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Changes of Cooperativity between *N*-Methylscopolamine and Allosteric Modulators Alcuronium and Gallamine Induced by Mutations of External Loops of Muscarinic M_3 Receptors

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ABSTRACT

To clarify the involvement of specific domains of muscarinic receptors in the action of allosteric modulators, muscarinic M_3 receptors (on which allosteric interactions are weak) were genetically modified to become more similar to M_2 receptors (on which allosteric interactions are strong) and were expressed in COS-7 cells. Affinity for allosteric modulator gallamine was enhanced 25- to 50-fold by modifications of the third external loop (o3) and the negative effect of gallamine on the affinity for classical antagonist *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) was augmented. Affinity for alcuronium became 3-fold higher after the o3 loop of M_3 receptors was made identical with the o3 loop of M_2 receptors, and alcuronium acquired positive influence on the affinity for [^{3}H]NMS. This is the first instance of inducing positive cooperativity on muscarinic receptors by genetic manipulation. Transferring whole o2 loop from M_2 to M_3 receptors substantially enhanced affinities for gallamine and alcuronium without augmenting their negative action on [^{3}H]NMS binding. In contrast, effects of simply adding two negative charges into the o2 loop of M_3 receptors were small. Removal of Arg from o1 loop abolished the negative effect of gallamine but not of alcuronium on [^{3}H]NMS binding at equilibrium. Data point to an important role of o3 loop in the mechanism of the positive and negative cooperativity between [^{3}H]NMS and alcuronium and gallamine, respectively, and in the binding of both modulators to M_2 receptors and reveal independence between mutation-induced changes in the affinity for a modulator and in the magnitude and direction of the allosteric effect of the modulator.

Allosteric modulation of muscarinic receptors has been much investigated (for review, see Tuček and Proška, 1995; Christopoulos et al., 1998; Holzgrabe and Mohr, 1998; Christopoulos, 2000), but the location and amino acid composition of the site(s) with which the allosteric modulators associate is not fully understood. Virtually nothing is known about the mechanism of the conformational changes that the allosteric modulators induce. Most of the known allosteric modulators compete for the same binding domain (Ellis and Seidenberg, 1992, 2000; Waelbroeck, 1994) and this concerns also gallamine and alcuronium (Proška and Tuček, 1995; Lanzafame et al., 1997), the two modulators applied in the present study. The existence of additional allosteric binding site(s) for certain compounds is likely (Lazareno et al., 2000).

To explain the effects of allosteric modulators on the kinetics of the association and dissociation of classical muscarinic ligands, Proška and Tuček (1994) proposed that the binding site for alcuronium, gallamine, and related compounds is located near but more superficially than the binding site for classical ligands. Such location is supported by data obtained in experiments with site-directed mutations of the M_1 to M_4 receptor subtypes (Leppik et al., 1994; Matsui et al., 1995; Gnagey et al., 1999) and with hybrid receptors combining parts of the M_2 and M_5 subtypes (Ellis et al., 1993; Ellis and Seidenberg, 2000), and also by data obtained on covalently modified receptors (Jakubík and Tuček, 1994, 1995).

To learn more about the likely participation of individual receptor domains and amino acid residues in the binding of allosteric modulators and in their cooperative effects, we performed modifications of the gene for the M_3 muscarinic receptor subtype, expressed the modified genes in COS-7 cells, and determined how the modifications affect the affinities of receptors for gallamine and alcuronium and the cooperativities between the binding of gallamine and alcuronium and of the orthosteric muscarinic antagonist *N*-methylscopolamine (NMS). The modifications that we performed concerned the first, second and third external loops (o1–o3) of the M_3 receptors.

Among the five muscarinic receptor subtypes (M_1–M_5), the M_2 subtype has the highest affinity for gallamine and alcuronium, whereas the affinities of the M_3 subtype for these modulators are low (Ellis et al., 1991; Jakubík et al., 1995). An important difference between the M_2 and M_3 subtypes consists in that the M_2 subtype (but not the M_3 subtype)

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ABBREVIATIONS: NMS, *N*-methylscopolamine; PCR, polymerase chain reaction.

displays positive cooperativity between the binding of [^{3}H]NMS and alcuronium (Tuček et al., 1990; Jakubík et al., 1995; Dong et al., 1995). We concentrated on the amino acids that are different between the M_3 and M_2 receptor subtypes (see Fig. 1 for comparison of amino acid sequences in the external loops of the two subtypes). Most of the modifications were designed to make the M_3 receptor more similar to the M_2 subtype, in the hope that this would help to reveal the domains and amino acid residues that are responsible for the high affinity of the M_2 receptors for the allosteric modulators or important for the allosteric control of the orthosteric binding site. Two modifications were designed to remove a positive charge from the external surface of the receptor, in the expectation that this might eliminate an obstacle hindering the association of positively charged modulators with the receptor. Two of the modifications made the amino acid sequence of the whole o2 loop (mutation designated o2M_2) or o3 loop (mutation designated DSKFN) of the engineered M_3 receptors completely identical with the amino acid sequence of wild-type M_2 receptors. We explored whether a change in the affinity for the modulator was necessarily accompanied by a similar change in the allosteric efficacy of that modulator (i.e., in the strength of its cooperative effect on the receptor's affinity for a classical ligand) and whether it was possible to generate in the M_3 receptors the positive cooperativity between alcuronium and NMS.

