Molecular Mechanisms of Methoctramine Binding and Selectivity at Muscarinic Acetylcholine Receptors

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ABSTRACT

Methoctramine (N,N'-bis[6-[(2-methoxyphenyl)-methyl]hexyl]-1,8-octane) diamine) is an M2-selective competitive antagonist of muscarinic acetylcholine receptors and exhibits allosteric properties at high concentrations. To reveal the molecular mechanisms of methoctramine binding and selectivity we took advantage of reciprocal mutations of the M2 and M3 receptors in the second and third extracellular loops that are involved in the binding of allosteric ligands. To this end we performed measurements of kinetics of the radiolabeled antagonists N-methylscopolamine (NMS) in the presence of methoctramine and its precursors, fluorescence energy transfer between green fluorescent protein (GFP)-conjugated precursor of methoctramine, and simulation of molecular dynamics of methoctramine association with the receptor. We confirm the hypothesis that methoctramine high-affinity binding to the M2 receptors involves simultaneous interaction with both the orthosteric binding site and the allosteric binding site located between the second and third extracellular loops. Methoctramine can bind solely with low affinity to the allosteric binding site on the extracellular domain of NMS-occupied M3 receptors by interacting primarily with glutamate 175 in the second extracellular loop. In this mode, methoctramine physically prevents dissociation of NMS from the orthosteric binding site. Our results also demonstrate that lysine 523 in the third extracellular loop of the M3 receptors forms a hydrogen bond with glutamate 219 of the second extracellular loop that hinders methoctramine binding to the allosteric site at this receptor subtype. Impaired interaction with the allosteric binding site manifests as low-affinity binding of methoctramine at the M3 receptor.

Introduction

Five muscarinic receptor subtypes have been cloned (Bonner et al., 1987; Bonner, 1989). They all belong to the family of G-protein–coupled receptors with seven transmembrane helices. The binding site of acetylcholine and competitive agonists and antagonists is located deep in a pocket formed by transmembrane helices (Wess, 1996). All subtypes of muscarinic receptors share high homology in the transmembrane domains, making it difficult to discover competitive ligands that are selective for different receptor subtypes. However, a few such selective compounds have been identified. One example is methoctramine (N,N'-bis[6-[(2-methoxyphenyl)-methyl]hexyl]-1,8-octane) diamine), which binds to the M2 subtype of muscarinic receptors with high affinity (Melchiorre et al., 1987) and to the M3 subtype with low affinity (Caulfield, 1993). However, deviations from competitive behavior (slowdown of dissociation of orthosteric antagonists) at high concentrations of methoctramine were revealed soon after the discovery of its selectivity (Giraldo et al., 1988; Lee et al., 1989). Additional investigations have concluded that methoctramine interacts with muscarinic receptors both competitively and allosterically (Waelbroeck, 1994; Boselli and Grana, 1995), and the concept that methoctramine binds simultaneously to the orthosteric and allosteric receptor domains was proposed (Melchiorre et al., 1989). In the present work, we provide experimental support for this concept.

A large number of allosteric modulators selectively affect binding of the competitive ligands to M2 receptors (Tuček and Proška, 1995). Several lines of evidence suggest that muscarinic allosteric ligands (e.g., gallamine and alcuronium) bind to the extracellular domain of muscarinic receptors (Jakubík and Tuček, 1994; Leppik et al., 1994; Proška and Tuček, 1994), and that amino acid residues in the second and third extracellular loops are important for this mode of binding (Leppik et al., 1994; Matsui et al., 1995; Gnagey et al., 1999; Krejcí and Tuček, 2001; Jakubík et al., 2005). In contrast, virtually nothing is known on the nature of the selectivity of orthosteric muscarinic ligands.
In this study, we investigated whether the extracellular domains of muscarinic receptors are also involved in putative allosteric properties of methoctramine binding, and if they take part in high affinity of the M2 receptor for methoctramine. To achieve this aim, we avoided differences of methoctramine binding between the M2 and M3 receptor subtypes. We modified the gene of the M3 receptor in parts that encode the extracellular domains so that the resulting amino acid sequence resembles that in the M2 receptor. Using this approach, we demonstrate that methoctramine selectivity for the M3 receptor arises from its binding to glutamate residues in the second extracellular loop. A similar interaction at the M3 receptor is prevented by interaction with K523 in the third extracellular loop.

Materials and Methods

Chemicals

N-[methyl-3H]scopolamine ([3H]NMS) was from New England Nuclear Corporation (Boston, MA); 4-difluoracetoxy-N-methylpiperidine (4-DAMP), 11-2-[2-diethylamino]methyl]-1-piperidinyl[acetyl]-5, 11-dihydro-6H-pyrido[2,3-b][1,4] benzodiazepine-6-on (AF-DX 116), and methoctramine were from Tocris Cookson Ltd. (Avonmouth, UK); and 4-2(1,6-dimethyl-piperidin-2-yl)-vinyl]-3-methyl-decahydro-naphth[2,3-c]fur-an-1-on (himbacine) and N-methylisocapoline were from Sigma-Aldrich (Prague, Czech Republic). Succinimidyl ester of Alexa-555 carboxylic acid was from Life Technologies (Prague, Czech Republic).

Synthesis of methoctramine precursors (6-aminohexyl)[(2-methoxy-phenyl)methyl]amine and 8-amino-N-(6-[(2-methoxyphenyl)methyl]amino)hexyl)octanamide and their conjugation with fluorescent label Alexa-555 is described in Supplemental Figs. 1 and 2.

Mutagenesis and Expression

For the sake of brevity, names of mutants consist of receptor subtype followed by a list of mutated amino acids in the third extracellular (o3) loop. Amino acids in either the M2 or M3 receptors were always mutated to the corresponding residue in the other receptor subtype (Supplemental Fig. 3). For example, M2 P means that proline 415 of the M2 receptor was mutated to the corresponding serine in the M3 sequence, and M3 DSKFN means that five amino acids in the o3 loop of the M3 receptor were mutated to their corresponding residues in the M2 sequence.

The mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) containing the coding sequence of the human variants of M2 and M3 subtype of muscarinic acetylcholine receptors was obtained from Missouri SynTAC DNA Resource Center (Rolla, MO). Construction of M2 or M3 receptors with the substituted second extracellular (o2) loop and mutations (K252N, KFN, and DSKFN) in the o3 loop were previously described in Krejci and Tucek (2001). Additional mutants were generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies Company, Santa Clara, CA). To replace serine 519 with proline, a plasmid containing wild-type M3 receptor as template and 5'-TGAGA CCT TTT GTT ACC CCT GCA TAC CCA AAA CCT TTT GG-3' primer were used. To obtain DKFN mutant, plasmid containing KFN mutant as template and 5'-GTTG ACG ACC TTT TGT GGC ACC AGC ATA CCC AAT ACC-3' primer were used. To obtain SK mutant plasmid containing K252N mutant as template and 5'-TGAGA CCT TTT GTT ACC CCT GCA TAC CCA ATA CCT TTT GG-3' primer were used. To obtain SKFKN mutant, plasmid containing K252N mutant and 5'-TGAGA CCT TTT GTT ACC CCT GCA TAC CCA ATA CCT TTT GG-3' primer were used. To replace proline 415 of the M3 receptor with serine, a plasmid containing wild-type M3 receptor as template and 5'- AAC ACC TTT TGT GCA TCT TGC ATC CCC AAC ACT GTG-3' primer were used.

Chimeric proteins used in this study consist of three parts: chicken α7 nicotinic signal peptide, emerald green fluorescent protein (GFP), and human muscarinic M2 (hM2) or M3 (hM3) receptor. The DNA sequence of chicken α7 nicotinic signal peptide (a peptide 31 amino acids long with extracellular protease cleavage site included; Ilien et al., 2003) was fused directly to stop-codon-free GFP (Invitrogen) at its 5' end to ensure extracellular localization of fluorescent protein. cDNA for the hM2 receptor (cloned into pcDNA3.1+ vector) were mutated (QuikChange II Site-Directed Mutagenesis Kit) to obtain Agel restriction site at the 5' cDNA end. EcoRI and Agel (New England Biolabs, Ipswich, MA) double digestion opened the vector and created two cohesive ends for ligation. T4 ligation (New England Biolabs) reaction included opened hM2 receptor vector, signal peptide–GFP complex, and short linker hM2 cDNA was substituted with hM3 cDNA to obtain GFP-tagged hM3 chimeric protein. All constructs were sequenced before use. Chimeric protein expression in eukaryotic cell lines was verified under a fluorescence microscope and via [3H]NMS binding (described later). All mutated DNAs were sequenced with the dideoxy method by Dr. J. Felsberg (Academy of Sciences, Institute of Microbiology, Prague, Czech Republic).

COS-7 cells were transfected using the DEAE-dextran method. They were grown in 10-cm Petri dishes in Iscove's modified Dulbecco's medium (Sigma-Aldrich) with 10% fetal calf serum. On day 1, 2×106 cells were seeded per dish. On day 3, after washing with phosphate-buffered saline, the transfection mix (2 μg of plasmid DNA and 0.27 mg of DEAE-dextran in 1 ml of phosphate-buffered saline) was applied for 2 hours. Serum-free Iscove's modified Dulbecco's medium with chloroquine (80 μM in 7 ml of medium) was then added for an additional 3 hours. Transfection medium was then removed and fresh medium supplemented with 10% fetal calf serum was applied. Cells were harvested 72 hours after transfection.

Radioligand Binding Experiments

Radioligand binding experiments were performed on membranes of COS-7 cells. Membranes were obtained by dilution of freshly harvested COS-7 cells in a medium composed of 136 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM EDTA, and 10 mM Na2HEPES (pH = 7.4) to a final concentration of 107 cells/ml. Cell suspension was homogenized using an Ultra-Turrax homogenizer (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) by two 30-second strokes. Homogenate was centrifuged for 5 minutes at 1000 × g. The resulting supernatant was centrifuged for 30 minutes at 30,000 × g and the supernatant was discarded. Pellet was resuspended in 10 times the original volume (before centrifugation), left at 4°C for 30 minutes, and then the latter centrifugation step was repeated. Membranes were kept frozen at −20°C for a maximum of 1 month. Binding experiments to membranes from 1 to 2 million cells per tube were performed as described earlier (Jakubík et al., 1995). The medium used for incubation was the same as described earlier without EDTA, and the incubation volume was 0.8 ml. Incubations performed at 25°C were terminated by filtration through Whatman GF/C glass fiber filters in a Brandel filtration apparatus (Sensat, Herts, UK). Nonspecific binding was determined in the presence of 1 μM NMS.

The affinity of wild-type and mutated muscarinic receptors for [3H]NMS was measured in saturation binding experiments (1-hour incubation with [3H]NMS at concentrations ranging from 32 pM to 1 nM) and expressed as Kd[NMS] (equilibrium dissociation constant for the binding of [3H]NMS). The inhibition constants Ki of receptors for methoctramine, 4-DAMP, AF-DX 116, and himbacine were determined in competition experiments in which membranes were incubated in the presence of a fixed 200 pM concentration of [3H]NMS and increasing concentrations of the competitor. The incubation lasted 5 hours to achieve full equilibrium. In dissociation experiments, membranes were preincubated for 1 hour with 750 pM [3H]NMS. Dissociation was induced by adding 50 μl of NMS to a final concentration of 1 μM NMS was added either alone or in mixture with methoctramine or methoctramine precursors at final concentrations ranging from 10 pM to 1 mM.

Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) between GFP-M2 or GFP-M3 receptors and conjugate of long methoctramine precursor (LMP) and Alexa-555 (Alexa-555–LMP) was measured in 96-well
Data were processed and analyzed with open-source software OpenOffice 3.4 (OpenOffice Foundation, www.openoffice.org) and Grace 5.1.12 (Grace Development Team, plasma-gate.weizmann.ac.il/Grace) on Scientific Linux (www.scientificlinux.org).

