Allosteric interactions with muscarinic acetylcholine receptors:
Complex role of the conserved tryptophan M2^{422}Trp in a critical cluster of amino acids for baseline affinity, subtype selectivity, and cooperativity

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Abbreviations:
M₂,M₅: M₂, M₅ subtype of the muscarinic acetylcholine receptor, NMS: N-methylscopolamine, o₂, o₃: second, third outer (extracellular) loop of the receptor, TM: transmembrane region of the receptor, PB: sodium-potassium phosphate buffer (5 mM, pH 7.4), W84: hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide.
Abstract

Generally, the M\textsubscript{2} subtype of muscarinic acetylcholine receptors has the highest sensitivity for allosteric modulators and the M\textsubscript{5} subtype the lowest. The M\textsubscript{2}/M\textsubscript{5} selectivity of some structurally diverse allosteric agents is known to be completely explained by M\textsubscript{2}\textsuperscript{177}Tyr and M\textsubscript{2}\textsuperscript{423}Thr in receptors whose orthosteric site is occupied by the conventional ligand N-methylscopolamine (NMS). This study explored the role of the conserved M\textsubscript{2}\textsuperscript{422}Trp and the adjacent M\textsubscript{2}\textsuperscript{423}Thr in the binding of alkane-bisammonio type modulators, gallamine, and diallylcaracurine V. Experiments were performed with human M\textsubscript{2} or M\textsubscript{5} receptors or mutants thereof. It was found that M\textsubscript{2}\textsuperscript{422}Trp and M\textsubscript{2}\textsuperscript{423}Thr independently influenced allosteric agent binding. The presence of M\textsubscript{2}\textsuperscript{423}Thr may enhance the affinity of binding, depending on the allosteric agent, either directly or indirectly (by avoiding sterical hindrance through its M\textsubscript{5} counterpart \textsuperscript{478}His). Replacement of M\textsubscript{2}\textsuperscript{422}Trp and of the corresponding M\textsubscript{5}\textsuperscript{477}Trp by alanine revealed a pronounced contribution of these epitopes to subtype independent baseline affinity in NMS-bound and NMS-free receptors for all agents except diallylcaracurine V. In a few instances, this tryptophan also influenced cooperativity and subtype selectivity. Docking simulations using a three-dimensional M\textsubscript{2} receptor model revealed that the aromatic rings of M\textsubscript{2}\textsuperscript{177}Tyr and M\textsubscript{2}\textsuperscript{422}Trp, in a concerted action, may fix one of the aromatic moieties of alkane-bisammonio compounds between them. Thus, M\textsubscript{2}\textsuperscript{422}Trp and the spatially adjacent M\textsubscript{2}\textsuperscript{177}Tyr, as well as M\textsubscript{2}\textsuperscript{423}Thr, form a cluster of amino acids within the allosteric binding cleft that is pivotal for both M\textsubscript{2}/M\textsubscript{5} subtype selectivity and baseline affinity of allosteric agents.
All five subtypes of muscarinic acetylcholine receptors contain an allosteric site apart from the orthosteric site that is addressed by acetylcholine and conventional muscarinic agonists and antagonists. Binding of an allosteric modulator allows formation of ternary complexes consisting of the allosteric agent, the orthosteric ligand and the receptor protein. Through ternary complex formation, allosteric agents may evoke particular actions that cannot be induced by orthosteric ligands alone and that may have therapeutic potential. For instance, allosteric modulators may increase the binding of orthosteric agonists or antagonists (positive cooperativity) or they may inhibit orthosteric ligand binding (negative cooperativity). In either case, the magnitude of the cooperativity will define an intrinsic limit on the magnitude of the positive or negative effect, in marked contrast to the unconstrained action of orthosteric agonists and antagonists. It is also possible for allosteric modulators to leave orthosteric ligand binding unchanged (neutral cooperativity), while nevertheless changing the kinetics of binding (Ellis, 1997; Christopoulos and Kenakin, 2002; Krejčí et al., 2004; Soudijn et al., 2004; Birdsall and Lazareno, 2005; Wess, 2005). Finally, in addition to modulating orthosteric ligand binding properties, allosteric agents also may modulate agonist induced intrinsic efficacy (Zahn et al., 2002). A better understanding of the molecular topology and mechanisms of allosteric agent binding and action will help to design new allosteric agents with improved properties and will lead to a better insight into the principles of muscarinic receptor function. The M2 subtype of muscarinic receptors generally displays highest affinity for allosteric modulators, whereas the M5 subtype has lowest sensitivity. A rather good insight into the allosteric binding area has now been achieved by combining three strategies, i.e. development of allosteric agents with high affinity and selectivity for M2 receptors that presumably fit tightly in a fixed position at the receptor protein (Mohr et al., 2003),
receptor mutagenesis starting from M$_2$/M$_5$ chimeric receptor constructs to identify essential epitopes for allosteric agent binding (Ellis et al., 1993; Gnagey et al., 1999; Buller et al., 2002; Huang et al., 2005), and generation of a three-dimensional M$_2$ receptor model based on the crystal structure of the inactive bovine rhodopsin (Jöhren and Höltje, 2002; Voigtländer et al., 2003). This approach has allowed the visualization of different binding topologies for typical and atypical allosteric agents (Tränkle et al., 2005; Wess, 2005). However, the mode by which certain epitopes affect binding affinity of allosteric agents is still in question. For instance, an amino acid may directly serve as a docking point or alternatively constitute a steric hindrance, or it may indirectly contribute to ligand binding by governing the conformation of amino acid strands that contain a relevant point of attachment. Previously, we found that two amino acids are sufficient to account completely for the hundred-fold M$_2$/M$_5$ selectivity of structurally different allosteric agents (Voigtländer et al., 2003). These amino acids are M$_2$$^{177}$Tyr and M$_2$$^{423}$Thr, corresponding to the M$_5$ amino acids 184Gln and 478His. The receptor model suggested M$_2$$^{423}$Thr to be a direct docking point for caracurine V-type agents. For alkane-bisammonio type compounds such as W84 (Fig. 1), however, the model suggested an indirect influence, in that M$_2$$^{423}$Thr induces a favorable spatial adjustment of the adjacent M$_2$$^{422}$Trp for its interaction with one of the phthalimide moieties of W84. The involvement of M$_2$$^{422}$Trp is supported by a broad mutagenesis study by Matsui et al. (1995), who found the corresponding tryptophan of M$_1$ to be relevant to the binding of the allosteric agent gallamine. Therefore, we set out to clarify the role of this conserved tryptophan and its neighboring M$_2$$^{423}$Thr or M$_5$$^{478}$His, respectively. As a major outcome of this study we found that this tryptophan is of crucial importance for M$_2$/M$_5$ subtype independent baseline affinity of alkane-bisammonio type allosteric modulators and of gallamine.
Thus, an epitope of high relevance to M₂/M₅ subtype independent baseline affinity of allosteric agents has been discovered. Furthermore we found that this tryptophan in certain instances may provide subtype selectivity for allosteric agents or may modulate their cooperativity with an orthosteric antagonist. Taken together the findings reveal that relevant epitopes for subtype dependent and subtype independent allosteric agent binding are clustered in close spatial proximity at the junction between the allosteric and the orthosteric binding areas of muscarinic acetylcholine receptors.
Materials and Methods

Materials.

Atropine sulfate, gallamine triethiodide, and N-methylscopolamine bromide were obtained from Sigma-Aldrich (Steinheim, Germany). W84 is commercially available from Tocris Cookson Inc. (St. Louis, MO, USA). Dimethyl-W84 (Tränkle et al., 1998), naphmethonium (Muth et al. 2003) and diallylcaracurine V (Zlotos et al., 2000) were generously provided by Prof. Dr. Ulrike Holzgrabe, Dr. Mathias Muth, and Dr. Darius P. Zlotos (Institute of Pharmacy, University of Würzburg, Germany). The orthosteric radioligand $[^3H]$NMS ($[^3H]$N-methylscopolamine chloride, 81 Ci/mmol) was purchased from NEN-DuPont (Homburg, Germany).

Mutagenesis and Expression.

Point mutated $M_2$ and $M_5$ receptors were generated as described previously (Voigtländer et al., 2003; Huang et al., 2005) using the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands). pCD-plasmids including wild type genes for human $M_2$ and $M_5$ muscarinic receptors served as templates in PCR (polymerase chain reaction). Mutations were inserted by addition of synthetic oligonucleotide primers containing the required triplett changes (Sigma Genosys, Steinheim, Germany). Primers were elongated during temperature cycling by a high-fidelity DNA-polymerase. After amplification over twelve to sixteen cycles the parental DNA was digested by a methylation-specific endonuclease, thus selecting the mutated plasmids. The PCR-products were transformed into supercompetent Escherichia coli cells. Bacteria were plated on ampicillin containing agar and incubated over night. Clones with the putative mutation were isolated and grown up in 500 ml Luria-Bertani medium (Sigma-Aldrich). Plasmids were extracted using the
Quiagen Plasmid Maxi kit (Quiagen, Hilden, Germany) and mutation was confirmed by sequencing. Subsequently, wild type or mutant plasmid-DNA was transiently transfected into COS-7 cells by lipofection using PolyFect transfection reagent (Quiagen).