Experimental Procedures

Materials. *N*-[methyl-^{3}H]methylscopolamine ([^{3}H]NMS) was from Amersham Pharmcia Biotech (Little Chalfont, Buckinghamshire, UK), alcuronium was kindly provided by Hoffmann-La Roche (Basel, Switzerland), gallamine was from Sigma (Prague, Czech Republic).

Mutagenesis and Expression. Wild-type genes for human muscarinic M_2 and M_3 receptors incorporated into pCD vectors (kindly provided by Professor N. Buckley, University of Leeds, Leeds, UK) were used. DNA sequences of these genes are deposited in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers M16404 (Bonner et al., 1987) and U29589 (Bonner et al., 1988). Single-point (R133G, K213T, P217D, K523N), two-point (P217D+P218E) and three point [KFN (i.e., K523N+F525V+N527T)] mutations of the M_3 receptor were performed on whole plasmids by PCR with Pfu Turbo polymerase, using Stratagene QuikChange site directed mutagenesis kit according to the producer's instructions (Stratagene, La Jolla, CA). HPLC-purified primers (37–50 bases, 1–4 mismatches) were obtained from VBC Genomics (Vienna, Austria). PCR consisted of 17 cycles at 95°C (30 s), 55°C (1 min), and 68°C (13 min), and Dpn I was applied to remove the unmutated template (2 h at 37°C). XL-1 blue strain of *Escherichia coli* was used for heat-shock transformation with the PCR mixture. Plasmids originating from a single ampicillin resistant colony were isolated using QIAGEN Mini or Midi purification kits (QIAGEN, Chatsworth, CA), sequenced, and used for transfection.

To obtain M_3 genes with five mutated amino acid residues in the o3 loop (DSKFN mutation, D518A+S519P+ K523N+F525V+N527T), a two-step procedure was applied. In the first step, we obtained the three-point KFN mutant described above, after which we performed the additional two-point (D518A+S519P) mutation.

The substitution of the o2 loop of the M_2 receptor for the o2 loop of the M_3 receptor (the o2M_2 mutation) involved several steps. 1) A NarI restriction site was introduced in the M_3 gene in positions coding for W^{200}-A^{201}-P^{202} (base positions 801–806). This was a silent mutation and did not require subsequent repair. 2) A *Bgl*II restriction site was introduced in the M_3 gene within the sequence coding for Thr^{230}-Ile^{231}-Thr^{232} (base positions 891–897). This change required subsequent repair. 3) The o2 loop from the mutated M_3 vector was excised with *Nar*I and *Bgl*II restriction enzymes and the vector was purified in agarose gel. 4) Part of the M_2 gene coding for the o2 loop (including codons for amino acids in positions 158–184) was amplified by PCR using Pfu Turbo polymerase and primers that simultaneously introduced restriction sites for *Nar*I and *Bgl*II in the 5′ and 3′ ends, respectively, of the amplified segment. The amplified segment was trimmed by incubation with *Nar*I and *Bgl*II, purified by polyacrylamide gel electrophoresis, and used for overnight ligation into the previously cut and purified M_3 vector (see 3 above) using T4 DNA ligase (USB). 5) After transformation and ampicillin selection of XL-1 blue *E. coli* strain, plasmids originating from a single ampicillin resistant colony were checked by sequencing. 6) The *Bgl*II restriction site at the 3′ end of the o2 loop was then corrected by PCR using the Stratagene Quikchange kit as described above with primers corresponding partly to M_2 and partly to M_3 constructed to add two mutations in the beginning of the fifth transmembrane segment, namely T230A and I231V.

All mutated DNAs were sequenced with the dideoxy method by Dr. J. Felsberg (Academy of Sciences, Institute of Microbiology, Prague, Czech Republic) or by Generi Biotech Co. (Hradec Králové, Czech Republic).

COS-7 cells were transfected with the use of DEAE-dextran method. They were grown on Petri dishes (2×10^6 cells per 10-cm dish) in Iscove's modified Dulbecco's medium (Sigma) with 10% fetal calf serum. After washing with phosphate-buffered saline, the transfection mix (2 μg of plasmid and 5.5 mg of DEAE-dextran in 0.9 ml of phosphate-buffered saline) was applied for 2 h. Iscove's modified Dulbecco's medium with 10% fetal calf serum and 10 μM chloroquine was then added for an additional 4 h. After that, fresh medium was applied. Cells were harvested 72 h after transfection.

Sequences of external loops in human M_2 and M_3 subtypes of muscarinic receptors

o1																								
M_2	T^{81}	L	Y	T	V	I	G	Y	W	P	L	G	P	V	V	C	D^{97}							
M_3	T^{126}	T	Y	I	I	M	N	R	W	A	L	G	N	L	A	C	D^{142}							
o2																								
M_2	Q^{163}	F	I	V	G	V	R	T	V	E	D	G	E	C	Y	I	Q	F	F	S	N	A^{184}	A	V
M_3	Q^{208}	Y	F	V	G	K	R	T	V	P	P	G	E	C	F	I	Q	F	L	S	E	P^{229}	T	I
o3																								
M_2	N^{409}	T	F	C	A	P	C	I	P	N	T	V	W	T^{423}										
M_3	N^{514}	T	F	C	D	S	C	I	P	K	T	F	W	N^{527}										

Fig. 1. Sequences of amino acids in the external loops (o1–o3) of the M_2 and M_3 subtypes of human muscarinic receptors. Data are taken from Bonner et al. (1988); the first two amino acids of the fifth transmembrane segment (as delineated by Bonner et al.) have been added to the o2 sequence because they had been also changed in the M_3 receptors by the o2M_2 mutation, as described in the text.

