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Allosteric Site on Muscarinic Acetylcholine Receptors: A Single Amino Acid in Transmembrane Region 7 Is Critical to the Subtype Selectivities of Caracurine V Derivatives and Alkane-Bisammonium Ligands

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ABSTRACT

Diverse muscarinic allosteric ligands exhibit greatest affinity toward the M_2 receptor subtype and lowest affinity toward M_5. In this study, we evaluated the potencies with which two groups of highly M_2/M_5 selective allosteric agents modulate the dissociation of [^{3}H]*N*-methylscopolamine from M_2/M_5 chimeric and point-mutated receptors. These allosteric ligands included two alkane-bisammonium compounds and a series of caracurine V derivatives, which are structurally closely related to (but stereochemically different from) the prototype allosteric ligand alcuronium. Like alcuronium, the caracurine V and alkane-bisammonium compounds displayed significantly increased affinities compared with M_5 toward the chimera that included the M_2 second outer loop (o2) plus surrounding regions. Unlike alcuronium, the compounds had enhanced affinities for a chimera with M_2 sequence in transmembrane region (TM) 7; site-directed mutagenesis in wild-type and chimeric receptors indicated that the threonine residue at M_2^{423} was entirely responsible for the sensitivity toward TM7. Subsequent studies demonstrated that this TM7 epitope is likewise present in the M_4 receptor, as M_4^{436}serine. The M_2^{423}threonine residue is near the M_2^{419}asparagine identified previously to influence gallamine binding. These studies demonstrate that a stereochemical difference can be sufficient to translate into divergent epitope sensitivities. Nonetheless, these allosteric ligands seem to derive affinity from two main regions of the receptor: o2 plus flanking regions and o3/TM7. These two epitopes are sufficient to explain the M_2/M_5 selectivity of the presently investigated compounds; this is the first time that the subtype selectivity of muscarinic allosteric agents has been completely accounted for by distinct receptor epitopes.

The five subtypes of muscarinic acetylcholine receptors are members of the superfamily of G protein-coupled receptors. The binding site for acetylcholine and conventional agonists and antagonists on muscarinic receptors seems to be located within a pocket formed by the seven α-helical transmembrane domains characteristic of all G protein-coupled receptors (Hulme et al., 1990; Wess, 1993). There is a high degree of conservation of amino acid sequence in the regions that are considered to bind agonists and antagonists. This may be a reason for the difficulty in developing agonists and antagonists that are highly subtype selective.

Another feature of muscarinic receptors is the presence of a second, allosteric, binding site (Lee and El-Fakahany, 1991; Lazareno and Birdsall, 1995; Tucek and Proska, 1995; Ellis, 1997; Christopoulos et al., 1998; Holzgrabe and Mohr, 1998). Several observations have suggested that muscarinic allosteric ligands bind to more extracellular and presumably less conserved regions of the receptor, which may allow for greater selectivities. All five muscarinic receptor subtypes are subject to allosteric modulation (Ellis et al., 1991), and a wide array of structurally very different allosteric agents has been demonstrated to modulate the binding of conventional ligands to the five subtypes. Interestingly, among the allosteric agents that exhibit a high degree (>10-fold) of allosteric subtype selectivity, all have the greatest affinity toward the M_2 subtype and the lowest affinity toward M_5 (Ellis and Seidenberg, 2000). With regard to the M_2 receptor, gallamine, alcuronium and a number of other allosteric ligands have been demonstrated to interact competitively with the probe obidoxime providing strong experimental evidence for

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ABBREVIATIONS: CR, chimeric receptor; TM, transmembrane region of the receptor; o2, the second outer (extracellular) loop of the receptor; o3, the third outer (extracellular) loop of the receptor; NMS, *N*-methylscopolamine; PB, sodium-potassium phosphate buffer (5 mM), pH 7.4.

a common allosteric site (Ellis and Seidenberg, 1992, 2000; Tränkle and Mohr, 1997).

Studies of chimeric and mutant receptors have begun to identify receptor domains and amino acids that may constitute the muscarinic allosteric site. Almost every mutagenic study published so far has focused on gallamine as the allosteric modulator (Lee et al., 1992; Ellis et al., 1993; Leppik et al., 1994; Matsui et al., 1995; Gnagey et al., 1999). Of these studies, three have taken advantage of the subtype selectivity of gallamine by investigating chimeric substitutions. In studies with M_2/M_5 chimeric receptors, the affinity of gallamine was exclusively enhanced, relative to M_5, when the chimeric receptor included the M_2 third extracellular loop (Ellis et al., 1993) due to an asparagine residue at M_2^{419} (Gnagey et al., 1999). Substitutions that did not include this epitope were without effect. Nonetheless, insertion of this epitope in M_5 did not restore M_2 affinity completely. Chimeric studies including other muscarinic subtypes have demonstrated that gallamine also interacts with acidic residues in the region of the second outer loop of both the M_2 and the M_5 receptor (Leppik et al., 1994; Ellis, 1997; Gnagey et al., 1999).

Recently, the mutagenic studies were extended to other muscarinic allosteric ligands (Ellis and Seidenberg, 2000). In M_2/M_5 chimeric receptors, the affinity of alcuronium, another prototype allosteric modulator, was not dependent on the source of the third outer loop of the chimera, but was exclusively sensitive to the source of the second outer loop plus flanking transmembrane regions. As in the gallamine study above, this epitope, although clearly very important, did not confer the entire M_2 affinity. Finally, even though these different allosteric agents seemed to derive affinity from different receptor epitopes, they nevertheless were shown to compete for the obidoxime-sensitive site (Tränkle and Mohr, 1997; Ellis and Seidenberg, 2000).

Diallylcaracurine V, structurally closely related to alcuronium, has recently been identified as an allosteric agent with a similar high binding affinity for the M_2 subtype (Zlotos et al., 2000). The structural formulae of these two compounds look almost identical (Fig. 1), but their stereochemistry is considerably different (Zlotos, 2000). Using this tool, we aimed to find out whether the stereochemical difference, relative to alcuronium, is sufficient to translate into divergent epitope sensitivities. Two other caracurine V derivatives and two flexible alkane-bisammonium-type allosteric modulators were included in our studies.