The equations for nonlinear regression analysis were as follows:

**For Saturation Binding Experiments.** After subtraction of nonspecific binding, eq. 1 was fitted to the data:

\[ Y = B_{\text{MAX}} - \frac{X}{(K_D + X)} \]  

where \( Y \) is [\(^3\)H]NMS binding at concentration \( X \) of free [\(^3\)H]NMS, \( K_D \) is the equilibrium dissociation constant, and \( B_{\text{MAX}} \) is the number of binding sites.

**For Competition Experiments.** After subtraction of nonspecific binding and normalization (to express the binding of [\(^3\)H]NMS in the presence of competitor [methoctramine, 4-DAMP, AF-DX 116, or himbacine] as a percentage of the binding in the absence of competitor), eq. 2 was fitted to the data:

\[ Y = 100 - 100/(1 + 10^{X-K_i}) \]  

where \( Y \) is [\(^3\)H]NMS binding at logarithm of concentration of competitor \( X \), and \( K_i \) is the concentration of competitor that results in 50% of maximal inhibition. Inhibition constant \( K_i \) was calculated from the IC\(_{50}\) value according to Cheng and Prussof (1973):

\[ K_i = \frac{IC_{50}}{(1 + [NMS]/K_D)} \]  

where \([NMS]\) is the actual concentration of [\(^3\)H]NMS used in the experiment, and \( K_D \) is the equilibrium dissociation constant of [\(^3\)H]NMS.

**Dissociation Experiments.** After subtraction of nonspecific binding, eq. 4 was fitted to the data:

\[ Y = 100 \times e^{-K_{\text{off}}(\text{obs})X} \]  

where \( Y \) is [\(^3\)H]NMS binding at time \( X \), and \( K_{\text{off}}(\text{obs}) \) is the observed dissociation rate constant. Apparent equilibrium dissociation constant \( K_D \) for methoctramine based on dissociation experiments was obtained according to Lazareno and Birdsall (1995):

\[ Y = k_0 \times K_D / (X + K_D) \]  

where \( Y \) is the observed rate of dissociation \( k_{\text{off}}(\text{obs}) \) at concentration \( X \) of the allosteric ligand [methoctramine or (6-aminohexyl)[(2-methoxyphenyl)methyl]amine, short methoctramine precursor (SMP)], and \( k_0 \) is the observed dissociation rate in the absence of the allosteric ligand.

The error distributions for individual constants were verified according to Christopoulos (1998). IC\(_{50}\) (eq. 2), and consequently \( K_i \) (eq. 3), and \( K_D \) (eq. 5) have log-normal error distribution. Error distributions for NMS K\(_{50}\) (eq. 1) and \( k_{\text{off}}(\text{obs}) \) (eq. 4) conform to normal Gaussian distribution.

Parameters of binding kinetics from FRET measurements were obtained by fitting a tandem two-site model (Jakubík et al., 2000) to the experiment, and KD is the equilibrium dissociation constant of [\(^3\)H]NMS.

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>M(_2)</th>
<th>M(_3)</th>
<th>M(_3(\text{o2M}_2))</th>
<th>M(_3(\text{o3M}_2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-DAMP</td>
<td>7.72 ± 0.06</td>
<td>8.50 ± 0.09</td>
<td>6.84 ± 0.05(^*)</td>
<td>8.35 ± 0.07</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>7.18 ± 0.04</td>
<td>5.68 ± 0.05</td>
<td>5.54 ± 0.05</td>
<td>5.68 ± 0.06</td>
</tr>
<tr>
<td>Himbacine</td>
<td>7.56 ± 0.08</td>
<td>6.93 ± 0.03</td>
<td>6.83 ± 0.08</td>
<td>6.91 ± 0.06</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>7.29 ± 0.04</td>
<td>5.69 ± 0.12</td>
<td>5.89 ± 0.11</td>
<td>7.17 ± 0.04(^*)</td>
</tr>
</tbody>
</table>

\(^*\)Significantly different from wild-type M\(_2\) (\(P < 0.01\) by Student’s two-tailed \(t\) test).
Mechanism of Methoctramine Binding

Inhibition of \(^{3}H\)NMS binding to \(M_2\), \(M_3\), and mutant \(M_3\) receptors by methoctramine. Binding of \(^{3}H\)NMS to membranes expressing wild-type \(M_2\) or \(M_3\) or mutant \(M_3\) receptors was measured at increasing concentrations of methoctramine. Binding is expressed as the percentage of \(^{3}H\)NMS binding to the membranes incubated in the absence of methoctramine (950–2300 dpm). Data are means \(\pm\) S.E.M. of four to six independent experiments performed in quadruplicates. For the mutants nomenclature, see Materials and Methods and Supplemental Fig. 3.

The pooled data with subtracted background values using the program COPASI (www.copasi.org) (Hoops et al., 2006). Initial parameter estimates and background values were obtained by fitting two exponential growth and two exponential decay functions, respectively, to the individual data sets using the program Grace.

Molecular Modeling

Preparation of Structures and Systems. The structure of methoctramine (CID 4108) was downloaded from the PubChem database (pubchem.ncbi.nlm.nih.gov) and processed with Schrödinger’s (Schrödinger LLC, New Portland, OR) LigPrep. Crystal structures of \(M_2\) (Haga et al., 2012) (PDB code 3UON) and \(M_3\) (Kruse et al., 2012) (PDB code 4DAJ) receptors were downloaded from the RCSB Protein Data Bank (www.rcsb.org), and were preprocessed with Schrödinger’s Protein Preparation Wizard to remove nonreceptor parts, fill missing side chains, and energy minimize structures in the OPLSA 2005 force field. A system consisting of receptor, 1,2-dipalmitoylphosphatidylcholine membrane, water, and 0.15 M NaCl with or without methoctramine randomly placed close to the receptor extracellular domain was built with the Desmond System Builder (Bowers et al., 2006).

