3; Table 2). However, a substitution from ethoxy to methoxy (**2** to **4**) is significant if a methionine is present at position

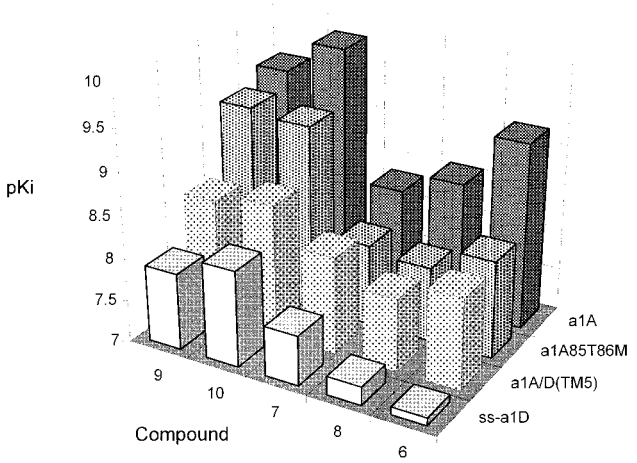


FIGURE 4: Plots of the variation of the average pK_is of 4-piperidyloxazole analogues **6**−**10** which differ only in the substituent at the 4-position of the oxazole ring. Solid bars depict results for α_{1A}, and open bars are for ss-α_{1D}. The compounds are ordered, from left to right, based on the increasing size of the 4-substituents. The pK_is for α_{1A}, α_{1A}85T86M, $\alpha_{1A/D}$(TM5), and ss-α_{1D} are shown.

86 in α_{1A} (e.g., α_{1D} or α_{1A}86M), but there is no change in affinity with a phenylalanine at this position (e.g., α_{1A} or α_{1A}85T). The presence of an Ile or Thr at position 85 in α_{1A} does not affect this result. This suggests that methionine at position 86 in α_{1A} may exhibit lower binding affinity due to diminished hydrophobic contact.

Comparing the pK_is for **1** and **5**, a simple change from hydroxy to methoxy at the 4-position in the phenethyl ring reduced the affinities toward ss-α_{1D} as well as α_{1A}85T86M by 15- to 30-fold, while the affinity for α_{1A} was reduced only 2-fold. These data may indicate that there is a larger hydrophobic pocket near residue 86 in α_{1A} relative to α_{1D}. It is possible that phenylalanine at position 86 may provide a more hydrophobic environment as well as being more rigid than methionine (*29*). Alternately, a phenylalanine residue at position 86 may alter helix packing, leading to a larger pocket in this region of the ligand binding site. This would allow bulkier substituents at the 3- and 4-positions of the phenethyl ring to be accommodated more readily.

Modification at 4-Position of the Oxazole Ring. Various modifications at the 4-position of the oxazole ring were also studied to probe their effects on subtype selectivity. Oxazole ring substitutions had less dramatic impact on receptor binding and selectivities versus the phenylethyl ring substitutions discussed above. Five compounds, **6**−**10**, with various modifications at the 4-position of the oxazole ring were tested against the chimeric receptors, $\alpha_{1A/D}$(TM5) and α_{1A}85T86M (Figure 4). The relative affinities of these compounds for $\alpha_{1A/D}$(TM5) and ss-α_{1D} were similar. The relative affinities of the compounds **6**−**10** for α_{1A}85T86M and α_{1A} were also comparable. However, there were no clear trends in receptor subtype selectivity versus 4-oxazole substituents for these compounds.

These data suggest that the oxazole end of the ligands interact with residues in TM5, but the relationship between the substituents and their effects on selectivity is complex. For α_{1D}, the highest binding affinities were achieved with small substituents such as methyl (**10**) or hydrogen (**9**), while methoxymethyl (**7**) and larger substituents resulted in reduced affinities (Table 2; Figure 4). The relationship between affinity and size of the 4-oxazole substituent was less straightforward for α_{1A}, as methyl, hydrogen, and trifluoromethoxymethyl (**6**) substituents resulted in the highest affinities, followed by methoxymethyl, ethoxymethyl (**8**), and butyloxymethyl (**11**) substituents, which all had comparable, lower affinities. Although smaller substituents such as methyl and hydrogen resulted in the highest affinities for α_{1A} and α_{1D}, selectivity for α_{1A} versus α_{1D} was optimized with the introduction of a trifluoromethoxymethyl group (**6**). The smallest difference in selectivity was observed with a methoxymethyl substituent (**7**). Clearly, there is no simple correlation between substituent size and subtype selectivity.