Radioligand Binding Experiments. Experiments with radioligand binding were performed on suspensions of freshly harvested COS-7 cells or on homogenates of these cells using procedures described previously (Proška and Tuček, 1994; Jakubík et al., 1995). The medium used for homogenization (Ultra-Turrax) and for incubation consisted of 100 mM NaCl and 20 mM Na-HEPES, pH 7.4. Incubations performed at 25°C were terminated by filtration through Whatman GF/B glass fiber filters in a Brandel filtration apparatus. Nonspecific binding was determined in the presence of 5 μM atropine.

The affinity of wild-type and mutated muscarinic receptors for [^{3}H]NMS was measured in saturation binding experiments on homogenates (3 h incubation with [^{3}H]NMS at concentrations corresponding to 0.125- to 8-fold of the anticipated K_d value), and expressed as K_{NMS} (equilibrium dissociation constant for the binding of [^{3}H]NMS). The affinities of receptors for gallamine and alcuronium were determined in pseudocompetitive experiments (Ehlert, 1988) in which suspended cells were incubated in the presence of a fixed concentration of [^{3}H]NMS (corresponding to the K_{NMS} determined for each particular mutant) and increasing concentrations (mostly $10^{-6.5}$–$10^{-3.5}$ M) of the allosteric modulator. The modulator was added after 1 h preincubation with [^{3}H]NMS and the incubation was continued for 20 h to achieve full equilibration. Values for K_A (equilibrium dissociation constant for the binding of the allosteric ligand to free receptors) and cooperativity factor α (corresponding to the ratio of K_{NMS} values in the presence and the absence of the allosteric modulator) were computed according to Ehlert's (1988) equation 6 with the use of GraphPad Prism (version 3.0; GraphPad, San Diego, CA).

The influence of gallamine and alcuronium on the rate of [^{3}H]NMS dissociation was examined in a procedure modified from Lysíková et al. (1999). Cell homogenates were preincubated for 2 h with [^{3}H]NMS at a concentration corresponding to twice the K_{NMS} value for each particular mutant. Dissociation was induced by adding 5 μM atropine alone or 5 μM atropine simultaneously with 10^{-7}–10^{-3} M gallamine or alcuronium. It was stopped after intervals of 5 to 30 min (selected to achieve 65 to 90% dissociation) and k_{off} values based on single-point determinations were computed for each concentration of the allosteric modulator. The pK_{diss} values (corresponding to the negative logarithm of the molar concentration of the allosteric modulator which caused a 50% decrease of the k_{off} determined in the absence of the allosteric modulator) were computed as described previously (Lysíková et al., 1999).

Results

Binding Properties of Wild-Type Muscarinic M_3 Receptors and the Effect of R133G Mutation in Their First Extracellular Loop. The binding properties of wild-type muscarinic M_3 receptors expressed in COS-7 cells were similar to those of the M_3 receptors expressed in other types of cells. They had high affinity for [^{3}H]NMS (K_{NMS} = 0.29 nM) and the binding of [^{3}H]NMS became diminished in the presence of muscarinic allosteric modulators gallamine (α = 4.3) and alcuronium (α = 2.6) (Table 1). Both gallamine and alcuronium slowed the rate of [^{3}H]NMS dissociation. The value of pK_{diss} for gallamine (3.91), representing the affinity of gallamine for receptors occupied by NMS, was clearly lower than the corresponding pK_A value for gallamine (4.7), representing the affinity of gallamine for free receptors. The difference of 0.79 on a logarithmic scale suggested a 6.2-fold difference in affinity, which should be compared with the α value of 4.3 determined in experiments with [^{3}H]NMS binding at equilibrium. In the case of alcuronium, the difference between the values of pK_{diss} and pK_A was small (3.70 versus 3.9), suggesting a 1.6-fold difference between the affinity of alcuronium for NMS-occupied and free receptors. This should be compared with the α value of 2.6 determined in experiments with [^{3}H]NMS binding at equilibrium.

Replacement of arginine in position 133 (close to the center of the o1 external loop) by glycine (R133G mutation) diminished the affinity for [^{3}H]NMS less than 2-fold. It had virtually no effect on the allosteric interaction between alcuronium and [^{3}H]NMS but profound effect on the interaction between gallamine and [^{3}H]NMS because the binding of

TABLE 1

Effects of genetic modifications on the binding properties of muscarinic M_3 receptors

Data are means ± S.E.M. of three to nine experiments; individual results have been shown where $n < 3$.