Simulation of Molecular Dynamics. Molecular dynamics of full membrane systems was simulated using Desmond (Bowers et al., 2006). First, systems were relaxed to 300 K using standard Desmond protocol for membrane systems and subsequently 120 ns of free (without restraints) molecular dynamics (MD) (ensemble class NVE, Coulombic short-range method: cutoff with radius 9 Å, long-range method: smooth particle mesh Ewald) was simulated using Desmond-GPU.

Six systems with methoctramine were built and used for simulation of methoctramine association. After system relaxation prior to free MD simulation, 25 ps of steered MD was run, during which acceleration of 500 pm ps \(^{-2}\) toward the center of the orthosteric binding site (defined by D103 and N404) was applied to one anisole group of methoctramine. Steered MD and free MD were repeated until the distance between the centers of the anisole group and the orthosteric binding site reached 5 Å. Then 500 ns of free MD was simulated.

Results

Equilibrium Experiments: Methoctramine. In preliminary experiments, we measured the effects of replacement of the entire \(o2\) or \(o3\) loop of the \(M_2\) receptor with the corresponding \(M_2\) sequence on the binding of the muscarinic \(M_2\)-selective ligands AF-DX 116, himbacine, and methoctramine (Fig. 1) and the \(M_3\)-selective ligand 4-DAMP. Replacement of the \(o2\) loop of the \(M_3\) receptor with that of the \(M_2\) receptor did not change the affinity of the tested ligands except for 4-DAMP, where affinity of the hybrid receptor fell far below the value of the \(M_2\) wild-type receptor (Table 1). Replacement of the \(o3\) loop of the \(M_2\) receptor with that of the \(M_2\) receptor did not change the affinity of 4-DAMP, AF-DX 116, or himbacine but increased the affinity of methoctramine to a value close to that at the \(M_2\) receptor (Fig. 2).

In subsequent experiments, we measured binding of methoctramine to the seven mutant receptors with mutations in the \(o3\) loop. Mutation of serine 519 (M3 numbering) to corresponding

### TABLE 2

Binding parameters of wild-type \(M_2\) and \(M_3\) and mutated \(M_3\) receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>(^{3}H)NMS (K_d)</th>
<th>Methoctramine (pK_a)</th>
<th>SMP (pK_a)</th>
<th>LMP (pK_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M_2) wt</td>
<td>0.32 (\pm) 0.01</td>
<td>5.69 (\pm) 0.12</td>
<td>4.41 (\pm) 0.15</td>
<td>5.43 (\pm) 0.13</td>
</tr>
<tr>
<td>(M_2) M</td>
<td>0.78 (\pm) 0.04</td>
<td>7.29 (\pm) 0.04</td>
<td>5.25 (\pm) 0.12</td>
<td>6.78 (\pm) 0.08</td>
</tr>
<tr>
<td>Mutations in (o3) of (M_2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(M_2) S</td>
<td>0.32 (\pm) 0.02</td>
<td>5.95 (\pm) 0.15</td>
<td>4.58 (\pm) 0.14</td>
<td>5.64 (\pm) 0.12</td>
</tr>
<tr>
<td>(M_2) K</td>
<td>0.34 (\pm) 0.02</td>
<td>6.49 (\pm) 0.11</td>
<td>4.96 (\pm) 0.13</td>
<td>6.15 (\pm) 0.12</td>
</tr>
<tr>
<td>(M_2) KFN</td>
<td>0.51 (\pm) 0.03</td>
<td>6.51 (\pm) 0.09</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>(M_2) DKFN</td>
<td>0.49 (\pm) 0.03</td>
<td>6.51 (\pm) 0.16</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>(M_2) SK</td>
<td>0.34 (\pm) 0.02</td>
<td>7.02 (\pm) 0.19</td>
<td>5.33 (\pm) 0.09</td>
<td>6.65 (\pm) 0.09</td>
</tr>
<tr>
<td>(M_2) SKFN</td>
<td>0.68 (\pm) 0.05</td>
<td>7.15 (\pm) 0.15</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>(M_2) DSDFN</td>
<td>0.76 (\pm) 0.05</td>
<td>7.17 (\pm) 0.14</td>
<td>5.20 (\pm) 0.14</td>
<td>6.68 (\pm) 0.08</td>
</tr>
<tr>
<td>Mutations in (o3) of (M_2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(M_2) P</td>
<td>0.62 (\pm) 0.05</td>
<td>6.31 (\pm) 0.12</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., not determined.

*For the mutants nomenclature, see Materials and Methods and Supplemental Fig. 3.

*(Significantly different from \(M_2\) wt \(P < 0.01\), multiparametric one-way analysis of variance with Tukey-Kramer post-test).

*(Significantly different from \(M_2\) K \(P < 0.01\), multiparametric one-way analysis of variance with Tukey-Kramer post-test).

*(Significantly different from \(M_2\) wt \(P < 0.01\), multiparametric one-way analysis of variance with Tukey-Kramer post-test).
proline (M3 S) only slightly increased the affinity of methoctramine. Mutation of lysine 523 (M3 K) increased the affinity of methoctramine more than six times. Combination of the M3 K mutation with the mutation of phenylalanine 525 to valine, asparagine 527 to threonine, and aspartate 518 to alanine, i.e., M3 KFN and M3 DSKFN, respectively, did not further change the affinity of methoctramine. Double-mutation M3 SK increased the affinity of methoctramine to a value close to that at the wild-type M2 receptor. Combination of the double-mutation M3 SK with mutation of F525, N527, and D518, i.e., M3 SKFN and M3 DSKFN, did not further change the affinity of methoctramine (Table 2). Taken together, only the M3 S and M3 K mutations increased affinity of methoctramine. Mutation M3 K contributed most to affinity increase. In accordance with these observations, mutation of proline 415 (M2 numbering) in o3 loop of the M2 receptor, i.e., M2 P, brought about a 10-fold decrease in affinity of methoctramine (Table 2).