Interestingly, we found that the affinities of the caracurine V and alkane-bisammonium compounds were sensitive to common epitopes. Like alcuronium, they had higher affinity for receptors that possessed M_2 sequence in the second outer loop and flanking regions. However, their affinities were also dependent on an epitope in the seventh transmembrane domain. Using site-directed mutagenesis, we identified the essential epitope in TM7 as M_2^{423}threonine. This epitope is also present in the M_4 receptor, as M_4^{436}serine. We have found that these two epitopes completely account for the M_2/M_5 selectivity of the caracurine V and alkane-bisammonium compounds. The simultaneous insertion of both M_2 epitopes into the M_5 receptor fully attained M_2 affinity. Some of these data have been reported previously in abstract form (Buller et al., 2001).

Experimental Procedures

Materials. Atropine sulfate was obtained from Sigma Chemical Co. (St. Louis, MO). Alcuronium was obtained from Roche Laboratories (Nutley, NJ). The bisquaternary caracurine V derivatives were synthesized as described previously (Zlotos et al., 2000). W84 was synthesized by Dr. Joachim Pfeffer (Department of Pharmacology, University of Kiel, Kiel, Germany), according to the method of Wassermann (1970). Dimethyl-W84 was generously provided by Prof. Dr. Ulrike Holzgrabe (Tränkle et al., 1998). [^{3}H]*N*-methylscopolamine chloride ([^{3}H]NMS; 70–82 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA).

Mutagenesis and Expression. The preparation of the chimeric receptors has been described previously (Ellis et al., 1993); schematic diagrams are shown in Figs. 2 and 3. The exact sequence compositions are as follows: CR3: hM_5 1–162, hM_2 156–300, hM_5 336–532; CR4: hM_5 1–445, hM_2 391–421, hM_5 477–532; CR5: hM_2 1–155, hM_5 163–532; and CR6: hM_2 1–69, hM_5 77–445, hM_2 391–466. Site-directed mutagenesis was performed with the QuikChange kit (Stratagene, La Jolla, CA). Briefly, oligonucleotides containing the desired base changes were synthesized and allowed to anneal with pcD plasmids containing the appropriate muscarinic receptor DNA sequence. A high-fidelity polymerase then extended the synthetic oligonucleotides in a thermocycled reaction. The parental DNA was digested by a methylation-specific endonuclease before transformation of bacteria. Mutations were confirmed by sequencing. Plasmids containing the human M_2 or M_4 wild-type or mutated receptor genes were purified from bacterial cultures and transiently transfected into COS-7 cells by calcium phosphate precipitation. M_5 wild-type receptors were stably expressed in CHO cells. For binding assays, cells were harvested by scraping into 5 mM Na,K,P_i buffer, pH 7.4 (PB). After homogenization and centrifugation at 50,000*g* for 20 min, membranes were resuspended in 5 mM PB and stored as aliquots at −70°C.

Dissociation Binding Assays. Binding assays were conducted in 5 mM PB, pH 7.4, at 23°C. Membranes were prelabeled with 1 nM [^{3}H]NMS for 30 min. Measurement of [^{3}H]NMS dissociation was then initiated by the addition of 3 μM atropine, with or without the indicated concentration of allosteric modulator. After the appropriate time interval, the incubation was terminated by filtration through S&S 32 glass fiber filters (Schleicher & Schuell, Keene, NH), followed by two rinses with 40 mM PB (0°C). Membrane-bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined by the inclusion of 3 μM atropine during the labeling period.

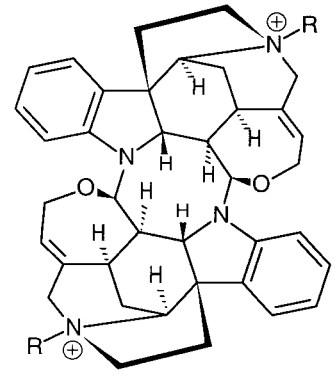
Dissociation assays were used throughout, because they guarantee that allosteric effects are being measured; data from the dissociation assays were transformed to rate constants. The apparent rate constant (k_{obs}) for the dissociation of [^{3}H]NMS was determined in the presence of each indicated concentration of allosteric modulator and divided by the control rate constant (k_0), determined in the presence of 3 μM atropine only. Curve fitting was based on a four parameter logistic function as described previously (Tränkle and Mohr, 1997) and led to concentration-effect curves for the delay of [^{3}H]NMS dissociation, which correspond to the occupancy curves for the allosteric agent at the ligand-occupied receptor (Lazareno and Birdsall, 1995; Ellis, 1997; Tränkle et al., 1998). Thus, the concentration inducing a half-maximal effect on radioligand dissociation ($EC_{50,diss}$) indicates the equilibrium dissociation constant (K_D) of alloster binding to the ligand-occupied receptor and serves as a measure of affinity of the allosteric modulator for the ligand-occupied receptor. Curve-fitting was performed using the Prism program (version 3.00; GraphPAD Software, San Diego, CA).

Results

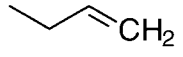
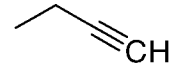
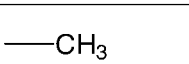
The structures of the muscarinic allosteric ligands investigated in this study are shown in Fig. 1. All of the investigated compounds exhibited much greater allosteric affinity

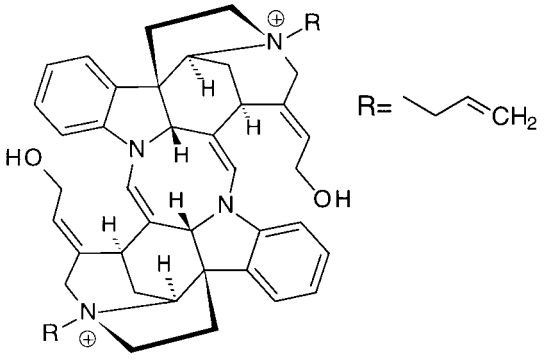
toward the M_2 subtype than the M_5 subtype. The ratio of these affinities ranged from about 70-fold to more than 300-fold higher affinity for the M_2 subtype. Dissociation assays have been used extensively to ensure that only effects mediated by the allosteric binding site are being observed. The effect on ligand dissociation results from an interaction of the modulator with the ligand-occupied receptor and is thus indicative of an interaction with a recognition site distinct from the ligand binding site. The concentration-dependent effects of an allosteric ligand on [^{3}H]NMS dissociation should be proportional to the occupancy of the allosteric site (Ellis, 1997) and thus reflect the affinity of the allosteric ligand at the NMS-occupied receptor (Lazareno and Birdsall, 1995). To investigate the structural basis of their subtype selectivity, the compounds have been evaluated at M_2/M_5 chimeric receptors in which segments of low-affinity M_5 receptor were systematically replaced with analogous segments of high-affinity M_2 receptor. Subsequently, site-directed mutagenesis was performed with the aim of attributing their selectivities to specific residues.

