Supplemental Material can be found at:
http://molpharm.aspetjournals.org/content/suppl/2006/04/27/mol.106.023481.DC1.html

0026-895X/06/7001-181–193$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 70:181–193, 2006

Vol. 70, No. 1
23481/3124530
Printed in U.S.A.

MOL PHARM
MOLECULAR PHARMACOLOGY

Allosteric Interactions with Muscarinic Acetylcholine Receptors: Complex Role of the Conserved Tryptophan M_2^{422}Trp in a Critical Cluster of Amino Acids for Baseline Affinity, Subtype Selectivity, and Cooperativity[S]

Stefanie Prilla, Jasmin Schrobang, John Ellis, Hans-Dieter Höltje, and Klaus Mohr

Department of Pharmacology and Toxicology, Institute of Pharmacy, Rheinische Friedrich-Wilhelms-Universität Bonn, Germany (S.P., K.M.); Institute of Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-Universität Düsseldorf, Germany (J.S., H.-D.H.); and Departments of Psychiatry and Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania (J.E.)

Received February 13, 2006; accepted April 26, 2006

ABSTRACT

In general, the M_2 subtype of muscarinic acetylcholine receptors has the highest sensitivity for allosteric modulators and the M_5 subtype the lowest. The M_2/M_5 selectivity of some structurally diverse allosteric agents is known to be completely explained by M_2^{177}Tyr and M_2^{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_2^{422}Trp and the adjacent M_2^{423}Thr in the binding of alkane-bisammonio type modulators, gallamine, and diallylcaracurine V. Experiments were performed with human M_2 or M_5 receptors or mutants thereof. It was found that M_2^{422}Trp and M_2^{423}Thr independently influenced allosteric agent binding. The presence of M_2^{423}Thr may enhance the affinity of binding, depending on the allosteric agent, either directly or indirectly (by avoiding sterical hindrance through its M_5 counterpart ^{478}His). Replacement of M_2^{422}Trp and of the corresponding M_5^{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_2 receptor model revealed that the aromatic rings of M_2^{177}Tyr and M_2^{422}Trp, in a concerted action, might fix one of the aromatic moieties of alkane-bisammonio compounds between them. Thus, M_2^{422}Trp and the spatially adjacent M_2^{177}Tyr, as well as M_2^{423}Thr, form a cluster of amino acids within the allosteric binding cleft that is pivotal for both M_2/M_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

This work was supported by the Deutsche Forschungsgemeinschaft (Mo821/1–4), and by United States Public Health Service grant R01-AG05214 (to J.E.).
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.023481.

ABBREVIATIONS: M_2, M_2 subtype of the muscarinic acetylcholine receptor; M_5, M_5 subtype of the muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine; o2, second outer (extracellular) loop of the receptor; o3, third outer (extracellular) loop of the receptor; PB, sodium-potassium phosphate buffer (5 mM, pH 7.4); W84, hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

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 M_2 subtype of muscarinic receptors generally displays highest affinity for allosteric modulators, whereas the M_5 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 M_2 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 sterical hindrance, or it may indirectly contribute to ligand binding by governing the conformation of amino acid strands that contain a relevant point of attachment. We have found that two amino acids are sufficient to account completely for the 100-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 ^{184}Gln and ^{478}His. 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. 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 M_2/M_5 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.

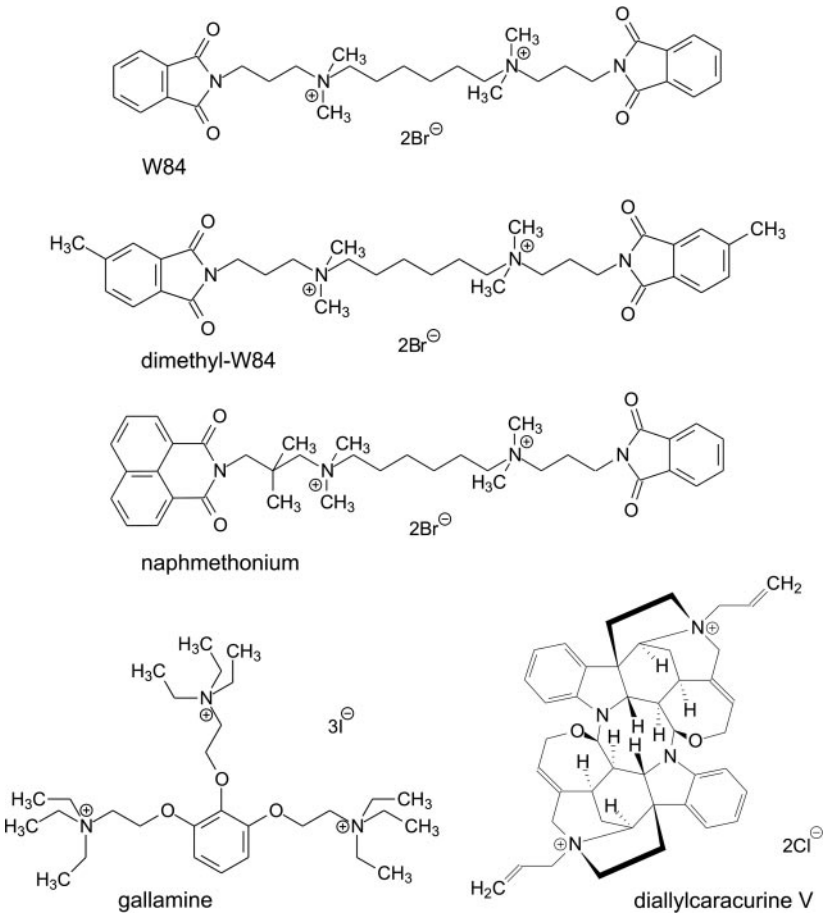

Fig. 1. Structures of the applied allosteric agents.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

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). 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, Würzburg, Germany). The orthosteric radioligand [^{3}H]NMS ([^{3}H]*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 QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). pCD plasmids, including wild-type genes for human M_2 and M_5 muscarinic receptors, served as templates in polymerase chain reaction. Mutations were inserted by addition of synthetic oligonucleotide primers containing the required triplet changes (Sigma Genosys, Steinheim, Germany). Primers were elongated during temperature cycling by a high-fidelity DNA polymerase. After amplification over 12 to 16 cycles, the parental DNA was digested by a methylation-specific endonuclease, thus selecting the mutated plasmids. The polymerase chain reaction products were transformed into supercompetent *Escherichia coli* cells. Bacteria were plated on ampicillin-containing agar and incubated overnight. Clones with the putative mutation were isolated and grown up in 500 ml of Luria-Bertani medium (Sigma-Aldrich). Plasmids were extracted using the Plasmid Maxi kit (QIAGEN, Hilden, Germany) and mutation was confirmed by sequencing. Thereafter, wild-type or mutant plasmid-DNA was transiently transfected into COS-7 cells using PolyFect transfection reagent (QIAGEN).

Cell Culture and Membrane Preparation

COS-7 cells were cultured at 37°C under humidified air supplemented with 5% CO_2 in Dulbecco's modified Eagle's medium containing 1% penicillin-streptomycin and 10% fetal bovine serum. One day before transfection, cells were grown in 10-cm dishes by seeding 1.6×10^6 cells per dish. Forty-eight hours after transfection, cells were harvested and homogenized in 5 mM sodium/potassium phosphate buffer (PB; 4 mM Na_2HPO_4 and 1 mM KH_2PO_4), pH 7.4. After centrifugation, membranes were resuspended in 5 mM PB and stored in aliquots at −80°C.

Dissociation Binding Assays

Two-point [^{3}H]NMS dissociation experiments were performed in 5 mM PB, pH 7.4, at 23°C as described previously (Voigtländer et al., 2003; Huang et al., 2005). Membranes with the respective wild-type or mutant M_2 or M_5 receptor were prelabeled with 1 nM radioligand for 30 min. Measurement of [^{3}H]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 [^{3}H]NMS dissociation half-time of the respective receptor, dissociation was terminated by filtration using a Brandel cell harvester and GMF2 glass microfiber 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. Nonspecific binding of [^{3}H]NMS was determined in the presence of 3 μM atropine.

