Asparagine, Valine, and Threonine in the Third Extracellular Loop of Muscarinic Receptor Have Essential Roles in the Positive Cooperativity of Strychnine-Like Allosteric Modulators

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ABSTRACT

We have investigated allosteric interactions of four closely related strychnine-like substances: Wieland-Gumlich aldehyde (WGA), propargyl Wieland-Gumlich aldehyde, strychnine, and brucine with \(N\)-methylscopolamine (NMS) on \(M_3\) subtype of muscarinic receptor genetically modified in the second or the third extracellular loop to corresponding loops of \(M_2\) subtype (\(M_3\)o2 and \(M_3\)o3 chimeras). The \(M_3\)o2 chimeric receptor exhibited no change in either affinity of strychnine, brucine, and WGA or in cooperativity of brucine or WGA, whereas both parameters for propargyl-WGA changed. In contrast, there was a change in affinity of all tested modulators (except for brucine) and in their cooperativity in the \(M_3\)o3 chimera. Directions of affinity changes in both chimeras were always toward values of the donor \(M_2\) subtype, but changes in cooperativity were variable. Compared with the native \(M_3\) receptor, strychnine displayed a slight increase in positive cooperativity and propargyl-WGA a robust decrease in negative cooperativity at \(M_3\)o2 chimera. Similar changes were found in the \(M_3\)o3 chimera. Interestingly, cooperativity of brucine and WGA at the \(M_3\)o3 chimera changed from negative to positive. This is the first evidence of constitution of positive cooperativity of WGA by switching sequences of two parental receptors, both exhibiting negative cooperativity. Gradual replacement of individual amino acids revealed that only three residues (NVT of the \(o3\) loop of the \(M_2\) receptor) are involved in this effect. Data suggest that these amino acids are essential for propagation of a conformation change resulting in positive cooperativity induced by these modulators.

Muscarinic receptors belong to the superfamily of seven transmembrane spanning segment, \(G\) protein-coupled receptors (Fredriksson et al., 2003). Five subtypes of muscarinic receptors that share a high degree of homology in transmembrane segments while connecting extracellular loops displaying more diversity have been identified (Bonner et al., 1987, 1988; Peralta et al., 1987; Bonner, 1989a,b). Selectivity of individual subtypes for classical ligands and particularly for agonists is rather low because the orthosteric binding site is formed by highly homologous transmembrane segments (Lu et al., 2002; Hulme et al., 2003). Besides the classical binding site, muscarinic receptors possess one or more allosteric binding sites (Ellis and Seidenberg, 1989; Ellis et al., 1993; Lazareno et al., 2000; Christopoulos and Kenakin, 2002) whose binding determinants are located on less conserved extracellular loops. Binding of allosteric ligands can induce conformational changes of the receptor that result in changes in receptor affinity for orthosteric agonists and antagonists (Tuček et al., 1990, 1998; Proška and Tuček, 1994; Dong et al., 1995; Jakubík et al., 1995, 1997; Lazareno and Birdsall, 1995). These effects exhibit remarkable receptor subtype selectivity that is also reflected in the functional outcome of receptor stimulation by orthosteric agonists (Lazareno and Birdsall, 1995; Doležal and Tuček, 1998; Birdsall et al., 1999; Lazareno et al., 2004). Allosteric interactions are usually more pronounced at \(M_2\) and \(M_4\) than at \(M_1, M_3,\) or \(M_5\) subtypes of muscarinic receptors (Christopoulos et al., 1999, Christopoulos and Kenakin, 2002), and at least in the case of the allosteric modulator gallamine this difference between \(M_2\) and \(M_3\) subtypes is not due to preferential coupling with \(G\) protein (Tränkle et al., 2001), which is largely determined by the third intracellular loop. Allosteric modulators are thus useful for identifying muscarinic receptor subtype that mediate specific physiological functions in native systems and...
also offer a great promise for developing strategies of subtype-selective therapeutic interventions.