**Cell Culture and Membrane Preparation.**

COS-7 cells were cultured at 37°C under humidified air supplemented with 5 % CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) containing 1 % penicillin-streptomycin and 10 % fetal bovine serum. One day before transfection, cells were grown in 10 cm dishes by seeding 1.6 x 10⁶ cells per dish. 48 h after transfection cells were harvested and homogenized in 5 mM Na, K, P_i-buffer (PB; 4 mM Na₂HPO₄, 1 mM KH₂PO₄), pH 7.4. After centrifugation membranes were resuspended in 5 mM PB and stored in aliquots at -80°C.

**Dissociation Binding Assays.**

Two point [³H]NMS dissociation experiments were performed in 5 mM PB, pH 7.4, at 23°C as described before (Voigtländer et al., 2003; Huang et al., 2005). Membranes with the respective wild type or mutant M₂ or M₅ receptor were prelabeled with 1 nM radioligand for 30 min. Measurement of [³H]NMS dissociation was started by the addition of 3 µM atropine, with or without varying concentrations of allosteric modulator. After an appropriate time interval, depending on the [³H]NMS dissociation half time of the respective receptor, dissociation was terminated by filtration using a Brandel cell harvester and GMF2 glass microfibre sheets (Sartorius, Göttingen, Germany), saturated with 0.1 % polyethylenimine. Filtration was immediately followed by two rinses with ice cold 40 mM PB. Filter-bound radioactivity was
detected by liquid scintillation counting. Non specific binding of [3H]NMS was determined in the presence of 3 μM atropine.

**Equilibrium Binding Assays.**

[3H]NMS equilibrium binding was measured in the presence of 0.2 nM radioligand and the indicated concentrations of the allosteric agent, as described before (Voigtländer et al., 2003). Experiments were conducted in 5 mM PB, pH 7.4 at 23°C and incubation was carried on until equilibrium binding was achieved. The incubation time sufficient to attain equilibrium was calculated according to Lazareno and Birdsall (1995, eq. 31 therein).

The affinity of the orthosteric probe [3H]NMS for the wild type and mutant receptors was determined in homologous competition experiments. For this purpose, membranes were incubated with 0.2 nM radioligand and unlabeled NMS (1 pM to 30 nM) for the same time period, as applied in the equilibrium binding experiment with the allosteric agent. Filtration and quantification of membrane-bound radioligand were carried out as described above.

**Data Analysis.**

Data from dissociation experiments were converted into apparent rate constants assuming monoexponential decay. The apparent rate constant obtained in the absence of allosteric modulator served as control, and the rate constants determined in the presence of each concentration of allosteric agent were expressed as a percentage of that control value. This percentage was then plotted against the log concentration of the modulator. Curve fitting by nonlinear regression analysis was based on a four parameter logistic function as described by Tränkle and Mohr (1997).
The resulting concentration-effect curves for the allosteric delay of [\(^3\)H]NMS dissociation reflect the binding of the allosteric agent to the NMS-occupied receptors. When there was no significant difference (F-test, \(p \geq 0.05\)) between the top plateau of the curve and the control value \(k_1 = 100\%\), or between the bottom plateau of the curve and \(k_1 = 0\%\), plateaus were fixed at 100\% and 0\%, respectively. The negative logarithm of the concentration inducing a half-maximal reduction of the NMS dissociation rate, \(pEC_{0.5,\text{diss}}\), indicates the affinity of the allosteric agent for the NMS-occupied receptor.

[\(^3\)H]NMS equilibrium binding data were analyzed according to the ternary complex model of allosteric interactions (Stockton et al., 1983; Ehlert, 1988) using the following equation

\[
B_A = \frac{B_0 \cdot (10^{\log L} + 10^{\log K_L})^{\frac{\log K_A + 10^{\log A}}{\alpha}}}{10^{\log L} + 10^{\log K_L} + 10^{\log K_A} + 10^{\log A}}
\]

\(L\) and \(A\) indicate the concentration of the orthosteric ligand (\(L\)) and the allosteric agent (\(A\)), respectively. \(K_L\) and \(K_A\) are the equilibrium dissociation constants for the binding of [\(^3\)H]NMS and the allosteric agent, respectively, to the free receptor. The negative log values, \(pK_L\) and \(pK_A\), reflect the corresponding binding affinities. \(\alpha\) is the factor of cooperativity and serves as a measure of the magnitude and direction of the interaction between \(L\) and \(A\). In two cases of steep curves, equation 2 from Tränkle et al. (2003) was applied that was derived from Lazareno and Birdsall (1995) and that contains the slope factor as a variable. As a consequence of the allosteric delay of [\(^3\)H]NMS dissociation, rather long incubation periods can be required to attain equilibrium binding of [\(^3\)H]NMS, especially in the case of \(M_5\) receptors (control half
time of $[^3\text{H}]$NMS dissociation about two hours). W84 is known to be sensitive to spontaneous hydrolytic cleavage ($t_{1/2} = 11$ h; Schulz, 1998); the concentration values indicated for W84 represent effective concentrations calculated on the basis of the half time of the decay and the applied incubation time. In the case of almost neutral cooperativity ($\alpha \sim 1$) $[^3\text{H}]$NMS equilibrium binding remains nearly unchanged under the influence of increasing concentrations of the allosteric modulator. Under this condition, curve fitting with equation 1 does not work. Since the validity of the cooperativity model can be assumed (e.g. Ellis, 1997; Tränkle et al., 1998; Raasch et al., 2002) the affinity of the allosteric agent to the NMS-occupied receptors $pEC_{0.5,\text{diss}}$ is identical to $p(\alpha \cdot K_A)$. Therefore, we replaced $K_A$ in equation 1 by $EC_{0.5,\text{diss}} / \alpha$ (Raasch et al., 2002). $EC_{0.5,\text{diss}}$ was derived from preceding dissociation experiments and curve fitting yielded the cooperativity factor $\alpha$, which then served to calculate $K_A = EC_{0.5,\text{diss}} / \alpha$. Nonlinear regression analysis was performed using the Prism program (vers. 3.02, GraphPad Software, San Diego, CA).

Three-Dimensional Modeling and Docking Simulations.

**Homology Modeling.** As template for the model the latest X-ray structure of bovine rhodopsin (Protein Data Base accession no. 1U19; Okada et al., 2004) with a resolution of 2.2 Å was used. The sequences of the human $M_2$ receptor and the bovine rhodopsin were extracted from SwissProt (code no. P08172; Bonner et al., 1987; P02699; Nathans and Hogness, 1983, respectively). The sequence alignment was carried out with CLUSTALW (Thompson et al., 1994) as well as based on the pinpoints identified by Baldwin et al. (1997). Transmembrane regions of the $M_2$ receptor were detected using several secondary structure prediction methods. The extracellular and intracellular loops and the N terminus, respectively, were created by
the application of a loop search routine based on an alpha-carbon distance-matrix as implemented in the Homology module of Insight II 2000 (Accelrys, San Diego, USA). The three-dimensional coordinates for the C terminus were added in analogy to the X-ray structure of bovine rhodopsin.

**Receptor-Ligand Complex.** The orthosteric ligand N-methylscopolamine and the allosteric agent W84 were manually docked. The W84 conformation used in the docking procedure was the one which had been detected as the most favorable one in the course of a dynamical conformational search in aqueous environment (Voigtländer et al., 2003). Two steps were used to reach a good starting geometry for the following molecular dynamics simulation. First, the free volume located between the helices and the extracellular loops were calculated using the program SURFNET (Laskowski, 1995). In the second step the large cavities were analyzed using GRID interaction fields (Goodford, 1985). Different GRID-probes were applied to mimic the functional groups present in the ligands, and afterwards the ligands were manually docked according to the favorable positions detected by GRID.

**Molecular Dynamics Simulation.** For further refinement and structure validation molecular dynamics simulations were carried out using the software package GROMACS (Lindahl et al., 2001). For this purpose the model was embedded in a phospholipid bilayer with aqueous phases containing Na⁺ and Cl⁻ as counterions extra- as well as intracellularly. This procedure was recently described by Schlegel et al. (2005). Position restraints were initially set on the ternary complex with a force of 5000 kJ·mol⁻¹·nm⁻² to equilibrate the membrane and solvent molecules. Afterwards the position restraints were slowly reduced in ten steps of 100 ps, respectively, until
an unconstrained dynamics simulation over a period of 2500 ps was carried out. Frames were written out for every 2 ps.