The rates of dissociation of [^{3}H]NMS from wild-type, chimeric, and point-mutated receptors in the absence of allosteric modulator (k_0) are compiled in Table 1. The rates for the wild-type and chimeric receptors are in good agreement with previously reported data (Ellis et al., 1993; Gnagey et al., 1999). [^{3}H]NMS dissociated much more rapidly from the M_2 subtype than from M_5. The M_2/M_5 chimeric receptor CR3 exhibited a dissociation rate even slower than M_5 (factor of 2), whereas NMS dissociated from CR6 as fast as it did from the M_2 subtype itself. The subsequently studied point mutations in wild-type and chimeric receptors produced small but

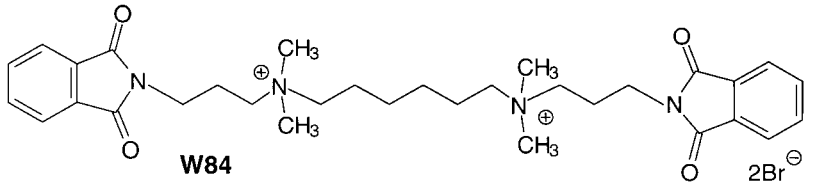


Caracurine V skeleton

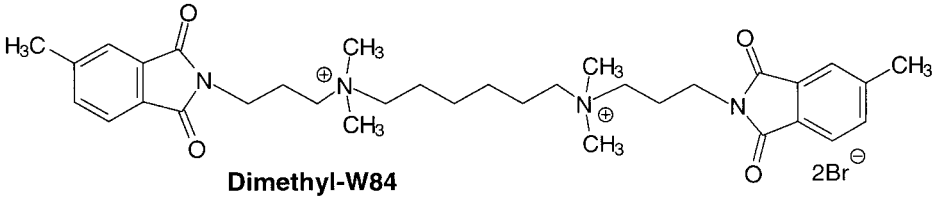
R	Modulator
	CARALL
	CARPROG
	CARMETH



Alcuronium



W84



Dimethyl-W84

Fig. 1. Structures of the muscarinic allosteric ligands investigated in this study.

noticeable changes in k_0 that did not exceed a factor of 3 (see *Discussion*).

M_2/M_5 Chimeric Receptors. In a first round, the affinity of the compounds toward the two M_2/M_5 chimeric receptors CR3 and CR6 was investigated. CR3 contains a segment of M_2 sequence that includes the extracellular half of TM4, the second outer loop, TM5, and a portion of the third intracellular loop. CR6 contains M_2 segments at the amino and carboxyl termini embedded in the M_5 receptor (schematic diagrams are given in Fig. 2; for exact sequence compositions of the chimeric receptors, see *Experimental Procedures*).

In agreement with data from previous studies (Ellis and Seidenberg, 2000), alcuronium displayed a greatly enhanced affinity (compared with M_5) toward the chimera CR3, whereas its affinity toward CR6 was only 2-fold higher than to M_5 (Fig. 2B). The caracurine V derivative CARALL, which is structurally very closely related to alcuronium but stereochemically different from it (Fig. 1), exhibited not only a considerably higher affinity toward CR3 but also a strongly enhanced affinity toward CR6, relative to M_5 (Fig. 2A). CARALL and alcuronium thus displayed remarkably divergent binding properties; in addition to an epitope in CR3, CARALL obviously derives affinity from a virtually equally important epitope in CR6. Like CARALL, CARPROG (Table 2) had similar affinity for CR3 and CR6, whereas CARMETH had significantly greater affinity for CR6 than for CR3 (Fig.

