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Allosteric Site on Muscarinic Acetylcholine Receptors: Identification of Two Amino Acids in the Muscarinic M_2 Receptor That Account Entirely for the M_2/M_5 Subtype Selectivities of Some Structurally Diverse Allosteric Ligands in *N*-Methylscopolamine-Occupied Receptors

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ABSTRACT

Two epitopes have been identified recently to be responsible for the high-affinity binding of alkane-bisammonium and caracurine V type allosteric ligands to *N*-methylscopolamine (NMS)-occupied M_2 muscarinic acetylcholine receptors, relative to M_5 receptors: the amino acid M_2-Thr^{423} at the top of transmembrane region (TM) 7 and an epitope comprising the second extracellular loop (o2) of the M_2 receptor including the flanking regions of TM4 and TM5. We aimed to find out whether a single amino acid could account for the contribution of this epitope to binding affinity. Allosteric interactions were investigated in wild-type and mutant receptors in which the orthosteric binding site was occupied by [^{3}H]NMS (5 mM Na,K,P_i buffer, pH 7.4, 23°C). Using M_2/M_5 chimeric and point-mutated receptors, the relevant epitope was narrowed down to M_2-Tyr^{177}. A double point-mutated M_2 receptor in which both M_2-Tyr^{177} and M_2-Thr^{423} were replaced by the corresponding amino acids of M_5 revealed that these two amino acids account entirely for the (approximately 100-fold) M_2/M_5 selectivity of the alkane-bisammonium and the caracurine V type allosteric ligands. At NMS-free M_2 receptors, the caracurine V derivative also displayed approximately 100-fold M_2/M_5 selectivity, but the double point mutation reduced the M_2 affinity by only ~10-fold; thus, additional epitopes may influence selectivity for the free receptors. A three-dimensional model of the M_2 receptor was used to simulate allosteric agent docking to NMS-occupied receptors. M_2-Tyr^{177} and M_2-Thr^{423} seem to be located near the junction of the allosteric and the orthosteric areas of the M_2 receptor ligand binding cavity.

Muscarinic acetylcholine receptors are members of the rhodopsin-like family of G protein-coupled receptors, which share general structural motifs, including seven hydrophobic transmembrane helices connected by intracellular and extracellular loops, an extracellular amino terminus, and a cytoplasmic carboxyl terminus. Molecular cloning studies revealed the existence of five (M_1-M_5) muscarinic acetylcholine receptors (Bonner et al., 1987), all of which are susceptible to allosteric modulation (Ellis et al., 1991). The orthosteric acetylcholine binding site seems to be lined by the transmembrane helices (Wess, 1993) and seems to be highly conserved among the five subtypes (Hulme et al., 1990). The allosteric binding site is located at the entrance of the ligand binding pocket (Ellis et al., 1993; Leppik et al., 1994) and is likely to be less well conserved than the orthosteric ligand binding site, thus potentially allowing the design of ligands with greater subtype selectivity (Tuček and Proška, 1995). The family of the muscarinic acetylcholine receptors has been widely studied as a model system for the interaction of allosteric modulators with G protein-coupled receptors (Christopoulos and Kenakin, 2002).

It is striking that muscarinic allosteric ligands exhibit generally the highest affinity to the M_2 receptor subtype (Lee and El-Fakahany, 1991; Ellis et al., 1991; Ellis and Seidenberg, 2000). A number of studies aimed to identify receptor

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3D complex coordinates are available on request from Kirstin Jöhren, Institute of Pharmaceutical Chemistry, University of Düsseldorf, Universitätsstr. 1, D-40225 Düsseldorf (E-mail: joehren@pharm.uni-duesseldorf.de).

ABBREVIATIONS: NMS, *N*-methylscopolamine; TM, transmembrane region of the receptor; o1, o2, o3, first, second, third outer (extracellular) loop of the receptor; PB, sodium-potassium phosphate buffer; 3D, three-dimensional.

epitopes that are critical for the binding of allosteric agents to the M_2 receptor (Leppik et al., 1994; Gnagey et al., 1999; Buller et al., 2002). The use of M_2/M_5 chimeric receptors has turned out to be an effective approach to gain more insight into receptor areas that confer the high affinity of allosteric ligands to the M_2 receptor subtype (Ellis et al., 1993; Gnagey et al., 1999; Ellis and Seidenberg, 2000). In these experiments, we have focused on the formation of ternary complexes that are the characteristic feature of allosteric interactions. For this purpose, the interaction of the allosteric agents was measured with receptors in which the orthosteric site was occupied by [^{3}H]*N*-methylscopolamine ([^{3}H]NMS). Receptor binding of the allosteric agents is reflected by an inhibition of [^{3}H]NMS dissociation. Using this procedure, we were able to identify two epitopes (Buller et al., 2002) that account fully for the high affinity of alkane-bisammonium–type and caracurine V-type allosteric ligands (Fig. 1) to the M_2 receptor, relative to the M_5 receptor. One of these epitopes consists of a single amino acid at the beginning of the seventh transmembrane region of the M_2 receptor, M_2-Thr^{423}. The other epitope has not been so precisely located but lies either within the second outer loop of the M_2 receptor or in adjacent regions of TM4 and TM5.

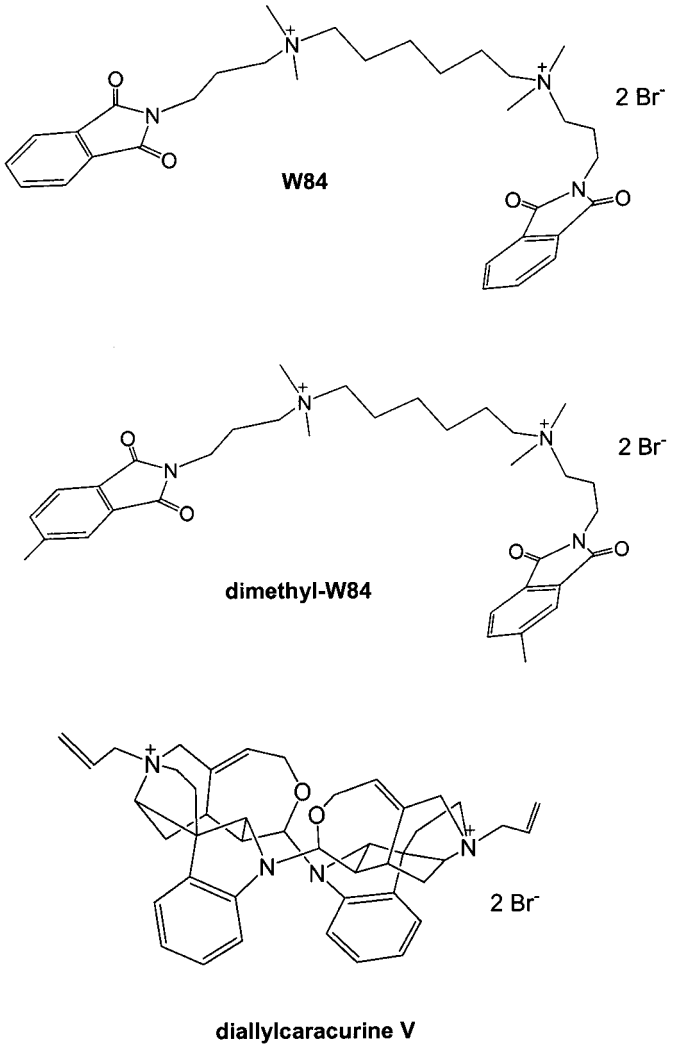

Fig. 1. Structures of the applied allosteric agents. The molecules are shown in an energetically favorable conformation.