Allosteric modulator-induced effects on orthosteric ligand affinity is characterized by a factor of cooperativity (α) that denotes direction and maximal change in binding affinity of the orthosteric ligand. The magnitude of α is dependent on the pair of allosteric/orthosteric ligands and the receptor subtype. Cooperativity can be positive (allosteric modulator increases affinity for a given classical ligand), negative (allosteric modulator decreases affinity), or neutral (allosteric modulator binds to the receptor but does not change affinity for a given classical ligand). Mutagenesis studies have been used to reveal sensor sequences important for allosteric modulator affinity and cooperativity with classical ligands. These studies have used two different approaches, i.e., site-directed mutagenesis of specific receptor residues (Leppik et al., 1994; Matsui et al., 1995; Gnagey et al., 1999) or construction of hybrid receptors exploiting pharmacological differences among individual receptor subtypes (Ellis et al., 1993; Ellis and Seidenberg, 2000; Krejčí and Tuček, 2001). Together, experimental evidence is consistent with the notion that the second and the third extracellular loop mainly determine properties of interaction of allosteric ligands with muscarinic receptors.

In recent experiments, we exploited the divergence of allosteric interactions at M2 and M3 subtypes (Krejčí and Tuček, 2001). We found that complete exchange of the extracellular o2 loop (o2) of the M3 subtype of muscarinic receptor by the o2 loop of the M2 subtype increased affinity of the chimeric receptor (M3o2 chimera) for gallamine and alcuronium, but had no effect on their cooperativity with N-methylscopolamine (NMS) binding. Similar substitution of the extracellular o3 loop (M3o3 chimera) also increased affinity of the chimeric receptor for both allosteric modulators. Replacement of the o3 loop augmented negative cooperativity of gallamine and inverted negative cooperativity of alcuronium at parent M3 receptor to positive cooperativity on the donor M2 receptor. This was the first example of reversal of the direction of cooperativity that was accomplished by exchange of the defined segment of muscarinic receptor subtype displaying negative cooperativity for corresponding segment of a subtype exhibiting positive cooperativity (Krejčí and Tuček, 2001).

Using the same approach in the present experiments, we investigated the influence of similar and additional mutations in the third extracellular loop (o3) of the M3 receptor subtype on mutual interactions of the classical ligand NMS and four closely related derivatives of strychnine (Fig. 1). We demonstrate that the o3 loop of the M3o3 chimera is responsible for the appearance of positive cooperativity between NMS and Wieland-Gumlich aldehyde (WGA) or brucine. Results of our experiments establish for the first time that substitution of as little as three amino acids in the third extracellular loop of the M3 subtype of muscarinic receptor by
corresponding residues of the M₂ subtype brings about positive cooperativity between NMS and WGA, whereas this compound exerts negative cooperativity on both parent subtypes. We show in addition that positive cooperativity of tested strychnine-like modulators on the chimeric receptor is conditioned by proper interaction with the o₂ loop.

Materials and Methods

Materials. WGA ([17R]-17,18-epoxy-17-hydroxy-19,20-didehydrocuranium) and propargyl-WGA ([4R,17R]-4-propargyl-17,18-epoxy-17-hydroxy-19,20-didehydrocuranium iodide) were synthesized by Dr. T. Elbert (Charles University, Prague, Czech Republic). [³H]NMS was from PerkinElmer Life and Analytical Sciences (Boston, MA). Brucine, strychnine, and atropine were from Sigma-Aldrich (Prague, Czech Republic).

Mutagenesis and Expression. Amino acids of the M₃ receptor were always mutated to the corresponding residues of the M₂ receptor (Fig. 2). Mutants of M₃ receptor containing entire o₂ or o₃ loops of M₂ sequence are named M₃o₂ and M₃o₃, respectively. For the sake of brevity, names of the rest of mutants consist of receptor subtype followed by a suffix of original amino acids in the third extracellular loop followed by the substituted amino acids. For example, M₃SKFN→PNVT means that four amino acids (serine 519, lysine 523, phenylalanine 525, and asparagine 527) in the third extracellular loop of M₃ receptor were mutated on proline, asparagine, valine, and threonine in M₂ sequence (Fig. 2).

