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Structural Basis of the Subtype Selectivity of Muscarinic Antagonists: A Study with Chimeric m2/m5 Muscarinic Receptors

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SUMMARY

The five muscarinic receptors (m1–m5), although structurally closely related, can be distinguished pharmacologically by the use of subtype-selective ligands. Various tricyclic muscarinic antagonists, including the AF-DX derivative AQ-RA 741 and the alkaloid himbacine, for example, have been shown to display up to 200-fold higher affinities for m2 and m4 than for m5 receptors. On the other hand, antagonists such as sila-hexocyclium and the pirenzepine derivative UH-AH 37 exhibit lower affinities for m2 than for m5 and all other muscarinic receptors. To identify receptor epitopes that contribute to the subtype selectivities of these antagonists, we prepared a series of chimeric m2/m5 muscarinic receptors in which regions of the m5 receptor were systematically replaced with the homologous regions of the m2 receptor. AQ-RA 741, himbacine, and sila-hexocyclium bound to the various chimeric receptors, expressed in COS-7 cells, with affinity profiles indicative of multiple receptor domains contributing to the subtype selectivities of these antagonists. On the other hand, the higher affinity of UH-AH 37 for m5 than for m2 receptors appears to be largely dependent on a short stretch of 31 amino acids comprising most of transmembrane region VI and the third extracellular loop, a region that does not contribute to the subtype selectivity of AQ-RA 741 and himbacine. Our data indicate that different receptor epitopes are involved in conferring subtype selectivity on structurally different muscarinic antagonists.

Molecular cloning studies have revealed the existence of five structurally closely related muscarinic receptor proteins (m1–m5) (1–5). As are all other members of the superfamily of G protein-coupled receptors, the five muscarinic receptors are predicted to be composed of seven hydrophobic TM helices (TM I–VII) connected by alternating cytoplasmic and extracellular loops, an extracellular amino-terminal domain, and a cytoplasmic carboxyl-terminal segment. Whereas sequences within the third cytoplasmic loop appear to be responsible for G protein recognition and activation (6–9), ligand binding to muscarinic receptors, in analogy to findings obtained with adrenergic receptors (for reviews, see Refs. 10 and 11), is thought to occur within a cavity formed by the seven TM helices (12, 13). mRNA mapping (14–16), as well as immunological studies using subtype-selective antibodies (17, 18), has shown that each muscarinic receptor displays a distinct pattern of distribution throughout the central and peripheral nervous systems.

As more information is being gathered about the physiological and pathophysiological roles of each of the muscarinic receptor subtypes, pharmacologists are focusing on the therapeutic potential of subtype-selective muscarinic drugs in a variety of central nervous system disorders. For example, antagonists that selectively block presynaptic m2 receptors mediating autoinhibition of acetylcholine release have been proposed for the treatment of memory disorders associated with Alzheimer's disease (19). On the other hand, selective m5 receptor antagonists, primarily based on the observation that m5 and D2 mRNAs are colocalized in discrete brain areas (16), may have clinical utility in the treatment of disorders characterized by an elevated dopaminergic tone (e.g., schizophrenia and Tourette's syndrome).

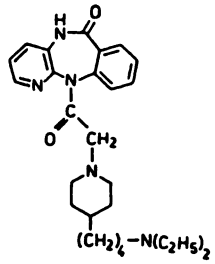
Although several subtype-selective muscarinic antagonists, such as pirenzepine, AF-DX 116, or hexahydro-sila-difenidol, have been developed during the last decade (20, 21), none of these antagonists shows a clear (>10-fold) selectivity for one receptor subtype over all other subtypes (22, 23). We have recently shown that various tricyclic compounds, including the

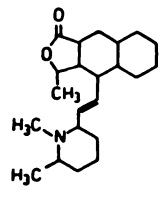
ABBREVIATIONS: G protein, guanine nucleotide-binding protein; TM, transmembrane; NMS, *N*-methylscopolamine chloride; AF-DX 116, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; AQ-RA 741, 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; UH-AH 37, 6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11*H*-dibenzo-[*b*,*e*][1,4]diazepine-11-one; PCR, polymerase chain reaction; kb, kilobase(s).