In the present study, chimeric and point mutated receptors were used to examine whether the second epitope likewise consists of a single amino acid that is pivotal for the binding of these allosteric agents. We identified M_2-Tyr^{177}, in the middle region of the second outer loop of the M_2 receptor, as the critical amino acid. A double point-mutated receptor revealed that these two amino acids, M_2-Tyr^{177} and M_2-Thr^{423}, are sufficient to completely account for the M_2/M_5 selectivity of the applied allosteric agents in the NMS-occupied receptors. To check whether these amino acids play a role for the interaction with NMS-free receptors, we carried out equilibrium binding experiments with [^{3}H]NMS that were analyzed according to the ternary complex model of allosteric interactions (Stockton et al., 1983; Ehlert, 1988). The findings suggest that the two amino acids are involved in the binding affinity of the allosteric modulators for the free M_2 receptor, but additional epitopes seem to contribute to the M_2/M_5-

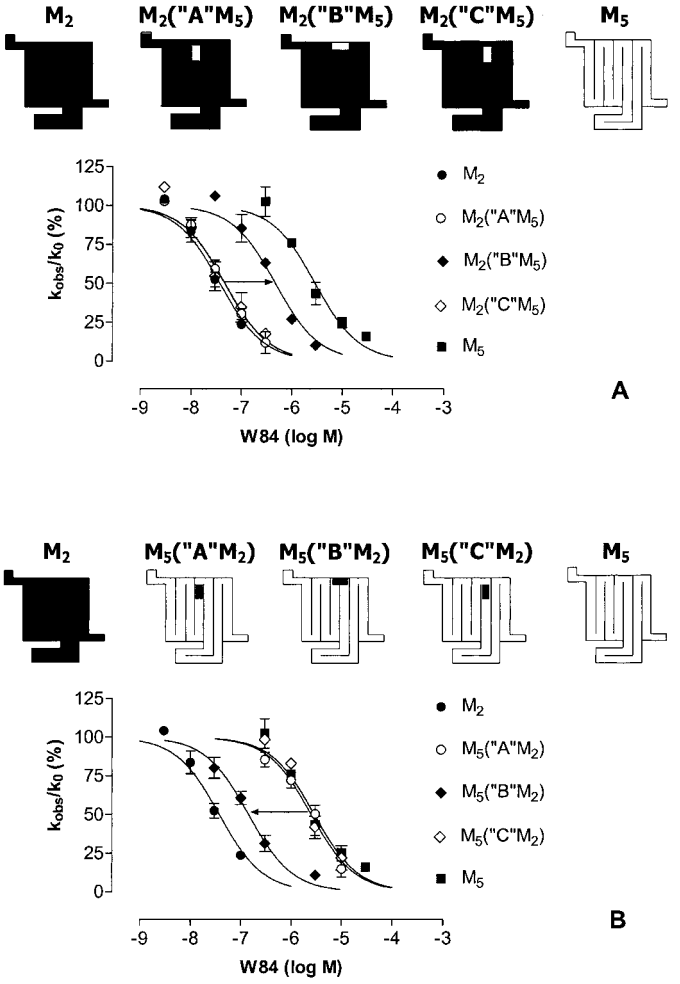

Fig. 2. Concentration-effect curves for the allosteric delay of [^{3}H]NMS dissociation induced by W84 at the indicated wild-type and mutant receptors. A, chimeric substitutions in o2 of the M_2 receptor. B, converse chimeric substitutions in o2 of the M_5 receptor. The receptors were prelabeled with [^{3}H]NMS, dissociation was then observed in the absence or presence of the allosteric modulator. Ordinate, apparent rate constant of [^{3}H]NMS dissociation in the presence of test compound (k_{obs}) divided by the parameter value found with [^{3}H]NMS alone (k_0), expressed in percent. Abscissa, log concentration of allosteric modulator. Experiments were carried out with membranes from transiently transfected COS-7 cells in 5 mM PB. Indicated are means ± S.E. of two to four separate experiments. Curve fitting based on a four parameter logistic function.

selectivity under this condition. A three-dimensional model of the M_2 receptor in the NMS-occupied state was built on the basis of the crystal structure of bovine rhodopsin. The model seems to be suitable to gain insight into the putative topography and the molecular mechanisms of allosteric-orthosteric interactions.

Materials and Methods

Receptor Mutagenesis and Expression

Site-directed mutagenesis was performed with the QuikChange kit (Stratagene, La Jolla, CA). In short, 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, leaving only the mutated product, which was confirmed by sequencing. Subsequently, the mutated products were transformed in *Escherichia coli* cells and amplified overnight. Plasmids containing the human M_2 or M_5 wild-type or mutated receptor genes were purified from bacterial cultures and transiently transfected into COS-7 cells by calcium phosphate precipitation. Cells were harvested 72 h after transfection by scraping into 5 mM Na,K,P_i buffer (PB), pH 7.4. After homogenization and centrifugation at 50,000*g* for 20 min, membranes were resuspended in 5 mM PB and stored as aliquots at −80°C. Schematic diagrams of the chimeric receptors are shown in Fig. 2. The exact sequences of the chimeras are as follows:

M_2("A"M_5): hM_2 1–160, hM_5 168–175, hM_2 169–466
M_2("B"M_5): hM_2 1–171, hM_5 179–184, hM_2 178–466
M_2("C"M_5): hM_2 1–180, hM_5 188–193, hM_2 187–466
M_5("A"M_2): hM_5 1–167, hM_2 161–168, hM_5 176–532
M_5("B"M_2): hM_5 1–178, hM_2 172–177, hM_5 185–532
M_5("C"M_2): hM_5 1–187, hM_2 181–186, hM_5 194–532

Binding Assays

Dissociation Binding Assays. Dissociation binding assays were conducted in 5 mM PB, pH 7.4, at 23°C. Membranes were preincubated with 1 nM [^{3}H]NMS for 30 min. Net dissociation of [^{3}H]NMS was initiated by the addition of 3 μM atropine, with or without the indicated concentrations of allosteric modulator. After the appropriate time interval, the dissociation was terminated by filtration through Whatman GF/B glass fiber filters that had been soaked in 0.1% polyethylenimine, using a Brandel cell harvester; the filtration was immediately followed by two rinses with 40 mM PB (0°C). The filter-bound radioactivity was quantitated by liquid scintillation counting. Nonspecific binding was defined as the radioactivity bound in the presence of 3 μM atropine.

Equilibrium Binding Assays. [^{3}H]NMS equilibrium binding was measured in 5 mM PB, pH 7.4, at 23°C. The [^{3}H]NMS concentration was 0.2 nM. The incubation time necessary for attaining equilibrium binding of [^{3}H]NMS in the presence of allosteric modulator was calculated according to Lazareno and Birdsall (1995; eq. 31 therein). Five observed half-life times of association were taken to be sufficient to attain equilibrium binding. Incubation times amounted for M_2 and the indicated double point mutant to 3 to 5 h and for M_5 to 24 h. Filtration and further processing was carried out as described above.

TABLE 1
Half-times of dissociation of [^{3}H]NMS from the indicated wild-type and mutant receptors in the absence of an allosteric agent
Data are presented as mean ± S.E. derived from three to four independent experiments.

Receptor/Mutation	Dissociation $t_{1/2}$ [^{3}H]NMS
	min
M_2 WT	5.1 ± 0.2
M_5 WT	93 ± 1
M_2('A'M_5)	6.3 ± 0.2
M_2('B'M_5)	4.6 ± 0.2
M_2('C'M_5)	4.5 ± 0.3
M_5('A'M_2)	115 ± 4
M_5('B'M_2)	239 ± 48
M_5('C'M_2)	276 ± 69
M_2-E^{172}→A	4.5 ± 0.2
M_2-D^{173}→L	3.7 ± 0.2
M_2-Y^{177}→Q	5.9 ± 0.2
M_2-T^{423}→H	15 ± 1
M_2-Y^{177}→Q + T^{423}→H	28 ± 1
M_5-Q^{184}→Y	151 ± 19
M_5-H^{478}→T	45 ± 2
M_5-Q^{184}→Y + H^{478}→T	58 ± 3

Data Analysis

Dissociation data were analyzed using the monoexponential decay equation, and apparent rate constants were obtained. The rate constant of [^{3}H]NMS dissociation in the presence of a given concentration of allosteric modulator (k_{obs}) was divided by the rate constant for the control condition [i.e., [^{3}H]NMS dissociation in the absence of the allosteric modulator (k_0)]. Curve fitting was based on a four-parameter logistic function as described previously (Tränkle and Mohr, 1997). The concentration-effect curve for the delay of [^{3}H]NMS dissociation reflects the binding curve of the allosteric agent at the NMS-occupied receptor (Lazareno and Birdsall, 1995; Ellis, 1997; Tränkle et al., 1998). The concentration for a half-maximum effect on [^{3}H]NMS dissociation ($EC_{0.5,diss}$) can be taken as a measure of affinity of the allosteric modulator for the NMS-occupied receptor.