Equilibrium Binding Assays

[^{3}H]NMS equilibrium binding was measured in the presence of 0.2 nM radioligand and the indicated concentrations of the allosteric agent, as described previously (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 equation 31 in Lazareno and Birdsall (1995).

The affinity of the orthosteric probe [^{3}H]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 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, $pEC_{0.5,diss}$, 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

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

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. α 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; this equation was derived from Lazareno and Birdsall (1995) and 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}H]NMS dissociation approximately 2 h). 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 ($\alpha \approx 1$), [^{3}H]NMS equilibrium binding remains nearly unchanged under the influence of increasing concentrations of the allosteric modulator. Under this condition, curve fitting with eq. 1 does not work. Because 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 $pEC_{0.5,diss}$ is identical to $p(\alpha \cdot K_A)$. Therefore, we replaced K_A in eq. 1 with $EC_{0.5,diss}/\alpha$ (Raasch et al., 2002). $EC_{0.5,diss}$ was derived from preceding dissociation experiments, and curve fitting yielded the cooperativity factor α,

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

which then served to calculate $K_A = EC_{0.5,diss}/\alpha$. 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 Bank accession 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 [codes P08172 (Bonner et al., 1987) and 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 α-carbon distance-matrix as implemented in the Homology module of Insight II 2000 (Accelrys, San Diego, CA). 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 that had been detected as the most favorable one in the course of a dynamic 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 was 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; afterward, 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 counter-ions both extra- and intracellularly. Schlegel et al. (2005) recently described this procedure. 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. Afterward the position restraints were slowly reduced in 10 steps of 100 ps each 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 M_2 receptor and the possible interplay with the spatially adjacent amino acids M_2^{177}Tyr and M_2^{423}Thr (Fig. 2). To probe the influence of these M_2 and the respective M_5 amino acids on the binding of allosteric modulators, these residues were either replaced by the corresponding amino acids of the counterpart receptor subtype (M_2/M_5 or M_5/M_2 substitution) or by alanine as a neutral residue. M_2/M_5 substitution and M_5/M_2 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 M_2 compared with M_5, 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 first 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 [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) as an orthosteric ligand. The observed alteration, generally a delay, of the [^{3}H]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 [^{3}H]NMS. The half-times of [^{3}H]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 M_2 receptors ranged from 5.6 min to 56 min. At M_5, dissociation was much slower than at M_2, 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.

M_2^{422}Trp and the Corresponding M_5^{477}Trp Confer Sensitivity to W84 Independent of Whether the Adjacent Downstream Amino Acid Is Threonine. The previous M_2 receptor model predicted the conserved residue

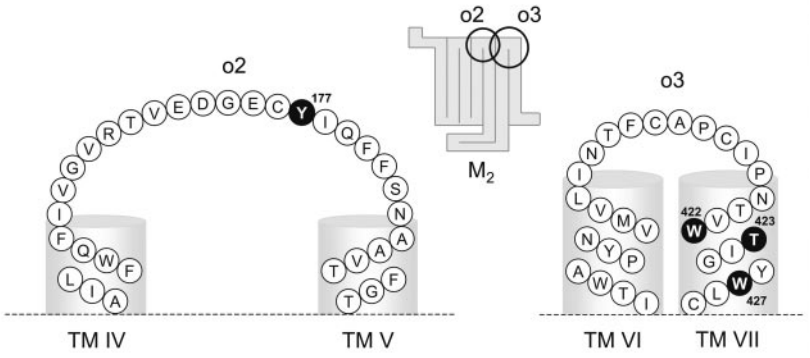

Fig. 2. Schematic presentation of the second and third outer loop (o2, o3) of the M_2 receptor along with the flanking α-helical regions. The pictograph in between shows the respective positions of the loops in the M_2 receptor protein. The adjacent α-helical domains were set according to the M_2 receptor model and referred to as transmembrane domains (TM). Amino acids mutated in this study are highlighted.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet

M_2^{422}Trp to interact directly with W84, whereas the adjacent amino acid M_2^{423}Thr relative to the corresponding M_5^{478}His was suspected to be essential for the proper orientation of the side chain of this tryptophan (Voigtländer et al., 2003). If so, replacement of M_2^{422}Trp by alanine should reduce the potency of W84. Moreover, substitution of M_2^{423}Thr by the corresponding M_5^{478}His, which was suspected to be unfavorable, should diminish the role of M_2^{422}Trp for the binding of W84. In the M_5 receptor, however, mutation of M_5^{477}Trp should have less effect on the binding of W84 if the absence of a neighboring threonine causes an unfavorable conformation of ^{477}Trp in M_5. To check this concept, we generated mutant M_2 and M_5 receptors. The effects of the substitutions in M_2 on the allosteric potency of W84 are illustrated in Fig. 3. As anticipated, mutation of M_2^{422}Trp to alanine led to a pronounced loss of potency of W84, compared with M_2 wild type; the $pEC_{0.5,diss}$-value at the M_2^{422}W→A mutant was reduced by −1.51 log units (Table 2). The double mutant M_2^{422}W→A+^{423}T→H displayed a further decrease of sensitivity for W84, compared with the single mutant M_2^{423}T→H. This finding was unexpected, because the absence of M_2^{423}Thr was thought to induce an unfavorable orientation of the adjacent M_2^{422}Trp and thus to diminish its contribution to the potency of W84. It is noteworthy that this unexpected further reduction of potency (−1.47 log units relative to M_2^{423}T→H) was almost equal to the loss of sensitivity observed at the M_2^{422}W→A mutant relative to M_2 (−1.51 log units). The effects of both single mutations were additive and thus seem to be independent of each other. At M_5 (data compiled in Table 2), substitution of the corresponding tryptophan ^{477}Trp by alanine resulted in an impressive loss of potency of W84 by −2.17 log units. This was also unexpected, because the presence of M_5^{478}His instead of threonine was thought to force M_5^{477}Trp into an unfavorable orientation. As shown previously (Voigtländer et al., 2003), replacement of the neighboring M_5^{478}His by the corresponding threonine of M_2 increases W84's potency by 0.77 log units compared with M_5. Additional mutation of ^{477}Trp in the M_5^{478}H→T mutant reduced the potency of W84 by −0.78 log units relative to M_5; compared with the M_5^{478}H→T mutant, affinity was diminished by −1.55 log units. This value is similar to the affinity shift found in M_5^{477}W→A relative to M_5 wild type (−2.17 log units). Taken together, the findings indicate that the conserved tryptophans M_2^{422}Trp and M_5^{477}Trp, 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, M_2^{423}Thr does not seem to induce a proper orientation of the adjacent ^{422}Trp for W84 binding.

M_5^{478}His Hinders NMS Binding Kinetics. The aforementioned data showed that the loss of potency of W84 observed at the M_2^{423}T→H mutant is not caused by an impaired orientation of the adjacent tryptophan. To further elucidate the role of this residue, 423 in M_2 and 478 in M_5, respectively, we introduced alanine instead of the respective M_2 and M_5 amino acid. The binding parameters of [^{3}H]NMS for wild-type and mutant receptors are given in Table 1. Whereas replacement of M_2^{423}Thr by the corresponding amino acid of M_5, histidine, led to a 3-fold increase of the [^{3}H]NMS dissociation half-time, insertion of alanine instead of threonine had only a minor effect on the [^{3}H]NMS dissociation. Conversely, substitution of the corresponding residue ^{478}His in M_5 by either alanine or threonine reduced the NMS dissociation half-time by approximately 2- to 3-fold. Because none of these mutations had much effect on the binding affinity of [^{3}H]NMS (pK_D values in Table 1), compared with the respective wild-type receptor, it can be concluded that the

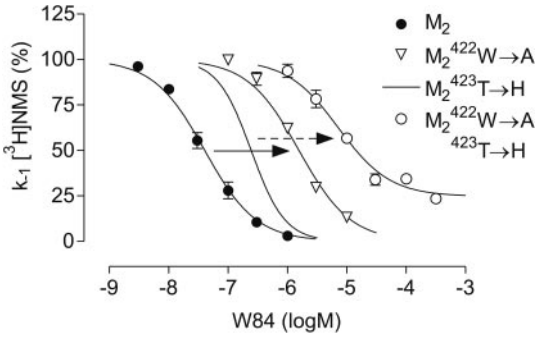

Fig. 3. Role of M_2^{422}Trp in the absence and presence of M_2^{423}Thr for the interaction of W84 with the NMS-occupied M_2 receptor. Ordinate, apparent rate constant of [^{3}H]NMS dissociation, expressed as percentage of the control value. Abscissa, log concentration of W84. Indicated are mean values ± S.E. of three to four separate experiments. The curve without data points is replotted from Voigtländer et al. (2003).