**Model Quality Check.** The molecular dynamics simulation was checked for equilibration of the protein and its stability. The intramolecular interaction energy of the protein and the root-mean-square deviation of the entire and helical backbone and of W84, respectively, were taken as measures for complex equilibration. The program g_cluster implemented in GROMACS was applied to attain a representative structure from the trajectory after equilibration. This structure was minimized using the steepest descent algorithm and the protein geometry was checked with PROCHECK (Laskowski et al., 1993).
Results

In this study we aimed at characterizing the role for allosteric action of two conserved tryptophans located in the essential region of the allosteric binding domain of the M₂ receptor and the possible interplay with the spatially adjacent amino acids M₂¹⁷⁷Tyr and M₂⁴²³Thr (Fig.2). To probe the influence of these M₂ and the respective M₅ amino acids on the binding of allosteric modulators, these residues were either replaced by the corresponding amino acids of the counterpart receptor subtype (M₂/M₅ or M₅/M₂ substitution) or by alanine as a neutral residue. M₂/M₅ substitution and M₅/M₂ substitution, respectively, provide insight into the contribution of an amino acid of interest relative to its counterpart amino acid to the high affinity of the allosteric agents at M₂ compared with M₅, whereas substitution against alanine serves to elucidate the role of the amino acid by itself and was applied throughout in the case of conserved residues.

We firstly investigated the effect of point mutations on the formation of ternary complexes, which is the main characteristic of allosteric interactions. For this purpose, we employed dissociation binding experiments, using [³H]N-methylscopolamine ([³H]NMS) as an orthosteric ligand. The observed alteration, generally a delay, of the [³H]NMS dissociation rate is strictly indicative of an allosteric action, because it results from an interaction of the modulator with receptors, whose orthosteric site is occupied by [³H]NMS. The half times of [³H]NMS dissociation at the diverse wild type and mutant receptors in the absence of allosteric agents are shown in Table 1. Dissociation half times at the M₂ receptors ranged from 5.6 min to 56 min. At M₅, dissociation was much slower than at M₂, which is in accordance with data presented previously (Ellis et al., 1993; Buller et al., 2002;
Voigtländer et al., 2003; Huang et al., 2005). Mutation-induced changes of the half times are mentioned in the subsequent paragraphs.

**M₂⁴²²Trp and the corresponding M₅⁴⁷⁷Trp confer sensitivity to W₈₄ independent of whether the adjacent downstream amino acid is threonine.**

The previous M₂ receptor model predicted the conserved residue M₂⁴²²Trp to interact directly with W₈₄, while the adjacent amino acid M₂⁴²³Thr relative to the corresponding M₅⁴⁷⁸His was suspected to be essential for the proper orientation of the side chain of this tryptophan (Voigtländer et al., 2003). If so, replacement of M₂⁴²²Trp by alanine should reduce the potency of W₈₄. Moreover, substitution of M₂⁴²³Thr by the corresponding M₅⁴⁷⁸His, which was suspected to be unfavorable, should diminish the role of M₂⁴²²Trp for the binding of W₈₄. In the M₅ receptor, however, mutation of M₅⁴⁷⁷Trp should less affect the binding of W₈₄, provided that the absence of a neighboring threonine causes an unfavorable conformation of M₅⁴⁷⁷Trp in M₅. In order to check this concept, we generated mutant M₂ and M₅ receptors. The effects of the substitutions in M₂ on the allosteric potency of W₈₄ are illustrated in Fig. 3. As anticipated, mutation of M₂⁴²²Trp to alanine led to a pronounced loss of potency of W₈₄, compared with M₂ wild type; the pEC₀.₅ₐ₃₅-value at the M₂⁴²²W→A mutant was reduced by -1.51 log units (Table 2). The double mutant M₂⁴²²W→A⁺⁴²³T→H displayed a further decrease of sensitivity for W₈₄, when compared with the single mutant M₂⁴²³T→H. This finding was unexpected, since the absence of M₂⁴²³Thr was thought to induce an unfavorable orientation of the adjacent M₂⁴²²Trp and thus to diminish its contribution to the potency of W₈₄. Notably, this unexpected further reduction of potency (-1.47 log units relative to M₂⁴²³T→H) was almost equal to the loss of sensitivity observed at the M₂⁴²²W→A mutant relative to
M₂ (-1.51 log units). The effects of both single mutations were additive and thus appear to be independent of each other. At M₅ (data compiled in Table 2), substitution of the corresponding tryptophan ⁴⁷⁷Trp by alanine resulted in an impressive loss of potency of W84 by -2.17 log units. This was also unexpected, because the presence of M₅ ⁴⁷⁸His instead of threonine was thought to force M₅ ⁴⁷⁷Trp into an unfavorable orientation. As shown before (Voigtländer et al., 2003), replacement of the neighboring M₅ ⁴⁷⁸His by the corresponding threonine of M₂ increases W84’s potency by 0.77 log units compared to M₅. Additional mutation of ⁴⁷⁷Trp in the M₅ ⁴⁷₈H→T mutant reduced the potency of W84 by -0.78 log units relative to M₅; compared with the M₅ ⁴⁷₈H→T mutant affinity was diminished by -1.55 log units. This value is similar to the affinity shift found in M₅ ⁴⁷⁷W→A relative to M₅ wild type (-2.17 log units). Taken together, the findings indicate that the conserved tryptophans M₂ ⁴²²Trp and M₅ ⁴⁷⁷Trp, respectively, are important for the binding of W84 irrespective of whether a neighboring threonine is present or not. Thus, in contrast to the previous concept, M₂ ⁴²₃Thr does not appear to induce a proper orientation of the adjacent ⁴²²Trp for W84 binding.

M₅ ⁴⁷₈His hinders NMS binding kinetics.

The aforementioned data showed that the loss of potency of W84 observed at the M₂ ⁴²₃T→H mutant is not caused by an impaired orientation of the adjacent tryptophan. In order to further elucidate the role of this residue, 423 in M₂ and 478 in M₅, respectively, we introduced alanine instead of the respective M₂ and M₅ amino acid. The binding parameters of [³H]NMS for wild type and mutant receptors are given in Table 1. While replacement of M₂ ⁴²₃Thr by the corresponding amino acid of M₅, histidine, led to a three-fold increase of the [³H]NMS dissociation half time,
insertion of alanine instead of threonine had only a minor effect on the [3H]NMS dissociation. Conversely, substitution of the corresponding residue 478His in M5 by either alanine or threonine reduced the NMS dissociation half time by about two-fold to three-fold, respectively. Since none of these mutations had much effect on the binding affinity of [3H]NMS (pK_D-values in Table 1), compared with the respective wild type receptor, it can be concluded that the association of NMS at these mutants was affected to the same extent as the dissociation. In conclusion, 423Thr is located near the entrance of the orthosteric site of M2, but is itself rather unimportant for the kinetics of [3H]NMS binding at M2, while histidine at the corresponding position either in M2 or in M5 appears to be an obstacle for the passage of NMS to and from the orthosteric binding pocket.

**Low potency binding of W84 at the M2**

423T→H mutant and at M5 wild type is due to a detrimental effect of histidine.

Since M5 478His has been shown above to slow NMS binding kinetics and is part of the allosteric site, we hypothesized that the histidine at this position might be an obstacle for the binding of W84 to the NMS-liganded receptor. Concentration-effect curves, displaying the allosteric interaction of W84 with the different receptor mutants, are shown in Fig. 4. It has been reported previously (Voigtländer et al., 2003) that insertion of histidine in M2 instead of 423Thr lowers the potency of W84 (-0.79 log units; Fig. 4, Table 2). The present findings show that replacement of M2 423Thr by alanine does not affect the potency of W84, compared with the wild type receptor. The corresponding mutations in M5, M5 478H→A and M5 478H→T, both resulted in an equal increase in sensitivity for W84 (0.70 and 0.77 log units). We conclude that the beneficial role of M2 423Thr for the M2/M5 selectivity of W84 is not an
“active” contribution to W84 binding but “merely” reflects the replacement of an affinity lowering amino acid, i.e. M5478His.

In case of diallylcaracurine V, M2423Thr enhances potency directly.