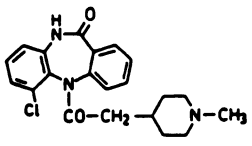
AF-DX derivative AQ-RA 741 (24) and the alkaloid himbacine (25) (Fig. 1), display up to 200-fold higher affinities for m2 and m4 than for m5 receptors (23). On the other hand, antagonists such as sila-hexocyclium (22) and the pirenzepine derivative UH-AH 37 (26) (Fig. 1) exhibit lower affinity for m2 than for m5 and all other muscarinic receptor subtypes. To explore the structural basis underlying the binding selectivities of these antagonists, we prepared a series of chimeric m2/m5 muscarinic receptors in which regions of the human m5 receptor were systematically replaced with the homologous regions of the human m2 receptor (Figs. 2 and 3). Excluding their highly divergent amino-termini and third cytoplasmic loops from a sequence comparison, the two receptors are 68% identical in amino acid sequence (5) (Fig. 3). The antagonist binding properties of the expressed chimeric receptors suggest that multiple receptor domains are involved in conferring subtype selectivity on AQ-RA 741, himbacine, and sila-hexocyclium. On the other hand, the subtype selectivity of UH-AH 37 appears to be largely dependent on a short receptor segment comprising most of TM VI and the third extracellular loop.

Experimental Procedures

Construction of chimeric receptor genes. Chimeric m2/m5 muscarinic receptor genes were constructed by using the human m2 (Hm2pCD) and human m5 (Hm5pCDp1) expression plasmids described previously (4, 5). The various constructs were created by replacing specific segments of Hm5pCDp1 with homologous segments of Hm2pCD prepared by the PCR. Each Hm2pCD segment was amplified from 1 ng of Hm2pCD template DNA by 35 cycles, consisting of 1 min at 94°, 2 min at 37°, and 3 min at 72°, as described by the manufacturer (Cetus). To allow the insertion of the amplified Hm2pCD segments into Hm5pCDp1, linker sequences encoding rare restriction sites present in Hm5pCDp1 were added to the 5′ portions of both PCR primers. The amplified Hm5pCDp1 fragments were digested at both ends with the proper restriction enzymes and gel purified. The homologous sequences were cut out from Hm2pCD by using the same enzymes and were replaced with the corresponding PCR fragments. To create CR 1 to CR 6, the following restriction fragments were removed from Hm5pCDp1: CR1, *Nco*I-*Bgl*II 0.59-kb; CR 2, *Bgl*II-*Apa*I 0.26-kb; CR 3, *Apa*I-*Eco*RI 0.52-kb; CR 4, *Bst*XI-*Dra*III 0.09-kb; CR 5, *Nco*I-*Apa*I 0.85-kb; CR 6, *Nco*I-*Bgl*II 0.59-kb and *Bst*XI-*Kpn*I 0.45-kb. Four additional hybrid m2/m5 receptor genes were constructed, which contained varying lengths of carboxyl-terminal m2 sequences (amino acid composition): m5 1–162/m2 156–466, m5 1–337/m2 301–466, m5 1–445/m2 391–466, and m5 1–476/m2 422–466. However, no specific [^{3}H]NMS binding could be detected in COS-7 cells transfected with any of these four chimeric constructs. The identity of the various chimeric receptor genes was confirmed by sequencing of the PCR-derived sequences (27) and by restriction endonuclease analysis.







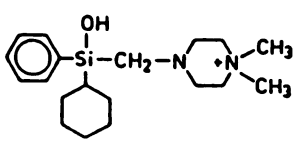


Fig. 1. Chemical structures of subtype-selective muscarinic antagonists used in this study.