TABLE 1
[^{3}H]NMS binding parameters in wild-type and mutant M_2 and M_5 receptors
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.

	Dissociation $t_{1/2}$	k_{-1}	pK_D ([^{3}H]NMS)
	min	*min*$^{-1}$	
M_2 wild type	6.8 ± 0.2	0.1031 ± 0.0027	10.13 ± 0.06
M_2^{422}W→A	13.0 ± 0.4	0.0504 ± 0.0025	9.61 ± 0.07
M_2^{423}T→A	8.5 ± 0.3	0.0823 ± 0.0029	9.89 (9.88; 9.91)
M_2^{423}T→H	22.9 ± 0.9	0.0316 ± 0.0012	9.90 ± 0.08
M_2^{427}W→A	35.9 ± 1.3	0.0192 ± 0.0001	9.88 ± 0.16
M_2^{177}Y→Q + ^{422}W→A	5.6 ± 0.1	0.1253 ± 0.0018	9.62 (9.56; 9.67)
M_2^{422}W→A + ^{423}T→H	45.0 ± 1.1	0.0155 ± 0.0004	9.82 (9.76; 9.87)
M_2^{422}W→A + ^{427}W→A	56.4 ± 1.5	0.0126 ± 0.0003	9.86 ± 0.06
M_5 wild type	121.7 ± 3.4	0.0058 ± 0.0002	9.91 ± 0.08
M_5^{477}W→A	110.9 ± 4.8	0.0061 ± 0.0002	9.97 (9.98; 9.96)
M_5^{478}H→A	63.8 ± 0.6	0.0109 ± 0.0001	9.77 (9.80; 9.74)
M_5^{478}H→T	41.7 ± 1.6	0.0167 ± 0.0007	9.89 (9.92; 9.86)
M_5^{482}W→A	83.6 ± 2.2	0.0084 ± 0.0003	9.92 ± 0.08
M_5^{477}W→A + ^{478}H→T	50.8 ± 2.6	0.0142 ± 0.0008	9.91 (9.93; 9.89)
M_5^{477}W→A + ^{482}W→A	117.1 ± 7.7	0.0056 ± 0.0002	9.62 ± 0.06

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.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet

association of NMS at these mutants was affected to the same extent as the dissociation. In conclusion, ^{423}Thr is located near the entrance of the orthosteric site of M_2 but is itself rather unimportant for the kinetics of [^{3}H]NMS binding at M_2, whereas histidine at the corresponding position either in M_2 or in M_5 seems to be an obstacle for the passage of NMS to and from the orthosteric binding pocket.

Low Potency Binding of W84 at the M_2^{423}T→H Mutant and at M_5 Wild Type Is Due to a Detrimental Effect of Histidine. Because M_5^{478}His has been shown in the preceding paragraph 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 M_2 instead of ^{423}Thr lowers the potency of W84 (−0.79 log units; Fig. 4, Table 2). The present findings show that replacement of M_2^{423}Thr by alanine does not affect the potency of W84 compared with the wild-type receptor. The corresponding mutations in M_5, M_5^{478}H→A and M_5^{478}H→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 M_2^{423}Thr for the M_2/M_5 selectivity of W84 is not an "active" contribution to W84 binding but "merely" reflects the replacement of an affinity lowering amino acid (i.e., M_5^{478}His).

In the Case of Diallylcaracurine V, M_2^{423}Thr Enhances Potency Directly. In the case of diallylcaracurine V, the former M_2 receptor model proposed a direct interaction between M_2^{423}Thr and the allosteric agent (Voigtländer et al., 2003). In that case, replacement of this M_2 residue by alanine should result in a loss of the allosteric potency. Figure 5 displays the sensitivities of the different receptors for diallylcaracurine V. Replacement of M_2^{423}Thr 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 M_5 mutants M_5^{478}H→A and M_5^{478}H→T exhibited significantly higher sensitivity for diallylcaracurine V than the wild-type receptor. It is noteworthy that diallylcaracurine V benefits to a greater extent from the replacement of M_5^{478}His by threonine (0.98 log units) than by alanine (0.58 log units). These findings support the notion that M_2^{423}Thr has a direct beneficial effect for the interaction of diallylcaracurine V with the allosteric site.

Kinetics of NMS Binding at M_2 Are Accelerated by the Conserved Tryptophan M_2^{427}Trp, Whereas the Binding of W84 Does not Depend on This Residue. In the M_2 receptor, there is a second conserved tryptophan next to M_2^{422}Trp at position 427 that might be involved in the binding of W84. To check this assumption, we introduced alanine instead of M_2^{427}Trp. We also created the double mutant M_2^{422}W→A+^{427}W→A. Table 1 indicates the consequences of these mutations on the binding of [^{3}H]NMS in the absence of an allosteric agent. NMS dissociation was remarkably retarded by the substitution of M_2^{427}Trp by alanine (5-fold), whereas the mutation of M_2^{422}Trp to alanine increased the dissociation half-

TABLE 2

Potencies ($pEC_{0.5,diss}$) of W84 to retard [^{3}H]NMS dissociation from the indicated wild-type and mutant receptors

$\Delta pEC_{0.5,diss}$ 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.

	$pEC_{0.5,diss}$	$\Delta pEC_{0.5,diss}$ (Mutant-Wild Type)
M_2 wild type	7.40 ± 0.03	
M_2^{422}W→A	5.89 ± 0.04	−1.51
M_2^{423}T→A	7.44 ± 0.04	+0.04
M_2^{423}T→H	6.61 ± 0.05[a]	−0.79
M_2^{422}W→A + ^{423}T→H	5.14 ± 0.07	−2.26
M_5 wild type	5.41 ± 0.04	
M_5^{477}W→A	3.24 ± 0.54	−2.17
M_5^{478}H→A	6.11 ± 0.02	+0.70
M_5^{478}H→T	6.18 ± 0.10[a]	+0.77
M_5^{477}W→A + ^{478}H→T	4.63 ± 0.08	−0.78

[a] Data are from Voigtländer et al. (2003).

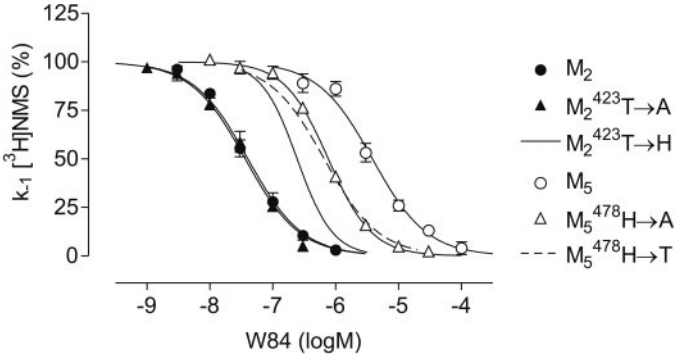

Fig. 4. Role of M_2^{423}Thr and the corresponding M_5^{478}His for the interaction of W84 with NMS-liganded M_2 and M_5 receptors. For sake of comparison, effects of substitution of M_2^{423}Thr and M_5^{478}His by the corresponding amino acids of M_5 or M_2, respectively, are also shown. Indicated are mean values ± S.E. of three to four separate experiments. Curves without data points are replotted from Voigtländer et al. (2003).