In the case of diallylcaracurine V, the former M2 receptor model proposed a direct interaction between M2423Thr and the allosteric agent (Voigtländer et al., 2003). In that case, replacement of this M2 residue by alanine should result in a loss of the allosteric potency. Fig. 5 displays the sensitivities of the different receptors for diallylcaracurine V. Replacement of M2423Thr by either alanine or histidine reduced the modulator’s potency to the same extent (-0.66 and -0.68 log units, respectively; Table 3). The corresponding M5 mutants M5478H→A and M5478H→T exhibited significantly higher sensitivity for diallylcaracurine V than the wild type receptor. Notably, diallylcaracurine V benefits to a greater extent from the replacement of M5478His by threonine (0.98 log units) than by alanine (0.58 log units). These findings support the notion that M2423Thr has a direct beneficial effect for the interaction of diallylcaracurine V with the allosteric site.

Kinetics of NMS binding at M2 are accelerated by the conserved tryptophan M2427Trp, while the binding of W84 does not depend on this residue.

In the M2 receptor, there is a second conserved tryptophan next to M2422Trp at position 427 that might be involved in the binding of W84. In order to check this assumption, we introduced alanine instead of M2427Trp. We also created the double mutant M2422W→A+427W→A. Table 1 indicates the consequences of these mutations on the binding of [3H]NMS in the absence of an allosteric agent. NMS dissociation was remarkably retarded by the substitution of M2427Trp by alanine (five-fold),
whereas the mutation of M$_2^{422}$Trp to alanine increased the dissociation half time only slightly (two-fold). Both effects were nearly additive, as the NMS dissociation half time was increased by the factor eight at the double mutant. The pK$_D$-value of [³H]NMS was reduced at most by a factor of three by the aforementioned mutations (Table 1). Thus we conclude that [³H]NMS association at these receptors was also retarded. Therefore, M$_2^{427}$Trp and to a minor degree M$_2^{422}$Trp seem to be important for speeding up the access of NMS to and its egress from the orthosteric binding pocket of the M$_2$ receptor.

In the case of W84, as mentioned above, substitution of M$_2^{422}$Trp by alanine led to a pronounced loss of the modulator´s allosteric potency. In contrast to this, replacement of M$_2^{427}$Trp by alanine did not change the potency of the modulator significantly, compared with the wild type receptor (Fig. 6, upper panel; Table 4A, left panel). Accordingly, the double mutant M$_2^{422}$W→A$^+$427W→A displayed the same reduction of sensitivity for W84 as the single mutant M$_2^{422}$W→A. Hence, only M$_2^{422}$Trp conveys allosteric potency to W84, while M$_2^{427}$Trp does not seem to be involved in the binding of the modulator (although it does alter the cooperativity between W84 and NMS quite strikingly, as discussed below).

**M$_2^{422}$Trp plays a crucial role for other allosteric modulators at the NMS-occupied M$_2$ receptor.**

We also explored the role of both conserved tryptophans in M$_2$ receptors for the other allosteric agents (Fig. 1). We chose two additional representatives of alkane-bisammonio type agents, i.e. dimethyl-W84, which has been developed as a radioalloster (Tränkle et al., 1998), and naphmethonium, which is an enhancer of NMS binding (Muth et al., 2003). Furthermore, we included gallamine, which is an
archetypal allosteric agent (Clark and Mitchelson, 1976; Stockton et al., 1983), and
diallylcaracurine V (Zlotos et al., 2000). Allosteric potencies of the modulators,
expressed as $\text{pEC}_{0.5,\text{diss}}$, are compiled in Table 4A (left panel). The alkane-
bisammonio type agents (dimethyl-W84 and naphmethonium) and gallamine were
quite similar to W84 in terms of their patterns of epitope dependency at $M_2$. That is,
replacement of $M_2^{422}\text{Trp}$ by alanine was always deleterious for the binding of the
modulators, whereas mutation of $M_2^{427}\text{Trp}$ had no effect. However, in contrast with
W84, there was actually an increase in potency toward diallylcaracurine V when
either $M_2^{422}\text{Trp}$ or both tryptophans were substituted by alanine (Table 4a, left panel).
This gain in potency was mainly caused by the replacement of $M_2^{422}\text{Trp}$. It is
intriguing that diallylcaracurine V appears to be exceptional in two aspects: firstly, this
agent depends directly on $M_2^{423}\text{Thr}$ and, secondly, this agent is negatively influenced
by $M_2^{422}\text{Trp}$.

Taken together, the amino acid $^{422}\text{Trp}$ is critical, either in a positive or in a negative
fashion, for allosteric agent binding at the NMS-occupied $M_2$ receptor; depending on
the agent it may provide affinity or impair binding.

Also in NMS-free $M_2$ receptors $^{422}\text{Trp}$ is often critical for allosteric agent
binding.

The results presented above refer to the formation of ternary complexes, i.e. the
ability of allosteric test compounds to interact with NMS-occupied receptors. In order
to gain insight into the role of the two conserved tryptophans for allosteric agent
binding at NMS-free receptors, we carried out equilibrium binding experiments with
$[^3\text{H}]\text{NMS}$. These experiments also reveal the type of cooperativity between the
allosteric agents and the orthosteric probe $[^3\text{H}]\text{NMS}$. $pK_D$-values, indicating the
binding affinity of [3H]NMS at the diverse wild type and mutant receptors in the absence of allosteric modulator, are compiled in Table 1. Binding of [3H]NMS was hardly affected by mutations in M2 or by mutations in M5. The wild type pK_D was maximally changed by a factor of three; this effect being found in receptors that contained the M2\(^{422}W\rightarrow A\) mutant. Fig. 6 shows the effect of W84 on the equilibrium binding of [3H]NMS at M2 and at the tryptophan M2 receptor mutants (lower panel).

Affinity values (pK_A) for the allosteric agents at the unoccupied receptors and measures of cooperativity with [3H]NMS (p\(\alpha\)) were derived from curve fitting based on the ternary complex model of allosteric interactions as outlined in Materials and Methods and are displayed in Table 4A (right panel). W84 revealed a negative cooperativity with [3H]NMS at M2 and in higher concentrations at the M2\(^{422}W\rightarrow A\) mutant, too (Fig. 6, lower panel). Thus, replacement of M2\(^{427}Trp\) by alanine led to a pronounced reduction of the binding affinity of W84 for the NMS-free receptors (Table 4A, right panel). When M2\(^{427}Trp\) was replaced by alanine, [3H]NMS equilibrium binding remained unaffected by W84 at concentrations which fully inhibited the dissociation kinetics of [3H]NMS binding (Fig. 6, upper panel). Thus, the lack of effect of W84 on [3H]NMS equilibrium binding reflects neutral cooperativity between these ligands. The change from negative (M2 wild type) to neutral cooperativity at the M2\(^{427}W\rightarrow A\) mutant is accompanied by a small loss of affinity of W84 to the NMS-free receptor mutant relative to the NMS-free wild type receptor (Table 4A, pK_A-values, right panel).

The affinity of naphmethonium, an enhancer of the binding of [3H]NMS at M2 (curves not shown), was considerably reduced at the M2\(^{422}W\rightarrow A\) mutant, whereas the mutant M2\(^{427}W\rightarrow A\) showed the same sensitivity for naphmethonium as the NMS-free
wild type M2 receptor (Table 4A, right panel). Naphmethonium continued to be positively cooperative with NMS at both singly and doubly mutated receptors.

Gallamine revealed a significantly reduced binding affinity both at the NMS-free M2\(^{422W\rightarrow A}\) mutant and at the M2\(^{427W\rightarrow A}\) mutant relative to M2, albeit the effects were not additive (Table 4A, right panel). The underlying \(^{[3H]}\)NMS inhibition curves (not shown) were rather steep (curve slopes \(n_H = -2.46 \pm 0.38\) and \(n_H = -2.33 \pm 0.30\), respectively; means ± S.E., \(n = 4-5\)). \(\alpha\)-values indicate a strong negative cooperativity between gallamine and \(^{[3H]}\)NMS at any M2 receptors tested in this study. Currently, we have no explanation why the sum of \(\alpha\) and \(pK_A\) often was considerably different from \(pEC_{0.5,diss}\); further investigation is needed to understand this phenomenon. In the case of diallylcaracurine V, there was no significant change of binding affinity at the NMS-free receptor mutants relative to the M2 wild type receptor.

Taken together, the role of the tryptophans at NMS-free M2 receptors is more complex than at the NMS-liganded M2 receptor, but in general M2\(^{422}\)Trp is more important for allosteric agent binding than M2\(^{427}\)Trp.

In M5 receptors the tryptophan corresponding to M2\(^{422}\)Trp plays also a key role for allosteric agent action.