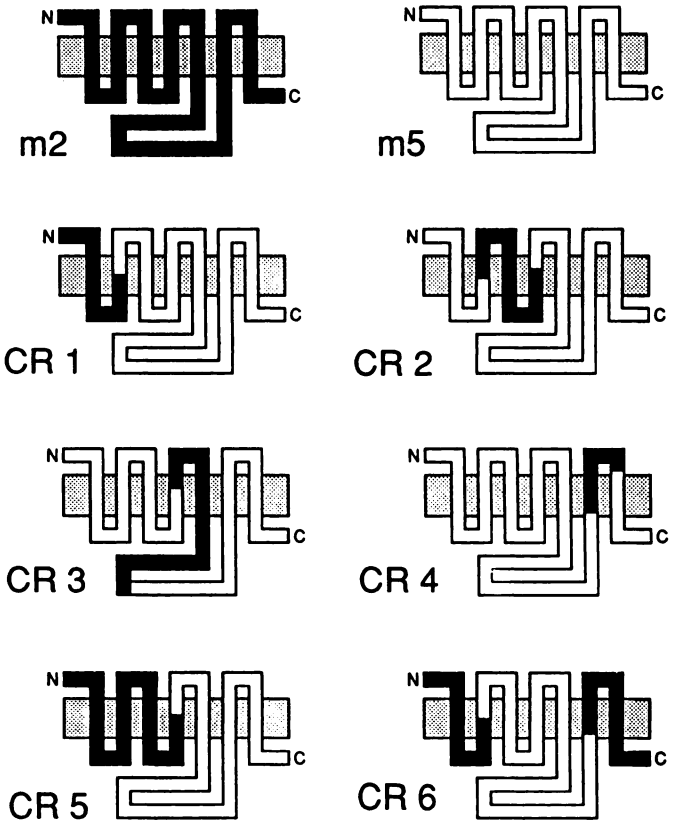


Fig. 2. Structure of chimeric muscarinic receptors (CR 1 to CR 6) composed of human m2 (■) and human m5 (□) sequences. The amino terminus (*N*) is thought to be located extracellularly, whereas the carboxyl terminus (*C*) is predicted to be located on the cytoplasmic side of the plasma membrane (shown as *grey area*). The fact that the m2 receptor differs from the m5 receptor in the lengths of the amino-terminal portion preceding the first TM domain (−7 amino acids), the large third cytoplasmic loop (−48 amino acids), and the carboxyl-terminal domain following the seventh TM domain (−11 amino acids) is ignored. In total, the human m2 and m5 receptors are composed of 466 and 532 amino acids, respectively (4, 5). The individual chimeric receptors are composed as follows (amino acid numbers): CR 1, m2 1–69/m5 77–532; CR 2, m5 1–76/m2 70–155/m5 163–532; CR 3, m5 1–162/m2 156–300/m5 336–532; CR 4, m5 1–445/m2 391–421/m5 477–532; CR 5, m2 1–155/m5 163–532; CR 6, m2 1–69/m5 77–445/m2 391–466.

Expression of chimeric receptors and binding studies. For transient expression of the various receptors, COS-7 cells were transfected, in 10-cm plates, with 20 μg of plasmid DNA, by calcium phosphate precipitation (28). Cells were harvested 72 hr after transfections, and membrane homogenates were prepared as described (22). Protein concentrations were determined according to the method of Bradford (29), using a Bio-Rad protein assay kit.