	[^{3}H]NMS		Gallamine			Alcuronium		
	K_d	k_{off}	pK_{diss}	pK_A	α	pK_{diss}	pK_A	α
	nM	*min^{-1}*	*M*			*M*		
Wild-type M_3	0.29 ± 0.006	0.05 ± 0	3.91 ± 0.02	4.7 ± 0.08	4.3 ± 0.6	3.70 ± 0.04	3.9 ± 0.09	2.6 ± 0.3
Wild-type M_2	0.75 ± 0.05*	0.34 ± 0.02*	4.80 ± 0.19*	6.4 ± 0.03*	11 ± 0.4*	6.90 ± 0.10*	6.1 ± 0.2*	0.3 ± 0.05*
Modifications in o1 of M_3								
R133G	0.49 (0.50; 0.48)	0.07 ± 0.002*	4.44 ± 0.08*	NM	Close to 1#	3.71 ± 0.18	3.9 ± 0.03	1.8 ± 0.2
Modifications in o2 of M_3								
K213T	0.33 (0.32; 0.34)	0.05 ± 0.002	4.01 ± 0.01	4.5 ± 0.18	2.9 ± 0.4	3.79 ± 0.05	4.1 ± 0.08	1.6 ± 0.03
P217D	0.31 (0.31; 0.31)	0.06 ± 0.002*	4.30 ± 0.04*	4.8 ± 0.01	4.2 ± 1.0	4.03 ± 0.15	4.1 ± 0.05	2.4 ± 0.5
P217D + P218E	0.39 (0.39; 0.39)	0.06 ± 0.002*	4.23 ± 0.06*	4.6 ± 0.06	4.0 ± 0.8	3.95 ± 0.02*	4.2 ± 0.03	2.1 ± 0.4
o2M_2	0.27 ± 0.03	0.06 ± 0.003*	4.75 ± 0.04*	5.8 ± 0.05*	4.8 ± 0.5	4.72 ± 0.01*	4.8 ± 0.04*	3.1 ± 0.8
Modifications in o3 of M_3								
K523N	0.65 (0.65; 0.66)	0.15 ± 0.003*	4.54 ± 0.003*	6.1 ± 0.06*	16.0 ± 1.9*	4.69 ± 0.04*	4.7 ± 0.20*	1.8 ± 0.3
KFN	0.49 ± 0.11	0.22 ± 0.02*	4.12 ± 0.07*	6.4 ± 0.17*	46.5 ± 12.0*	4.72 ± 0.12*	NM	Close to 1#
DSKFN	0.74 ± 0.12*	0.21 ± 0.01*	4.21 ± 0.04*	6.3 ± 0.11*	25.1 ± 3.8*	4.67 ± 0.07*	4.4 ± 0.12*	0.3 ± 0.03*

*, Significantly different from wild-type M_3 ($p < 0.05$ by Student's two-tailed t test); #, Although clearly different from wild-type M_3, significance could not be evaluated numerically because of flat binding curves.

NM, not measurable because the binding curves were flat.

[^{3}H]NMS at equilibrium was no longer diminished by gallamine (Table 1, Fig. 2). Therefore, it was impossible to determine the pK_A value for gallamine in equilibrium binding experiments. A slight inhibitory effect of alcuronium on the binding of [^{3}H]NMS persisted. The loss of the allosteric inhibition of [^{3}H]NMS binding by gallamine was not caused by a loss of gallamine association with the mutated receptors. This is evident because gallamine (as well as alcuronium) preserved its inhibitory action on the rate of [^{3}H]NMS dissociation, and the affinity of gallamine for receptors occupied by NMS was even higher on the R133G than on the wild-type receptors (pK_{diss} values of 4.44 and 3.91, respectively), apparently reflecting the loss of negative cooperativity between NMS and gallamine in the mutant.

Modifications of the Second Extracellular Loop. Four types of modifications have been performed (Table 1, Fig. 3): 1) replacement of lysine 213 by threonine (K213T mutation); 2) replacement of proline 217 by aspartate (P217D mutation); 3) simultaneous replacement of proline 217 by aspartate and of proline 218 by glutamate (P217D+P218E mutation); 4) total replacement of the o2 loop of the M_3 receptor by the corresponding portion of the M_2 receptor, associated with the mutation of the first two amino acids of the fifth transmembrane segment (o2M_2 mutation). Because of the o2 M_2 mutation, the amino acid sequence in positions 199 to 241 of the mutated M_3 receptor became identical with the amino acid sequence of the corresponding portion of the wild-type M_2 receptor (positions 154–196). Specifically, this involved changes of the following amino acids: Y209F, F210I, K213V, P217E, P218D, F222Y, L226F, E228N, P229A, T230A, and I231V.

Neither the replacement of the positively charged lysine by neutral threonine (K213T) nor the introduction of one negative charge (P217D) or two negative charges (P217D+P218E) into the o2 loop brought about any substantial change in the affinities of unoccupied receptors for [^{3}H]NMS, gallamine, or alcuronium, as determined in equilibrium binding experiments. The P217D and P217D+P218E mutants displayed a slightly faster dissociation of [^{3}H]NMS and slightly higher affinity for gallamine as determined according to its effect on [^{3}H]NMS dissociation.

On the other hand, the difference between the o2M_2 mutant and the parent M_3 receptor was striking. The affinity for gallamine was 13-fold higher (pK_A 5.8 versus 4.7) on free and 7-fold higher (pK_{diss} 4.75 versus 3.91) on NMS-occupied o2M_2 mutant receptors, compared with the wild-type M_3 receptor. The affinities for alcuronium were similarly enhanced. In contrast, the affinity for [^{3}H]NMS was not significantly affected consequent to mutation, and the extent of the allosteric interactions between gallamine and NMS and alcuronium and NMS (as evaluated by the cooperativity factors α) was not affected.

Modifications of the Third Extracellular Loop. Three modifications have been performed (Table 1, Fig. 4): 1) replacement of lysine 523 by asparagine (K523N mutation); 2) combined replacement of lysine 523 by asparagine, phenylalanine 525 by valine, and asparagine 527 by threonine (KFN mutation); 3) combined replacement of aspartate 518 by alanine, serine 519 by proline, lysine 523 by asparagine, fenylalanine 525 by valine, and asparagine 527 by threonine (DSKFN mutation). All newly introduced amino acid residues corresponded to those that are present in matching positions of the M_2 muscarinic receptor subtype. In the case of DSKFN mutation, the amino acid sequence of the entire third external loop (o3) of the M_3 receptor (from N514 to N527) became identical with that of the corresponding segment of the M_2 receptor (positions 410–423 in M_2 receptor).