Equilibrium binding data were analyzed according to the ternary complex model of allosteric interactions (Ehlert, 1988) using the equation

$$B_A = \frac{B_0 \cdot (10^{\log L} + 10^{\log K_L})}{10^{\log L} + 10^{\log K_L} \cdot \left(\frac{10^{\log K_A} + 10^{\log A}}{10^{\log K_A} + \frac{10^{\log A}}{\alpha}} \right)} \quad (1)$$

where L and A denote the concentration of the orthosteric ligand and the allosteric agent, respectively; K_L and K_A indicate the equilibrium dissociation constants for the free receptors of the ligand L and of the allosteric modulator A, respectively; and α is the factor of cooperativity representing the change in affinity of the orthosteric ligand for binding to the receptor occupied by an allosteric agent compared with the free receptor and vice versa. If an allosteric agent alters [^{3}H]NMS equilibrium binding only slightly, curve fitting with α and K_A as variable parameters is difficult. In that instance, we replaced K_A in eq. 1 by $EC_{0.5,diss}/\alpha$ assuming that $\alpha \times K_A/EC_{0.5,diss} = 1$ (Raasch et al., 2002). $EC_{0.5,diss}$ was known from the dissociation experiments and the curve fit yielded α. K_A was then calculated as $EC_{0.5,diss}/\alpha$. Nonlinear regression analysis was performed using the Prism program (ver. 3.02; GraphPad Software, San Diego, CA).

Chemicals

Atropine sulfate and polyethylenimine were obtained from Sigma Chemical (München, Germany). Diallylcaracurine V (Zlotos et al., 2000) and dimethyl-W84 (Tränkle et al., 1998) were generously provided by Prof. Dr. Ulrike Holzgrabe and Dr. Darius P. Zlotos (Institute of Pharmacy, University of Würzburg, Germany). W84 is commercially available at Tocris Cookson Inc. (Ellisville, MO). [^{3}H]NMS (83.5 Ci/mmol) was purchased from PerkinElmer Life Sciences Inc. (Boston, MA).

Three-Dimensional Modeling and Docking Simulations

Homology Modeling. The model is based on the X-ray structure of bovine rhodopsin (Protein Data Base accession no. 1F88/1HZX; Palczewski et al., 2000); the sequence of the human M_2 receptor was

TABLE 2

Potencies ($pEC_{0.5,diss}$) of the allosteric agents to slow the rate of [^{3}H]NMS dissociation from the indicated wild-type and mutant receptors

$pEC_{0.5,diss}$ is the -log concentration reducing [^{3}H]NMS dissociation to half of the control rate. Data are presented as mean ± S.E. of two to four separate experiments.

	M_2	M_5	M_2('A'M_5)	M_2('B'M_5)	M_2('C'M_5)	M_5('A'M_2)	M_5('B'M_2)	M_5('C'M_2)
W84	7.42 ± 0.07	5.54 ± 0.06	7.33 ± 0.05	6.33 ± 0.07	7.32 ± 0.09	5.60 ± 0.06	6.84 ± 0.05	5.54 ± 0.08
Dimethyl-W84	8.09 ± 0.08	5.78 ± 0.10	8.19 ± 0.04	6.89 ± 0.03	8.26 ± 0.05	5.83 ± 0.04	6.98 ± 0.12	5.60 ± 0.05
Diallylcaracurine V	8.00 ± 0.06[a]	5.69 ± 0.07	8.15 ± 0.01	7.19 ± 0.02	7.95 ± 0.02	5.84 ± 0.05	6.62 ± 0.06	5.85 ± 0.11

extracted from SwissProt (code no. P08172; Bonner et al., 1987). Transmembrane regions of the M_2 receptor were detected using so-called pinpoints, identified by Baldwin et al. (1997). The extracellular and intracellular loops were created employing a combination of different methods [i.e., secondary structure prediction and the application of a loop search routine based on homology aspects, implemented in the Homology module of Insight II (Accelrys, San Diego, CA)]. 3D-coordinates for N and C termini were built in analogy to the X-ray structure of bovine rhodopsin. A detailed description of the modeling procedure has been given elsewhere (Jöhren and Höltje, 2003).

Receptor-Ligand Complexes. The geometries of interaction for the allosteric modulators diallylcaracurine V and W84 were found in a stepwise manner. In a first step, the free volume located between the extracellular loops and the upper part of the transmembrane helices was explored using the program SURFNET (Laskowski, 1995). In the next step, the large cavity, which was found, was investigated using different GRID-probes, imitating the functional groups present in the ligands. Subsequently, the allosteric modulators were manually docked into this cavity according to the favorable positions detected by GRID (Stoll et al., 2002). The two resulting wild-type complexes (diallylcaracurine V + NMS, W84 + NMS) were virtually mutated (M_2-Tyr^{177}→Gln, M_2-Thr^{423}→His, M_2-Tyr^{177}→Gln + Thr^{423}→His), and all newly generated complexes were minimized to obtain energetically acceptable geometries.

Molecular Dynamics Simulation. To check whether the protocol for the molecular dynamics simulation was suitable, identical simulations for the X-ray structure of bovine rhodopsin as well as for the M_2 receptor model were performed. The result was confirmed that a backbone tether of 200 kcal/Å^2, a distance dependent dielectric constant of 2 × r and the use of the physiological pH of 7.4 give acceptable results when using the consistent valence force field (Accelrys). A simulation time of 300 ps is adequate for equilibration and simulation of receptor-ligand-complexes.

Model Quality Check. All molecular dynamics simulation runs were checked for equilibration of the system and protein stability. The potential energy and the root-mean-square deviation were taken as measures for complex equilibration. During the molecular dynamics simulation, the protein geometry, which was monitored with PROCHECK (Laskowski et al., 1993), was stable. The program NMRCLUST (Kelley et al., 1996) was applied to achieve representative structures of the protein-ligand complex after equilibration. The interaction energies between the allosteric ligands and the M_2 receptor during molecular dynamics simulation were determined using the DeCipher module in Insight II (Accelrys).

Results

In a first step, we aimed to narrow down the epitope in o2 by dividing the amino acid strand into three parts: A, B, and C. Thus, M_2/M_5 chimeric receptors were created, in which part A, B, or C of the M_2 receptor was substituted with the corresponding sequences of the M_5 receptor and vice versa. Schematic representations of the chimeric receptors are shown in Fig. 2, and the exact sequences are given under *Materials and Methods*.

The chimeric receptors were investigated for their sensitivities to the allosteric actions of the test compounds. The experiments were performed with receptors in which the orthosteric site was occupied by [^{3}H]NMS. The allosteric delay of [^{3}H]NMS-dissociation served to assess the binding

M_2: ^{172}Glu^{173}Asp^{174}Gly^{175}Glu^{176}Cys^{177}Tyr

M_5: ^{179}Pro^{180}Leu^{181}Asp^{182}Glu^{183}Cys^{184}Gln

Fig. 3. Amino acid sequences of the corresponding "parts B" of the human M_2 and M_5 receptor. Sequences are from Bonner et al. (1987).

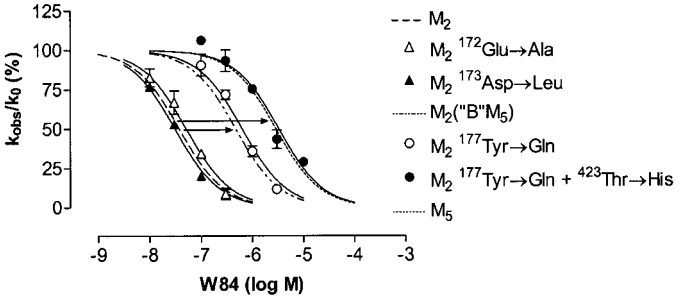

Fig. 4. Allosteric effects of W84 at the indicated wild-type and point mutated receptors. Experiments were conducted and analyzed as described in the legend to Fig. 2. Values are means ± S.E. of two to four separate experiments.