Wild-type genes for human muscarinic M₃ and M₃ receptors incorporated in pCD vector (kindly provided by Professor N. Buckley, University Leeds, Leeds, UK) were used. Construction of M₃ receptor with the substituted M₃ o₂ or o₃ loop and mutations (M₃K→N and M₃KFN→NVT) in the o₃ loop was described in Krejcí and Tucek (2001). Additional mutants were generated using QuikChange kit (Stratagene, La Jolla, CA). To replace serine 519 with proline, plasmid containing wild-type M₃ receptor as template and 5'-GTAACA CCT TTT GTG ACC CCT GCA TAC CCA AAA CTT GG-3' primer were used. To obtain M₃DKFN→ANVT, mutant plasmid containing M₃KFN→NVT mutant as template and 5'-GTCG AAC ACC TTT TGT GCC ACC TTC GTA ATC CAA AAC ACC-3' primer were used. To obtain M₃SK→PN and M₃SK→PNVT, mutant plasmid containing M₃SK→PN and M₃SK→PNVT mutant, as template and 5'-TGAACA CCT TTT GTG ACC CCT GCA TAC CCA ATA CTT GG-3' primer were used. To obtain M₃KFN→PNVT, mutant plasmid containing M₃KFN→NVT mutant and 5'-TGAACA CCT TTT GTG ACC CCT GCA TAC CCA ATA

Fig. 2. Sequences of amino acids in the second and third extracellular loop of the M₂ and M₃ subtypes of human muscarinic receptors and nomenclature of the mutants. Data are taken from Bonner et al. (1988). Amino acids in the o₂ or o₃ extracellular loop of M₂ receptor were mutated to the corresponding residue of M₃ sequence. Chimeric M₃ receptors incorporating entire sequence of o₂ or o₃ loop of M₂ receptor are named M₃o₂ and M₃o₃, respectively. Accordingly, names of individual mutants consist of receptor subtype name followed by list of mutated amino acids in single letter code. For example, M₃K→N indicates that the lysine 523 of the third extracellular loop of the M₃ receptor was mutated to the corresponding asparagine of M₂ sequence, and M₃SKFN→PNVT means that four amino acids in the third extracellular loop of M₃ receptor were mutated to their corresponding residues in M₂ sequence.

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Data Treatment. Data were processed and analyzed with open source software OpenOffice 1.0.2 (OpenOffice Foundation; www.openoffice.org) and Grace 5.1.12 (Grace Development Team; plasma-gate.weizmann.ac.il/Grace) on Mandrake distribution of Linux (www.mandrake.com). The equations for nonlinear regression analysis were as follows.

Saturation binding experiments. After subtraction of nonspecific binding, eq. 1 was fitted to the data:

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

**Competition-type experiments.** After subtraction of nonspecific binding and normalization (to express the binding of $[^3H]$NMS in the presence of allosteric ligand as percentage of the binding in the absence of allosteric ligand), eq. 2 was fitted to the data:

$$Y = 100 \times \frac{(N + K_d)/(N + K_d \times (K_a + X/(K_a + X \alpha)))}{(K_a + X/(K_a + X \alpha))}$$

(2)

where $Y$ is $[^3H]$NMS binding at concentration of allosteric ligand $X$, $N$ is concentration of $[^3H]$NMS, $K_d$ is the equilibrium dissociation constant obtained from saturation experiment, $K_a$ is apparent equilibrium dissociation constant of allosteric ligand, and $\alpha$ is the cooperativity factor between allosteric ligand and $[^3H]$NMS (Ehrlert, 1988).

**Dissociation experiments.** After subtraction of nonspecific binding and normalization (to express the binding of $[^3H]$NMS at a given time as percentage of the binding at time 0), eq. 3 was fitted to the data:

$$Y = 100 \times e^{(-k_{off} \times X)}$$

(3)

where $Y$ is $[^3H]$NMS binding at time $X$, and $k_{off}$ is rate dissociation constant.