Replacement of lysine 523 by asparagine diminished the affinity for [^{3}H]NMS and accelerated [^{3}H]NMS dissociation. At the same time, it strongly enhanced the affinity of free receptors for gallamine (shift of pK_A from 4.7 to 6.1, corresponding to a 25-fold increase in affinity). The affinity of free receptors for alcuronium was also enhanced (shift of pK_A from 3.9 to 4.7, corresponding to 6.3-fold increase in affinity). The negative allosteric effect gallamine on the binding of [^{3}H]NMS became much stronger in the mutated receptor (shift of α from 4.3 to 16.0), whereas the negative allosteric effect of alcuronium was very low (shift of α from 2.6 to 1.8).

Both KFN and DSKFN mutations diminished the affinity of receptors for [^{3}H]NMS and accelerated [^{3}H]NMS dissociation. In KFN mutants, the affinity for gallamine was enhanced by 50-fold (pK_A 6.4 versus 4.7) and the negative effect of gallamine on the binding of [^{3}H]NMS became much stronger (α of 46.5 versus 4.3). On the other hand, alcuronium lost its inhibitory effect on [^{3}H]NMS binding in KFN mutants, and the flat concentration-response curves did not allow determination of the affinity of free receptors for alcuronium. The ability of alcuronium to decelerate [^{3}H]NMS dissociation from the KFN mutants was preserved, however, and the affinity of alcuronium for the NMS-occupied M_3 receptors with KFN mutation was 10-fold higher than that for the wild-type M_3 receptors.

The effects of DSKFN mutation were similar to those of the KFN mutation with regard to interactions involving gallamine. The affinity for alcuronium was 9.3-fold higher on NMS-occupied receptors with DSKFN mutation than on

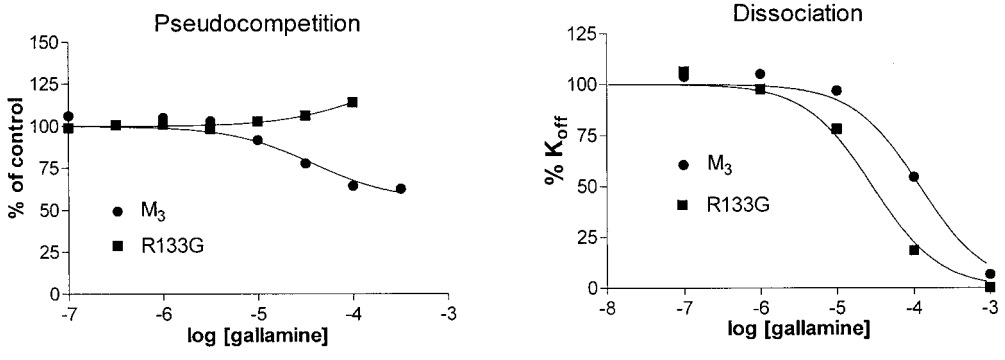

Fig. 2. Effects of gallamine on the binding of [^{3}H]NMS to wild-type M_3 receptors and their R133G mutant at equilibrium (left) and on the rate of [^{3}H]NMS dissociation from these receptors (right). Abscissa, concentration (log M) of gallamine. Left ordinate, binding in the presence of gallamine as percentage of binding in its absence. Right ordinate, k_{off} in the presence of gallamine as percentage of k_{off} in its absence. Shown are representative experiments which have been repeated three times with similar results (see Table 1).

NMS-occupied wild-type M_3 receptors (pK_{diss} of 4.67 versus 3.70), and 3.2-fold higher on free receptors with DSKFN mutation than on free wild-type receptors (pK_A of 4.4 versus 3.9). However, the direction of the allosteric interaction between alcuronium and [^{3}H]NMS was reversed from negative to positive after DSKFN mutation and the affinity for [^{3}H]NMS became enhanced (rather than diminished) by alcuronium on DSKFN mutants (α = 0.3).

To obtain additional evidence that positive cooperativity between alcuronium and [^{3}H]NMS does indeed occur in M_3 receptors with DSKFN mutation, we determined the effect of alcuronium not only in 'competition-type' experiments, ac-