**Kinetic Experiments: Methoctramine.** Kinetic binding experiments were performed to ascertain putative allosteric properties of methoctramine. Membranes were first preincubated with [3H]NMS for 1 hour, then dissociation was started by the addition of unlabeled NMS to a final concentration of 1 μM, either alone or in mixture with various concentrations of methoctramine. In the absence of methoctramine, dissociation of [3H]NMS from the M2 receptors is about six times faster than that from the M3 receptors (Fig. 3; Table 3). In the presence of 100 μM methoctramine, the dissociation from the M2 receptor was slowed down >50-fold (Fig. 3, top panel; Table 3). In contrast, the observed dissociation rate of [3H]NMS from the M3 receptor did not significantly differ in the presence of 100 μM methoctramine (Fig. 3, bottom panel; Table 3).

Interestingly, all tested mutations in the o3 loop of the M3 receptor, except M3 S, accelerated [3H]NMS dissociation in the absence of methoctramine (second column of Table 3). The rate of [3H]NMS dissociation at M3 KFN, M3 SKFN, and M3 DSKFN receptors was the same as that at the wild-type M3 receptor. Moreover, there was a significant difference in [3H]NMS dissociation rate at M3 KFN and M3 DSKFN versus M3 K. Also, [3H]NMS dissociation from M3 SKFN and M3 DSKFN mutants was significantly faster than from M3 SK, but there was no difference in dissociation from M3 KFN, M3 SKFN, and M3 DSKFN as well as from M3 K and M3 SK. Taken together, although all mutations except M3 S accelerate [3H]NMS dissociation, mutations of F525 to valine and N527 to threonine have the most profound effect on dissociation of [3H]NMS.

[3H]NMS dissociation in the presence of 100 μM methoctramine was slower at all mutants (except for M3 S) than at the M3 wild type. In fact, the rate of radioligand dissociation at M3 KFN, M3 SKFN, and M3 DSKFN receptors was the same as at the wild-type M3 receptor. In other words, mutation of only a single residue, K523 to asparagine, elicited allosteric interaction between methoctramine and [3H]NMS on the M3 receptor, inferred from accelerating the speed of dissociation. In accordance with these observations, mutation of P415 in the o3 loop of the M2 receptor to serine, i.e., M2 P, did not change [3H]NMS dissociation significantly, either in the absence or in the presence of methoctramine (Table 3). Only mutations of K523, F525, and N527 (i.e., M3 KFN, M3 SKFN, and M3 DSKFN) significantly altered the ratio of Koff(obs) in the absence of methoctramine to Koff(boa) in the presence of 100 μM methoctramine (fourth column of Table 3). Again, this parameter was the same for the M3 SKFN, M3 DSKFN, and the M2 wt receptor.

In the next set of experiments, we measured the effects of various concentrations of methoctramine on [3H]NMS dissociation on wild-type receptors and all mutants. The observed dissociation rate constants [Koff(boa)] were calculated according to eq. 4. Calculated Koff(boa) were plotted as a function of methoctramine concentration (Fig. 4). Values of apparent equilibrium dissociation constants (Kp) for methoctramine binding to [3H]NMS-occupied receptors were obtained by fitting eq. 5 to the data in Fig. 4 (top panel) and are shown in Table 3. Except for the M3 S mutant, the calculated Kp values are close to the Kp of M2 wt. In other words, mutation of K523 (M3 K) is crucial for allosteric properties of methoctramine binding.

To gain deeper insight into the mechanisms underlying methoctramine slowing down of [3H]NMS dissociation, we measured [3H]NMS dissociation from M2 receptors in the presence of concentrations of methoctramine that saturate its...
binding to \(^{[3]H}\)NMS-occupied receptors (Fig. 4, bottom panel). Dependence of \(K_{\text{eff(o)}}\) on the concentration of methoctramine fits eq. 5 well [negative logarithm of equilibrium dissociation constant (pKD) fits eq. 5 well [negative logarithm of equilibrium dissociation constant (pKD)] = 5.2 ± 0.2 (mean ± S.E.M., \(n = 3\)]. A good fit to eq. 5 even at high methoctramine concentrations means that dissociation limits to 0, i.e., methoctramine prevents radioligand dissociation completely.

Equilibrium Experiments: Methoctramine Precursors. To gain insight into substructures of methoctramine responsible for interaction with the receptor, we synthesized a short and a long methoctramine precursor—namely, SMP and 8-amino-N-6-[(2-methoxyphenyl)methyl](methyl)amino)hexyl)octanamide (LMP) (Fig. 1). Binding of these precursors was measured at M2 and M3 wild-type receptors and M2 S, M3 K, M3 SK, and M3 DSKFN mutants. Affinity of SMP was lower than the affinity of methoctramine at both M2 and M3 wild-type receptors and M3 S, M3 K, M3 SK, and M3 DSKFN mutants. Affinity of SMP was lower than affinity of methoctramine only in the presence of 100 μM LMP was conjugated with Alexa-555 (Supplemental Methods).

Kinetics Experiments: FRET. For FRET measurements, we constructed emerald GFP attached to the N terminus of either M2 wt or M3 wt receptors as donors. As an acceptor, LMP was conjugated with Alexa-555 (Supplemental Methods). Attachment of GFP to the N termini of the receptors did not change their binding properties as assessed in \(^{[3]H}\)NMS saturation and competition experiments (Supplemental Table 1). The affinity of LMP–Alexa-555 in competition experiments with \(^{[3]H}\)NMS at M2 wt and M3 wt receptors was the same as that of LMP (Supplemental Table 1). Measurements of FRET allowed direct assessment of binding kinetics of LMP–Alexa-555 (Figs. 7 and 8). LMP–Alexa-555 was added to membranes from COS-7 cells transiently expressing GFP–M2 receptors (Figs. 7) at final concentrations of 30 (top panel), 100 (middle panel), and 300 nM (bottom panel), and FRET signal was followed for 4 hours (black). Dissociation was initiated by the addition of methoctramine at a final concentration of 1 mM after 5 (red), 60 (green), or 120 minutes (blue) of association. Association was biphasic with a very rapid initial phase followed by a phase with an observed association rate around 0.02 minute\(^{-1}\) (analytical data are in Supplemental Table 2). Non-specific signal was very strong, being about two-thirds of the total signal under equilibrium even at the lowest concentration. With increasing concentrations, specific signal increased as well as the proportion of fast association that rose from 15% at 30 nM to 53% at 300 nM. The observed association rate of the slower phase remained the same. Dissociation of LMP–Alexa-555 from M2 receptors was also biphasic. The rate of the fast dissociation phase could not be determined as it was below assay resolution. The rate of slow dissociation was around