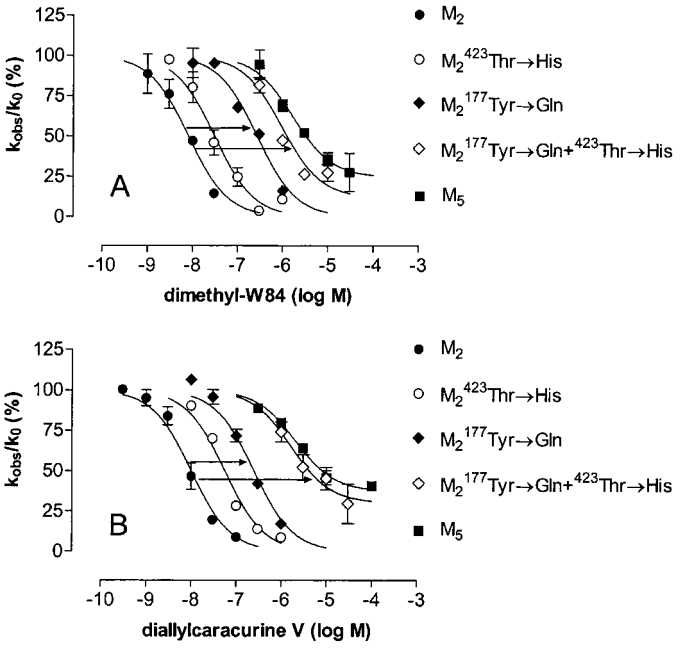

Fig. 5. Allosteric effects of dimethyl-W84 (A) and diallylcaracurine V (B) at the indicated wild-type and point mutated receptors. Experiments were carried out and analyzed as described in the legend to Fig. 2. Indicated are means ± S.E. of two to four separate experiments.

TABLE 2 (Continued)

	M_2-E^{172}→A	M_2-D^{173}→L	M_2-Y^{177}→Q	M_2-T^{423}→H	M_2-Y^{177}→Q + T^{423}→H	M_5-Q^{184}→Y	M_5-H^{478}→T	M_5-Q^{184}→Y + H^{478}→T
W84	7.29 ± 0.05	7.51 ± 0.02	6.20 ± 0.05	6.61 ± 0.05	5.54 ± 0.05	6.26 ± 0.10	6.18 ± 0.10	6.65 ± 0.03
Dimethyl-W84	7.63 ± 0.03	8.07 ± 0.03	6.57 ± 0.04	7.51 ± 0.07	6.02 ± 0.26	6.39 ± 0.11	6.48 ± 0.02[a]	6.87 ± 0.07
Diallylcaracurine V	N.D.	N.D.	6.61 ± 0.06	7.25 ± 0.06	5.72 ± 0.2	6.0 ± 0.18	6.55 ± 0.05[a]	6.82 ± 0.05

N.D., not determined.
[a] Data taken from Buller et al. (2002).

affinity of the allosteric test compounds for the NMS-occupied receptors. The half-lives of [^{3}H]NMS-dissociation measured in the absence of test compounds are compiled in Table 1. In agreement with previous studies (Ellis et al., 1991), [^{3}H]NMS dissociated much more rapidly from the M_2 subtype than from the M_5 subtype. The dissociation rates found for the M_2 receptors with small chimeric substitutions or point mutations were near the rate of the parent M_2 receptor. The dissociation rates for the M_5 wild-type and M_5 mutant receptors were much slower.

The dependence of the allosteric action of W84 on parts A to C of o2 is shown in Fig. 2. Only the replacement of part B of the M_2 receptor with the corresponding sequence of the M_5 receptor resulted in a significant loss of affinity of W84 (Fig. 2A). The converse mutation (Fig. 2B) (i.e., replacement of the corresponding part B of the M_5 receptor with part B of the M_2 receptor) led to a reciprocal effect; the affinity toward W84 is increased, relative to the M_5 wild-type receptor. In contrast, substitutions of part A or C had no effect on the affinity toward W84. The same pattern of effects was observed with the other allosteric modulators, dimethyl-W84 and diallylcaracurine V (Table 2). These results suggest that the crucial amino acid is located in part B. The sequences of part B in the M_2 and in the M_5 receptor are given in Fig. 3.

Subsequently, site-directed mutagenesis was applied to gain more insight into the contribution of the amino acids for the binding affinity of the allosteric agents. We focused on three amino acids of part B of the M_2 receptor: Glu^{172}, Asp^{173} and Tyr^{177}. The neutral amino acid Gly^{174} was not expected to contribute to the binding affinity of the allosteric ligands,

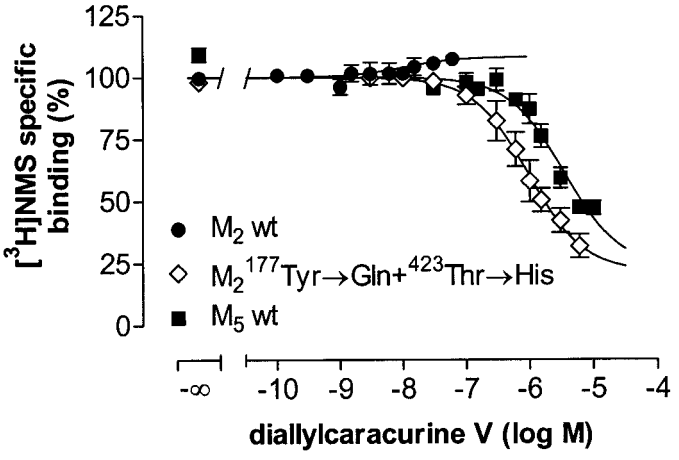

Fig. 6. Effects of diallylcaracurine V on the equilibrium binding of [^{3}H]NMS to the indicated wild-type and point mutated receptors. Ordinate, specific [^{3}H]NMS binding in percentage of the control value in the absence of the allosteric agent. Abscissa, concentration of the allosteric agent. Indicated are means ± S.E. of three to four separate experiments with duplicated values. Curve fitting based on the ternary complex model of allosteric interactions.

and Glu^{175} and Cys^{176} are conserved at the corresponding positions in the M_5 receptor.

In the cases of Asp^{173} and Tyr^{177} of M_2, the corresponding amino acids of the M_5 receptor were substituted. However, the corresponding amino acid of Glu^{172} is proline, which is known to disrupt the conformation of peptide strands (MacArthur and Thornton, 1991). Therefore, Glu^{172} was replaced by alanine. Thus, three point-mutated receptors were generated: M_2-Glu^{172}→Ala, M_2-Asp^{173}→Leu, and M_2-Tyr^{177}→Gln. Neither the replacement of Glu^{172} with Ala nor the substitution of Asp^{173} with Leu resulted in any significant change of affinity of W84 (Fig. 4), relative to the M_2 wild-type receptor. In contrast, replacement of M_2-Tyr^{177} by glutamine of M_5 markedly reduced the affinity of W84 (Fig. 4) relative to the M_2 wild-type receptor. The loss of affinity to this mutant receptor is equivalent to the loss of affinity observed when substituting the whole part B of the M_2 receptor by the corresponding part of M_5. Similar findings were obtained for dimethyl-W84 and diallylcaracurine V (Table 2). The converse mutation was also constructed, M_5-Gln^{184}→Tyr, and the result was an increase of affinity toward all of the allosteric modulators that were studied, relative to the M_5 wild-type receptor (Table 2). The loss of binding affinity that was caused by removing Tyr^{177} from the M_2 receptor and the gain of binding affinity caused by introducing tyrosine into the corresponding position of M_5 suggested that M_2-Tyr^{177} is the essential amino acid within the o2 epitope of the M_2 receptor.

In a preceding study, we found that M_2-Thr^{423} is a critical epitope for the M_2/M_5 selectivity of all the allosteric test compounds examined here. Therefore, we further investigated whether M_2-Thr^{423} and M_2-Tyr^{177} might fully account for the M_2/M_5 selectivity of these allosteric compounds. A

TABLE 3

Affinities (pK_A) of the allosteric agents to bind to the indicated wild-type and mutant receptors and factors of cooperativity ($p\alpha$) for the interaction with [^{3}H]NMS equilibrium binding

pK_A is the -log equilibrium dissociation constant for allosteric agent binding to NMS-free receptors. $p\alpha$ is the -log value of the cooperativity factor; a positive or negative $p\alpha$ reflects an elevation or reduction of [^{3}H]NMS equilibrium binding, respectively. Data are presented as means ± S.E. of three to four separate experiments with duplicated values.