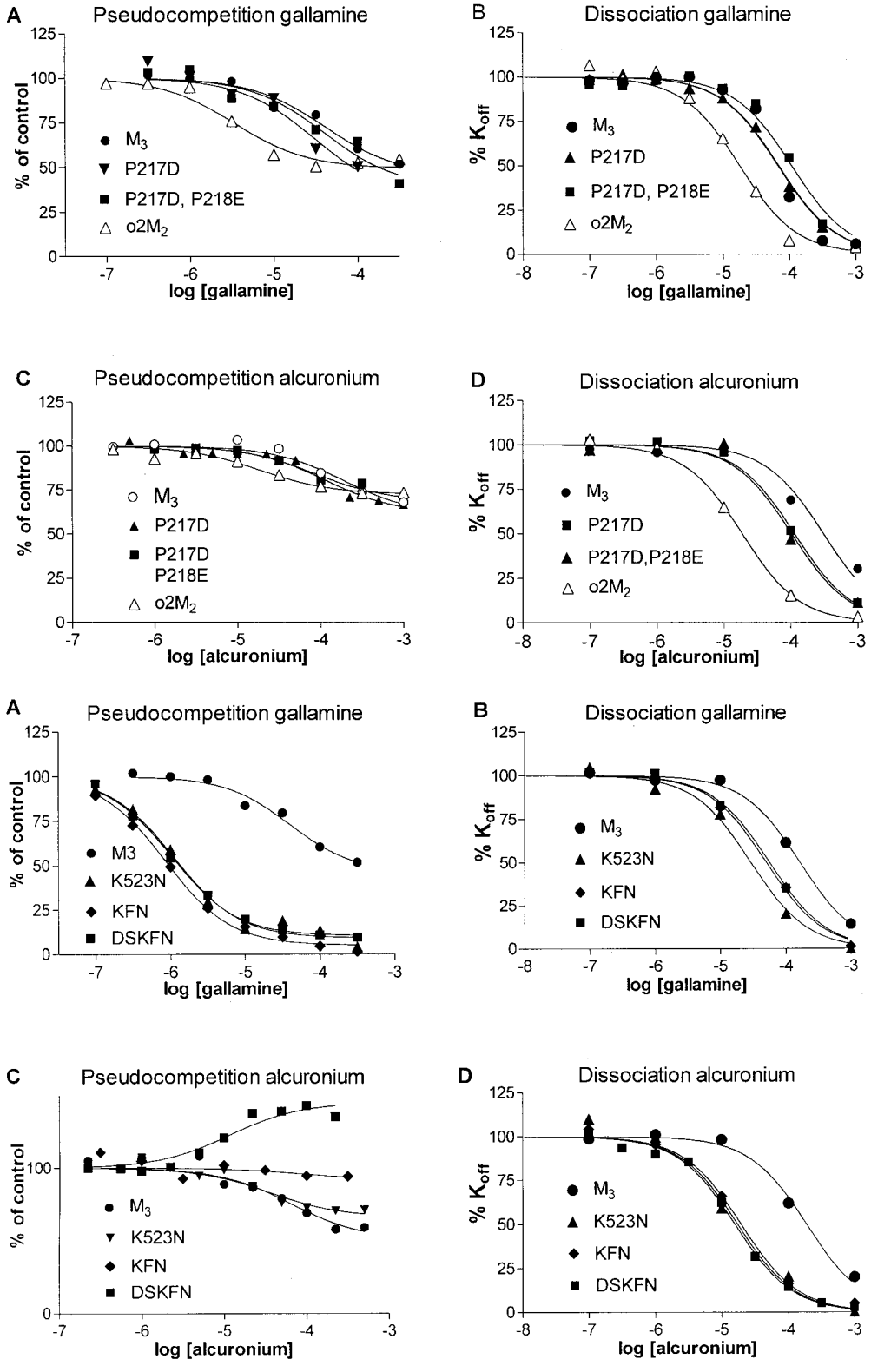

Fig. 3. Effects of gallamine (A, B) and alcuronium (C, D) on the binding of [^{3}H]NMS to wild-type M_3 receptors and their P217D, P217D+P218E and o2 M_2 mutants at equilibrium (A, C) and on the rate of [^{3}H]NMS dissociation from these receptors (B, D). Abscissa, concentration (log M) of gallamine (A, B) and alcuronium (C, D). Ordinates in A and C, binding in the presence of gallamine or alcuronium as percentage of binding in their absence. Ordinates in B and D, k_{off} in the presence of gallamine or alcuronium as percentage of k_{off} in their absence. Shown are representative experiments that have been repeated three to five times with similar results (see Table 1).

Fig. 4. Effects of gallamine (A, B) and alcuronium (C, D) on the binding of [^{3}H]NMS to wild-type M_3 receptors and their K523N, KFN, and DSKFN mutants at equilibrium (A, C) and on the rate of [^{3}H]NMS dissociation from these receptors (B, D). Abscissa, concentration (log M) of gallamine (A, B) and alcuronium (C, D). Ordinates in A and C, binding in the presence of gallamine or alcuronium as percentage of binding in their absence. Ordinates in B and D, k_{off} in the presence of gallamine or alcuronium as percentage k_{off} in their absence. Shown are representative experiments that have been repeated three to six times with similar results (see Table 1).

cording to Ehlert (1988) (i.e., in binding experiments with a fixed concentration of [^{3}H]NMS and increasing concentrations of alcuronium), but also in 'Scatchard-type' saturation binding experiments (i.e., in binding experiments with increasing concentrations of [^{3}H]NMS, performed in the absence and the presence of a fixed concentration of alcuronium). On homogenates from four different transfections, the mean K_{NMS} value (±S.E.M.) was 0.65 ± 0.06 nM in the absence of alcuronium and 0.33 ± 0.05 nM in the presence of 100 μM alcuronium ($p = 0.002$ by Student's paired t test), whereas the B_{max} value was not altered by alcuronium.

Discussion

An approach frequently used in studies of binding sites consists in replacing potentially important amino acid residues and measuring the resulting *loss* of the binding affinity. With regard to the allosteric binding sites on muscarinic receptors, such approach has been applied by Leppik et al. (1994), who demonstrated the importance of negatively charged amino acid residues in the o2 loop of M_2 receptors, and by Matsui et al. (1995), who revealed the roles of two tryptophan residues in M_1 receptors. Our approach was different in that we looked for amino acid residues that might *enhance* the affinity for allosteric modulators. We have chosen such amino acids that might make the molecule of the low-affinity (with regard to gallamine and alcuronium) M_3 receptor more similar to the high-affinity M_2 receptor. A similar principle has been applied by Ellis et al. (1993) and Ellis and Seidenberg (2000), who investigated which segments of the M_2 receptor induce high affinity for gallamine and other modulators when inserted into the low-affinity M_5 receptor. The work by Ellis and colleages concentrated on determinations of the potency with which the modulators decelerated NMS dissociation from receptors, providing information on changes in the affinity of NMS-occupied receptors for allosteric modulators. We investigated the effects of allosteric modulators both at equilibrium and during NMS dissociation, which enabled us to see not only whether the mutations altered the affinity of the receptor for the modulator, but also whether they affected the allosteric interaction between the modulator and the classical ligand (NMS).