### TABLE 3

**Effect of methoctramine on \(^{[3]H}\)NMS dissociation**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>NMS (K_{\text{eff(o)}})</th>
<th>Methoctramine</th>
<th>SMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio pKD</td>
<td>Ratio pKD</td>
<td></td>
</tr>
<tr>
<td>M2 wt</td>
<td>0.053 ± 0.002</td>
<td>0.046 ± 0.003</td>
<td>0.046 ± 0.004</td>
</tr>
<tr>
<td>M3 wt</td>
<td>0.33 ± 0.01</td>
<td>0.0062 ± 0.0002</td>
<td>0.0006 ± 0.0005</td>
</tr>
<tr>
<td>Mutations in (\alpha_3) of M3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3 S</td>
<td>0.070 ± 0.004(^{b})</td>
<td>0.074 ± 0.004(^{b})</td>
<td>0.071 ± 0.004(^{b})</td>
</tr>
<tr>
<td>M3 K</td>
<td>0.12 ± 0.01(^{b})</td>
<td>0.016 ± 0.001(^{b})</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>M3 KFN</td>
<td>0.32 ± 0.01(^{b})</td>
<td>0.018 ± 0.003(^{b})</td>
<td>n.d.</td>
</tr>
<tr>
<td>M3 DKFN</td>
<td>0.15 ± 0.01(^{b, c})</td>
<td>0.012 ± 0.002(^{b, c})</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>M3 SKFN</td>
<td>0.31 ± 0.02(^{b})</td>
<td>0.0097 ± 0.0005(^{b})</td>
<td>n.d.</td>
</tr>
<tr>
<td>M3 DSKFN</td>
<td>0.32 ± 0.01(^{b})</td>
<td>0.0084 ± 0.0003(^{b})</td>
<td>0.0085 ± 0.0007</td>
</tr>
</tbody>
</table>

*Significantly different from M3 wt (\(P < 0.01\) by multiparametric one-way analysis of variance with Tukey-Kramer post-test). For more statistical analysis, see Results.

*Significantly different from M3 wt (\(P < 0.01\) by multiparametric one-way analysis of variance with Tukey-Kramer post-test). For more statistical analysis, see Results.

n.c., not calculated; n.d., not determined.

For the mutants nomenclature, see Materials and Methods and Supplemental Fig. 3.
0.007 minute\(^{-1}\). The proportion of slow dissociation increased with both concentration of LMP and the length of association. The rate of slow dissociation was independent of both the duration of association and concentration used. Fitting a receptor-antagonist complex isomerization model (Järv et al., 1979) to the kinetic data in Fig. 7 using program COPASI provided the following results: 

\[
k_1 = \frac{5 \times 10^8}{C_2} \text{M}^{-1}\text{min}^{-1}; k_{-1} = 5 \times 10^2 \text{minute}^{-1}; k_2 = 0.07 \text{minute}^{-1}; k_{-2} = 0.008 \text{minute}^{-1}.
\]

Higher concentrations of LMP–Alexa-555 were used in FRET measurements at GFP–M3 receptors because of the lower affinity of methoctramine and its precursors at the wild-type M3 receptor (Fig. 8). Even still, higher concentrations of LMP–Alexa-555 would be required to achieve comparable occupancy at the M3 receptors. However, at a 3 μM concentration of LMP–Alexa-555, the background signal was already too strong and background noise obscured a specific signal. Due to slower kinetics of LMP–Alexa-555 at M3 than M2 receptors, dissociation was initiated after 60 minutes (green), 2 hours (blue), and 4 hours (yellow) of association. Association was biphasic with a very rapid initial phase followed by a phase with an observed association rate around 0.01 minute\(^{-1}\) (analytical data are in Supplemental Table 3). Dissociation of LMP–Alexa-555 from M3 receptors was also biphasic. The rate of the fast dissociation cannot be determined as it is below assay resolution. The rate of slow dissociation was around 0.004 minute\(^{-1}\). Fitting a receptor-antagonist complex isomerization model (Järv et al., 1979) to the kinetic data in Fig. 8 using program COPASI gave the following results: 

\[
k_1 = 2 \times 10^5 \text{M}^{-1}\text{min}^{-1}; k_1 = 100 \text{ minute}^{-1}; k_{-1} = 0.07 \text{ minute}^{-1}; k_2 = 0.008 \text{ minute}^{-1}.
\]

**Molecular Modeling.** Molecular dynamics of membrane systems with either the M2 or M3 receptor were run as described in Materials and Methods and analyzed with Desmond Simulation Interactions Diagram. In analysis of free molecular dynamics without methoctramine, we focused on the o2 and o3 loops. Although in the crystal structure of the M3 receptor (4DAJ) K523 forms a hydrogen bond with Y127 in the transmembrane α-helix (TM) II (Fig. 9, top-left panel), simulation of molecular dynamics shows that, at 59% of time, K523 forms a hydrogen bond with the conserved glutamate E219 (Fig. 9, top-right panel). In addition, K523 binds to E219 by ionic interaction at 18% of time and by water bridge at 21% of time (Supplemental
N419 at the M2 receptor corresponds to K523 of the M3 receptor. The side chain of N419 is free in the M2 crystal structure (Fig. 9, bottom-left panel). Simulation of molecular dynamics indicated that N419 is free most of the time. This also applies to E175 (corresponding residue to E219 in the M3 sequence) (Fig. 9, bottom-right panel; Supplemental Figs. 6 and 7).