To study the contribution of the fifth transmembrane domain for α_1-subtype selectivity in greater detail, another α_{1A} chimeric receptor, $\alpha_{1A/B}$(TM5), containing the fifth transmembrane domain from α_{1B}, was constructed. The chimeras $\alpha_{1A/B}$(TM5) and $\alpha_{1A/D}$(TM5) had similar affinities for compounds modified at the 4-position of the oxazole ring (Table 2). With respect to α_{1A}, $\alpha_{1A/D}$(TM5) has nine amino acid substitutions and $\alpha_{1A/B}$(TM5) has four substitutions (Table 3). Three of the four substitutions found in $\alpha_{1A/B}$(TM5), V185A, A189S, and I199V, were also present in $\alpha_{1A/D}$(TM5). Since $\alpha_{1A/B}$(TM5) and $\alpha_{1A/D}$(TM5) exhibited similar affinities for the oxazole compounds, we assumed that the determinant for selectivity resides in one or more of these three residues (Table 2). The residue at α_{1A} position 199 was not considered further as it has been predicted to lie near the cytoplasmic end of helix 5. The residue at position 185 in α_{1A} has been predicted to lie at the top of TM5 near the extracellular surface and has been shown to affect subtype selectivity of α_1 agonists (*6*). It has also been reported that the residue analogous to Val185 in the neurokinin-1 receptor (His197 based on sequence alignments—data not shown) interacts directly with the aromatic groups of a non-peptide antagonist (*30*). Thus, α_{1A} Val185 was selected as a single point mutation site. The mutant receptor, α_{1A}185A, was tested against 10 4-piperidyloxazole compounds and the results are presented along with pK_is for α_{1A}, α_{1D}, and $\alpha_{1A/D}$(TM5) in Figure 5. For the six compounds containing a trifluoromethoxymethyl group at position 4 of the oxazole ring, α_{1A}185A displayed reduced affinities versus α_{1A} and comparable affinities to $\alpha_{1A/D}$(TM5). Thus, the approximate 10-fold difference in affinity among these six compounds appears to be related to direct or indirect contacts between the trifluoromethoxymethyl group and Val185 in α_{1A}. To examine this position in more detail, Ala185 in the chimeric receptor $\alpha_{1A/D}$(TM5) was mutated to determine if the high affinity of α_{1A} could be recovered by substituting a valine at position 185, $\alpha_{1A/D}$(TM5-185V). This A185V substitution in $\alpha_{1A/D}$(TM5) enhanced binding affinity to levels comparable to, and in some cases exceeding, those of α_{1A} (Table 2). These results also suggest that Val185 interacts with the trifluoromethoxymethyl substituent at position 4 on the oxazole ring.

For compounds without a trifluoromethoxymethyl substituent (**7**, **8**, **9**, and **10**), the mutant receptor α_{1A}185A exhibited significantly higher affinities versus $\alpha_{1A/D}$(TM5), with no clear trends in comparison to α_{1A} (Table 2). These results suggest the determinant of selectivity for these four compounds may not be related to the residue at α_{1A} position 185. However, the substitution of valine at position 185 in the chimeric receptor $\alpha_{1A/D}$(TM5−185V) (Figure 5) resulted

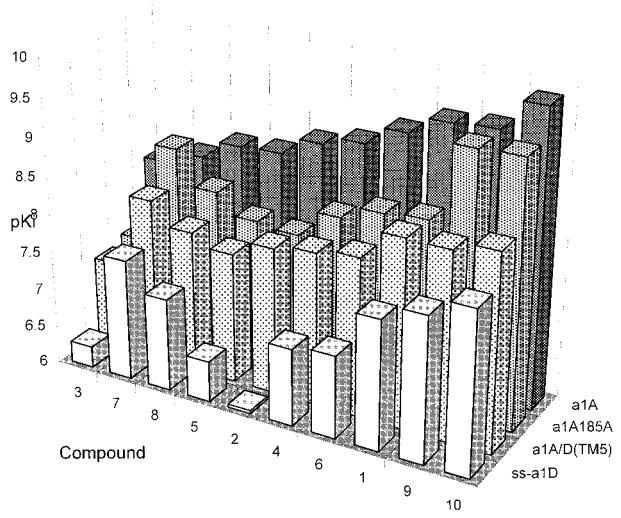


FIGURE 5: Representation of the average pK_is of 4-piperidyloxazole analogues **1**−**10** measured against α_{1A}185A and $\alpha_{1A/D}$(TM5) compared to those obtained against α_{1A} and ss-α_{1D}. The compounds are ordered based on increasing pK_is for α_{1A}.

in affinities that exceeded α_{1A} for both **7** and **8**, suggesting that Val185 may interact with methylmethoxy or ethylmethoxy substituents at position 4 of the oxazole.