Technically, determinations of the effect of allosteric modulators of muscarinic receptors on equilibrium binding of classical ligands are connected with two difficulties. 1) Because the allosteric modulators slow the association and the dissociation of classical ligands, binding equilibrium is approached only after long incubations at high concentrations of the modulators. We used 20-h incubations for equilibration. 2) Affinities for allosteric modulators are easy to determine when their allosteric efficacy is high and the concentration-response curves are steep, as is the case with alcuronium or gallamine acting on the M_2 receptors (e.g., Proška and Tuček, 1995). The accuracy with which the binding parameters are determined is lower on the non-M_2 subtypes, where the concentration-response curves are flat. Minor inaccuracies associated with these two difficulties tend to impair quantitative fit between the predictions of the allosteric model of antagonist-receptor-modulator interaction (Ehlert, 1988; Lazareno and Birdsall, 1995) and the parameters computed from experimental data.

Among the observations described under *Results*, the following seem to be of particular interest:

Mutations in the o3 Loop Substantially Enhanced the Affinity of Free M_3 Receptors for Both Gallamine and Alcuronium. The affinity for gallamine was enhanced 25-fold after a simple replacement of Lys^{523} by Asn (K523N mutation) and 50-fold after the complex DSKFN mutation, which made the sequence of the entire o3 loop of the M_3 subtype identical to that of the M_2 subtype. Our finding concerning the effect of K523N mutation on the affinity of free M_3 receptors for gallamine agrees with the observation made by Gnagey et al. (1999) with regard to M_3 receptors occupied by NMS. Although we cannot completely exclude the possibility that the enhancement of the affinity of M_3 receptors for alcuronium and gallamine consequent to K523N mutation was caused by the removal of the positive charge on lysine, it seems more likely to us that the insertion of asparagine into this particular position in the o3 loop was the decisive factor. This is suggested by the finding of Gnagey et al. (1999) that the affinity of the NMS-occupied M_5 receptors for gallamine was similarly enhanced by an analogous mutation substituting asparagine for valine. It is likely that the corresponding domain of the M_2 receptors (including their Asn^{419}) plays an important role in the binding of both gallamine and alcuronium to the M_2 subtype. It is worth noting, however, that the high affinity of the M_2 receptors for gallamine was further enhanced 4-fold by their N419D mutation (Gnagey et al., 1999).

Positive Cooperativity between Alcuronium and [^{3}H]NMS Occurred on the M_3 Receptors Modified So That Their o3 Loop Became Identical with That of the M_2 Receptors. So far, the nature of the conformational change of the receptor that underlies the allosteric enhancement of its affinity for a classical ligand is not at all understood. The DSKFN mutation of the M_3 receptor represents the first instance in which positive cooperativity has been "created" on a muscarinic receptor by genetic modification. The same mutation also remarkably enhanced the negative cooperativity between gallamine and NMS. These findings point to a direct involvement of the o3 loop in the intimate mechanism of both the positive and the negative cooperativity. Contrary to our results with the o3 loop, the transfer of the o2 loop from M_2 to M_5 receptors (Ellis and Seidenberg, 2000) or to M_3 receptors (present experiments) failed to reproduce the positive cooperative action of alcuronium in the engineered receptors.

An Increase in the Affinity of Receptors for Gallamine or Alcuronium Was Not Always Accompanied by an Increase in the Allosteric Efficacy of the Modulators. Replacement of the whole o2 loop of the M_3 receptors by the o2 loop of the M_2 receptors (o2M_2 mutation) enhanced the affinity of free receptors for gallamine by 13-fold and that for alcuronium by 8-fold, but the strength of the negative allosteric effect of the modulators on the receptors' affinity for [^{3}H]NMS did not change. This contrasts with the simultaneous enhancement of both the affinity for gallamine and the negative allosteric effect of gallamine induced by the DSKFN mutation. The observations indicate that there is no direct relation between the affinity and the allosteric efficacy of an allosteric modulator.

Increasing the Number of Negative Charges in the o2 Loop Did Not Enhance the Affinity of M_3 Receptors for Allosteric Modulators, but a Total Replacement of the o2 Loop by That from the M_2 Receptors Did. Leppik

et al. (1994) demonstrated that the presence of the highly acidic EDGE sequence in the o2 loop of M_2 receptors (positions 172–175) is necessary for their high affinity for gallamine. Our expectation that adding one or two negatively charged amino acid residues into the corresponding positions in the o2 loop of M_3 receptors (P217D and P217D+P218E mutations) would enhance the affinity of M_3 receptors for gallamine and alcuronium has not been borne out by experiments. On the other hand, total substitution of the o2 loop from M_2 receptors for the o2 loop of M_3 receptors was accompanied by 8- to 13-fold increases in the affinities for both modulators. This discrepancy indicates that, in addition to the presence of three acidic residues in the EDGE sequence, other features in the o2 loop of the M_2 receptors contribute to the high affinity of M_2 receptors for allosteric modulators.