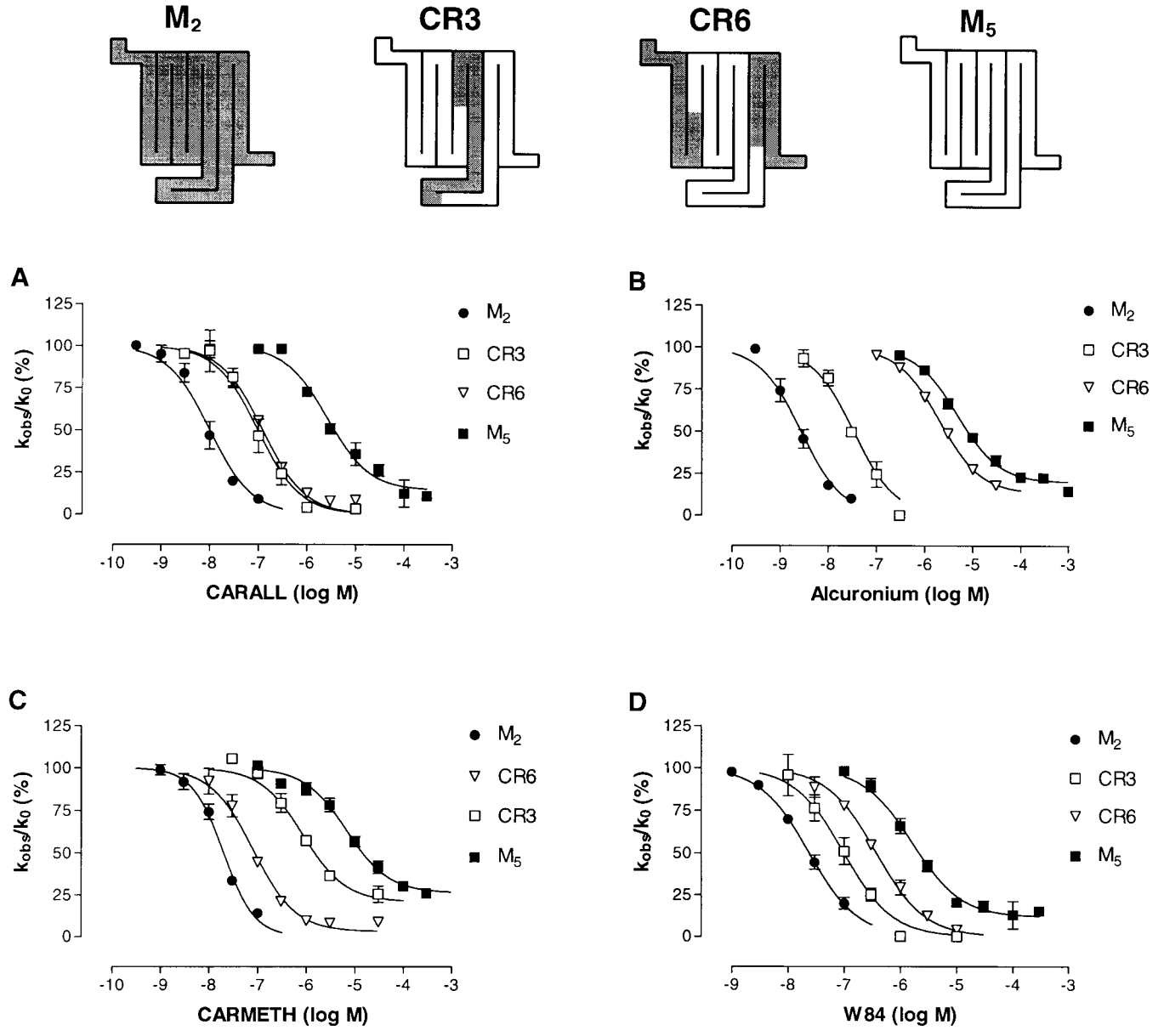


Fig. 2. Allosteric effect at M_2 and M_5 wild-type and the chimeric receptors CR3 and CR6. Schematic representation of the chimeric receptors CR3 and CR6 (top). A through D, concentration-effect curves for the allosteric delay of [^{3}H]NMS dissociation for the compounds CARALL, Alcuronium, CARMETH, and W84. The receptors were prelabeled with [^{3}H]NMS and the rate of dissociation of the labeled ligand was then determined in the absence or presence of the indicated allosteric modulator. Ordinate, ratio of the rate of [^{3}H]NMS dissociation in the presence of each concentration of allosteric modulator (k_{obs}) to the rate of dissociation of [^{3}H]NMS alone (k_0), expressed in percent. Abscissa, concentration of allosteric modulator. Experiments were conducted in 5 mM PB. The data presented are mean values ± S.E. of two to four separate experiments and the curves are the best fits to the model described under *Experimental Procedures*.

2C). On the other hand, the alkane-bisammonium compounds W84 (Fig. 2D; Table 2) and dimethyl-W84 (Table 2) had higher affinity for CR3 than for CR6. Although the respective ratios vary, all of the caracurine V and alkane-bisammonium compounds displayed significantly greater affinities toward both of these chimeric constructs, relative to M_5.

Subsequent studies were performed with the chimeric receptors CR5 and CR4 (schematic diagrams in Fig. 3). In CR5, a great part of the M_5 receptor is substituted by an M_2 segment from the N terminus of the receptor to the middle of TM4; this M_2 segment covers the entire N-terminal M_2 sequence in CR6. CR4 contains M_2 sequence from the beginning of TM6 to the end of the third extracellular loop, and this piece of M_2 sequence is completely included in the larger C-terminal M_2 segment in CR6. Thus, the chimeric substitutions in CR4 and CR5 partly overlap those of CR6. Interestingly, none of the compounds was found to have significantly greater affinity toward CR4 or CR5, relative to M_5 (see Table 2 and CARALL data in Fig. 3). The differential affinities of

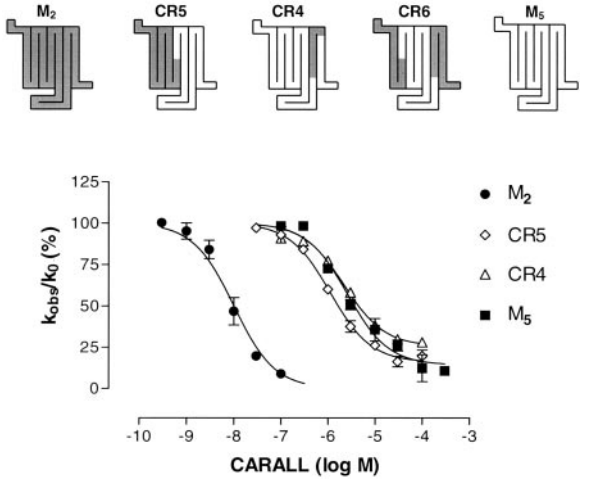


Fig. 3. Allosteric effects of CARALL at overlapping chimeric receptors. Schematic representation of the chimeric receptors CR4 and CR5 (top), concentration-effect curves for the allosteric effect of CARALL at M_2 and M_5 wild-type and the chimeric receptors CR4 and CR5 (bottom). Experiments were conducted as in Fig. 2. Indicated are mean values ± S.E. of three to four separate experiments.

TABLE 1

Half-times of dissociation of [^{3}H]NMS from mutant and wild-type receptors

Values are the means ± S.E. in the absence of allosteric modulator, derived from three or more independent experiments. The point mutations studied in M_2, M_4, M_5, CR3 and CR6 were all located at the position homologous to M_2^{423}.

Receptor construct	Dissociation $t_{½}$ ([^{3}H]NMS)
	min
M_2	4.3 ± 0.07
CR3	125 ± 3
CR4	57 ± 2
CR5	64 ± 2
CR6	3.8 ± 0.1
M_5	58 ± 4
M_2^{423} Thr→His	14 ± 0.5
M_5^{478} His→Thr	46 ± 0.8
CR6 Thr→His	11 ± 0.8
CR3 His→Thr	57 ± 1.8
M_4	46 ± 2.1
M_4^{436} Ser→Thr	23 ± 0.4
M_4^{436} Ser→His	94 ± 2.8

TABLE 2.

Potencies with which the compounds slow the rates of dissociation [^{3}H]NMS from the various wild-type and mutant receptors

Potencies are given as $pEC_{50,diss}$, which is the negative logarithm of the concentration that reduced [^{3}H]NMS dissociation to half of the control rate. As in Table 1, all point mutations are at the position homologous to M_2^{423}. Indicated are mean values ± S.E. of two to four separate experiments.