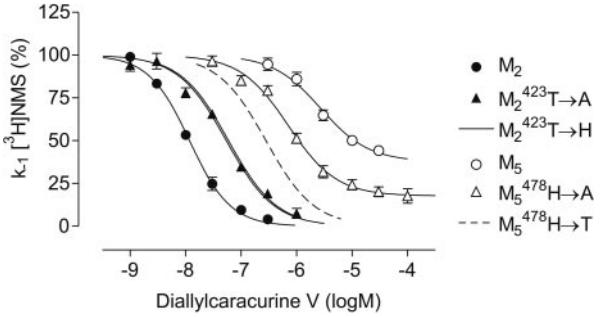

Fig. 5. Role of M_2^{423}Thr and the corresponding M_5^{478}His for the interaction of diallylcaracurine V with NMS-liganded M_2 and M_5 receptors. For sake of comparison effects of substitution of M_2^{423}Thr and M_5^{478}His by the corresponding amino acids of M_5 or M_2, respectively, are also shown. Indicated are mean values ± S.E. of three to four separate experiments. Curves without data points are replotted from Voigtländer et al. (2003).

TABLE 3

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

$\Delta pEC_{0.5,diss}$ indicates 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.

	$pEC_{0.5,diss}$	$\Delta pEC_{0.5,diss}$ (Mutant-Wild Type)
M_2 wild type	7.93 ± 0.03	
M_2^{423}T→A	7.27 ± 0.04	−0.66
M_2^{423}T→H	7.25 ± 0.06[a]	−0.68
M_5 wild type	5.57 ± 0.09	
M_5^{478}H→A	6.15 ± 0.05	+0.58
M_5^{478}H→T	6.55 ± 0.05[a]	+0.98

[a] Data are from Voigtländer et al. (2003).

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

time only slightly (2-fold). Both effects were nearly additive, in that the NMS dissociation half-time was increased by a factor of 8 at the double mutant. The pK_D-value of [^{3}H]NMS was reduced at most by a factor of 3 by the aforementioned mutations (Table 1). Thus we conclude that [^{3}H]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, top; Table 4, left). Therefore, the double mutant M_2^{422}W→A+^{427}W→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, whereas 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 recep-

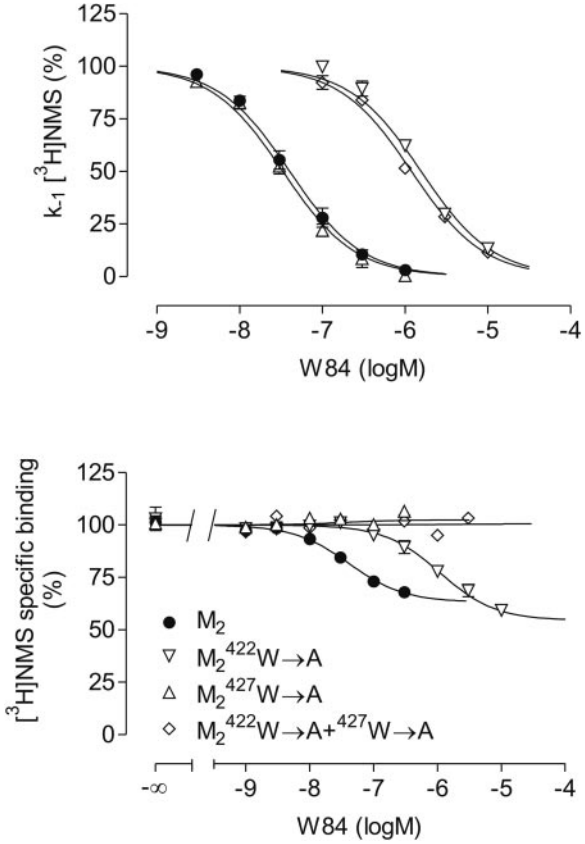

Fig. 6. Allosteric effects of W84 at the indicated wild type and point mutated M_2 receptors. Top, concentration-effect curves for the allosteric delay of [^{3}H]NMS dissociation. Bottom, effects on the equilibrium binding of [^{3}H]NMS. Ordinate, specific [^{3}H]NMS binding as a percentage of the control value. Abscissa, log concentration of W84. Experiments were conducted and analyzed as described under *Materials and 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→A were all on the control level and are not shown for the sake of identical concentration ranges in both panels.

TABLE 4
Interaction of the indicated allosteric agents with wild-type M_2 receptors and related mutants
Minus log values of the factors of cooperativity (pα) are indicated in parentheses on the right of the table. Indicated are mean values ± S.E. of three to ten separate experiments with duplicated values.

	pEC$_{0.5,diss}$				pK_A (pα)			
	M_2	M_2^{422}W→A	M_2^{427}W→A	$M_{2\ 427}^{422}$W→A W→A	M_2	M_2^{422}W→A	M_2^{427}W→A	M_2^{422}W→A + ^{427}W→A
W84	7.40 ± 0.03	5.89 ± 0.04	7.48 ± 0.04	5.92 ± 0.03	7.82 ± 0.02 (−0.50 ± 0.02)	6.27 ± 0.13 (−0.38 ± 0.03)	7.46 ± 0.03[a] (0.02 ± 0.03)	5.92 ± 0.01[a] (0.01 ± 0.01)
Dimethyl-W84	8.14 ± 0.02	6.66 ± 0.03	8.26 ± 0.03	6.65 ± 0.03	N.D.	N.D.	N.D.	N.D.
Naphmethonium	9.04 ± 0.04	7.71 ± 0.05	9.12 ± 0.03	7.65 ± 0.05	8.19 ± 0.11 (0.75 ± 0.13)	6.58 ± 0.11 (0.61 ± 0.14)	8.26 ± 0.18 (0.55 ± 0.11)	6.61 ± 0.06 (0.37 ± 0.08)
Gallamine	6.96 ± 0.05	5.93 ± 0.06	6.86 ± 0.08	5.79 ± 0.06	7.70 ± 0.07 (<-2)	6.04[b] ± 0.07 (−1.01 ± 0.01)	6.39[b] ± 0.09 (−0.80 ± 0.05)	6.05 ± 0.05 (−0.89 ± 0.06)
Diallylcaracurine V	7.93 ± 0.03	8.52 ± 0.04	8.11 ± 0.04	8.74 ± 0.03	7.70 ± 0.07[a] (0.23 ± 0.07)	7.52 ± 0.06 (0.43 ± 0.06)	7.96 ± 0.03[a] (0.15 ± 0.03)	7.54 ± 0.11 (0.73 ± 0.08)

pEC$_{0.5,diss}$, minus log concentration reducing [^{3}H]NMS dissociation to half of the control rate; pK_A, minus log value of the equilibrium dissociation constant for allosteric agent binding to free receptors; N.D., not determined.
[a] EC$_{0.5,diss}$ was used for curve fitting because of nearly neutral cooperativity as described under *Materials and Methods*.
[b] Equation with a variable slope factor used for curve fitting; see text.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

tors 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 $pEC_{0.5,diss}$, are compiled in Table 4, left. The alkane-bisammonio type agents (dimethyl-W84 and naphmethonium) and gallamine were quite similar to W84 in terms of their patterns of epitope dependence at M_2. That is, replacement of M_2^{422}Trp by alanine was always deleterious for the binding of the modulators, whereas mutation of M_2^{427}Trp had no effect. However, in contrast with W84, there was actually an increase in potency toward diallylcaracurine V when either M_2^{422}Trp or both tryptophans were substituted by alanine (Table 4, left). This gain in potency was caused mainly by the replacement of M_2^{422}Trp. It is intriguing that diallylcaracurine V seems to be exceptional in two aspects: 1) it depends directly on M_2^{423}Thr, and 2) it is negatively influenced by M_2^{422}Trp. Taken together, the amino acid ^{422}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.