As M2\(^{422}\)Trp and M2\(^{427}\)Trp are conserved among all five muscarinic receptor subtypes, we aimed to find out in how far the corresponding tryptophans M5\(^{477}\)Trp and M5\(^{482}\)Trp are also involved in the action of allosteric agents at this subtype. Therefore, we replaced these tryptophans by alanine. \(^{[3H]}\)NMS binding parameters under control conditions are given in Table 1. Similar to the results obtained with M2 receptors, the binding affinity toward NMS was hardly changed, only with the double
mutant $M_5^{477}W\rightarrow A + 482W\rightarrow A$ a small loss of affinity by a factor of 2 was seen. However, unlike the case with $M_2$, the kinetics of NMS dissociation remained almost unchanged; only in $M_2^{482W\rightarrow A}$, $k_1$ was increased by a factor of 1.4 relative to $M_5$ wild type. Remarkably, the double mutation, which slowed dissociation of NMS eight-fold in $M_2$, had no effect at all in $M_5$. With regard to allosteric agent binding, we firstly investigated the influence of both residues at NMS-occupied receptors. As mentioned above, replacement of $M_5^{477}Trp$ by alanine resulted in a pronounced reduction of potency of W84 (Fig. 7, upper panel). Furthermore, we found a pronounced loss of allosteric efficacy of W84, i.e. dissociation of the orthosteric ligand could hardly be inhibited, even in the presence of highest concentrations of the allosteric modulator (lower plateau at 87%; maximum effects found in this study are compiled in Supplemental Table 1 online). This suggests that the egress of NMS from the ternary complex is considerably facilitated when the aromatic residue of $M_5^{477}Trp$ is absent. Essentially the same findings were made with the double mutant, whereas the $M_5^{482W\rightarrow A}$ mutant had the same sensitivity for W84 as the $M_5$ wild type receptor.

The allosteric actions of dimethyl-W84 and naphmethonium on $[^3H]NMS$ dissociation were likewise affected by mutation of the two tryptophans (Table 4B, left panel). However, at the $M_5^{477W\rightarrow A}$ mutant both modulators revealed a more pronounced efficacy than W84 to inhibit NMS dissociation with maximum reduction of the apparent $k_1$-value to a level of 73% (dimethyl-W84) and 57% (naphmethonium) (curves not shown). Notably, gallamine had no visible effect up to a concentration of 3 mM on the NMS dissociation at this mutant. Furthermore, the $M_5^{482W\rightarrow A}$ mutant exhibited significantly lower sensitivity for gallamine than the wild type receptor. Diallylcaracurine V showed no change of potency to retard $[^3H]NMS$ dissociation at the diverse $M_5$ receptor mutants.
In equilibrium binding experiments, W84 had no effect on the binding of [\(^3\)H]NMS at M\(_5\) wild type and at the M\(_5^{482}W\rightarrow A\) mutant; i.e., the cooperativity was neutral (Fig. 7, lower panel). In contrast, replacement of M\(_5^{477}\)Trp by alanine resulted in a pronounced inhibition of NMS equilibrium binding by W84, i.e. the cooperativity was negative. The pK\(_A\)-values shown in Table 4B reveal that the NMS-free mutant receptor M\(_5^{477}W\rightarrow A\) and the double mutant, but not M\(_5^{482}W\rightarrow A\) displayed significantly lower sensitivity for W84 than M\(_5\) wild type, but this loss of sensitivity was less pronounced than at the NMS-occupied receptor. Accordingly, W84 exhibited strong negative cooperativity at these mutants (Table 4B, p\(\alpha\)-values, right panel). In the case of gallamine, only substitution of 477Trp affected the agent’s binding affinity to the NMS-free M\(_5\) receptors (Table 4B, right panel). The effect of gallamine on [\(^3\)H]NMS equilibrium binding, i.e. the extent of negative cooperativity, was almost left unaffected by the diverse mutations. Diallylcaracurine V again hardly revealed any affinity-shift at the mutants compared with M\(_5\) wild type (Table 4B, right panel), but a change toward weaker negative cooperativity was noted when M\(_5^{477}\)Trp was substituted.

Taken together, in the NMS-bound as well as in the NMS-free M\(_5\) receptor 477Trp is important for the receptor’s sensitivity to allosteric agents.

The high potency interaction between W84 and M\(_2\) is mediated to a great extent by M\(_2^{177}\)Tyr and M\(_2^{422}\)Trp.

Preceding studies (Buller et al., 2002; Voigtländer et al., 2003; Huang et al., 2005) suggested the subtype selective amino acid M\(_2^{177}\)Tyr to interact directly via \(\pi-\pi\) interaction with W84. Here, we identified the conserved M\(_2^{422}\)Trp as a second amino acid that contributes to a major extent to the allosteric potency of W84. In order to
check whether the contribution of both amino acids for the action of W84 is additive, we generated the double mutant M2\textsuperscript{177Y→Q,422W→A} and measured the effect of W84 on [\textsuperscript{3}H]NMS-liganded receptors (Fig. 8). As mentioned above, both single mutants M2\textsuperscript{177Y→Q} (Voigtländer et al., 2003; pEC\textsubscript{0.5,diss}: 6.20 ± 0.05) and M2\textsuperscript{422W→A} showed clearly reduced sensitivities for W84, compared with M2 wild type. Combined replacement of both residues led to a further decrease of the potency of W84 (pEC\textsubscript{0.5,diss}: 4.97 ± 0.04). The effects of the single mutants were almost additive. Hence, about 2.5 log units of allosteric potency of W84 at M2 can be attributed to only two amino acids, i.e. M2\textsuperscript{177Tyr} and M2\textsuperscript{422Trp}.

**Molecular modeling of the W84 docking to the M2 receptor.**

To gain more insight into the topography of allosteric agent binding to the M2 receptor, recently a three-dimensional model of the receptor protein was built (Jöhren and Höltje, 2002; Voigtländer et al., 2003). The availability of an actual and revised bovine rhodopsin X-ray structure showing higher resolution (Okada et al., 2004) as well as progress in computational techniques made it necessary to generate a new and improved M2 receptor model. In contrast to the preceding model, the receptor is now embedded in a phospholipid bilayer composed of dipalmitoylphosphatidylcholine with aqueous phases containing sodium ions and chloride ions as counterions extracellularly as well as intracellularly (Fig. 9). The new model was not only in good agreement with previous results but in addition seemed to be more suitable to explain experimental data reported before (Voigtländer et al., 2003). Due to the fact that the template of this model, the X-ray structure of bovine rhodopsin, appears in its inactive state, the resulting geometry of the homology model should also be in this state and should thus be appropriate to simulate an antagonist- (NMS-) liganded receptor. As
described before (Voigtländer et al., 2003; Tränkle et al., 2005) the orthosteric binding site is located between the transmembrane helices TM3-TM6 and is connected through a narrow channel with the allosteric binding site which is formed by the extracellular loops.

To model the ternary complex characteristic for allosteric interactions, NMS was placed in the orthosteric site. To obtain a realistic low energy conformation for the highly flexible W84 molecular dynamics simulations in an aqueous environment were carried out as reported before (Voigtländer et al., 2003). The conformation resulting from the dynamical treatment of W84 was docked to the NMS-occupied receptor. The system showed a stable geometry during the molecular dynamics simulation. The representative structure from the molecular dynamics simulation was of good quality. The Ramachandran-plot, calculated by PROCHECK, showed no disallowed residues near the orthosteric or allosteric binding site. The position of the orthosteric ligand NMS is consistent with the interaction fields detected by the different probes implemented in GRID. W84 fills the cavity (white transparent silhouette in Fig. 10A) between the extracellular loops to a great extent. Remarkably, the phthalimide group diving down into the ligand binding cavity of the receptor (Fig. 9) is positioned between Tyr^{177} and Trp^{422} (Fig. 10A) almost in a sandwich like manner. This position would allow the formation of π-π interactions between the aromatic residues of the two amino acids and the phthalimide moiety of W84. Hence, an interaction of W84 with both residues at the same time is possible. The narrow corridor between the orthosteric site and the allosteric site is closed and NMS (partially visible at the bottom of Fig. 10A) cannot dissociate from the M_{2} receptor.

In some instances, even maximally effective concentrations of an allosteric agent did not induce complete inhibition of [[H]NMS dissociation (bottom k_{1} significantly higher
than $k_1 = 0\%$). This phenomenon is known for some allosteric agents such as obidoxime and hexamethonium even at wild type receptors (e.g. Tränkle et al., 1997; Tränkle et al., 2005). We used the double mutant $M_2^{422W\rightarrow A+423T\rightarrow H}$, in which W84 reduced the probability of $[^3H]NMS$-dissociation at maximum only 4-fold (bottom $k_1 = 24.7\%$, cf. Supplemental Table 1 online), to find out whether the model gives a clue as to why $[^3H]NMS$ dissociation is not completely prevented. A molecular dynamics simulation revealed a higher flexibility of the outer loops, especially o3, and a movement of W84 away from the critical cluster of amino acids. The receptor did not stabilize during the relatively long 6000 ps-simulation. A free volume (in the sense of a channel allowing escape of NMS from the W84 occupied receptor) initially was formed, but did not persist permanently during the simulation.