Binding studies were carried out essentially as described (23). Binding buffer consisted of 25 mM sodium phosphate (pH 7.4) containing 5 mM $MgCl_2$. Assays were conducted in 1-ml total volume. In the [^{3}H]NMS saturation experiments, six to eight different concentrations of the radioligand (12.5–1600 pM) were used. In the [^{3}H]NMS displacement studies, 10 different concentrations of the unlabeled ligands were used. The [^{3}H]NMS concentration in the inhibition binding experiments was 200 pM. Nonspecific binding was determined in the presence

Fig. 3. Comparison of the amino acid sequences of the human m2 and m5 receptors. Sequences were taken from Refs. 4 and 5. The seven TM domains (*I–VII*), as well as the three intracellular (*i1–i3*) and three extracellular (*o1–o3*) loops, are indicated. Only the membrane-proximal portions of the highly divergent third intracellular loops (*i3*) are shown. Restriction endonuclease sites present in the m5 receptor gene that were used for the construction of the various chimeric receptors are indicated above the sequences. *, Amino acid identities.

of 1 μM atropine. Incubations were carried out at 22° for 3 hr. Data were analyzed by a nonlinear least squares curve-fitting procedure, as described (23), using the program Dataplot run on a VAX II computer. IC_{50} values were converted to K_i values according to the method of Cheng and Prusoff (30).

Ligands. [^{3}H]NMS (78.9 Ci/mmol) was purchased from New England Nuclear. AQ-RA 741 and UH-AH 37 were kindly donated by the Dr. Karl Thomae GmbH (Biberach, FRG). Sila-hexocyclium (31) and himbacine were generous gifts of Dr. G. Lambrecht (University of Frankfurt, FRG).

Results

To investigate the structural basis underlying the subtype selectivity of certain muscarinic antagonists, we created a series of chimeric m2/m5 muscarinic receptors (Fig. 2). The antagonist binding properties of these receptors were studied after their transient expression in COS-7 cells.

[^{3}H]NMS binding studies. Transfected COS-7 cells were first examined for their ability to specifically bind the nonselective muscarinic antagonist [^{3}H]NMS. Whereas no binding activity was found in nontransfected cells, specific [^{3}H]NMS binding could be detected in cells transfected with the various wild-type and chimeric receptor genes (Table 1). All [^{3}H]NMS binding isotherms were characterized by Hill numbers not significantly different from unity (Table 1), consistent with the presence of a single muscarinic binding site. In agreement with previous studies in stably transformed Chinese hamster ovary cell lines (23), [^{3}H]NMS bound to the wild-type m2 and m5 receptors with equally high affinities (K_D = 75 ± 9 and 69 ± 3 pM, respectively) (Table 1). The various chimeric m2/m5 muscarinic receptors studied (CR 1 to CR 6) displayed [^{3}H]NMS binding affinities that differed <3-fold from the affinities determined for the two wild-type receptors.

Binding studies with subtype-selective antagonists. Four muscarinic antagonists were studied in [^{3}H]NMS competition binding experiments because of their known ability to discriminate between m2 and m5 receptors. Whereas AQ-RA 741 and himbacine have been shown to exhibit higher affinities for m2 than for m5 receptors (23), the reverse selectivity profile has been described for sila-hexocyclium (22) and UH-AH 37 (26). The inhibition binding curves obtained for the four antagonists at the different wild-type and chimeric receptors studied were characterized by Hill coefficients close to unity (Fig. 4; Table 1), consistent with a simple competitive receptor-ligand interaction.

In this study, AQ-RA 741 and himbacine were found to bind to m2 receptors with 282- and 28-fold higher affinities, respectively, than those for m5 receptors (Table 1; Figs. 4 and 5). None of the chimeric receptors studied gained the ability to bind these two ligands with the same high affinity as did the m2 receptor. However, replacement of several domains of the m5 receptor with analogous segments of the m2 receptor resulted in significant affinity increases (compared with m5). With the exception of himbacine binding to CR 2, AQ-RA 741 and himbacine bound to CR 1, CR 2, and CR 3 with 3–5-fold higher affinities than those for the m5 receptor (Table 1; Fig. 5). However, no affinity gain was observed in case of CR 4, which bound both antagonists with low "m5-like" affinities. CR 5 and CR 6, in which larger domains of the m2 receptor were substituted for homologous m5 receptor sequences, also displayed more pronounced affinity increases, compared with CR1 to CR 3 (8–23-fold increases, compared with m5) (Table 1; Fig. 5).