In simulations of molecular dynamics of association of methoctramine with the M2 receptor, methoctramine was placed into the extracellular vestibule by steered molecular dynamics. In continuing molecular dynamics simulations, methoctramine nitrogen in atom position 10 forms a hydrogen bond with E175 at the o2 loop within the first 30 ns (Fig. 10, left panel; Fig. 11, top panel; Supplemental Figs. 8 and 11). After four cycles of alternating free and steered molecular dynamics (480 ns), the anisole group of methoctramine reached the orthosteric binding site defined by D103 and N404. During subsequent free molecular dynamics, methoctramine nitrogen in atom position 10 forms a hydrogen bond with D103, the anisole ring interacts with Y104 in the orthosteric site by π-π stacking interaction, and methoctramine nitrogen in atom position 33 forms a hydrogen bond with E175 in o2 loop (Figs. 10 and 11, middle panel; Supplemental Figs. 9 and 12). Two cycles of free molecular dynamics (total 240 ns) showed the methoctramine anisole group moving toward TM VI to interact with Y403 by π-π stacking interaction, and that the methoctramine oxygen in atom position 7 forms a hydrogen bond with N404 (Fig. 10, right panel; Fig. 11, bottom panel; Supplemental Figs. 10 and 13). Additionally, methoctramine nitrogen in atom position 17 forms a hydrogen bond with D103 in the orthosteric binding site, and nitrogen in atom position 33 forms a hydrogen bond with either E172 or E175 in the o2 loop (E172 59% and E175 35% of the time).
In this study, we delineated the molecular mechanism of methoctramine (Fig. 1) binding and selectivity toward M2 muscarinic receptors. We show that interaction of methoctramine with both the orthosteric and allosteric binding sites is responsible for its high affinity at the M2 receptor. Further, we show that K523 in the third extracellular (o3) loop of the M3 receptor interacts with E219 in the second extracellular (o2) loop and hinders interaction of methoctramine with the allosteric site, which results in low affinity at the M3 receptor.

Our results confirm earlier findings that, in addition to the high-affinity competitive interaction with [3H]NMS at the M2 muscarinic receptors, methoctramine also binds to an allosteric domain on the M2 muscarinic receptor. It has been noted that methoctramine slows down [3H]NMS dissociation from cardiac membranes (Giraldo et al., 1988; Waelbroeck, 1994), and that methoctramine noncompetitively inhibits carbachol-induced phosphoinositide hydrolysis (Lee et al., 1989) and left atria functional response to carbachol (Boselli and Grana, 1995). Our results directly demonstrate an allosteric feature of methoctramine binding at the M2 receptor and its lack at M3 receptors (Fig. 3).

To elucidate the amino acid residues involved in high-affinity orthosteric and allosteric binding of methoctramine, we genetically modified the M3 receptors to resemble the M2 receptors. We focused on the extracellular domains where allosteric ligands such as alcuronium or gallamine (Krejcí and Tuček, 2001) have been proposed to bind. We found that replacement of the whole o3 loop of the M3 receptor with the corresponding sequence of the M2 receptor increases the affinity of methoctramine and is thus responsible for high-affinity methoctramine binding. On the other hand, finding that this modification of the M3 receptor does not influence the affinity of two other M2-selective ligands, AF-DX 116 and himbacine, and the M3-selective ligand 4-DAMP (Table 1) proves that the o3 loop is not involved in M2 selectivity in a general sense, and means that different ligands gain their selectivity by interacting with different regions on the receptor. Replacement of the o2 loop of the M3 receptor by the o2 loop of the M2 receptor does not influence the affinity of methoctramine. This mutation causes a decrease in affinity of the M3-selective 4-DAMP, indicating involvement of the o2 loop in its high affinity for the M3 receptor. These observations demonstrate that at least two additional domains exist which can determine ligand affinity besides the orthosteric site.

Sequence of the o3 loop of the M2 and M3 receptors differs in five positions (Supplemental Fig. 3). Mutation of just K523 of the M3 receptor to asparagine increases the affinity of methoctramine (Fig. 2) and uncovers allosteric properties of methoctramine, similar to those observed at the M2 receptor (Fig. 4). Mutation K523N is thus sufficient for methoctramine binding to an allosteric site from which it slows down [3H]NMS dissociation. We deduce that methoctramine interacts directly with the extracellular domain of the M2 receptor, and that this interaction is contingent on the presence of N419 in the o3 loop. This notion is further supported by the observation that the mutation M3 P decreases affinity of the M2 receptor for both [3H]NMS and methoctramine (Table 2).

Equilibrium binding experiments on the M3 receptor mutated at individual positions to corresponding amino acids of the M2 sequence indicate that the double mutation of K523N and S519P is virtually sufficient to equalize the affinity of methoctramine at the mutated M3 and the wild-type M3 receptors (Fig. 2). The mutation S519P at the o3 loop of the M3 receptor does not change affinity for methoctramine and does not reveal allosteric binding properties of methoctramine. These observations show that N419 of the M2 receptor is solely required for methoctramine binding to an allosteric site from which it slows down [3H]NMS dissociation. We deduce that methoctramine interacts directly with the extracellular domain of the M2 receptor, and that this interaction is contingent on the presence of N419 in the o3 loop. This notion is further supported by the observation that the mutation M2 P decreases affinity of the M2 receptor for both [3H]NMS and methoctramine (Table 2).

Analysis of [3H]NMS dissociation from the M2 receptors in the presence of methoctramine shows that the rate of [3H]NMS dissociation concentration-dependently decreases...
and approaches a limit of zero at concentrations above those necessary to saturate binding of methoctramine to its secondary allosteric binding site (Fig. 4). This demonstrates that, similar to other allosteric modulators (Proška and Tuček, 1994), methoctramine sterically prevents [3H]NMS dissociation.

To gain insight into substructures involved in methoctramine high- and low-affinity binding, we tested interactions of two methoctramine precursors, the short and the long methoctramine precursors (SMP and LMP) (Fig. 1). Both precursors have lower affinity than methoctramine at the M₂ receptor.

Fig. 9. Interaction of K522 at the M₃ receptor and lack of interaction of corresponding N419 in the M₂ receptor. Extracellular view of initial structures (left panel) and average structures (right panels) of simulation of free molecular dynamics of the M₃ (top panels) and M₂ (bottom panels) receptors. Red, TM II; white, α2 loop; blue, α3 loop and TM VII; yellow, hydrogen bond. Elements: cyan, carbon; red, oxygen; blue, nitrogen; white, hydrogen.