## Results

**Binding of $[^3H]$NMS to Wild-Type Receptors and $M_3$ Mutants.** Plasmids containing cDNA of the $M_3$ receptor were mutated in the region encoding the second (o2) or the third extracellular (o3) loops of the receptor so that the corresponding amino acid residue or residues (Fig. 2) of the $M_3$ receptor were expressed. $M_2$ wild-type ($M_2wt$) and $M_3$ wild-type ($M_3wt$) receptors and all seven mutants of the $M_3$ receptor were transiently expressed in COS-7 cells. Saturation binding experiments with $[^3H]$NMS indicated that all constructs are expressed at a comparable level of 170 to 210 fmol of binding sites per milligram of protein (not shown). Although $[^3H]$NMS is generally considered to be nonselective among muscarinic receptor subtypes, the affinity of $M_2$ receptors for $[^3H]$NMS ($K_d = 0.32 \pm 0.01; n = 6$) expressed in COS-7 cells was approximately 2.4 times higher than affinity of $M_3$ receptor ($K_d = 0.78 \pm 0.04; n = 6$) (Table 1). This is in concert with data obtained using Chinese hamster ovary cells (Buckley et al., 1989; Lyskova et al., 1999) and COS-7 cells (Krejci and Tucek, 2001). Mutation of three ($M_2$KFN) or more residues in the o3 loop of the $M_3$ receptor increased the $K_d$ value for $[^3H]$NMS, and exchange of the complete o3 loop led to a $K_d$ value for $[^3H]$NMS similar to that of the $M_2wt$ receptor. However, exchange of the complete second extracellular loop had no influence on affinity (Table 1). This is in agreement with a previous finding (Krejci and Tucek, 2001).

**Interaction of $[^3H]$NMS and Allosteric Modulators.** We tested four closely related chemicals: strychnine, its dimethoxy derivate brucine, strychnine synthesis precursor WGA, and its derivative propargyl-WGA as allosteric modulators of $[^3H]$NMS binding to $M_2wt$, $M_3wt$, and mutated $M_3$ subtypes of muscarinic receptors (Fig. 1). These compounds displayed a variety of cooperativities with $[^3H]$NMS. Thus, there was relatively strong negative cooperativity on both wild subtypes by propargyl-WGA with weaker negative cooperativity by WGA on both subtypes. In contrast, brucine exhibited weak negative cooperativity on $M_2wt$ and positive cooperativity on $M_3wt$ receptors, whereas strychnine had

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<th>Propargyl-WGA</th>
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<th>$K_d$</th>
<th>$K_a$</th>
<th>$K_{off}$</th>
</tr>
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<td>$M_2wt$</td>
<td>9.11 $\pm$ 0.02</td>
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<td>$M_3wt$</td>
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**Notes:** $[^3H]$NMS was obtained from saturation experiments, and apparent equilibrium dissociation constants ($K_d$) and factors of cooperativity of allosteric modulators were obtained from competition-like experiments. Data represent mean ± S.E.M of four to six or three independent experiments done in triplicates for wild-type and chimeric receptors and for receptors with mutations in the o3 loop, respectively.

† Significantly different from $M_3wt$ and $M_2wt$ ($P < 0.05$; one-way analysis of variance with Kramer-Tukey post test.

P 9.49 3
N 9.47 3
PNVT 9.17 3
ANVT 9.40 3

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positive cooperativity on both receptor subtypes (Table 1; Fig. 3). Tested modulators also differed in affinities for M2wt and M3wt receptors. Propargyl-WGA had 30 times higher affinity for the M3wt receptor than for the M2wt receptor, whereas affinity of WGA was only 2 times higher at the M2 than the M3 receptor. Affinity of brucine was equal at both receptor subtypes, and the affinity of strychnine was slightly (2 times) lower on the M2 than on the M3 subtype (Table 1).