^{422}Trp Is Often Critical for Allosteric Agent Binding in NMS-Free M_2 Receptors. 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). 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}H]NMS. These experiments also reveal the type of cooperativity between the allosteric agents and the orthosteric probe [^{3}H]NMS. pK_D values, indicating the binding affinity of [^{3}H]NMS at the diverse wild-type and mutant receptors in the absence of allosteric modulator, are compiled in Table 1. Binding of [^{3}H]NMS was hardly affected by mutations in M_2 or by mutations in M_5. The wild-type pK_D was maximally changed by a factor of 3; this effect was found in receptors that contained the M_2^{422}W→A mutant. Figure 6, bottom, shows the effect of W84 on the equilibrium binding of [^{3}H]NMS at M_2 and at the tryptophan M_2 receptor mutants. Affinity values (pK_A) for the allosteric agents at the unoccupied receptors and measures of cooperativity with [^{3}H]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 4, right. W84 revealed a negative cooperativity with [^{3}H]NMS at M_2 and in higher concentrations at the M_2^{422}W→A mutant, too (Fig. 6, bottom). Thus, replacement of M_2^{422}Trp by alanine led to a pronounced reduction of the binding affinity of W84 for the NMS-free receptors (Table 4, right). When M_2^{427}Trp was replaced by alanine, [^{3}H]NMS equilibrium binding remained unaffected by W84 at concentrations which fully inhibited the dissociation kinetics of [^{3}H]NMS binding (Fig. 6, top). Thus, the lack of effect of W84 on [^{3}H]NMS equilibrium binding reflects neutral cooperativity between these ligands. The change from negative (M_2 wild type) to neutral cooperativity at the M_2^{427}W→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 4, pK_A values, right).

The affinity of naphmethonium, an enhancer of the binding of [^{3}H]NMS at M_2 (curves not shown), was considerably reduced at the M_2^{422}W→A mutant, whereas the mutant M_2^{427}W→A showed the same sensitivity for naphmethonium as the NMS-free wild-type M_2 receptor (Table 4, right). 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 M_2^{422}W→A mutant and at the M_2^{427}W→A mutant relative to M_2, although the effects were not additive (Table 4, right). The underlying [^{3}H]NMS inhibition curves (not shown) were rather steep (curve slopes for M_2^{422}W→A, $n_H = -2.46 \pm 0.38$; for M_2^{427}W→A, -2.33 ± 0.30; means ± S.E., $n = 4$–5). $p\alpha$-values indicate a strong negative cooperativity between gallamine and [^{3}H]NMS at any M_2 receptors tested in this study. We have no explanation why the sum of $p\alpha$ and pK_A was often 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 M_2 wild-type receptor. Taken together, the role of the tryptophans at NMS-free M_2 receptors is more complex than at the NMS-liganded M_2 receptor, but in general M_2^{422}Trp is more important for allosteric agent binding than M_2^{427}Trp.

In M_5 Receptors, the Tryptophan Corresponding to M_2^{422}Trp Also Plays a Key Role for Allosteric Agent Action. Because M_2^{422}Trp and M_2^{427}Trp are conserved among all five muscarinic receptor subtypes, we aimed to find out in how far the corresponding tryptophans M_5^{477}Trp and M_5^{482}Trp are also involved in the action of allosteric agents at this subtype. Therefore, we replaced these tryptophans by alanine. [^{3}H]NMS binding parameters under control conditions are given in Table 1. Similar to the results obtained with M_2 receptors, the binding affinity toward NMS was hardly changed; with the double mutant M_5^{477}W→A+^{482}W→A, a small loss of affinity by a factor of 2 was seen. However, unlike M_2, the kinetics of NMS dissociation remained almost unchanged; only in M_2^{482}W→A, k_{-1} was increased by a factor of 1.4 relative to M_5 wild type. It was remarkable that the double mutation, which slowed dissociation of NMS by 8-fold in M_2, had no effect at all in M_5. With regard to allosteric agent binding, we first 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, top). 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^{482}W→A mutant had the same sensitivity for W84 as the M_5 wild type receptor. The allosteric actions of dimethyl-W84 and naphmethonium on [^{3}H]NMS dissociation were likewise affected by mutation of the two tryptophans (Table 5, left). However, at the M_5^{477}W→A mutant, both

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet

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). It is noteworthy that gallamine had no visible effect up to a concentration of 3 mM on the NMS dissociation at this mutant. Furthermore, the $M_5^{482}W{\rightarrow}A$ mutant exhibited significantly lower sensitivity for gallamine than the wild-type receptor. Diallylcaracurine V showed no change of potency to retard [^{3}H]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, bottom). 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 5 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. Therefore, W84 exhibited strong negative cooperativity at these mutants (Table 5, pα-values, right). In the case of gallamine, only substitution of ^{477}Trp affected the agent's binding affinity to the NMS-free M_5 receptors (Table 5, right). The effect of gallamine on [^{3}H]NMS equilibrium binding (i.e., the extent of negative cooperativity) was left almost unaffected by the diverse mutations. Diallylcaracurine V again hardly revealed any affinity-shift at the mutants compared with M_5 wild type (Table 5, right), but a change toward weaker negative cooperativity was noted when M_5^{477}Trp was substituted.

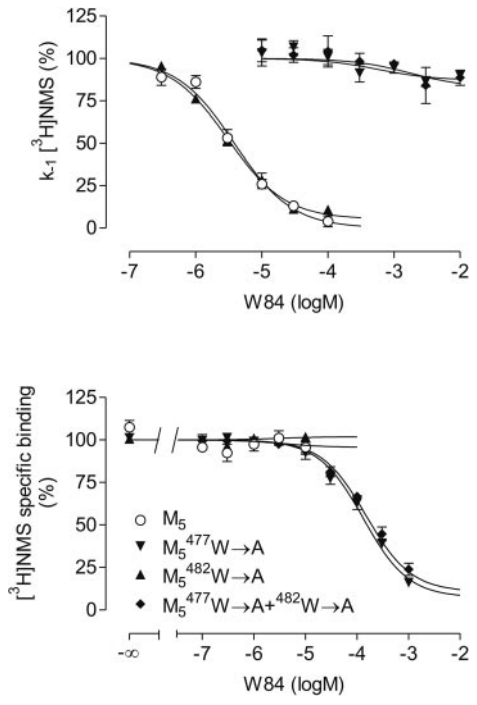

Fig. 7. Allosteric effects of W84 at the indicated wild type and mutant M_5 receptors. Top, concentration-effect curves for the allosteric delay of [^{3}H]NMS dissociation. Bottom, effects on the equilibrium binding of [^{3}H]NMS. Indicated are means ± S.E. of three to five separate experiments. Data points for lower concentrations at $M_5^{482}W{\rightarrow}A$ and $M_5^{477}W{\rightarrow}A{+}^{482}W{\rightarrow}A$ were all on the control level and are not shown for the sake of identical concentration ranges in both panels.

TABLE 5
Interaction of the indicated allosteric agents with wild type M_5 receptors and related mutants
Minus log values of the factors of cooperativity (pα) are indicated in parentheses on the right of the table. Indicated are mean values ± S.E. of three to ten separate experiments with duplicated values.

	pEC$_{0.5,diss}$				pK_A (pα)			
	M_5	$M_5^{477}W{\rightarrow}A$	$M_5^{482}W{\rightarrow}A$	$M_5^{477}W{\rightarrow}A + ^{482}W{\rightarrow}A$	M_5	$M_5^{477}W{\rightarrow}A$	$M_5^{482}W{\rightarrow}A$	$M_5^{477}W{\rightarrow}A + ^{482}W{\rightarrow}A$
W84	5.41 ± 0.04	3.24 ± 0.54	5.51 ± 0.04	2.75 ± 0.86	5.47 ± 0.05[a] (−0.05 ± 0.05)	4.72 ± 0.09 (−1.56 ± 0.15)	5.49 ± 0.03[a] (0.02 ± 0.04)	4.50 ± 0.07 (−1.23 ± 0.21)
Dimethyl-W84	5.40 ± 0.04	4.08 ± 0.29	5.52 ± 0.07	4.41 ± 0.24	N.D.	N.D.	N.D.	N.D.
Naphmethonium	6.22 ± 0.03	4.94 ± 0.17	6.20 ± 0.04	4.19 ± 0.29	N.D.	N.D.	N.D.	N.D.
Gallamine	5.41 ± 0.11	–[b]	3.49 ± 0.05	–	6.12 ± 0.03 (−1.45 ± 0.11)	5.05 ± 0.03 (−1.19 ± 0.03)	5.91 ± 0.15 (−1.35 ± 0.16)	4.90 ± 0.14 (−1.08 ± 0.10)
Diallylcaracurine V	5.57 ± 0.09	5.61 ± 0.05	5.43 ± 0.09	5.87 ± 0.08	6.18 ± 0.06 (−0.82 ± 0.08)	6.41 ± 0.11 (−0.46 ± 0.02)	6.13 ± 0.04 (−1.05 ± 0.01)	6.26 ± 0.15 (−0.40 ± 0.03)

pEC$_{0.5,diss}$, minus log concentration reducing [^{3}H]NMS dissociation to half of the control rate; pK_A, minus log value of the equilibrium dissociation constant for allosteric agent binding to free receptors; N.D., not determined.
[a] EC$_{0.5,diss}$ was used for curve fitting because of nearly neutral cooperativity as described under *Materials and Methods*.
[b] –, no effect up to minus log concentration = 2.5.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet

Taken together, in the NMS-bound as well as in the NMS-free M_5 receptor, ^{477}Trp 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 that the subtype-selective amino acid M_2^{177}Tyr interacts directly via π-π 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. To determine whether the contribution of both amino acids for the action of W84 is additive, we generated the double mutant M_2^{177}Y→Q+^{422}W→A and measured the effect of W84 on [^{3}H]NMS-liganded receptors (Fig. 8). As mentioned above, both single mutants M_2^{177}Y→Q (Voigtländer et al., 2003; $pEC_{0.5,diss}$, 6.20 ± 0.05) and M_2^{422}W→A showed clearly reduced sensitivities for W84 compared with M_2 wild type. Combined replacement of both residues led to a further decrease of the potency of W84 ($pEC_{0.5,diss}$, 4.97 ± 0.04). The effects of the single mutants were almost additive. Hence, approximately 2.5 log units of allosteric potency of W84 at M_2 can be attributed to only two amino acids (i.e., M_2^{177}Tyr and M_2^{422}Trp).

Molecular Modeling of the W84 Docking to the M_2 Receptor. To gain more insight into the topography of allosteric agent binding to the M_2 receptor, 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 M_2 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 counter-ions extra- and intracellularly (Fig. 9). The new model was not only in good agreement with previous results but also seemed to be more suitable to explain experimental data reported previously (Voigtländer et al., 2003). Because 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 previously (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 were carried out in an aqueous environment as reported previously (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 (Fig. 10A, white transparent silhouette) between the extracellular loops to a great extent. It is remarkable that 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 in Fig. 10A, bottom) cannot dissociate from the M_2 receptor.

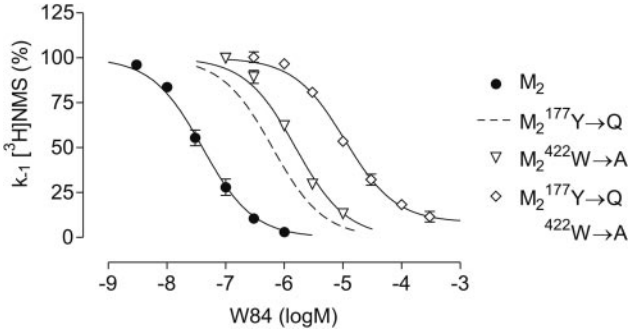

Fig. 8. Additive contribution of M_2^{177}Tyr and the conserved M_2^{422}Trp to the allosteric potency of W84. Indicated are means ± S.E. of three to five independent experiments. The curve without data points is taken from Voigtländer et al. (2003).

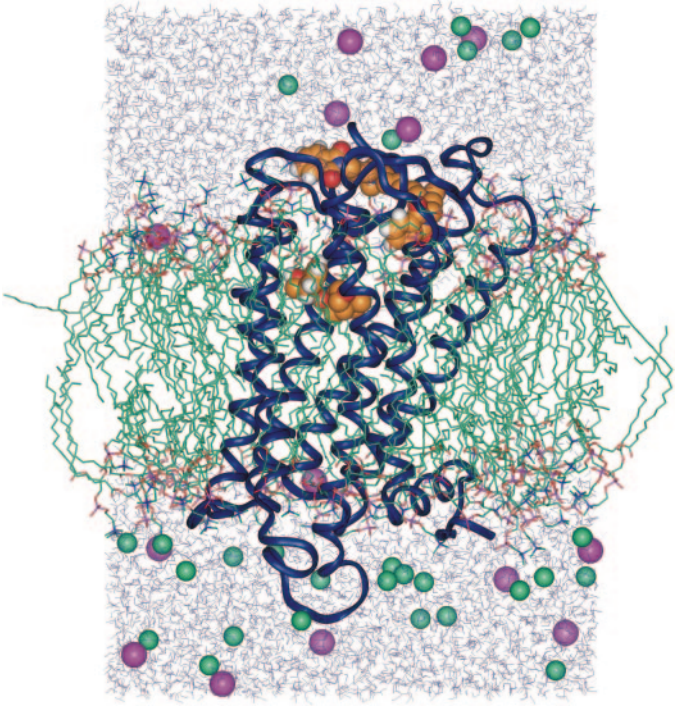

Fig. 9. Representative structure from the molecular dynamics simulation. Side view of the M_2 receptor in its membrane environment. Protein: blue ribbon. The orthosteric ligand NMS and the allosteric agent W84 (above NMS) are shown as solid surfaces. W84/NMS: carbon, orange. Phospholipid bilayer: carbon, green. Aqueous phase: water, blue; sodium, green; chloride, magenta. Color code of the other atoms: hydrogen, white; nitrogen, blue; oxygen, red; phosphorus, magenta.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

In some instances, even maximally effective concentrations of an allosteric agent did not induce complete inhibition of [^{3}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 and Mohr, 1997; Tränkle et al., 2005). We used the double mutant M_2^{422}W→A+^{423}T→H, in which W84 reduced the probability of [^{3}H]NMS-dissociation at maximum only 4-fold (bottom k_{-1} = 24.7%; see Supplemental Table 1 online), to find out whether the model gives a clue about why [^{3}H]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) was initially formed but did not persist permanently during the simulation.

Figure 10A shows that M_2^{423}Thr lines W84 lying in the allosteric cavity. To gain more insight into the postulated detrimental effect of the mutation M_2^{423}T→H on W84 binding (see Fig. 4), M_2^{423}Thr 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^{478}His seems to decrease the binding affinity of W84 sterically. Furthermore, the slowing of the [^{3}H]NMS binding kinetics by histidine (see Table 1) 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.

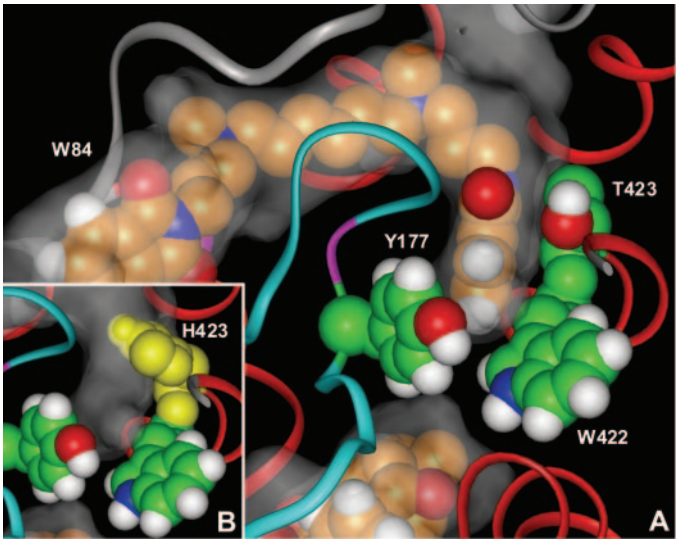

Fig. 10. A, M_2 wild type: interaction between W84 and the adjacent amino acids M_2^{177}Tyr, M_2^{422}Trp, and M_2^{423}Thr; view from the top of the receptor protein occupied by W84 and NMS (partially visible at the bottom of the figure). Helices, red; o2, cyan; disulfide bridge, magenta; other, gray. W84/NMS: carbon, orange; amino acids: carbon, green. Other atoms: hydrogen, white; nitrogen, blue; oxygen, red. Volumes of the binding sites: white, transparent silhouette. 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: Color code as above; ^{423}His: all atoms yellow. Volumes of the binding sites: white, transparent silhousette. Note the unfavorable spatial orientation of histidine in M_2^{423}T→H.