Sila-hexocyclium and UH-AH 37 bound with about 6.5-fold higher affinities to m5, compared with m2, receptors (Table 1; Figs. 4 and 5). CR 1, in which the amino-terminal 76 amino acids of the m5 receptor were replaced with the corresponding m2 receptor sequence, bound both ligands with affinities that were virtually identical to those of the wild-type m5 receptor. Sila-hexocyclium displayed intermediate affinities for all other chimeric receptors studied (Table 1; Fig. 5). A completely different pattern was observed for UH-AH 37. The affinities of this antagonist for CR 2, CR 3, and CR 5 differed by <1.6-fold

TABLE 1

Antagonist binding properties of chimeric m2/m5 muscarinic receptors expressed in COS-7 cells

Affinity constants (K_D) for [^{3}H]NMS were determined in direct binding studies. Inhibition constants (K_i) for the four subtype-selective muscarinic antagonists were obtained in competition binding experiments, as described in Experimental Procedures. Hill numbers (given in parentheses) were not significantly different from unity. Data are presented as means ± standard errors of two to four independent experiments, each performed in duplicate.

Receptor	m2 K_D = m5 K_D		m5 K_i > m2 K_i		m2 K_i > m5 K_i	
	B_{max}	[^{3}H]NMS K_D	AQ-RA 741 K_i	Himbacine K_i	Sila-hexocyclium K_i	UH-AH 37 K_i
	fmol/mg	pM	nM	nM	nM	nM
m2	1180 ± 160	75 ± 9 (1.05 ± 0.07)	1.8 ± 0.2 (0.97 ± 0.04)	11 ± 1 (0.97 ± 0.05)	9.0 ± 0.2 (1.03 ± 0.03)	33 ± 2 (0.99 ± 0.01)
m5	610 ± 210	69 ± 3 (1.07 ± 0.04)	507 ± 5 (1.08 ± 0.04)	305 ± 8 (1.09 ± 0.04)	1.4 ± 0.1 (0.92 ± 0.05)	5.0 ± 0.2 (0.96 ± 0.05)
CR 1	1090 ± 140	94 ± 6 (0.92 ± 0.07)	165 ± 4 (1.00 ± 0.06)	116 ± 16 (1.05 ± 0.14)	1.4 ± 0.1 (0.94 ± 0.04)	4.9 ± 0.5 (0.96 ± 0.02)
CR 2	260 ± 70	96 ± 31 (1.01 ± 0.08)	108 ± 10 (0.96 ± 0.06)	241 ± 37 (1.10 ± 0.06)	3.1 ± 0.4 (0.93 ± 0.04)	8.2 ± 1.4 (0.95 ± 0.03)
CR 3	330 ± 60	59 ± 1 (1.08 ± 0.04)	170 ± 13 (1.03 ± 0.04)	94 ± 21 (1.02 ± 0.09)	2.3 ± 0.1 (1.00 ± 0.01)	6.8 ± 0.4 (0.98 ± 0.06)
CR 4	470 ± 80	153 ± 16 (0.97 ± 0.02)	772 ± 10 (0.94 ± 0.05)	321 ± 19 (0.97 ± 0.03)	3.7 ± 0.1 (0.91 ± 0.06)	25 ± 1 (0.91 ± 0.05)
CR 5	960 ± 150	59 ± 5 (0.92 ± 0.06)	35 ± 7 (0.97 ± 0.02)	38 ± 3 (0.96 ± 0.02)	3.7 ± 0.2 (0.96 ± 0.03)	4.0 ± 0.1 (0.95 ± 0.03)
CR 6	470 ± 80	204 ± 14 (1.03 ± 0.05)	22 ± 4 (1.01 ± 0.02)	35 ± 2 (0.93 ± 0.03)	3.4 ± 0.3 (0.91 ± 0.11)	24 ± 1 (0.92 ± 0.05)