	M_5	M_2	CR3	CR4	CR5	CR6	M_2^{423} T→H	M_5^{478} H→T	CR6 T→H	CR3 H→T	M_4	M_4^{436} S→T	M_4^{436} S→H
CARALL	5.59 ± 0.08	8.00 ± 0.06	7.01 ± 0.07	5.66 ± 0.07	5.95 ± 0.04	6.91 ± 0.03	7.28 ± 0.10	6.55 ± 0.05	5.52 ± 0.06	7.84 ± 0.06			
CARPROG	5.66 ± 0.06	8.15 ± 0.05	7.03 ± 0.05	5.25 ± 0.11	6.01 ± 0.05	6.77 ± 0.03	7.19 ± 0.09	6.49 ± 0.09	5.35 ± 0.09	7.75 ± 0.08	7.61 ± 0.02	7.51 ± 0.03	6.03 ± 0.06
CARMETH	5.17 ± 0.07	7.70 ± 0.03	6.07 ± 0.08	4.75 ± 0.12	5.16 ± 0.07	7.08 ± 0.06	6.50 ± 0.08	6.64 ± 0.03	4.70 ± 0.21	7.71 ± 0.03	7.18 ± 0.06	7.29 ± 0.06	5.24 ± 0.09
W84	5.79 ± 0.06	7.62 ± 0.03	7.00 ± 0.07	5.66 ± 0.03	5.90 ± 0.03	6.43 ± 0.03	7.11 ± 0.03	6.49 ± 0.05	5.95 ± 0.03	7.47 ± 0.03			
Dimethyl-W84	5.71 ± 0.07	8.17 ± 0.03	7.22 ± 0.03	5.62 ± 0.06	5.93 ± 0.05	6.50 ± 0.04	7.51 ± 0.11	6.48 ± 0.02	6.07 ± 0.12	8.06 ± 0.18	7.31 ± 0.01	7.24 ± 0.02	6.68 ± 0.06
Alcuronium	5.32 ± 0.04	8.57 ± 0.05	7.47 ± 0.07	5.35 ± 0.12	5.72 ± 0.06	5.70 ± 0.03	8.00 ± 0.05	5.78 ± 0.10	5.68 ± 0.10	6.94 ± 0.04			

the caracurine V derivatives, W84, and dimethyl-W84 toward CR4, CR5, and CR6 suggest that the responsible element in CR6 lies between the top of TM7 and the carboxyl terminus of the receptor.

Aiming to identify a specific residue that might be responsible for the enhanced affinities toward CR6, we refined our chimeric approach to site-directed mutagenesis. Because many observations from different laboratories suggest that muscarinic allosteric ligands bind to the more extracellular region of the receptor (Jakubik and Tucek, 1994; Leppik et al., 1994; Matsui et al., 1995; Gnagey et al., 1999), attention was directed to amino acids near the top of TM7.

Point Mutations in TM7. There is in general a high degree of conservation in TM7, but there is a striking divergence at the position corresponding to the $M_2{}^{423}$threonine residue; M_5 has a histidine residue at this position (Fig. 4). To investigate whether this threonine residue makes a crucial contribution to the M_2 selectivity over M_5, a single point mutation was introduced into the M_2 receptor, replacing threonine^{423} with histidine. This point mutation markedly reduced the affinity toward the caracurine V derivatives and the compounds W84 and dimethyl-W84 (Fig. 5A, representatively for CARALL; Table 2). Interestingly, the affinity of this point mutant was about the same as that of the chimera CR3.

The converse mutation, that is replacement of the corresponding $M_5{}^{478}$histidine with threonine in the M_5 wild-type receptor, resulted in the reciprocal effect; the affinity toward the above compounds was considerably increased and virtually reached the affinity observed with CR6 (Fig. 5B; Table 2). These studies indicated that the $M_2{}^{423}$threonine was pivotal for the higher affinity of the compounds toward CR6, relative to M_5. This was further examined in a third point mutation that was created in the chimera CR6 itself. The TM7 threonine in CR6 was removed and "changed back" to M_5 histidine, which led to a reduction in affinity to approximately that of M_5 (Fig. 5C; Table 2). This loss of affinity was greatest for the compound that displayed the greatest gain in affinity between M_5 and CR6, CARMETH; its affinity was reduced by a factor of 244 by this mutation. There are 145 amino acids swapped from M_5 sequence to M_2 sequence in CR6 (69 amino acids at the N-terminal and 76 amino acids at the C-terminal part of the receptor). This mutant demonstrated that swapping the other 144 amino acids produced no effect if that single histidine residue remained. These findings are entirely consistent with the suggestion that the differences in the affinities of these compounds for M_5 and CR6 are due to the threonine that corresponds to $M_2{}^{423}$.

As noted above, the affinities of all of the compounds were found to be enhanced by an epitope in CR3. Because it contains M_5 sequence in TM7, CR3 does not possess the ($M_2{}^{423}$) threonine residue. A fourth point mutation was therefore studied in CR3. The corresponding TM7 histidine in CR3 was

	TM7 ==>							
M_5	Trp	His^{478}	Leu	Gly	Tyr	Trp	Leu	Cys	Tyr ==>
M_2	Trp	Thr^{423}	Ile	Gly	Tyr	Trp	Leu	Cys	Tyr ==>
M_4	Trp	Ser^{436}	Ile	Gly	Tyr	Trp	Leu	Cys	Tyr ==>

Fig. 4. Amino acid sequences of the human M_2, M_4, and M_5 receptors in the beginning of TM7. The shaded residues lie in homologous positions. Sequences are from Bonner et al. (1988).