Discussion

Previous studies using the pronounced M_2/M_5 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 M_2 relative to M_5 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 M_2^{177}Tyr and parts of the third outer loop at the beginning of the seventh transmembrane region (o3/TM7) containing M_2^{423}Thr (Fig. 2) (Ellis and Seidenberg, 2000; Jöhren and Höltje, 2002). The M_2/M_5 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. Lipophilic pockets lying apart from the above-mentioned essential domain (i.e., o2 and o3/TM7) 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, ^{422}Trp in M_2 and ^{477}Trp in M_5, respectively, that plays a key role for the binding of allosteric agents and that is a key component in a pivotal cluster of amino acids additionally to M_2^{177}Tyr and M_2^{423}Thr, instead of being located outside the essential domain. Furthermore, we provide evidence that the adjacent M_2^{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 M_5.

The molecular dynamics simulations of the docking of W84 to the allosteric site of M_2 receptors, whose orthosteric site is blocked by an antagonist, indicate that one of its lateral phthalimido groups is enclosed by the aromatic residues of M_2^{177}Tyr and M_2^{422}Trp 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 ^{422}Trp and M_2/M_5 subtype selectivity by ^{177}Tyr 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 M_2^{177}Tyr either by glutamine of M_5 or by alanine (Huang et al., 2005) yielded almost the same loss of W84 binding affinity. The additivity of the contributions of M_2^{177}Tyr and M_2^{422}Trp to W84 binding affinity suggests that both amino acids interact independent of each other with the allosteric agent. Substitution of M_2^{422}Trp by alanine reduced binding affinity of the alkane-bisammonio compounds by approximately 1.5 log units and of gallamine by approximately 1 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, M_2^{422}Trp is even detrimental for this agent's affinity. In any case, these findings support the concept of a central role of M_2^{422}Trp for allosteric agent binding. In addition, in the NMS-occupied M_5 receptor, the corresponding tryptophan (M_5^{477}Trp) is important for the

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

aspet

MOL PHARM

MOLECULAR PHARMACOLOGY

binding of all applied alkane-bisammonio compounds. In the case of gallamine, the lack of effect on [^{3}H]NMS dissociation indicates either a pronounced loss of gallamine's affinity or a complete loss of gallamine's efficacy to inhibit [^{3}H]NMS dissociation. In any case, this tryptophan is essential for gallamine's action at M_5. With diallylcaracurine V, however, M_5^{477}Trp did not affect affinity, in contrast to the corresponding M_2^{422}Trp.

Most of the allosteric modulators tested in this study displayed a pronounced sensitivity to mutation of both M_2^{422}Trp and M_5^{477}Trp at the NMS-occupied receptor and also at the NMS-free receptor. In the search for the binding location of gallamine at the M_1 receptor, Matsui et al. (1995) mutated more than 20 residues in the extracellular region of the receptor and found that replacement of the corresponding M_1^{400}Trp by alanine reduced gallamine's affinity by approximately 10-fold in NMS-occupied and NMS-free receptors. These results are quite similar to our findings at M_2^{422}Trp and M_5^{477}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 conserved among subtypes, may not only provide subtype-independent baseline affinity but also contribute to subtype selectivity. For example, in the binding of both W84 and gallamine to NMS-free receptors, substitution of M_5^{477}Trp by alanine resulted in a lesser reduction of affinity than did replacement of M_2^{422}Trp by alanine. Therefore, this tryptophan seems to contribute to the M_2 preference of some allosteric agents. On the contrary, the subtype selectivity of diallylcaracurine V at the NMS-occupied receptor is reduced as a result of the detrimental effect of M_2^{422}Trp, because this ligand is insensitive to mutation of M_5^{477}Trp. In other words, the M_2/M_5 selectivity of diallylcaracurine V amounts to 2.4 log units in wild-type receptors, but to 2.9 log units in the corresponding M_2^{422}/M_5^{477}Trp mutants.

Furthermore, our findings reveal that M_2^{422}Trp, as well as its counterpart M_5^{477}Trp, may regulate cooperativity between allosteric and orthosteric ligands. Substitution of M_5^{477}Trp by alanine resulted in a switch from neutral to strongly negative cooperativity between W84 and NMS (Tables 4 and 5, pα values). Moreover, in the case of diallylcaracurine V, mutation of M_2^{422}Trp and the corresponding M_5^{477}Trp yielded enhanced positive cooperativity at M_2 and tempered negative cooperativity at M_5, respectively.

In contrast to M_2^{422}Trp and its counterpart M_5^{477}Trp, the downstream conserved tryptophans M_2^{427}Trp and M_5^{482}Trp generally have no relevance for the binding and action of allosteric agents. Exceptions were encountered with the M_2^{427}Trp→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 M_2 and 477 of M_5 located at the junction of o3 and the beginning of TM7 has to be taken into account when the epitope dependence 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 M_2 receptor protein are important for the cooperative interaction of alcuronium and gallamine with [^{3}H]NMS. Voigtländer et al. (2003) found a large influence of M_2^{177}Tyr and M_2^{423}Thr on the cooperativity between NMS and diallylcaracurine V and a smaller effect in the case of dimethyl-W84. Studying the allosteric interactions of strychnine-like modulators, Jakubik et al. (2005) reported an important role for the M_2 sequence of the third outer loop and especially of three amino acids (M_2^{419}Asp, M_2^{421}Val, and M_2^{423}Thr) that are adjacent to M_2^{422}Trp. We now provide evidence for an involvement of this tryptophan and its counterpart M_5^{477}Trp in the allosteric actions of several muscarinic allosteric modulators.

It is remarkable that the conserved tryptophan at position 422 of M_2 is also involved in the binding of orthosteric ligands. We found that substitution of the tryptophan by alanine reduced the affinity of NMS for M_2 receptors approximately 3-fold and slowed NMS dissociation by approximately 2-fold. In M_5, however, the corresponding mutation did not reduce NMS affinity and hardly affected NMS binding kinetics. For M_1, Matsui et al. (1995) showed that replacement of this tryptophan by alanine reduced [^{3}H]NMS dissociation by a factor of 2 and hardly affected NMS binding affinity, but reduced acetylcholine binding affinity by a factor of 10. The new M_2 model suggests that M_2^{422}Trp and M_2^{423}Thr are situated at the bottom of the allosteric binding cavity and concomitantly at the top of the orthosteric binding pocket. According to the M_2 receptor model, both conserved tryptophans, M_2^{422}Trp and in particular M_2^{427}Trp, 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. Because mutation of the corresponding tryptophans in M_5 did not change NMS binding affinity and kinetics, the M_5 receptor protein seems to possess a quite different overall conformation of this region compared with M_2.

In conclusion, the present study demonstrates that the conserved tryptophan at position 422 in M_2 is important for receptor interactions of structurally diverse allosteric agents in M_2 and M_5 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 M_2/M_5 selectivity providing epitopes M_2^{177}Tyr and M_2^{423}Thr, this tryptophan forms a pivotal docking point complex for allosteric agents.

Acknowledgments

It is gratefully indicated that dimethyl-W84, naphmethonium, and diallylcaracurine V were synthesized and provided by Prof. Dr. Ulrike Holzgrabe, Dr. Mathias Muth, and Dr. Darius Paul Zlotos, Institute of Pharmaceutical Chemistry, University of Würzburg, Germany. We are grateful to Dr. Christian Tränkle, Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn (Bonn, Germany), for valuable help with the data analysis.

References

Baldwin JM, Schertler GFX, and Unger VM (1997) An alpha carbon template for the transmembrane helices of the rhodopsin family of G-protein-coupled receptors. *J Mol Biol* **272:**144–164.