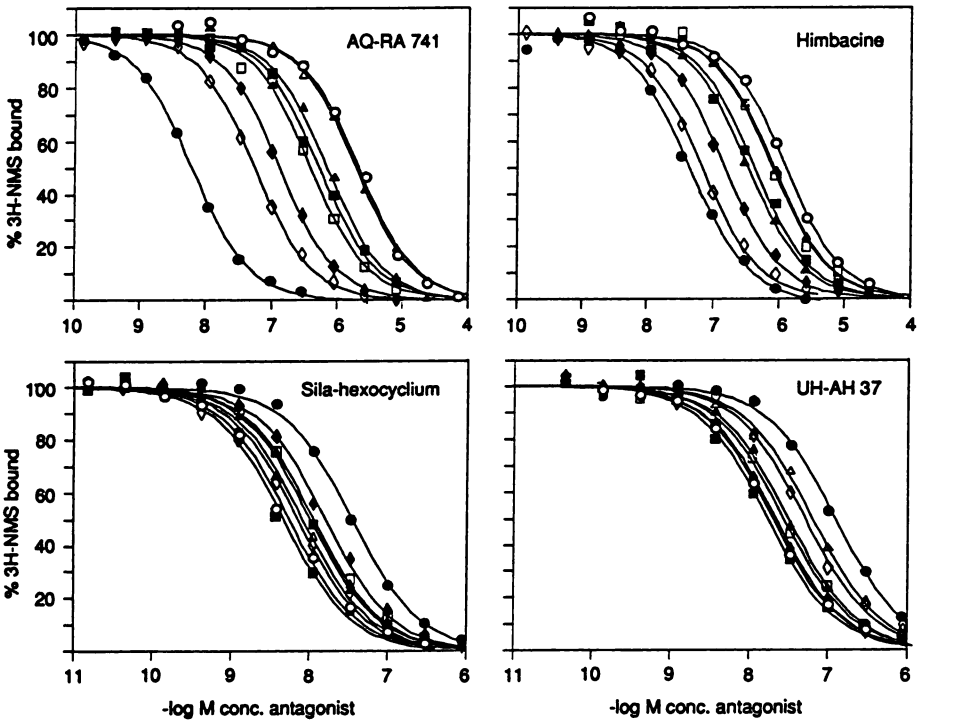


Fig. 4. Displacement of specific [^{3}H]NMS binding to chimeric m2/m5 muscarinic receptors by four subtype-selective muscarinic antagonists. Binding studies were carried out with membrane homogenates prepared from transfected COS-7 cells, as described in Experimental Procedures. The [^{3}H]NMS concentration used was 200 pM. Each *curve* is representative of two to four independent experiments, each carried out in duplicate. Curves were generated by computer fit, according to a one-binding site model.

from its affinity for the m5 receptor. However, CR 4 displayed a 5-fold decrease in UH-AH binding affinity (compared with m5), thus showing a phenotype similar to that of the wild-type m2 receptor (Table 1; Fig. 5). Interestingly, CR 4 contains only a short segment of the m2 receptor, which comprises most of TM VI and the third extracellular loop (Fig. 2). CR 6, which also contains this short domain, exhibited UH-AH binding properties similar to those of CR 4 (Table 1; Fig. 5).

Discussion

Each of the five muscarinic receptors (m1–m5) shows a distinct pattern of distribution throughout the central and peripheral nervous systems (14–18) and mediates a variety of different physiological responses (for reviews, see Refs. 32 and 33). Drugs acting selectively at the individual receptor subtypes are thought to have a therapeutic potential in several central nervous system diseases (19, 21).

The present study was designed to elucidate the molecular basis underlying the subtype selectivity of muscarinic antagonists that are able to discriminate between m2 and m5 muscarinic receptors. To this goal, chimeric m2/m5 muscarinic receptors were constructed and pharmacologically characterized in radioligand binding studies, using several subtype-selective antagonists.