Fig. 10A shows that $M_2^{423Thr}$ lines W84 lying in the allosteric cavity. In order to gain more insight into the postulated detrimental effect of the mutation $M_2^{423T\rightarrow H}$ on W84 binding (see above), $M_2^{423Thr}$ was replaced in the model by histidine. As shown in Fig. 10B, the histidine residue protrudes into the allosteric binding cavity yielding a reduction of space. Thus, $M_5^{478His}$ appears to decrease the binding affinity of W84 sterically. Furthermore, the slowing of the $[^3H]NMS$ binding kinetics by histidine (see above) could be nicely explained by its protrusion into the passage used by NMS to reach and leave the orthosteric site. In conclusion, the results of the docking and molecular dynamics simulation carried out with the current three-dimensional model correspond very well with the experimental findings from the binding assays in the wild type $M_2$ and the mutant $M_2$ receptors.
Discussion

Previous studies making use of the pronounced M2/M5 receptor subtype selectivity of muscarinic allosteric modulators have identified a domain near the entrance of the orthosteric ligand binding cavity that is essential for the high affinity binding of various allosteric modulators to muscarinic M2 relative to M5 receptors (Ellis and Seidenberg, 2000; Buller et al., 2002; Jöhren and Höltje, 2002; Voigtländer et al., 2003; Huang et al., 2005; Tränkle et al., 2005). This domain is lined by the second outer loop (o2) containing M2\textsuperscript{177}Tyr and parts of the third outer loop at the beginning of the seventh transmembrane region (o3/TM7) containing M2\textsuperscript{423}Thr (Fig. 2) (Ellis and Seidenberg, 2000; Jöhren and Höltje, 2002). The M2/M5 mutagenesis approach, however, cannot give direct insight into the contribution of conserved amino acids to allosteric agent binding that provide subtype independent “baseline” affinity for allosteric agents. Previously, lipophilic pockets lying apart from the abovementioned essential domain and containing highly conserved residues were speculated to provide such baseline affinity (Voigtländer et al., 2003). In the present study, however, we have identified a conserved amino acid, 422Trp in M2 and 477Trp in M5, respectively, that plays a key role for the binding of allosteric agents and that is, instead of being located outside the essential domain, a key component in a pivotal cluster of amino acids additionally to M2\textsuperscript{177}Tyr and M2\textsuperscript{423}Thr. Furthermore, we provide evidence that the adjacent M2\textsuperscript{423}Thr may sometimes be beneficial for allosteric agent affinity not because of a positive effect of its own but by lack of a negative spatial influence as exerted by the corresponding histidine of M5.

The molecular dynamics simulations of the docking of W84 to the allosteric site of M2 receptors, whose orthosteric site is blocked by an antagonist, indicate that one of its
lateral phthalimido groups is enclosed by the aromatic residues of M₂¹⁷⁷Tyr and M₂⁴²²Trp in a sandwich like manner (Fig. 10A). Forming π-π interactions these aromatic amino acids, like a pair of tongs, fix the aromatic phthalimido group between them. According to this model, the phthalimide moiety of W84 achieves both baseline affinity by ⁴²²Trp and M₂/M₅ subtype selectivity by ¹⁷⁷Tyr at the same time. The contributions of these amino acids to W84 affinity were nearly additive. In this context it may be mentioned that the substitution of M₂¹⁷⁷Tyr either by glutamine of M₅ or by alanine (Huang et al., 2005) yielded almost the same loss of W84 binding affinity. The additivity of the contributions of M₂¹⁷⁷Tyr and M₂⁴²²Trp to W84 binding affinity suggests that both amino acids interact independent of each other with the allosteric agent. Substitution of M₂⁴²²Trp by alanine reduced binding affinity of the alkane-bisammonio compounds by about 1.5 log units and of gallamine by about one log unit. This suggests that the contribution to affinity of this residue depends on the type of allosteric agent. In the case of diallylcaracurine V, M₂⁴²²Trp is even detrimental for this agent’s affinity. In any case, these findings support the concept of a central role of M₂⁴²²Trp for allosteric agent binding. Also in the NMS-occupied M₅ receptor, the corresponding tryptophan (M₅⁴⁷⁷Trp) is important for the binding of all applied alkane-bisammonio compounds. In case of gallamine, the lack of effect on [³H]NMS dissociation indicates either a pronounced loss of gallamine’s affinity or a complete loss of gallamine’s efficacy to inhibit [³H]NMS dissociation. In any case, this tryptophan is essential for gallamine’s action at M₅. With diallylcaracurine V, however, M₅⁴⁷⁷Trp did not affect affinity in contrast to the corresponding M₂⁴²²Trp.

Most of the allosteric modulators tested in this study displayed a pronounced sensitivity to mutation of both M₂⁴²²Trp and M₅⁴⁷⁷Trp at the NMS-occupied receptor and, additionally, also at the NMS-free receptor. In the search for the binding location
of gallamine at the M₁ receptor Matsui et al. (1995) mutated more than 20 residues in the extracellular region of the receptor and found that replacement of the corresponding M₁₄₀₀-Trp by alanine reduced gallamine’s affinity by about ten-fold in NMS-occupied and in NMS-free receptors. These results are quite similar to our findings at M₂₄₂₂-Trp and M₅₄₇₇-Trp. The similarity suggests that this conserved tryptophan may contribute to the baseline affinities of many allosteric agents for the muscarinic receptor family (diallylcaracurine V is certainly one exception to this rule).

Closer inspection of our findings reveals that this tryptophan, although being conserved among subtypes, may not only provide subtype independent baseline affinity but may also contribute to subtype selectivity. For example, in the binding of both W84 and gallamine to NMS-free receptors, substitution of M₅₄₇₇-Trp by alanine resulted in a lesser reduction of affinity than did replacement of M₂₄₂₂-Trp by alanine. Accordingly, this tryptophan appears to contribute to the M₂ preference of some allosteric agents. On the contrary, the subtype selectivity of diallylcaracurine V at the NMS-occupied receptor is reduced due to the detrimental effect of M₂₄₂₂-Trp, since this ligand is insensitive to mutation of M₅₄₇₇-Trp. In other words, the M₂/M₅ selectivity of diallylcaracurine V amounts to 2.4 log units in wild type receptors, but to 2.9 log units in the corresponding M₂₄₂₂ / M₅₄₇₇-Trp mutants.

Our findings furthermore reveal that M₂₄₂₂-Trp as well as its counterpart M₅₄₇₇-Trp may regulate cooperativity between allosteric and orthosteric ligands. Substitution of M₅₄₇₇-Trp by alanine resulted in a switch from neutral to strongly negative cooperativity between W84 and NMS (cf. pα-values Table 4). Moreover, in the case of diallylcaracurine V mutation of M₂₄₂₂-Trp and the corresponding M₅₄₇₇-Trp yielded
enhanced positive cooperativity at M₂ and tempered negative cooperativity at M₅, respectively.

In contrast to M₂₄²²Trp and its counterpart M₅₄⁷⁷Trp, the downstream conserved tryptophans M₂₄²⁷Trp and M₅₄⁸²Trp generally have no relevance for the binding and action of allosteric agents. The only exceptions were encountered with the M₂₄²⁷Trp→Ala mutant that clearly reduced the affinity of gallamine for the free receptor and that switched cooperativity between W84 and NMS from negative to neutral.

Taken together, the conserved tryptophan in position 422 of M₂ and 477 of M₅ located at the junction of o3 and the beginning of TM7 has to be taken into account when the epitope dependency of cooperative interactions is analyzed in muscarinic receptors. Using the approach of site-directed mutagenesis and the construction of hybrid receptors Krejčí and Tuček (2001) showed that the second and the third outer loop of the M₂ receptor protein are important for the cooperative interaction of alcuronium and gallamine with [³H]NMS. Voigtländer et al. (2003) found a large influence of M₂¹⁷⁷Tyr and M₂⁴²³Thr on the cooperativity between NMS and diallylcaracurine V and a smaller effect in the case of dimethyl-W84. Jakubík et al. (2005) recently reported for strychnine-like modulators an important role of the M₂ sequence of the third outer loop and especially of three amino acids (M₂⁴¹Asp, M₂⁴²¹Val and M₂⁴²³Thr) that are adjacent to M₂₄²²Trp. We now provide evidence for an involvement of this tryptophan and its counterpart M₅₄⁷⁷Trp in the allosteric actions of several muscarinic allosteric modulators.
It is remarkable that the conserved tryptophan at position 422 of M₂ is also involved in the binding of orthosteric ligands. We found that substitution of the tryptophan by alanine reduced the affinity of NMS for M₂ receptors about three-fold and slowed NMS dissociation about two-fold. In M₅, however, the corresponding mutation did not reduce NMS affinity and hardly affected NMS binding kinetics. For M₁, Matsui et al. (1995) showed that replacement of this tryptophan by alanine reduced [³H]NMS dissociation by a factor of two, hardly affected NMS binding affinity, but reduced acetylcholine binding affinity by a factor of ten. The new M₂ model suggests that M₂⁴²²Trp and M₂⁴²³Thr are situated at the bottom of the allosteric binding cavity and concomitantly at the top of the orthosteric binding pocket. According to the M₂ receptor model both conserved tryptophans, M₂⁴²²Trp and in particular M₂⁴²⁷Trp, may frame the pathway leading from the external surface of the receptor protein with its allosteric binding cleft to the orthosteric binding pocket and thereby facilitate the passage of orthosteric agents. Since mutation of the corresponding tryptophan in M₅ did not change NMS binding affinity and kinetics, the M₅ receptor protein appears to possess a quite different overall conformation of this region compared to M₂.