**Effects of Exchange of the Entire o2 or o3 Loop between M2 and M3 Receptors on Affinity and Cooperativity of Allosteric Modulators.** Strychnine displayed positive cooperativity with [3H]NMS on both M2wt and M3wt receptors and had higher affinity for M3wt than for M2wt receptors. Exchange of the entire o2 loop of the M2wt receptor by the M2 sequence (M2o2 chimera) had no effect on the affinity of strychnine and slightly enhanced positive cooperativity between strychnine and [3H]NMS. Replacement of the entire o3 loop of the M3wt receptor with the M2 sequence (M3o2 chimera) also decreased affinity of strychnine below the value of M2wt receptor and enhanced positive cooperativity between strychnine and [3H]NMS. Replacement of the o3 loop of M3wt receptor with the M2 sequence (M3o3 chimera) was significantly stronger than on M2wt receptor (Table 1; Fig. 3A).

Brucine had negative cooperativity with [3H]NMS binding on M2wt but positive cooperativity on M3wt. Both M2wt and M3wt receptors showed the same affinity for brucine. Exchange of the entire o2 loop had no effect either on affinity for brucine or cooperativity between brucine and [3H]NMS. Substitution of the entire o3 loop had no effect on affinity but reversed the negative cooperativity between brucine and [3H]NMS on parental M2wt receptor to positive cooperativity. As for strychnine, resulting positive cooperativity on chimeric M2o3 receptor was even stronger than on M2wt receptor (Table 1; Fig. 3B).

WGA exhibited negative cooperativity with [3H]NMS on both M2wt and M3wt receptors and had 2 times lower affinity for M2wt than for M3wt receptor. Exchange of the o2 loop had no effect on affinity of WGA or on its cooperativity with [3H]NMS. In contrast, exchange of the o3 loop increased affinity for WGA 4 times. Strikingly, the resulting M3o3 chimera exerted positive cooperativity between WGA and [3H]NMS, despite that parent M2wt as well as M2wt receptor demonstrated negative cooperativity. Because the negative cooperativity at the donor M2wt receptor is larger than that at the acceptor M2wt receptor, one would have expected enhanced negative cooperativity of WGA at the M3o3 chimeras (Table 1; Fig. 3C).

Propargyl-WGA showed strong negative cooperativity on both M2wt and M3wt receptors and had more than 30 times lower affinity for M3wt than for M2wt receptor. M2o2 chimera had more than 4 times higher affinity for propargyl-WGA than M3wt receptor. Cooperativity between propargyl-WGA and [3H]NMS on the M2o2 chimera was significantly weaker compared with both M2wt and M3wt receptors. Replacement of the o3 loop of M3 receptor brought about much stronger increase in affinity for propargyl-WGA than exchange of the o2 loop. Affinity of propargyl-WGA was about 40 times higher on M3o3 chimera than on M2wt and about 10 times higher than on M2o2 chimera. However, unlike other tested allosteric modulators, the effect of exchange of the o3 loop on coop-

**Fig. 3.** Modulation of [3H]NMS binding by strychnine (A), brucine (B), WGA (C), and propargyl-WGA (D) in M2wt, M3wt, and chimeric M2o2 and M3o3 receptors. Measurements of [3H]NMS binding and curve fitting were performed as described under Materials and Methods. Ordinate, [3H]NMS binding is expressed as percentage of control binding in the absence of indicated modulator, and each point represents mean ± S.E.M. of four to six independent experiments run in triplicates. Abscissa, concentration in log [M] of indicated allosteric modulator. Symbols denote M2 wild-type receptor (●), M3 wild-type receptor (○), M2o3 chimeric receptor (▼), and M3o2 chimeric receptor (▲). Calculated binding parameters are presented in Table 1. A, replacement of the o2 extracellular loop in M3 receptor slightly increases binding of [3H]NMS in the presence of strychnine compared with M2wt, whereas replacement of the entire o3 extracellular loop results in virtually identical binding curve with M2wt receptor. B, replacement of the second extracellular loop of M3 receptor has no effect on interaction with brucine whereas M3o3 chimeric receptor exhibits strong positive cooperativity that is even about 3 times higher than at M2wt receptor. C, WGA exhibits weak negative cooperativity with [3H]NMS binding at both wild-type receptors. In contrast to M2o2 mutation, which has no effect on the interaction of WGA with [3H]NMS binding, M3o3 chimera displays significant positive cooperativity between WGA and [3H]NMS binding. D, propargyl-WGA interactions compared with M2wt display substantial increase of affinity and decrease of cooperativity at both M2o2 and M3o3 chimeric receptors that in the latter case even exceeds values of M2wt receptor.
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in the β3 loop are propagated to other parts of the receptor where new intramolecular interactions may occur. Spreading of the conformational change is further supported by the decrease of affinity for the orthosteric antagonist [3H]NMS (Table 1) whose binding site is located deep within transmembrane segments. This notion is also consistent with the finding of Lee et al. (1992) who showed that mutation of aspartate 71 in the second transmembrane segment of the M1 receptor, an amino acid far away from the allosteric binding site, decreases affinity for the allosteric modulator gallamine. Similarly, changes of NMS dissociation rate from M2 receptor modified in the second and the third extracellular loops has been reported (Buller et al., 2002; Voigtlaender et al., 2003). Although muscarinic receptors are polytopic membrane proteins consisting of independent domains, the β3 loop cannot be considered an independent domain (Popot and Engelman, 1990; Maggio et al., 1993a,b; Novi et al., 2003). Therefore, observed effects of mutations in the β3 loop on cooperativity and affinity of the allosteric binding site should not be interpreted as indicative of a sole role of the β3 loop.