The nonselective muscarinic antagonist NMS bound to all chimeric receptors studied, with affinities comparable to those of the wild-type m2 and m5 receptors. This finding indicates

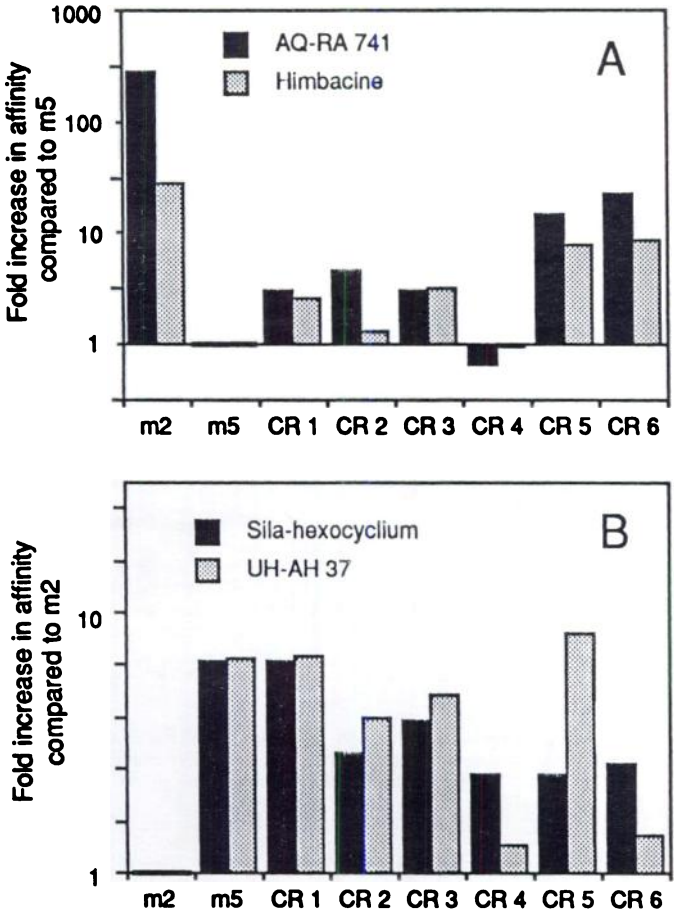


Fig. 5. Increase in binding affinities of subtype-selective antagonists for chimeric m2/m5 muscarinic receptors, compared with the wild-type m5 (A) and wild-type m2 (B) receptors. Affinity ratios were calculated by using the K_i values given in Table 1.

that the "primary" antagonist binding site on the muscarinic receptors is not adversely affected by the construction of the chimeric receptor proteins.

Several tricyclic muscarinic antagonists, including the AF-DX derivative AQ-RQ 741 and the alkaloid himbacine, have been shown to display a unique selectivity profile (23). These agents bind with high affinities to m2 and m4, with intermediate affinities to m1 and m3, and with drastically reduced affinities to m5 receptors (23). In the present study, both antagonists bound to all chimeric receptors investigated (except CR 4) with greater affinities than those for the wild-type m5 receptor. The most pronounced affinity increases were observed for CR 5 and CR 6, which contain the largest number of m2 receptor domains among all chimeric receptors studied. These findings suggest that multiple domains of the m2 receptor, rather than a distinct sequence element, are involved in conferring high affinity binding on AQ-RA 741 and himbacine. It seems, therefore, likely that the overall receptor conformation, resulting from the molecular interactions between the different TM and, possibly, extramembranous domains, may determine the subtype selectivity of these ligands.

Interestingly, AQ-RA 741 and himbacine showed very similar patterns of affinities for the various chimeric receptors, suggesting that their m2 selectivity may be based on a similar molecular mechanism. This similarity is also reflected in the chemical structures of these two antagonists, both of which contain a tricyclic core linked to a substituted piperidine ring system via a short electron-rich side chain. However, because these structural features are also present in other muscarinic antagonists, such as UH-AH 37, that bind to m2 receptors with low affinity (see below), additional structural parameters such as the substitution patterns of the different ring systems must be involved in defining the subtype selectivity of this class of antagonists.