	M_2	M_2-Y^{177}→Q + T^{423}→H	M_5
Diallylcaracurine V			
pK_A	7.76 ± 0.06[a]	6.93 ± 0.09	5.95 ± 0.06
$p\alpha$	0.24 ± 0.06	−1.47 ± 0.003	−0.99 ± 0.06
W84			
pK_A	8.15 ± 0.12	6.84 ± 0.06	N.D.
$p\alpha$	−0.81 ± 0.08	−1.01 ± 0.13	N.D.
Dimethyl-W84			
pK_A	9.02 ± 0.01	7.57 ± 0.05	N.D.
$p\alpha$	−1.04 ± 0.04	−1.61 ± 0.08	N.D.

N.D., not determined.
[a] $EC_{0.5,diss}$ was used for curve fitting as described under *Materials and Methods*.

double point-mutated receptor was generated, replacing the tyrosine and threonine of the M_2 receptor with the corresponding amino acids of the M_5 receptor, glutamine and histidine (M_2-Tyr^{177}→Gln + Thr^{423}→His). As shown in Fig. 4, the affinity of W84 toward this mutant receptor matched the affinity of W84 for the wild-type M_5 receptor. The double mutation in M_2 likewise reduced the affinities toward dimethyl-W84 and diallylcaracurine V to M_5 levels (Fig. 5 and Table 2). The reverse mutation, insertion of tyrosine and threonine in the M_5 receptor, resulted in a significant increase of affinity toward the allosteric agents, although the affinity level of M_2 was not obtained with any of the ligands (Table 2). This outcome is not altogether surprising. When an essential residue is removed from a receptor, its contribution is likely to be completely lost if the substituted residue is incapable of mimicking the lost interaction. On the other hand, the effectiveness of the insertion of such a residue may depend upon other aspects of the recipient receptor's structure (Ellis et al., 1993). In the present case, the proline at M_5^{179} might contribute to a less-than-optimal orientation of the inserted tyrosine at position 184, for example. Or there could be important structural features even further removed, as suggested by previous studies in which the entire second outer loop of M_2, along with TM4 and TM5 residues, was inserted into M_5; when the threonine was additionally inserted into TM7 of this chimera, affinities were observed for several allosteric ligands that were essentially like those for M2 (Buller et al., 2002).

The aforementioned experiments have all been conducted with NMS-occupied receptors. We aimed to check the role of

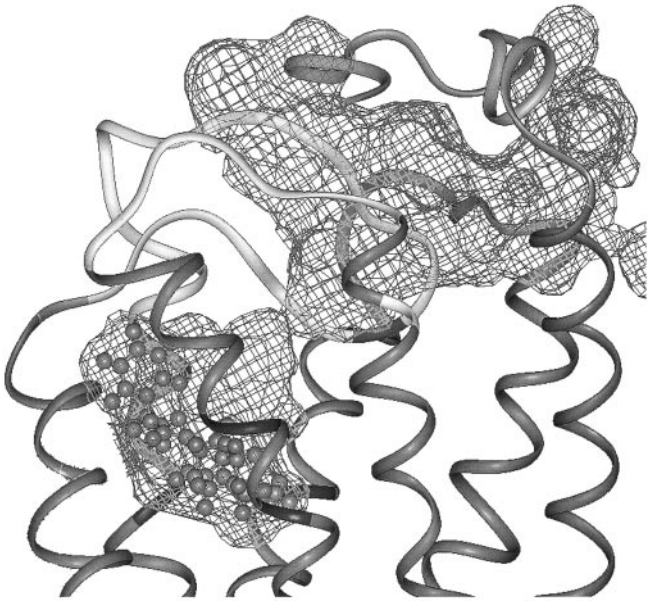

Fig. 7. Positions of the orthosteric binding site with bound *N*-methylscopolamine (NMS) and of the common allosteric binding site in the M_2 receptor model; view from the membrane. Helices are dark gray, extracellular loops are light gray, accessible volumes are in a gray grid, and NMS is a ball-and-stick model.

Tyr^{177} and Thr^{423} for allosteric agent binding in unoccupied M_2 receptors. Therefore, equilibrium binding experiments were carried out with [^{3}H]NMS. In the absence of test compound, the applied wild-type and mutant receptors revealed

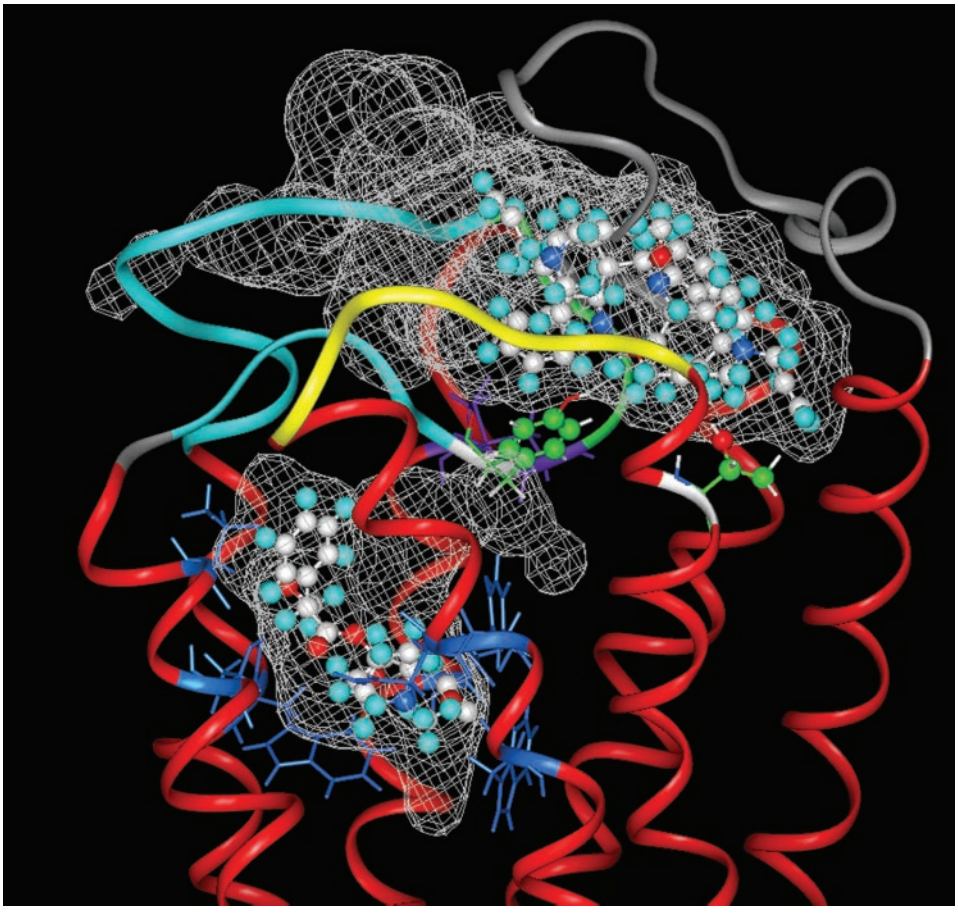

Fig. 8. Positions of NMS and diallylcaracurine V in the wild-type M_2 receptor; view from the membrane. Protein: helices, red; o1 and N terminus, gray; o2, cyan; EDGE (residues 172–175 in M_2) green; o3, yellow; disulfide bridge, magenta; orthosteric binding site, blue; free volumes, gray grid. Tyr^{177}/Thr^{423}: carbon, green; nitrogen, dark blue; oxygen, red; hydrogen, white. NMS/diallylcaracurine V: carbon, white; nitrogen, dark blue; oxygen, red; hydrogen, cyan.