Fig. 10. Simulation of molecular dynamic of methoctramine association with M₂ receptors. Three stages of molecular dynamics are displayed: initial (left panel), transient (middle panel), and final (right panel). The extracellular part of the M₂ receptor is up and TM IV and V are in front. The backbone of the receptor is colored by position in red to white to blue gradient. Side chains of D103, E172, E175, N404, and Y430 are displayed. Cyan, carbon; blue, nitrogen; green, carbon of methoctramine; red, oxygen; yellow, hydrogen bond. For high-resolution graphics, see Supplemental Figs. 8, 9, and 10.
Fig. 11. Ligand interaction diagrams of molecular dynamic simulation of methoctramine association with the M₃ receptor. Ligand interaction diagrams of initial (top panel), transient (middle panel), and final (bottom panel) stage of association are shown. Gray, solvent exposure; red, charged; blue, polar; green, hydrophobic; dotted arrow, H-bond to side chain; full arrow, H-bond to backbone; green connector, π-π stacking. For high-resolution graphics, see Supplemental Figs. 11, 12, and 13.
whereas only SMP affinity was lower at the M₃ receptor (Table 2). Mutations in the o3 loop of the M₃ receptor affected the affinity of both methoctramine precursors in the same way as it modified the affinity of methoctramine. SMP possesses allosteric properties as evidenced by the slowing down of [³H]NMS dissociation from the M₂ receptor and from the M₂ K, M₂ SK, and M₂ DSKF mutants (Fig. 6; Table 3). The equilibrium dissociation constant of SMP binding to [³H]NMS-occupied M₂ receptors is the same as that of methoctramine, indicating that this part of the methoctramine molecule is sufficient for binding to the allosteric domain. The observed lower affinity of SMP than that of methoctramine under equilibrium binding to the M₂ receptor indicates that both parts of the methoctramine molecule are involved in (needed for) methoctramine high-affinity binding.

FRET of binding kinetics of LMP at the M₂ receptors (Fig. 7) showed biphasic association as well as biphasic dissociation with the proportion of slow dissociation increasing with time of association. These results are compatible with at least two receptor-ligand interaction models: 1) isomerization of the receptor-antagonist complex (Járv et al., 1979) and 2) the tandem two-site model (Jakubík et al., 2000). The same pattern of binding kinetics of LMP at the M₂ receptors (Fig. 8) can be observed. Much higher concentrations of LMP were required at M₂ receptors for the same effect as at M₂ receptors, indicating dramatically lower affinity of LMP for the allosteric binding site at the M₂ than the M₂ receptors.

Simulations of molecular dynamics of methoctramine association with the M₂ receptor showed that binding is initiated by interaction with E175 at the o₂ loop (Fig. 10, left panel) followed by slow translocation to the orthosteric binding site (Fig. 10, middle panel) and methoctramine binding equilibration (Fig. 10, right panel). Thus, initial interaction with E175 (Fig. 11, top panel) represents the fast association step observed in FRET measurements (Fig. 7) and results in methoctramine low-affinity binding that prevents NMS dissociation. Slow translocation then represents the slow association step in FRET measurements (Fig. 7) and manifests itself as slowly dissociating sites whose proportion increases with time of association (proportion of occupied binding sites). Methoctramine interacts with both the orthosteric (D103, V403, N404) and allosteric (E172, E175) sites (Fig. 11, bottom panel), and this dual interaction results in methoctramine high-affinity binding. As is evident from the molecular model (Fig. 10, middle and right panels; Fig. 11, middle and bottom panels), SMP is too short to interact with both sites and therefore has lower affinity than methoctramine. At the M₂ receptors, methoctramine competes with K523 for interaction with E219, which is, unlike at the M₂ receptor, the only glutamate in the middle of the o₂ loop, and thus its binding to the allosteric site is impaired and contributes to low affinity of methoctramine binding at the M₂ receptors under equilibrium.

This work has practical implications. The major difficulty in producing muscarinic subtype-selective ligands is due to the structurally conserved orthosteric binding site. Understanding the molecular mechanisms of methoctramine binding may be useful for designing a novel family of selective compounds that combine elements of known high-affinity orthosteric ligands with known selective allosteric ligands in one molecule. Interaction of such hybrids with the orthosteric binding site would endow them with high affinity, whereas binding to the less conserved extracellular domain would give them subtype selectivity (Mohr et al., 2004; Antony et al., 2009). Modern methods using fluorescent ligands to detect ligand binding have been described (Ilien et. al., 2003; Daval et al., 2012). A common problem with fluorescent labeling of ligands is that fluorescent probe alters ligand affinity. Presented results show that ligand affinity need not be affected by fluorescent probe.

In summary, we demonstrate that the high-affinity methoctramine binding to the M₂ receptors is due to the simultaneous interaction with both the orthosteric and the allosteric binding sites. At the orthosteric binding site, methoctramine forms hydrogen bonds with D103 and N404, and interacts with Y403 via π−π stacking interaction. At the allosteric binding site, methoctramine forms a hydrogen bond alternating between E172 and E175. Methoctramine can bind to the NMS-occupied receptor with low affinity by interaction solely with the allosteric binding site. Although in such cases the interaction between methoctramine and NMS is allosteric (is not mutually exclusive), NMS cannot leave the complex in the presence of methoctramine, which physically prevents its dissociation. Lysine 523 in the o3 loop of the M₂ receptor interacts with E219 in the o₂ loop and hinders methoctramine binding to the allosteric site. It results in low affinity of methoctramine binding and lack of allosteric properties at the M₂ receptors.

**Authorship Contributions**

**Participated in research design:** Jakubík, El-Fakahany, Dolezal.

**Conducted experiments:** Jakubík, Zimek, Randakova.

**Contributed new reagents or analytic tools:** Fuksova.

**Performed data analysis:** Jakubík, Dolezal.

**Wrote or contributed to the writing of the manuscript:** Jakubík, El-Fakahany, Dolezal.

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