In addition, two antagonists, sila-hexocyclium (22) and UH-AH 37 (26), that have been shown to display higher affinity for m5 than for m2 receptors were studied. However, both compounds exhibit high affinity not only for m5 but also for m1, m3, and m4 receptors (22, 26). It is, therefore, conceivable that the m2 receptor lacks a sequence element (which is present in all other muscarinic receptors) that confers high affinity binding on these two ligands. In this study, sila-hexocyclium and UH-AH 37 showed virtually identical affinities for the wild-type m5 receptor and CR 1, suggesting that the amino-terminal receptor domains (comprising the extracellular amino terminus, TM I, the first intracellular loop, and the amino-terminal portion of TM II) do not determine the selectivity of these two antagonists. Interestingly, the same domain contributed to a significant extent to the subtype selectivity shown by the m2 (m4)-selective ligands described above. Sila-hexocyclium bound to all chimeric receptors (except CR 1) with affinities that were intermediate between those found for the m2 and the m5 receptors. In analogy with the results obtained with AQ-RA 741 and himbacine, this finding suggests that multiple receptor domains may determine the binding selectivity of this antagonist.

The affinity pattern observed for UH-AH 37 clearly differed from those of all other compounds investigated in this study. Whereas the majority of chimeric receptors studied bound this ligand with "m5-like" affinities, CR 4 and CR 6 bound UH-AH 37 with "m2-like" low affinity. The subtype selectivity of this antagonist, therefore, appears to be largely dependent on a short receptor domain (which is present in both CR 4 and CR 6), comprising most of TM VI and the third extracellular loop. Because considerable evidence suggests that ligand binding to muscarinic (12, 13), adrenergic (10, 11), and related G protein-coupled receptors occurs within a cavity formed by the seven TM domains, one might speculate that residues within TM VI are primarily involved in conferring binding selectivity on UH-AH 37. Within this domain, m2 and m5 differ in only three amino acids (Ala^{401}-Thr^{456}, Val^{405}-Ile^{460}, and Ile^{409}-Val^{464}), which, therefore, represent potential targets for future mutagenesis studies. However, we cannot exclude the possibility that the less well conserved third extracellular loop may also affect ligand binding selectivity by exerting indirect conformational effects on the primary ligand binding site formed by the hydrophobic receptor core.

In agreement with our findings, studies with chimeric β_1/β_2-adrenergic receptors also suggest that several TM domains are involved in the determination of antagonist binding specificity (34–36). It has, therefore, been proposed that the overall conformation of the β receptors, rather than a few distinct points of drug-receptor interaction, may determine their pharmacological specificity (35). Consistent with our findings, the results of a recent mutagenesis study also suggest that different domains of the β-adrenergic receptors may be involved in the binding selectivity of structurally different ligands (36).

In contrast, the pharmacological analysis of chimeric α_2/β_2-adrenergic receptors indicated that a more discrete region (TM VII and adjacent sequences) may be the major structural determinant of α_2/β_2 antagonist binding selectivity (37). Similarly, a relatively small domain (TM IV and adjacent sequences) appears to be largely responsible for determining β_1 versus β_2 agonist binding selectivity (34), although a more systematic mutagenesis study failed to reveal the direct involvement of distinct amino acids (35).

In conclusion, we have shown that the pharmacological analysis of chimeric muscarinic receptors may help define receptor domains that are critically involved in conferring antagonist binding selectivity. Our data suggest that the subtype selectivity of structurally different antagonists may be dependent on their interaction with different muscarinic receptor domains. It is hoped that the information obtained through this approach will eventually lead to the development of highly selective antagonists that may become clinically useful in a variety of pathological conditions.

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