the following pK_D-values for NMS binding (single values derived from separate [^{3}H]NMS/NMS competition experiments): M_2, 10.40 and 10.32; M_5, 9.89 and 9.88; M_2-Tyr^{177}→Gln + Thr^{423}→His, 10.80 and 10.14. Diallylcaracurine V was chosen to study the M_2/M_5 selectivity in the unoccupied receptors because the compound is stable (Wedig et al., 2002) in contrast to the alkane-bisammonium compounds, which are prone to undergo spontaneous degradation over the long incubation periods (24 h in this study) that are required to reach binding equilibrium in the presence of allosteric agents in M_5 receptors ($t_{1/2}$ of W84 degradation in PB is 10–14 h; Schulz, 1998). As shown in Fig. 6, the caracurine derivative slightly enhanced [^{3}H]NMS binding in M_2, whereas in M_5, the [^{3}H]NMS binding was reduced, and concentrations of the modulator ~100-fold higher were needed for this effect. To verify that the upper plateau of [^{3}H]NMS binding is stable in M_2, we carried out two additional experiments and included concentrations of diallylcaracurine V at which equilibrium binding was reached within an incubation time of 24 h (instead of 3 h). As expected according to the ternary complex model of allosteric interactions, the level of [^{3}H]NMS equilibrium binding was maintained at the higher concentrations of up to 3×10^{-7} M (data not shown). Figure 6 indicates that both the binding affinity of diallylcaracurine V and its cooperativity with [^{3}H]NMS are subtype-selective. The pK_A values of diallylcaracurine V binding to the free receptors and the factors of cooperativity with NMS are compiled in Table 3. In the double mutant receptor, the cooperativity with NMS was at least as negative as in M_5, whereas the affinity of diallylcaracurine V binding remained halfway between M_2 and M_5. Additional epitopes seem to be involved in the M_2/M_5 selectivity of the binding of this allosteric agent to unoccupied receptors. In cases of W84 and dimethyl-W84, the double point mutation of the M_2 receptor led to losses in binding affinity of >10-fold relative to the wild-type M_2 receptor; the cooperativity with NMS was negative. Taken together, the findings indicate that the pair of amino acids plays a major role for the binding of the alkane-bisammonium–type and the caracurine V-type allosteric agents in free M_2 receptors. Future studies will have to elucidate the relative contribution of the single amino acids to the loss of binding affinity seen with all agents and to the shift in cooperativity seen with the caracurine V type agent. Furthermore, it will be interesting to find out in how far the reverse mutation M_5-Gln^{184}→Tyr + His^{478}→Thr induces a gain of binding affinity for the free receptor and restores cooperativity.

To gain more insight into the topography of allosteric agent binding to the M_2 receptor, a 3-dimensional model of the receptor protein was built (Jöhren and Höltje, 2003). The model was derived from the crystal structure of bovine rhodopsin in the inactive state. Thus, the transmembrane helices should be in the same position as if stabilized through an orthosteric antagonist. For the modeling, NMS as the orthosteric agent offers favorable features: 1) it is relatively large and rigid and 2) the amino acids, which are important for its

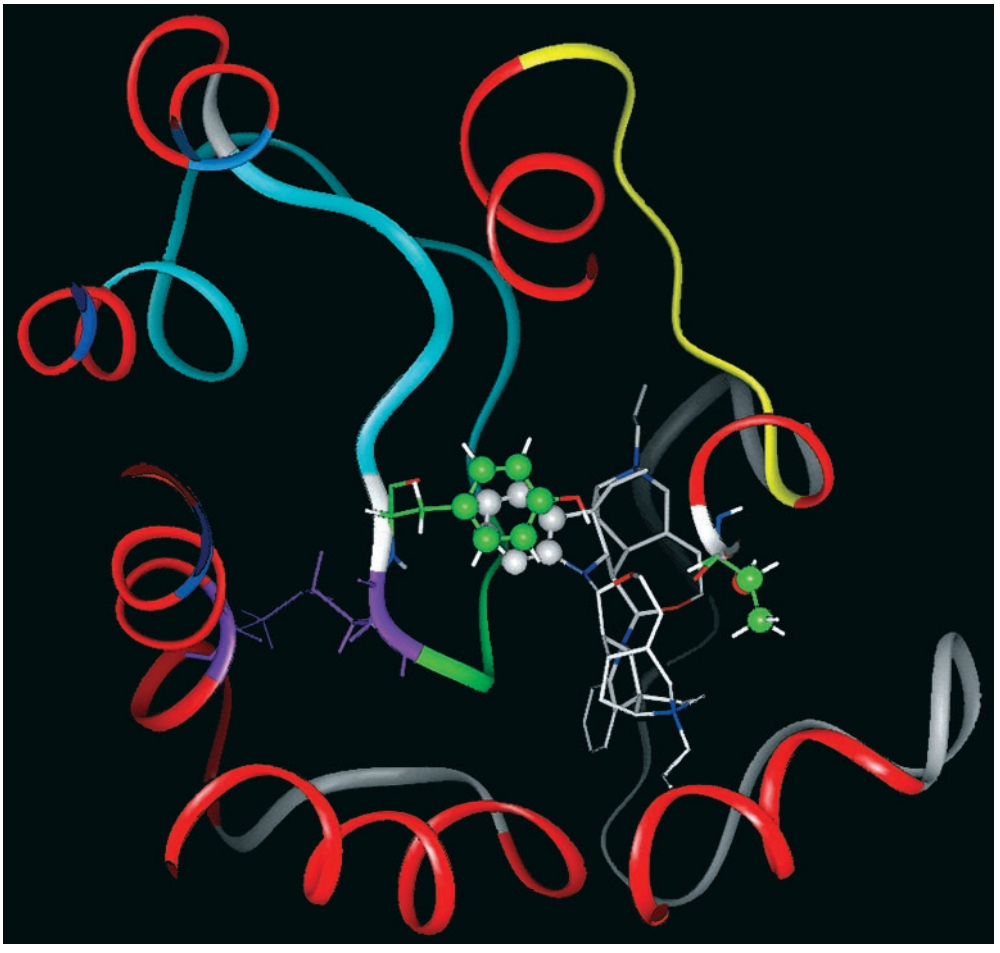

Fig. 9. π-π interaction between M_2-Tyr^{177} and diallylcaracurine V; view from the bottom of the ligand binding cavity. Protein: helices, red; o2, cyan; EDGE, green; o3, yellow; disulfide bridge, magenta. Tyr^{177}/Thr^{423}: carbon, green; nitrogen, dark blue; oxygen, red; hydrogen, white. Diallylcaracurine V: carbon, white; nitrogen, dark blue; oxygen, red; hydrogen, not shown.

binding, are well known but different from the amino acids important for agonist binding (Heitz et al.,1999; Lu et al., 2002). The model of the ligand binding cavity of the M_2 receptor is shown in Fig. 7. The extracellular loops form a cleft-like vestibule that is connected through a narrow corridor with a chamber in which the orthosteric binding site is located, lined by the transmembrane helices.

To model the ternary complex characteristic for allosteric interactions, NMS was placed in the orthosteric site as described previously (Vogel et al.,1997; Heitz et al., 1999). Then diallylcaracurine V in the conformation illustrated in Fig. 1, which is based on the 3D structure of caracurine V as determined by Zlotos (2000), could easily be docked into the entrance of the ligand binding cavity (Fig. 8). The molecular dynamics simulation performed subsequently indicated that the aromatic ring of M_2-Tyr^{177} is prone to make an upward movement toward the allosteric agent. Thereby, the aromatic ring of M_2-Tyr^{177} approaches the adjacent aromatic ring of the allosteric agent, leading to the formation of a π-π interaction (Fig. 9). The movement of M_2-Tyr^{177} takes place at the expense of free volume in the region connecting the two binding sites; consequently, the corridor between the orthosteric site and the allosteric site is closed and NMS is trapped (Fig. 8). In the NMS-liganded mutant M_2-Tyr^{177}→Gln, the conformation of the allosteric site is not changed compared with the wild-type M_2. In this mutant, docking of diallylcaracurine V is followed by a movement of Gln away from the allosteric agent [i.e., into the opposite direction compared with the movement of Tyr in the wild-type receptor (Fig. 10)]. This is accompanied by a considerable loss of interaction energy (Fig. 11, top).

We have also studied the M_2-Thr^{423}→His mutant receptor. M_2-Thr^{423} is located near one of the ether oxygens of diallylcaracurine V. In the mutant, the interaction energy converts from an attractive to a repulsive term (Fig. 11, bottom). Taken together, these results are in line with the experimental findings; i.e., the loss of binding affinity of diallylcaracurine V is greater in M_2-Tyr^{177}→Gln than in M_2-Thr^{423}→His. Investigation of the double mutant (M_2-Tyr^{177}→Gln + Thr^{423}→His) yielded both effects described above (data not shown), in analogy to the increased loss of affinity seen in the binding experiments.