Our data point to an essential role of the β3 loop in the observed reversal of negative cooperativity between NMS and WGA or brucine. Replacement of the β2 loop in the M3wt receptor does not influence either affinity for WGA or cooperativity of WGA with NMS, or affinity for NMS. In the case of brucine that has the same affinity for both wild receptor subtypes, complete replacement of the β2 or the β3 extracellular loops and any mutations in the β3 loop expectedly do not influence its affinity. Similar to WGA, the exchange of the β2 loop has no influence on moderate negative cooperativity of brucine with NMS at M3wt receptor. Although tested strychnine-like allosteric modulators display both negative and positive cooperativity at M2wt receptor, the transposition of its β3 loop to M3wt receptor in all cases shifts cooperativity in one direction (Fig. 4A), i.e., decreases original negative or increases positive cooperativity. Out of five amino acids that differ between the β3 loops of the two receptors, the most important amino acids of the β3 loop of M2 receptor for this shift to become apparent are asparagine, valine, and threonine. Participation of these residues that are close to the seventh transmembrane domain corroborate the importance of this segment in allosteric interactions, namely, in determining affinity of allosteric modulator binding (Matsui et al., 1995; Buller et al., 2002; Voigtlaender et al., 2003). However, in this context it is worth noting that the pattern of changes of cooperativity between [3H]NMS and strychnine-like allosteric modulators induced by mutations does not overlap with the pattern of their changes of affinity, e.g., gradual decrease of affinity of strychnine is accompanied by increase of cooperativity. Similarly, large increase of cooperativity of brucine is not associated with changes of affinity, and the large decrease of affinity for propargyl-WGA observed between mutants M3DKFN and M3SKFN is not accompanied with change of cooperativity (Fig. 4). This lack of correlation further demonstrates that amino acid residues essential for transmission of conformational change are not necessarily the same as those that form the binding site of allosteric modulators. A common transduction mechanism of cooperativity changes of all tested strychnine-like modulators is further supported by similar pattern of effects of gradual mutations (Fig. 4A).