Birdsall NJ and Lazareno S (2005) Allosterism at muscarinic receptors: ligands and mechanisms. *Mini Rev Med Chem* **5:**523–543.

Bonner TI, Buckley NJ, Young AC, and Brann MR (1987) Identification of a family of muscarinic acetylcholine receptor genes. *Science (Wash DC)* **237:**527–532.

Buller S, Zlotos DP, Mohr K, and Ellis J (2002) Allosteric site on muscarinic acetylcholine receptors: a single amino acid in transmembrane region 7 is critical

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

to the subtype selectivities of caracurine V derivatives and alkane-bisammonium ligands. *Mol Pharmacol* **61:**160–168.
Christopoulos A and Kenakin T (2002) G protein-coupled receptor allosterism and complexing. *Pharmacol Rev* **54:**323–374.
Clark AL and Mitchelson F (1976) The inhibitory effect of gallamine on muscarinic receptors. *Br J Pharmacol* **58:**323–331.
Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol Pharmacol* **33:**187–194.
Ellis J, Seidenberg M, and Brann MR (1993) Use of chimeric muscarinic receptors to investigate epitopes involved in allosteric interactions. *Mol Pharmacol* **44:**583–588.
Ellis J (1997) Allosteric binding sites on muscarinic receptors. *Drug Dev Res* **40:**193–204.
Ellis J and Seidenberg M (2000) Interactions of alcuronium, TMB-8 and other allosteric ligands with muscarinic acetylcholine receptors: studies with chimeric receptors. *Mol Pharmacol* **58:**1451–1460.
Gnagey AL, Seidenberg M, and Ellis J (1999) Site-directed mutagenesis reveals two epitopes involved in the subtype selectivity of the allosteric interactions of gallamine at muscarinic acetylcholine receptors. *Mol Pharmacol* **56:**1245–1253.
Goodford PJ (1985) A computational procedure for determining energetically favorable binding sites on biologically important macromolecules. *J Med Chem* **28:**849–857.
Huang XP, Prilla S, Mohr K, and Ellis J (2005) Critical amino acid residues of the common allosteric site on the M_2 muscarinic acetylcholine receptor: more similarities than differences between the structurally divergent agents gallamine and bis(ammonio)alkane-type hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide. *Mol Pharmacol* **68:**769–778.
Jakubík J, Krejčí and Doležal V (2005) Asparagine, valine and threonine in the third extracellular loop of muscarinic receptor have essential roles in the positive cooperativity of strychnine-like allosteric modulators. *J Pharmacol Exp Ther* **313:**688–696.
Jöhren K and Höltje HD (2002) A model of the human M_2 muscarinic acetylcholine receptor. *J Comput Aided Mol Des* **16:**795–801.
Krejčí A and Tuček S (2001) Changes of cooperativity between *N*-methylscopolamine and allosteric modulators alcuronium and gallamine induced by mutations of external loops of muscarinic M_3 receptors. *Mol Pharmacol* **60:**761–767.
Krejčí A, Michal P, Jakubík J, Říčný J and Doležal V (2004) Regulation of signal transduction at M_2 muscarinic receptor. *Physiol Res* **53(Suppl 1):**S131–S140.
Laskowski RA (1995) SURFNET: A program for visualizing molecular surfaces, cavities and intermolecular interactions. *J Mol Graph* **13:**323–328.
Laskowski RA, Macarthur MW, Moss DS, and Thornton JM (1993) PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Cryst* **26:**283–291.
Lazareno S and Birdsall NJ (1995) Detection, quantitation and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol Pharmacol* **48:**362–378.
Lindahl E, Hess B, and van der Spoel D (2001) GROMACS 3.0: a package for molecular simulation and trajectory analysis. *J Mol Model* **7:**306–317.
Matsui H, Lazareno S, and Birdsall NJ (1995) Probing of the location of the allosteric site on M_1 muscarinic receptors by site-directed mutagenesis. *Mol Pharmacol* **47:**88–98.
Mohr K, Tränkle C, and Holzgrabe U (2003) Structure/activity relationships of M_2 muscarinic allosteric modulators. *Recept Channels* **9:**229–240.
Muth M, Bender W, Scharfenstein O, Holzgrabe U, Balatková E, Tränkle C, and Mohr K (2003) Systematic development of high affinity bis(ammonio)alkane-type allosteric enhancers of muscarinic ligand binding. *J Med Chem* **46:**1031–1040.
Nathans J and Hogness DS (1983) Isolation, sequence-analysis and intron exon arrangement of the gene encoding bovine rhodopsin. *Cell* **34:**807–814.
Okada T, Sugihara M, Bondar AN, Elstner M, Entel P, and Buss V (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 Å crystal structure. *J Mol Biol* **342:**571–583.
Raasch A, Scharfenstein O, Tränkle C, Holzgrabe U, and Mohr K (2002) Elevation of ligand binding to muscarinic M_2 acetylcholine receptors by bis(ammonio)alkane-type allosteric modulators. *J Med Chem* **45:**3809–3812.
Schlegel B, Sippl W, and Holtje HD (2005) Molecular dynamics simulations of bovine rhodopsin: influence of protonation states and different membrane-mimicking environments. *J Mol Model* **12:**49–64.
Schulz U (1998) Antagonismus-Untersuchungen zur Topographie der Bindung allosterischer Modulatoren an muskarinischen Acetylcholinrezeptoren des Subtyps M_2. Doctoral Thesis, Faculty of Mathematics and Natural Sciences, University of Bonn.
Soudijn W, Van WI, and IJzerman AP (2004) Allosteric modulation of G protein-coupled receptors: perspectives and recent developments. *Drug Discov Today* **9:**752–758.
Stockton JM, Birdsall NJ, Burgen AS, and Hulme EC (1983) Modification of the binding properties of muscarinic receptors by gallamine. *Mol Pharmacol* **23:**551–557.
Thompson JD, Higgins DG, and Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:**4673–4680.
Tränkle C, Dittmann A, Schulz U, Weyand O, Buller S, Johren K, Heller E, Birdsall NJ, Holzgrabe U, Ellis J, et al. (2005) Atypical muscarinic allosteric modulation: cooperativity between modulators and their atypical binding topology in muscarinic M_2 and M_2/M_5 chimeric receptors. *Mol Pharmacol* **68:**1597–1610.
Tränkle C, Mies-Klomfass E, Botero Cid MH, Holzgrabe U, and Mohr K (1998) Identification of a [^{3}H]ligand for the common allosteric site of muscarinic acetylcholine M_2 receptors. *Mol Pharmacol* **54:**139–145.
Tränkle C and Mohr K (1997) Divergent modes of action among cationic allosteric modulators of muscarinic M_2 receptors. *Mol Pharmacol* **51:**674–682.
Tränkle C, Weyand O, Voigtländer U, Mynett A, Lazareno S, Birdsall NJ, and Mohr K (2003) Interactions of orthosteric and allosteric ligands with [^{3}H]dimethyl-W84 at the common allosteric site of muscarinic M_2 receptors. *Mol Pharmacol* **64:**180–190.
Voigtländer U, Jöhren K, Mohr M, Raasch A, Tränkle C, Buller S, Ellis J, Höltje HD, and Mohr K (2003) 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. *Mol Pharmacol* **64:**21–31.
Wess J (2005) Allosteric binding sites on muscarinic acetylcholine receptors. *Mol Pharmacol* **68:**1506–1509.
Zahn K, Eckstein N, Tränkle C, Sadée W, and Mohr K (2002) Allosteric modulation of muscarinic receptor signaling: alcuronium-induced conversion of pilocarpine from an agonist into an antagonist. *J Pharmacol Exp Ther* **301:**720–728.
Zlotos DP, Buller S, Tränkle C, and Mohr K (2000) Bisquaternary caracurine V derivatives as allosteric modulators of ligand binding to M_2 acetylcholine receptors. *Bioorg Med Chem Lett* **10:**2529–2532.

Address correspondence to: Dr. Klaus Mohr, Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, 53121 Bonn (Germany). E-mail: k.mohr@uni-bonn.de

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012