In conclusion, the present study demonstrates that the conserved tryptophan at position 422 in M₂ is important for receptor interactions of structurally diverse allosteric agents in M₂ and M₅ receptors. Depending on the allosteric agent and the receptor, this epitope is important for baseline affinity, subtype selectivity and cooperativity. Together with spatially closely related epitopes of o₂ and o₃/TM7 such as the M₂/M₅ selectivity providing epitopes M₂¹⁷⁷Tyr and M₂⁴²³Thr, this tryptophan forms a pivotal docking point complex for allosteric agents.
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References


Footnotes

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Figure legends

**Fig. 1:** Structures of the applied allosteric agents.

**Fig. 2:** Schematic presentation of the second and third outer loop (o2, o3) of the M₂ receptor along with the flanking α-helical regions. The pictograph in between shows the respective positions of the loops in the M₂ receptor protein. The adjacent α-helical domains were set according to the M₂ receptor model and referred to as transmembrane domains (TM). Amino acids mutated in this study are highlighted.

**Fig. 3:** Role of M₂⁴²²Trp in the absence and presence of M₂⁴²³Thr for the interaction of W84 with the NMS-occupied M₂ receptor. *Ordinate*, apparent rate constant of [³H]NMS dissociation, expressed as percentage of the control value. *Abscissa*, log concentration of W84. Indicated are mean values ± S.E. of three to four separate experiments. The curve without data points is taken from Voigtländer et al., 2003.

**Fig. 4:** Role of M₂⁴²³Thr and the corresponding M₅⁴⁷⁸His for the interaction of W84 with NMS-liganded M₂ and M₅ receptors. For sake of comparison effects of substitution of M₂⁴²³Thr and M₅⁴⁷⁸His by the corresponding amino acids of M₅ or M₂, respectively are also shown. Indicated are mean values ± S.E. of three to four separate experiments. Curves without data points are taken from Voigtländer et al., 2003.

**Fig. 5:** Role of M₂⁴²³Thr and the corresponding M₅⁴⁷⁸His for the interaction of diallylcaracurine V with NMS-liganded M₂ and M₅ receptors. For sake of comparison effects of substitution of M₂⁴²³Thr and M₅⁴⁷⁸His by the corresponding amino acids of
M$_5$ or M$_2$, respectively, are also shown. Indicated are mean values ± S.E. of three to four separate experiments. Curves without data points are taken from Voigtländer et al., 2003.

**Fig. 6:** Allosteric effects of W84 at the indicated wild type and point mutated M$_2$ receptors. **Upper panel:** Concentration-effect curves for the allosteric delay of [$^3$H]NMS dissociation. **Lower panel:** Effects on the equilibrium binding of [$^3$H]NMS. **Ordinate,** specific [$^3$H]NMS binding in percent of the control value. **Abscissa,** log concentration of W84. Experiments were conducted and analyzed as described in Methods. Indicated are means ± S.E. of three to five separate experiments. Data points for lower concentrations at M$_2$ wild type and M$_2^{427}W\rightarrow A$ were all on the control level and are not shown for the sake of identical concentration ranges in both panels.

**Fig. 7:** Allosteric effects of W84 at the indicated wild type and mutant M$_5$ receptors. **Upper panel:** Concentration-effect curves for the allosteric delay of [$^3$H]NMS dissociation. **Lower panel:** Effects on the equilibrium binding of [$^3$H]NMS. Indicated are means ± S.E. of three to five separate experiments. Data points for lower concentrations at M$_5^{482}W\rightarrow A$ and M$_5^{477}W\rightarrow A+^{483}W\rightarrow A$ were all on the control level and are not shown for the sake of identical concentration ranges in both panels.

**Fig. 8:** Additive contribution of M$_2^{177}$Tyr and the conserved M$_2^{422}$Trp to the allosteric potency of W84. Indicated are means ± S.E. of three to five independent experiments. The curve without data points is taken from Voigtländer et al., 2003.
**Fig. 9:** Representative structure from the molecular dynamics simulation. Side view of the M_2 receptor in its membrane environment. The protein is rendered by secondary structure: helices red, turns dark blue, random coil green. The orthosteric ligand NMS and the allosteric agent W84 (above NMS) are shown as orange solid surfaces. Color code of the phospholipid bilayer: carbon yellow, oxygen red, phosphorus magenta. Color code of the aqueous phase: water blue, sodium magenta, chloride green.

**Fig. 10:** (A) M_2 wild type: Interaction between W84 and the adjacent amino acids M_2^{177}Tyr, M_2^{422}Trp and M_2^{423}Thr; view from the top of the receptor protein occupied by W84 and NMS (partially visible at the bottom of the figure). Color codes: Protein helices red, o1, o3 and N terminus grey, o2 cyan, disulfide bridge magenta; ^{177}Tyr / ^{422}Trp / ^{423}Thr carbon green, nitrogen dark blue, oxygen red, hydrogen white. Volumes of the binding sites: white, transparent silhouette; W84 / NMS color code: carbon orange, nitrogen dark blue, oxygen red, hydrogen white. Note the sandwich like arrangement of M_2^{177}Tyr, the phthalimide residue of W84 and M_2^{422}Trp. (B) M_2^{423}T→H mutant: ^{177}Tyr / ^{422}Trp: color code as above; ^{423}His: all atoms yellow. Volumes of the binding sites: white, transparent silhouette. Note the unfavorable spatial orientation of histidine in M_2^{423}T→H.
Table 1: [[^3]^H]NMS binding parameters in wild type and mutant M2 and M5 receptors. Dissociation $t_{1/2}$: half time of dissociation of [[^3]^H]NMS in the absence of allosteric modulator. $k_1$: rate constant of [[^3]^H]NMS dissociation. pK$_D$: minus log value of the equilibrium dissociation constant of [[^3]^H]NMS binding. Data represent mean values ± S.E. of three or more independent experiments or mean values of two independent experiments along with the single values, respectively.