The divergence of allosteric properties between strychnine, brucine, and WGA on one side and propargyl-WGA on the other side may be due to a difference in the locations to which they bind. Exchange of the entire β2 loop of the M3wt receptor with M2 sequence has no influence on affinity for NMS.
and thus in itself, unlike replacement of the o3 loop, does not induce a conformational change propagated to the orthosteric binding site. This modification, however, increases affinity for propargyl-WGA, hence indicating difference in binding of this allosteric modulator with the o2 loop of M3 and M6 receptors. The important role of the o2 loop of M3 receptor in the affinity for alcuronium, a compound structurally similar to a dimer of propargyl-WGA, and gallamine, a compound structurally dissimilar to tested strychnine-like modulators, was demonstrated (Gnagey et al., 1999; Krejci and Tucek, 2001). Similarly, interaction of these ligands with the o2 and the o3 loop of muscarinic M3 receptor was shown (Jakubik and Weiss, 1999). Involvement of the o2 loop in high-affinity binding of propargyl-WGA on M2 receptor is in line with reported role of the o2 loop together with tyrosine 423 in the o3 loop in high-affinity binding of structurally different caracurine derivatives and alkane-bisammonium compounds to M2 receptor (Buller et al., 2002; Voigtlander et al., 2003). Together, these findings are compatible with and further support the view of muscarinic receptors as proteins with multiple allosteric binding sites (Lazareno et al., 2000; Birdsall et al., 2001).

Results of our experiments seem to be explained most easily on the assumption that an interplay of the o2 and the o3 extracellular loops plays a specific role in a conformational shift induced by binding of tested strychnine-like allosteric modulators and resulting cooperativity with [3H]NMS. WGA and brucine, whose binding to the o2 loop does not differ between M3 wt and M3 wt, display positive cooperativity with NMS at chimeric M3 o3 receptor regardless of cooperativity they have on parent wild-type receptors. Strychnine and propargyl-WGA, whose binding is affected by exchange of the o2 loop, show more positive cooperativity at M3 o3 in comparison with M3 wt when parent receptors have positive cooperativity (strychnine) or less negative cooperativity when parent receptors have negative cooperativity (propargyl-WGA). Replacement of the o2 loop (chimera M3 o2) slightly but significantly increases positive cooperativity of strychnine that is, however, much weaker than its positive cooperativity at M3 o3 chimera. These observations suggest that the interaction of allosteric ligands with the o3 loop that was transposed from M3 wt to create M3 o3 chimera always induces conformational change, manifesting itself as positive cooperativity provided that there is no difference in interaction of allosteric modulator with o2 loop of M3 and M6 receptors as in the case of strychnine, brucine, and WGA. Different binding of propargyl-WGA (exhibiting strong negative cooperativity at parent wild-type receptors) to o2 loop of M3 and M6 receptor impedes this presumed conformational change and results in only reduction of negative cooperativity at M3 o3 chimera. In line with this notion are results of experiments using M3 o2 chimeric receptors. Complete replacement of the o2 loop of the M3 wt receptor with that of M6 wt does not change affinity for strychnine, brucine, and WGA and has little or no effect on cooperativity with NMS. On the other hand, propargyl-WGA displays higher affinity and a decrease in negative cooperativity with NMS at M6 o2 chimera, which is significantly more pronounced than that at M3 o3 chimera (more than 2 times).

In conclusion, the positive cooperativity of studied strychnine-like allosteric modulators with the binding of [3H]NMS is mainly determined by the o3 loop of the receptor, namely, by three amino acids—asparagine, valine, and threonine—located close to the seventh transmembrane domain of M3 wt receptor. Their incorporation into the o3 loop of M6 receptor leads to manifestation of positive cooperativity between WGA and [3H]NMS, despite that both M3 wt and M6 wt receptors exert negative cooperativity. The reversal of original negative cooperativity at M6 wt receptor to positive at M6 o3 chimera is confined to WGA and brucine. Propargyl-WGA that has similarly to chemically closely related WGA negative cooperativity at both wild-type receptors does not exhibit this switch. Our data suggest that different epitopes on the outward oriented surface of muscarinic receptor are involved in binding of different allosteric modulators, whereas structures underlying the mechanism of allosteric interaction between allosteric modulators and orthosteric antagonist are similar. The dissimilarity of WGA and propargyl-WGA in binding to receptor is most likely due to the propargyl group and quaternary nitrogen (Fig. 1) that are apparently involved in interaction with the o2 loop and lead to high affinity for M3 wt. However, differences in chemical structure among other tested strychnine-like modulators responsible for distinct allosteric interactions are not clear. To clarify this issue, more studies of allosteric modulators of various chemical structures and fitting structures of these modulators to a molecular model of muscarinic receptor will be required.

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