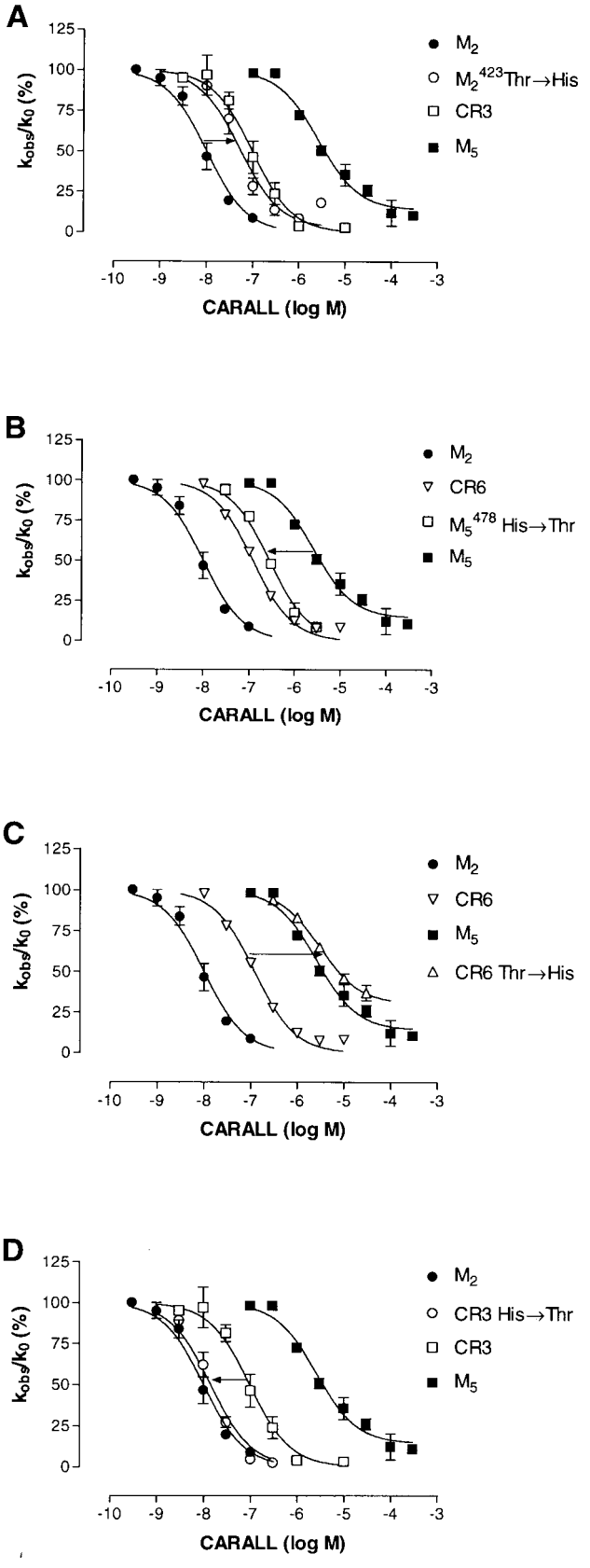


Fig. 5. Effects of point mutations in TM7 on the allosteric effects of CARALL. Reciprocal mutations were made in the M_2 and M_5 receptors, and in the chimeras CR3 and CR6, at the position homologous to $M_2{}^{423}$, as indicated. Experiments were conducted and analyzed as in Fig. 2. Indicated are mean values ± S.E. of two to four separate experiments.

replaced by threonine, resulting in markedly increased affinities toward the test compounds. These affinities were consistently increased to approximately that of the M_2 receptor (Table 2; CARALL data also shown in Fig. 5D). Consequently, two epitopes seem to be entirely responsible for the M_2/M_5 selectivity of the caracurine V derivatives and the alkane-bisammonium compounds: an epitope in CR3 and M_2^{423}threonine.

Given the lack of significant difference between the affinities of alcuronium for M_5 and CR6 (Fig. 2; Ellis and Seidenberg, 2000), the mutations in TM7 would not be expected to alter the affinity of this ligand very much. Indeed, in marked contrast to the large and consistent effects of those point mutations on the affinities of the other compounds in this study, the effects on the affinity of alcuronium were small and inconsistent. Notably, the replacement of the TM7 histidine by threonine in CR3 did not result in an affinity near that of M_2; rather, it slightly decreased the affinity toward alcuronium (Table 2). Furthermore, the opposite mutation in CR6 had no effect on the affinity of alcuronium.

Analogous Point Mutations in M_4. At the position corresponding to M_2^{423}threonine, the M_4 receptor has a serine residue (M_4^{436}serine; Fig. 4). In view of the similarity of these amino acids, it was hypothesized that the M_4 serine would be correspondingly involved in the selectivity of M_4 over M_5. To test this, two point mutations in the M_4 receptor were created. In one, M_4^{436}serine was replaced with the corresponding (M_2) threonine; in the other, M_4^{436}serine was replaced with (M_5) histidine. The compounds CARPROG, CARMETH, and dimethyl-W84 were investigated at the M_4 wild-type and mutant receptors. For all compounds, the M_4 wild-type affinity was somewhat lower than that of M_2, but nevertheless considerably higher than that of M_5 (Fig. 6, A–C). As expected, replacement of M_4^{436}serine with threonine did not produce any significant effect on affinity toward the above compounds (Table 2). However, analogously to M_2^{423}Thr→His, the mutation M_4^{436}Ser→His resulted in a marked loss of affinity toward the test compounds. There seems to be a correlation between the extent of affinity loss seen with M_4^{436}Ser→His and M_2^{423}Thr→His (Fig. 6, A–C). That is, CARMETH, which displayed the highest sensitivity toward M_2^{423}threonine, correspondingly showed the greatest loss in affinity (87-fold) when replacing M_4 serine with histidine. On the other hand, dimethyl-W84 was the least sensitive to the presence of M_2^{423}threonine among the compounds studied and also showed the smallest loss in affinity when M_4^{436}serine was mutated to histidine (4-fold). Thus, the M_2 and M_4 subtypes seem to contain a common epitope for the interaction with these allosteric ligands. This epitope is missing in M_5 and thus makes a crucial contribution to the M_2/M_5 and the M_4/M_5 subtype selectivities.

Discussion

Recent studies with M_2/M_5 chimeric receptors provided evidence that structurally different allosteric ligands may derive affinity from different receptor epitopes (Ellis and Seidenberg, 2000). We have investigated derivatives of the bisquaternary *strychnos* alkaloid caracurine V that are closely related to the prototype allosteric ligand alcuronium, in particular the *N*,*N*′-diallyl derivative CARALL (Fig. 1). However, the stereochemistry of alcuronium and CARALL is considerably different (Zlotos, 2000). Here, we show that a stereochemical difference is sufficient to translate into divergent epitope sensitivities. The two flexible alkane-bisammonium allosteric ligands W84 (Tränkle et al., 1996) and dimethyl-W84 that strongly contrast the rigid and highly fused ring system of the caracurine V skeleton (Zlotos et al., 2000) were included in our studies. All of these compounds were highly M_2/M_5 selective and thus ideally suited for evaluation at M_2/M_5 chimeric receptors to study the specific epitopes that confer their subtype selectivity. Dimethyl-W84 is of additional interest because it has been developed and is cur-