The quaternary nitrogens and the allyl substituents of diallylcaracurine V are placed in two lipophilic pockets. One is formed by M_2-Ala^{13}, Val^{23}, Glu^{22}, Ile^{26}, and Val^{85}, the other one by Met^1, Asn^6, Thr^{170}, Val^{171}, and Ile^{417}. The charge of the quaternary nitrogen, which is dissipated on the surrounding aliphatic carbon atoms, interacts favorably with the negative charge of Glu^{22} or with the polar Asn^6. Except for the three nearest the N-terminal (Met^1, Asn^6, and Ala^{13}), the residues comprising these pockets lie in or near regions of the receptor that are significantly conserved across the muscarinic family. Furthermore, these residues are themselves highly conserved between M_2 and M_5 and throughout the family.

To assign a realistic low energy conformation for the highly flexible W84, a conformational analysis was performed. For this purpose molecular dynamics simulations in an aqueous environment were employed (Höltje and Folkers, 1996). This procedure leads to a conformation of W84 that differs from the S-shape low energy conformer detected by Holzgrabe and Hopfinger (1996) in the course of a systematic analysis in

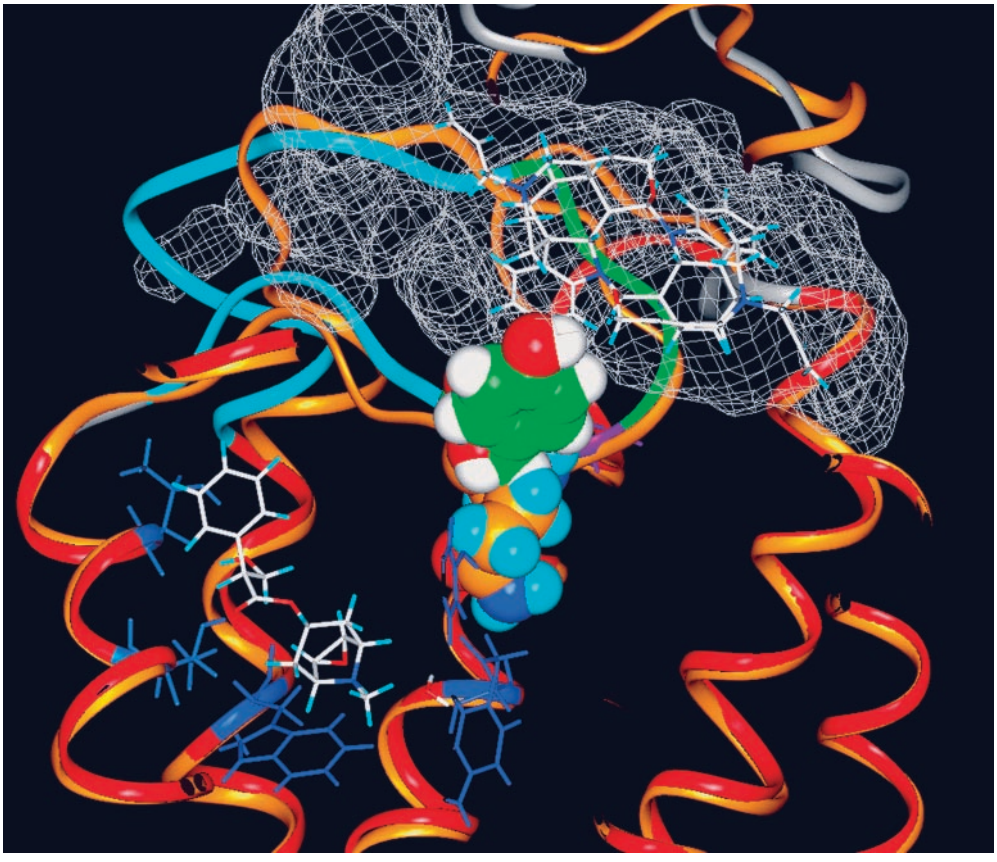

Fig. 10. Difference between the orientation of M_2 wild-type Tyr^{177} and mutant Gln^{177} in the ternary complex with NMS and diallylcaracurine V. Wild-type: helices, red; o1 and N terminus, gray; o2, cyan; EDGE, green; o3, yellow; disulfide bridge, magenta; orthosteric binding site, blue; free volumes, gray grid. Tyr^{177}: carbon, green; nitrogen, dark blue; oxygen, red; hydrogen, white. Tyr^{177}→Gln mutant: protein backbone, orange. Gln^{177}: carbon, orange; nitrogen, dark blue; oxygen, red; hydrogen, cyan.

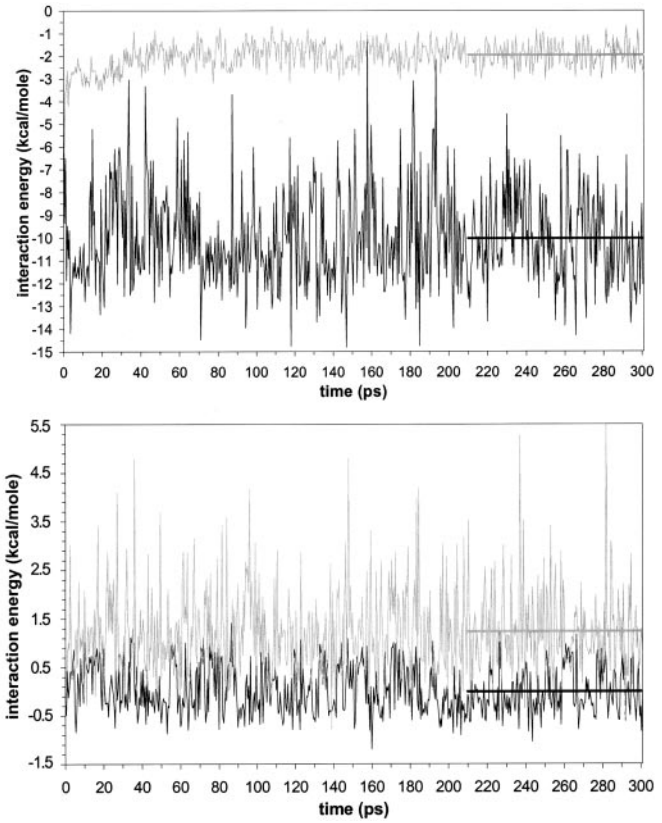

Fig. 11. Interaction energies during molecular dynamics simulations. Top, interaction between M_2 residue Tyr^{177} and diallylcaracurine V in the NMS-occupied M_2 receptor: Wild-type Tyr, black; Gln mutant, gray. Bottom, interaction between M_2 residue Thr^{423} (M_2) and diallylcaracurine V in the NMS-occupied M_2 receptor: wild-type Thr, black; His mutant, gray.

vacuo. The conformation resulting from the dynamical treatment of W84 was docked to the NMS-occupied receptor. W84 fills the ligand binding cavity and one phthalimido-group approaches M_2-Tyr^{177} and M_2-Thr^{423} (Fig. 12); the quaternary nitrogens are found in similar positions as described above for diallylcaracurine V. The M_2-Tyr^{177}→Gln mutant leads to a comparable loss of interaction energy and a geometry comparable to that described for diallylcaracurine V. Mutating M_2-Thr^{423} to His causes nearly no difference in the interaction energy in this position but reduces the interaction energy between W84 and the conserved tryptophans by influencing their side-chain conformation (data not shown). Taken together, the results of the docking simulations carried out with the three-dimensional model correspond with the experimental findings from the binding assays in the wild-type M_2 and the mutant M_2 receptors.

Discussion

In this study, we have identified M_2-Tyr^{177} in o2 as a second essential epitope for the high-affinity binding of some structurally diverse muscarinic allosteric agents in M_2 receptors whose orthosteric site is occupied by the muscarinic antagonist *N*-methylscopolamine. This amino acid, together with the previously identified M_2-Thr^{423} at the beginning of TM7 (Buller et al., 2002), account entirely for the M_2/M_5 selectivity. For each of the three ligands investigated in the present study, the substitution of M_2-Tyr^{177} resulted in a more pronounced loss of affinity than substitution of M_2-Thr^{423} (M_2-Y^{177}→Q, M_2-T^{423}→H, Table 2). Thus, M_2-Tyr^{177} seems to be more important for the binding affinity of these allosteric test compounds than the previously identified M_2-Thr^{423}. Yet, this is not a general phenomenon, because the allosteric affinity of the methyl analog of diallylcaracurine V has been shown to be affected much more by mutation at the M_2-Thr^{423} than by substitution of the entire o2 loop (Buller et al., 2002).