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<th>Dissociation $t_{1/2}$ (min)</th>
<th>$k_1$ (min$^{-1}$)</th>
<th>pK$_D$ ([[^3]^H]NMS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2 wild type</td>
<td>6.8 ± 0.2</td>
<td>0.1031 ± 0.0027</td>
<td>10.13 ± 0.06</td>
</tr>
<tr>
<td>M$_2^{422}W→A$</td>
<td>13.0 ± 0.4</td>
<td>0.0504 ± 0.0025</td>
<td>9.61 ± 0.07</td>
</tr>
<tr>
<td>M$_2^{423}T→A$</td>
<td>8.5 ± 0.3</td>
<td>0.0823 ± 0.0029</td>
<td>(9.89)</td>
</tr>
<tr>
<td>M$_2^{423}T→H$</td>
<td>22.9 ± 0.9</td>
<td>0.0316 ± 0.0012</td>
<td>9.90 ± 0.08</td>
</tr>
<tr>
<td>M$_2^{427}W→A$</td>
<td>35.9 ± 1.3</td>
<td>0.0192 ± 0.0001</td>
<td>9.88 ± 0.16</td>
</tr>
<tr>
<td>M$_2^{177}Y→Q + 422W→A$</td>
<td>5.6 ± 0.1</td>
<td>0.1253 ± 0.0018</td>
<td>9.62 (9.56; 9.67)</td>
</tr>
<tr>
<td>M$_2^{422}W→A + 423T→H$</td>
<td>45.0 ± 1.1</td>
<td>0.0155 ± 0.0004</td>
<td>9.82 (9.76; 9.87)</td>
</tr>
<tr>
<td>M$_2^{422}W→A + 427W→A$</td>
<td>56.4 ± 1.5</td>
<td>0.0126 ± 0.0003</td>
<td>9.86 ± 0.06</td>
</tr>
<tr>
<td>M5 wild type</td>
<td>121.7 ± 3.4</td>
<td>0.0058 ± 0.0002</td>
<td>9.91 ± 0.08</td>
</tr>
<tr>
<td>M$_5^{477}W→A$</td>
<td>110.9 ± 4.8</td>
<td>0.0061 ± 0.0002</td>
<td>9.97 (9.98; 9.96)</td>
</tr>
<tr>
<td>M$_5^{478}H→A$</td>
<td>63.8 ± 0.6</td>
<td>0.0109 ± 0.0001</td>
<td>9.77 (9.80; 9.74)</td>
</tr>
<tr>
<td>M$_5^{478}H→T$</td>
<td>41.7 ± 1.6</td>
<td>0.0167 ± 0.0007</td>
<td>9.89 (9.92; 9.86)</td>
</tr>
<tr>
<td>M$_5^{482}W→A$</td>
<td>83.6 ± 2.2</td>
<td>0.0084 ± 0.0003</td>
<td>9.92 ± 0.08</td>
</tr>
<tr>
<td>M$_5^{477}W→A + 478H→T$</td>
<td>50.8 ± 2.6</td>
<td>0.0142 ± 0.0008</td>
<td>9.91 (9.93; 9.89)</td>
</tr>
<tr>
<td>M$_5^{477}W→A + 482W→A$</td>
<td>117.1 ± 7.7</td>
<td>0.0056 ± 0.0002</td>
<td>9.62 ± 0.06</td>
</tr>
</tbody>
</table>
Table 2: Potencies (pEC_{0.5,diss}) of W84 to retard [³H]NMS dissociation from the indicated wild type and mutant receptors. ΔpEC_{0.5,diss} indicate the difference between the pEC_{0.5,diss} values for the mutant and for the respective wild type receptor. Values are means ± S.E. of three to five separate experiments. #: Data are taken from Voigtländer et al., 2003.

<table>
<thead>
<tr>
<th></th>
<th>pEC_{0.5,diss}</th>
<th>ΔpEC_{0.5,diss} (mutant - wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₂ wild type</td>
<td>7.40 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>M₂^{422}W→A</td>
<td>5.89 ± 0.04</td>
<td>-1.51</td>
</tr>
<tr>
<td>M₂^{423}T→A</td>
<td>7.44 ± 0.04</td>
<td>+0.04</td>
</tr>
<tr>
<td>M₂^{423}T→H</td>
<td>6.61 # ± 0.05</td>
<td>-0.79</td>
</tr>
<tr>
<td>M₂^{422}W→A +^{423}T→H</td>
<td>5.14 ± 0.07</td>
<td>-2.26</td>
</tr>
<tr>
<td>M₅ wild type</td>
<td>5.41 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>M₅^{477}W→A</td>
<td>3.24 ± 0.54</td>
<td>-2.17</td>
</tr>
<tr>
<td>M₅^{478}H→A</td>
<td>6.11 ± 0.02</td>
<td>+0.70</td>
</tr>
<tr>
<td>M₅^{478}H→T</td>
<td>6.18 # ± 0.10</td>
<td>+0.77</td>
</tr>
<tr>
<td>M₅^{477}W→A +^{478}H→T</td>
<td>4.63 ± 0.08</td>
<td>-0.78</td>
</tr>
</tbody>
</table>
Table 3: Potencies ($pE_{C_{0.5,diss}}$) of diallylcaracurine V to retard $[^3H]$NMS dissociation from the indicated wild type and mutant receptors. $\Delta pE_{C_{0.5,diss}}$ indicate the difference between the $pE_{C_{0.5,diss}}$ values for the mutant and for the respective wild type receptor. Values are means ± S.E. of three to five separate experiments. #: Data are taken from Voigtländer et al., 2003.

<table>
<thead>
<tr>
<th></th>
<th>$pE_{C_{0.5,diss}}$</th>
<th>$\Delta pE_{C_{0.5,diss}}$ (mutant - wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_2$ wild type</td>
<td>7.93 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>$M_2^{423T\rightarrow A}$</td>
<td>7.27 ± 0.04</td>
<td>-0.66</td>
</tr>
<tr>
<td>$M_2^{423T\rightarrow H}$</td>
<td>7.25# ± 0.06</td>
<td>-0.68</td>
</tr>
<tr>
<td>$M_5$ wild type</td>
<td>5.57 ± 0.09</td>
<td>-</td>
</tr>
<tr>
<td>$M_5^{478H\rightarrow A}$</td>
<td>6.15 ± 0.05</td>
<td>+0.58</td>
</tr>
<tr>
<td>$M_5^{478H\rightarrow T}$</td>
<td>6.55# ± 0.05</td>
<td>+0.98</td>
</tr>
</tbody>
</table>
Table 4: Interaction of the indicated allosteric agents with wild type M₂ and M₅ receptors (panel A and B, respectively) and related mutants. pEC_{0.5,diss}: minus log concentration reducing [³H]NMS dissociation to half of the control rate; pKₐ: minus log value of the equilibrium dissociation constant for allosteric agent binding to free receptors. Minus log values of the factors of cooperativity (pα) are indicated in brackets in the right hand panel. Indicated are mean values ± S.E. of three to ten separate experiments with duplicated values. n.d., not determined, *: EC_{0.5,diss} was used for curve fitting because of nearly neutral cooperativity as described in Materials and Methods, **: equation with a variable slope factor used for curve fitting, see text. #: No effect up to minus log concentration = 2.5.

<table>
<thead>
<tr>
<th></th>
<th>pEC_{0.5,diss}</th>
<th>pKₐ (pα)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M₂</td>
<td>M₂^{422}W→A</td>
</tr>
<tr>
<td>W84</td>
<td>7.40 ± 0.03</td>
<td>5.89 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-0.50 ± 0.02)</td>
</tr>
<tr>
<td>Dimethyl-W84</td>
<td>8.14 ± 0.02</td>
<td>6.66 ± 0.03</td>
</tr>
<tr>
<td>Naphmethonium</td>
<td>9.04 ± 0.04</td>
<td>7.71 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.75 ± 0.13)</td>
</tr>
<tr>
<td>Gallamine</td>
<td>6.96 ± 0.05</td>
<td>5.93 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&lt;-2)</td>
</tr>
<tr>
<td>Diallylcarcarine V</td>
<td>7.93 ± 0.03</td>
<td>8.52 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.23 ± 0.07)</td>
</tr>
<tr>
<td></td>
<td>pEC&lt;sub&gt;0.5,diss&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td>M&lt;sub&gt;5&lt;/sub&gt;</td>
<td>M&lt;sub&gt;5&lt;/sub&gt;&lt;sup&gt;477&lt;/sup&gt;W→A</td>
</tr>
<tr>
<td>W84</td>
<td>5.41 ± 0.04</td>
<td>3.24 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>(-0.05 ± 0.05)</td>
<td>(-1.56 ± 0.15)</td>
</tr>
<tr>
<td>Dimethyl-W84</td>
<td>5.40 ± 0.04</td>
<td>4.08 ± 0.29</td>
</tr>
<tr>
<td>Naphmethonium</td>
<td>6.22 ± 0.03</td>
<td>4.94 ± 0.17</td>
</tr>
<tr>
<td>Gallamine</td>
<td>5.41 ± 0.11</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>(-1.45 ± 0.11)</td>
<td>(-1.19 ± 0.03)</td>
</tr>
<tr>
<td>Diallylcaracurine V</td>
<td>5.57 ± 0.09</td>
<td>5.61 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(-0.82 ± 0.08)</td>
<td>(-0.46 ± 0.02)</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 4
Fig. 5
Fig. 6

The graphs depict the concentration-dependent inhibition of the binding of $[^3]$H]NMS using various W84 concentrations. The concentration of W84 is plotted on a log scale (W84 (logM)) on the x-axis, and the percentage of $[^3]$H]NMS bound is plotted on the y-axis.

The graphs show how the binding of $[^3]$H]NMS is affected by the presence of W84 at different concentrations. The data points for each concentration are shown as markers, with error bars indicating variability. The graphs illustrate the competitive inhibition of the binding of $[^3]$H]NMS by W84.

- **Upper Graph:** Shows the dissociation rate constant ($k_{-1}$) of $[^3]$H]NMS binding as a function of W84 concentration.
- **Lower Graph:** Shows the specific binding of $[^3]$H]NMS as a function of W84 concentration. Legend entries include:
  - **M2**
  - **M2 $^{422}$W→A**
  - **M2 $^{427}$W→A**
  - **M2 $^{422}$W→A $^{427}$W→A**

The graphs are consistent with a competitive binding model, where the presence of W84 decreases the binding of $[^3]$H]NMS to its receptor.
Fig. 7
Fig. 9