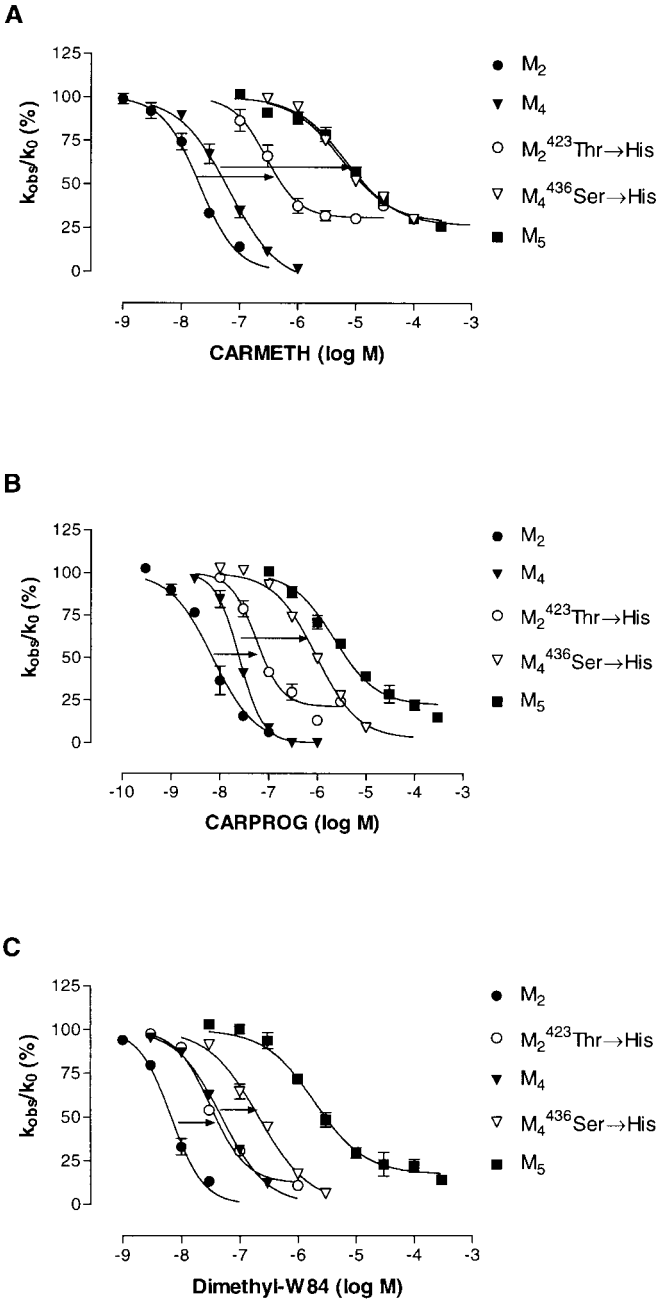


Fig. 6. Effects of mutation at M_4^{436}serine on the potencies of allosteric ligands. Concentration-effect curves for the allosteric effect of CARMETH, CARPROG, and dimethyl-W84 were carried out with M_2, M_4, and M_5 wild-type receptors and the indicated mutant receptors. Experiments were performed and analyzed as in Fig. 2. Data shown are mean values ± S.E. of two to three separate experiments.

rently used as a novel radioligand to label the allosteric site on M_2 receptors (Tränkle et al., 1998; 1999). Because the allosteric site seems to contain several potential epitopes for allosteric agent binding, we wondered whether the flexible agents could compensate for the removal of a point of attachment by switching to another conformation that would permit interaction with another epitope. If this were so, we would not expect to observe a clear-cut epitope dependence for the flexible compounds.

The affinity of alcuronium has been reported to be exclusively enhanced toward the M_2/M_5 chimera that includes the second outer loop and flanking regions of M_2, CR3 (Ellis and Seidenberg, 2000). However, the affinities of CARALL and the other caracurine V derivatives were enhanced not only at CR3 but also at CR6, which includes the seventh transmembrane domain of M_2. Thus, alcuronium and the caracurine V compounds may derive affinity from a common epitope in CR3, but they do not seem to share all points of attachment at the allosteric binding site. On the other hand, the flexible alkane-bisammonium compounds seem to be sensitive to the same epitopes that influence the binding of the caracurine V derivatives. With varying respective ratios, both groups of compounds had significantly greater affinities toward both CR3 and CR6, compared with M_5.

Our subsequent studies at other chimeric receptors and point mutations in wild-type and chimeric receptors demonstrated that the enhanced affinity of CR6 is caused entirely by a single amino acid at the top of TM7 (i.e., $M_2{}^{423}$threonine). This epitope was also found present at the corresponding position in the M_4 receptor, as $M_4{}^{436}$serine. Thus, both the M_2 and M_4 subtypes seem to contain a common epitope for the interaction with these allosteric ligands; absent in M_5, this epitope makes a crucial contribution to their M_2/M_5 and M_4/M_5 subtype selectivities. In addition, the data support the idea that these allosteric ligands adopt a similar orientation at the different subtypes.

In the present study we have taken the ability of the allosteric test compounds to slow the dissociation of [^{3}H]NMS as a measure of their allosteric effect. The great advantage of this approach is that it ensures that only allosteric effects are being observed. On the other hand, affinities for the free receptor cannot be extracted from these data. There are, however, some indications that suggest that there is a common binding mode for allosteric ligands at the free and NMS-liganded receptors (Ellis and Seidenberg, 2000; Schröter et al., 2000). Because [^{3}H]NMS dissociated much more rapidly from the M_2 subtype than from M_5, one could argue that high-affinity binding of the allosteric ligand is associated with a fast dissociation and low-affinity binding with slow dissociation of [^{3}H]NMS. However, the rate of dissociation of [^{3}H]NMS from the M_2/M_5 mutant receptors was not found to correlate with the potency of the allosteric agents. For instance, whereas M_2 and CR3 His→Thr were very similar in terms of affinity toward the compounds (see Table 2), the respective half-times for the dissociation of [^{3}H]NMS are clearly different (Table 1). A similar dichotomy can be appreciated by comparing M_5 and CR6 Thr→His. Several reasons have been previously discussed why rate constants (k_0) are not expected to be consistently tied to particular receptor epitopes (Ellis et al., 1993; Ellis and Seidenberg, 2000). Nonetheless, a certain consistency was observed in the way the threonine/histidine point mutations affected the respective half-times of [^{3}H]NMS dissociation. Replacement of threonine with histidine at $M_2{}^{423}$ and at the corresponding position of CR6 slowed the dissociation of [^{3}H]NMS (as did the replacement of $M_4{}^{436}$serine with histidine). On the other hand, replacement of the corresponding histidine with threonine in M_5 and CR3 accelerated [^{3}H]NMS dissociation (Table 1).