Multiple studies have indicated that residues in the second outer loop or near the junction of the third outer loop and the top of TM7 are important in the binding of muscarinic allosteric ligands. The EDGE sequence (residues 172–175 in M_2) markedly affects the binding of gallamine (Leppik et al., 1994; Gnagey et al., 1999). The asparagine residue at M_2^{419} has been implicated in the subtype selectivities of gallamine and alcuronium (Gnagey et al., 1999; Krejči and Tuček, 2001). Elimination of a tryptophan residue that is conserved among all five muscarinic receptor subtypes (located at position 422 in M_2) reduces the affinity of gallamine and other allosteric ligands (Matsui et al., 1995). More general studies using chimeric receptors have also suggested that these two regions of the receptor (i.e., o2 and o3) are critical to the binding or selectivity of many muscarinic allosteric ligands, although the relative importance of the regions varies from ligand to ligand (Ellis and Seidenberg, 2000). Nevertheless, many of these compounds seem to interact competitively at a common allosteric site (Ellis and Seidenberg, 1992; 2000; Tränkle and Mohr, 1997), perhaps reflecting the close proximity of the residues involved, either near the middle of o2 or near the o3/TM7 junction. In keeping with the above studies, the agents investigated in the present study were sensitive to residues in these two regions: the recently described threonine near o3/TM7 and the tyrosine in o2 that is the main finding of the present study. It remains to be seen whether another group of compounds, which does not seem to interact competitively with gallamine and the others (Tränkle and Mohr, 1997; Lazareno et al., 2000), may bind to regions other than o2 and o3.

The present findings made with diallylcaracurine V reveal that the pair of amino acids, M_2-Tyr^{177} and M_2-Thr^{423}, is also involved in the M_2/M_5 selectivity of the binding affinity for NMS-free receptors. The affinity of diallylcaracurine V for the NMS-free M_2 double mutant receptor does not reach the level of M_5. Thus, the pair of amino acids does not completely account for the M_2/M_5 selectivity in free receptors, and one or more additional epitopes seem to be involved.

Nevertheless, for NMS-occupied receptors, the present study is the first to identify epitopes that fully explain the M_2/M_5 selectivity of structurally different allosteric agents. The finding is a good starting point to develop a three-dimensional model of the M_2 receptor and to check its validity. Docking NMS into the orthosteric site has the advantage that NMS stabilizes an inactive conformation of the receptor protein; in addition, the possibility is excluded that the allosteric agent may use elements of the orthosteric site. The two amino acids identified in the present study can serve as probes to check the model in that these amino acids should be involved in a meaningful fashion in the docking of the allosteric agents. In fact, the model provides a hypothesis of how the alkane-bisammonium–type agent W84 and the caracurine derivative, although structurally diverse, could interact with the same two epitopes.

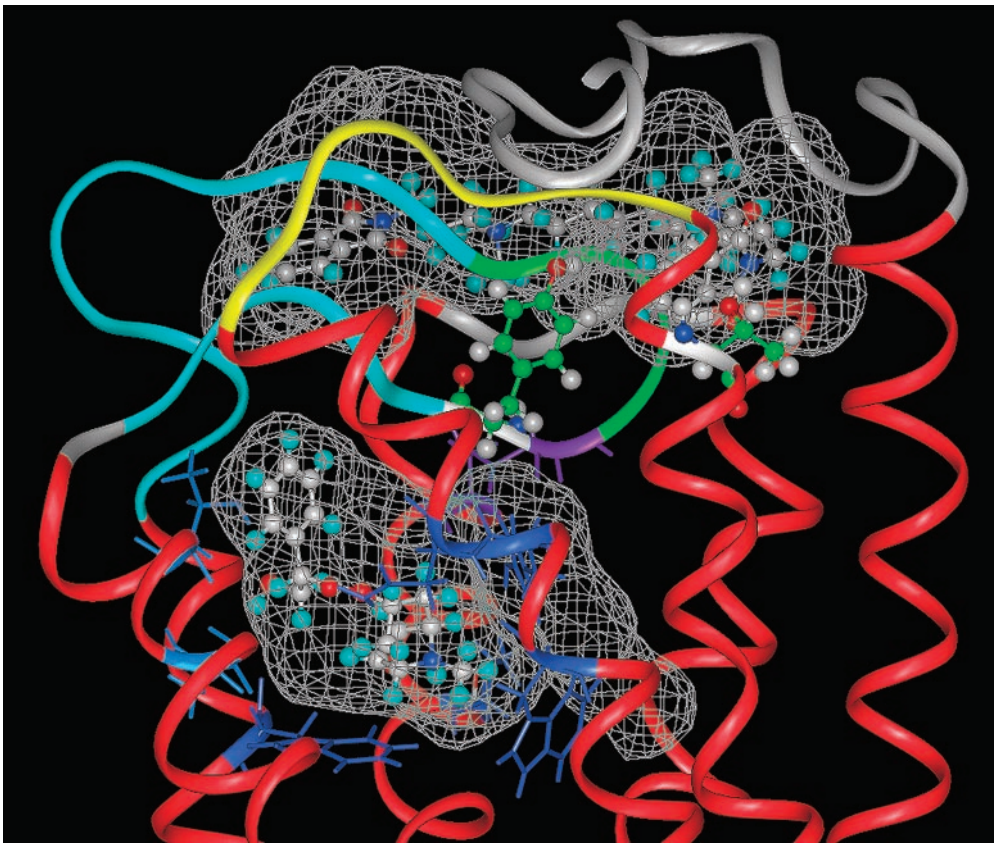

Fig. 12. Position of NMS and W84 in the wild-type M_2 receptor. Protein: helices, red; o1 and N terminus, gray; o2, cyan; EDGE, green; o3, yellow; disulfide bridge, magenta; orthosteric binding site, blue; free volumes, gray grid. Tyr^{177}/Thr^{423}: carbon, green; nitrogen, dark blue; oxygen, red; hydrogen, white. NMS/W84: carbon white; nitrogen, dark blue; oxygen, red; hydrogen, cyan.

In principle, the amino acids identified here as essential epitopes for the high binding affinity of the allosteric agents in M_2 relative to M_5 receptors could either be important for the overall conformation of the allosteric site or they could be docking points. In the 3D model, the exchange of M_2-Tyr^{177} by the corresponding Gln of M_5 did not affect the conformation of the allosteric site. In contrast, a mutation from the acidic M_2 sequence EDGE, located near M_2-Tyr^{177}, into the corresponding sequence PLDE of M_5 would change the proposed β-I′-turn and hereby the loop conformation and the allosteric binding site (data not shown). Upon docking of diallylcaracurine V to the wild-type M_2 receptor, M_2-Tyr^{177} makes a movement in the sense of an induced fit, whereas in the mutant receptor M_2-Tyr^{177}→Gln, the glutamine moves in the opposite direction (i.e., away from the allosteric agent). M_2-Thr^{423} is situated just between two tryptophans that are conserved in muscarinic receptors. The effect of the M_2-Thr^{423}→His mutation seems to be a direct interaction in the case of diallylcaracurine V, whereas in the case of W84, it is more likely to be a steric influence on the relative orientation of the two tryptophans. Taken together, the model recognizes M_2-Tyr^{177} and M_2-Thr^{423} as essential sites of attachment. At the same time, the allosteric ligands also possess significant affinity for the M_5 receptor subtype. We have speculated previously that a core binding region common to all of the receptor subtypes is likely to exist and that allosteric ligands bind to the different subtypes with similar orientations (Ellis, 1997; Buller et al., 2002). It remains to be seen whether the conserved residues that make up the lipophilic pockets, which we have identified in the model of the M_2 receptor, contribute to a core binding region of the allosteric site for the receptor family.

In summary, we have identified a second amino acid, M_2-Tyr^{177} in o2, which, together with M_2-Thr^{423} at the top of TM7, accounts entirely for the M_2/M_5 selectivity of alkane-bisammonium–type and caracurine V-type allosteric modulators at muscarinic acetylcholine receptors that are occupied by the antagonist *N*-methylscopolamine. In free receptors, the pair of epitopes clearly contributes to the binding affinity of the modulators for M_2 receptors, but it does not seem to fully account for the M_2/M_5 selectivity. A three-dimensional model of the NMS-occupied M_2 muscarinic acetylcholine receptor has been developed that accommodates the experimentally observed phenomena and may provide insight into the topography and the molecular mechanisms of allosteric-orthosteric-interactions.

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