Compounds with small substituents at the 4-oxazole position (**9**, **10**) showed the highest binding affinity for α_{1A} and ss-α_{1D} (Figure 4). For **9** and **10**, α_{1A}185A exhibited significantly higher binding affinities versus $\alpha_{1A/D}$(TM5) and comparable or higher affinities versus α_{1A}. These data suggest Val185 does not determine the selectivity of these two compounds, but there may be other residues in TM5 that do affect selectivity. Val185 has been reported to be a recognition site for α_{1A}-subtype selective agonists, and the point mutation A204V in α_{1B} (position 185 in α_{1A}) resulted in increased affinities for some agonists compared with both wild type α_{1A} and α_{1B} (*6*). It was proposed that a valine at α_{1A} position 185 increases affinity by making hydrophobic contacts with phenyl groups in the agonists phenylephrine, epinephrine, and norepinephrine. This increase in affinity by valine substitution was reversed by introducing a second mutation from leucine to methionine at α_{1B} position 314 in the sixth transmembrane domain (position 292 in α_{1A}). It was observed that the combination of valine and leucine in the neighboring positions of the fifth and sixth membrane domains (which does not occur in any wild-type α_1 receptors) resulted in affinities for agonists that exceeded those of wild-type receptors. The chimeric receptor $\alpha_{1A/D}$(TM5-185V) had the same combination of valine and methionine in the fifth and sixth membrane domains as found in α_{1A} yet still exhibited higher affinities versus α_{1A} (Table 2). The contacts in this region of the binding site may be more complex for this series of 4-piperidyloxazole antagonists than can be explained by only one or two amino acids. However, we must also be careful in interpreting these data as two residues may interact differently due to differences in helix−helix packing in a chimera such as $\alpha_{1A/D}$(TM5−185V), versus a receptor with a single or a double point mutation (*6*). It has also been reported that α_{1B} with a valine at position 204 (α_{1A}-185) was constitutively active (*31*), again suggesting that any structural changes introduced by mutagenesis must be considered with the dynamic nature of the receptors in mind. To better understand the increased affinities observed with various oxazole compounds, we will need to study the agonist binding and constitutive activity of $\alpha_{1A/D}$(TM5-185V) in more detail.

The serine at position 189 of the chimeric receptor $\alpha_{1A/D}$(TM5) was also mutated in order to determine if the high affinity of α_{1A} could be recovered by substituting an alanine at position 189, $\alpha_{1A/D}$(TM5-189A) (Table 2). The substitution of Ala189 in $\alpha_{1A/D}$(TM5-189A) did not produce any increase in affinity relative to $\alpha_{1A/D}$(TM5), suggesting that position 189 is not a determinant of selectivity. Residue 189 in α_{1A} is equivalent to Ser204 in the β_2 adrenergic receptor, which was found to be involved in a hydrogen bond with various ligands (*5*). In a recent study, three serines in the fifth transmembrane domain (Ser188, Ser189, and Ser192 in α_{1A}) in α_{1B} were mutated to alanines (*6, 32*). It was observed that a single mutation at these sites did not have a significant effect on agonist binding. However, the mutant containing alanines at positions 188 and 192 showed decreased affinity, suggesting that serines at 188 and 192, but not at 189, can provide a hydrogen bond sufficient for agonist binding. The results of our study are consistent with this result and the result of others in suggesting that position 189 (alanine or serine) in α_1 receptors does not contribute to subtype-selective interactions with ligands.

Clearly, there are no simple patterns observed for ligand selectivty for substituents at the 4-position of the oxazole ring relative to specific amino acid substitutions in TM5. The ligand binding data (Table 2; Figures 4,5) suggest that α_{1A} Val185 does interact with the substituent at position 4 of the oxazole ring, directly or indirectly, and indicate that the greatest selectivity between α_{1A} and α_{1D} is achieved with a trifluoromethoxymethyl group at this position. The specific ligand−receptor interactions in this region of the binding site are either more complex than can be identified with simple chimera and mutagenesis experiments, or else the correlation between subtype selectivity and oxazole position 4 substituents depends on factors other than substituent size, e.g., substituent hydrophobicity, polarizability, etc.

In summary, our experimental results and receptor−ligand complex models suggest that the 4-piperidyloxazole compounds bind to the α_1 receptors with the phenyl substituent of the 1-phenylethylpiperidine near the second transmembrane domain and the oxazole ring near the fifth transmembrane domain. In particular, 4-phenyl substituents are probably positioned close to residue 86 in the α_{1A} receptor, which appears to be a key determinant of α_1-subtype selectivity. Substituents at position 4 of the oxazole ring appear to interact with residues in the fifth transmembrane domain. The selectivity of the 4-piperidyloxazole compounds was increased for α_{1A} over α_{1D} by introducing a trifluoromethoxymethyl substituent at the 4-oxazole position. This increase in selectivity appears to be attributable to the presence of Val185 in α_{1A} versus an alanine at the corresponding position in α_{1D}. The specific interactions between Val185 in α_{1A} and the trifluoromethoxymethyl substituent are not completely clear, and residues in the fifth and sixth transmembrane domains will need to be examined in more detail to gain a better understanding of the determinants of α_1-subtype selectivity in this region of the receptor.

Although the first high resolution structure has been obtained for bacteriorhodopsin (*33*) it is likely to be some time before high-resolution structures become available for

other 7-transmembrane receptors such as the α_1-receptor−antagonist complexes. Until then, detailed mutational studies and biophysical experiments coupled with three-dimensional model construction will be critical to enhance our knowledge of these important receptors. Studies such as those outlined here can provide information that may be useful for the development of more selective therapeutic agents.

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SUPPORTING INFORMATION AVAILABLE

Tables of the *p* values for the pK_i data that are presented in Table 2 are provided (2 pages). Ordering information is given on any current masthead page.

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