The $M_2{}^{423}$threonine residue has previously been investigated in a different context. Liu et al. (1995) demonstrated that a series of M_2/M_5 chimeric receptors were expressed but were unable to bind muscarinic radioligands. The authors concluded that in the pharmacologically inactive receptors, a TM1 threonine residue ($M_5{}^{37}$Thr) faced a TM7 threonine residue ($M_2{}^{423}$Thr) that interfered with proper helix-helix packing. This might seem to imply that our $M_5{}^{478}$Thr mutant (and the mutant CR3 His→Thr) would suffer from the same helix-helix packing problem and be unable to bind [^{3}H]NMS. However, our studies found that both mutant receptors $M_5{}^{478}$His→Thr and CR3 His→Thr were expressed and gave the expected degree of [^{3}H]NMS binding. More detailed studies with $M_5{}^{478}$His→Thr revealed exactly the same NMS affinity as for the M_5 wild-type receptor (data not shown). Indeed, the M_2/M_3 chimeric receptor "m3N2" has the corresponding threonine residues in TM1 (from M_3) and in TM7 (from M_2), yet has been reported to bind [^{3}H]NMS with high affinity (Wess et al., 1990). In addition, the M_4 wild-type receptor has a serine residue at the corresponding position in TM7 ($M_4{}^{436}$serine) and a threonine residue in TM1 ($M_4{}^{39}$threonine). Thus, although it seems likely that the findings of Liu et al. (1995) do reflect interhelical interactions, the conflict may be unique to the particular receptor constructs that they studied.

Nearly every mutagenic study of the muscarinic allosteric binding site that has been published to date has focused on gallamine as the primary allosteric modulator (Lee et al., 1992; Ellis et al., 1993; Leppik et al., 1994; Matsui et al., 1995; Gnagey et al., 1999). Matsui et al. (1995) mutated conserved aromatic and polar residues in the extracellular loops and loop/transmembrane helix interfaces of the M_1 receptor. This study found that the $M_1{}^{400}$tryptophan residue at the junction of o3/TM7 was important for the binding of gallamine and that mutation to alanine reduced gallamine's affinity by about 10-fold. This tryptophan is conserved in all five subtypes and is adjacent to the $M_2{}^{423}$threonine and $M_4{}^{436}$serine residues identified in the present study (Fig. 4).

With regard to gallamine's subtype selectivity, previous studies with M_2/M_5 chimeric receptors have shown that gallamine derives affinity for the M_2 subtype from a region within the o3-chimera CR4 (Ellis et al., 1993), to which, so far, no other allosteric ligand has been found to be sensitive. Subsequent studies attributed gallamine's CR4 interaction to an asparagine residue in the third extracellular loop (i.e., $M_2{}^{419}$asparagine) (Gnagey et al., 1999), which lies four residues away from $M_2{}^{423}$threonine. Thus, within this region of the receptor, the presently investigated ligands and gallamine seem to derive affinity from receptor loci that are near each other but not identical.

On the other hand, the investigated ligands were found to have enhanced affinity toward the chimeric receptor CR3 that included the M_2 second extracellular loop (o2) plus surrounding regions. In a study by Leppik et al. (1994), the binding of gallamine was also shown to be sensitive to an

epitope in o2. The authors identified a four-residue sequence in o2 of the M_2 receptor (EDGE), which when mutated to its M_1 counterpart resulted in a significantly reduced affinity toward gallamine. However, gallamine had been found to bind equally well to the M_5 receptor and to the chimeric receptor CR3, which includes M_2 sequence in this region (Ellis et al., 1993). This apparent discrepancy has been explained by experiments that indicate that both the M_5 and M_2 receptor contain the essential epitope in this region, whereas M_1 lacks it (Gnagey et al., 1999). Nonetheless, this also implies that the ligands investigated in the present study, which did display significantly greater affinity toward CR3 than toward M_5, presumably derive affinity from an epitope that involves residues other than those that affect gallamine's affinity in this region of the receptor. This situation may be similar to that described above for the o3/TM7 region of the receptor. We are currently investigating smaller chimeric inserts to attempt to identify the responsible epitope(s) within CR3.

The present study confirms the suggestion that muscarinic allosteric ligands interact with different receptor loci. However, despite this apparent complexity, two main regions of the receptor stand out from which allosteric agents seem to derive affinity: 1) the second outer loop and/or adjacent regions of TM4 and TM5; and 2) the junction between the third outer loop and TM7. At the position corresponding to $M_2{}^{423}$threonine, the M_4 receptor was found to contain an analogous epitope for the interaction with these compounds (i.e., $M_4{}^{436}$serine). These findings are furthermore consistent with the notion that muscarinic allosteric ligands bind to regions that are located more extracellularly, relative to the classical ligand binding site. In TM7, Wess et al. (1991) identified two conserved tyrosine residues that seemed to be critical for muscarinic agonist binding. Relative to $M_2{}^{423}$threonine or $M_4{}^{436}$serine, these tyrosine residues are located about one and two helical turns down toward the intracellular side of the receptor. Recent studies have indicated the presence of a second muscarinic allosteric site, based on the lack of competition between two classes of allosteric ligands (Lazareno et al., 2000). The structural details of this site have not yet been investigated, but in view of the lack of sensitivity of the compounds investigated in the present study to substitutions in the N-terminal half of the receptor (i.e., CR5), one may speculate that this second allosteric site may be located in the N-terminal part of the receptor.

In summary, we have investigated a number of highly subtype-selective allosteric compounds at M_2/M_5 mutant receptors. The stereochemical difference between alcuronium and CARALL was found to translate into divergent epitope sensitivies. On the other hand, the rigid caracurine V derivatives and the flexible alkane-bisammonium compounds seemed to interact with the same receptor epitopes. In fact, the flexible compounds exhibited a substantially inflexible behavior with regard to their affinities toward the recombinant receptors; the respective mutations led to clear and predictable affinity shifts, indicating that they do not compensate for the removal of a point of attachment by changing their conformations. For the different allosteric ligands investigated in this study, two epitopes (the o2 region and $M_2{}^{423}$threonine at the top of TM7) are sufficient to account fully for their M_2 selectivity over M_5.

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