Mutating Arg^{133} in the o1 Loop to Gly (Mutation R133G) Abolished the Negative Effect of Gallamine on the Binding of [^{3}H]NMS without Abolishing the Binding of Gallamine. The R133G and K213T mutations of the o1 and o2 loops, respectively, had been performed to determine whether the positive charges on the respective residues of arginine and lysine diminish the affinity of the M_3 receptors for gallamine and alcuronium, presumably by repelling their positively charged molecules. The K213T mutation had no effect and the R133G mutation did not change the relation between the receptor and alcuronium. It did, however, abolish the negative cooperative effect of gallamine on the binding of [^{3}H]NMS. This was not due to a loss of gallamine binding, because gallamine continued to decelerate the dissociation of [^{3}H]NMS from the mutated receptor. A similar loss of a positive cooperative interaction had been observed by Jakubík and Tuček (1995) in experiments with alcuronium on cardiac M_2 receptors, as a result of a treatment with dithiothreitol, presumably disrupting the disulfide bond between Cys^{105} and Cys^{176} in the o1 and o2 loops, respectively. The decelerating effect of alcuronium on the dissociation of [^{3}H]NMS also remained preserved in those experiments. These observations confirm that there is considerable independence between the effects of mutations on the binding and the allosteric efficacy of the modulators. The role of the o1 loop in cooperative interactions between ligands of muscarinic receptors requires additional investigation.

In conclusion, our data point to the involvement of the o3 loop of muscarinic M_2 receptors in the intimate mechanism of positive cooperativity between alcuronium and NMS and of negative cooperativity between gallamine and NMS, to important roles for both the o2 and o3 external loops in the binding of gallamine and alcuronium, and to a role for the o1 loop in the negative cooperativity between gallamine and NMS.

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References

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.
Bonner TI, Young AC, Brann MR, and Buckley NJ (1988) Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1:**403–410.
Christopoulos A (2000) Overview of receptor allosterism, in *Current Protocols in Pharmacology* (Enna SJ, Williams M, Ferkany JW, Kenakin T, Porsolt RD, Sullivan JP eds) pp 21.1–1.21.45, Wiley, New York.
Christopoulos A, Lanzafame A, and Mitchelson F (1998) Allosteric interactions at muscarinic cholinoceptors. *Clin Exp Pharmacol Physiol* **25:**185–194.
Dong GZ, Kameyama K, Rinken A, and Haga T (1995) Ligand binding properties of muscarinic acetylcholine receptor subtypes (m1–m5) expressed in baculovirus-infected insect cells. *J Pharmacol Exp Ther* **274:**378–384.
Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol Pharmacol* **83:**187–194.
Ellis J and Seidenberg M (1992) Two allosteric modulators interact at a common site on cardiac muscarinic receptors. *Mol Pharmacol* **42:**638–641.
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.
Ellis J, Huyler J, and Brann MR (1991) Allosteric regulation of cloned m1–m5 muscarinic receptor subtypes. *Biochem Pharmacol* **42:**1927–1932.
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.
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.
Holzgrabe U and Mohr K (1998) Allosteric modulators of ligand binding to muscarinic acetylcholine receptors. *Drug Res Today* **3:**214–222.
Jakubík J and Tuček S (1994) Protection by alcuronium of muscarinic receptors against chemical inactivation and location of the allosteric binding site for alcuronium. *J Neurochem* **63:**1932–1940.
Jakubík J, and Tuček S (1995) Positive allosteric interactions on cardiac muscarinic receptors: efects of chemical modifications of disulphide and carboxyl groups. *Eur J Pharmacol* **289:**311–319.
Jakubík J, Bačáková L, El-Fakahany EE, and Tuček S (1995) Subtype selectivity of the positive allosteric action of alcuronium at cloned M1–M5 muscarinic acetylcholine receptors. *J Pharmacol Exp Ther* **274:**1077–1083.
Lanzafame L, Christopoulos A, and Mitchelson F (1997) Three allosteric modulators act at a common site, distinct from that of competitive antagonists, at muscarinic acetylcholine M_2 receptors. *J Pharmacol Exp Ther* **282:**278–285.
Lazareno S and Birdsall NJM (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.
Lazareno S, Popham A, and Birdsall JM (2000) Allosteric interactions of staurosporine and other indolocarbazoles with *N*-[methyl-^{3}H]scopolamine and acetylcholine at muscarinic receptor subtypes: identification of a second allosteric site. *Mol Pharmacol* **58:**194–207.
Leppik RA, Miller RC, Eck M, and Paquet JL (1994) Role of acidic amino acids in the allosteric modulation by gallamine of antagonist binding at the m2 muscarinic acetylcholine receptor. *Mol Pharmacol* **45:**983–990.
Lysíková M, Fuksová K, Elbert T, Jakubík J, and Tuček S (1999) Subtype-selective inhibition of [methyl-^{3}H]-*N*-methylscopolamine binding to muscarinic receptors by alpha-truxillic acid esters. *Br J Pharmacol* **127:**1240–1246.
Matsui H, Lazareno S, and Birdsall NJ (1995) Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. *Mol Pharmacol* **47:**88–98.
Proška J and Tuček S (1994) Mechanisms of steric and cooperative actions of alcuronium on cardiac muscarinic acetylcholine receptors. *Mol Pharmacol* **45:**709–717.
Proška J and Tuček S (1995) Competition between positive and negative allosteric effectors on muscarinic receptors. *Mol Pharmacol* **48:**696–702.
Tuček S and Proška J (1995) Allosteric modulation of muscarinic acetylcholine receptors. *Trends Pharmacol Sci* **16:**205–212.
Tuček S, Musílková J, Nedoma J, Proška J, Shelkovnikov S, and Vorlíček J (1990) Positive cooperativity in the binding of alcuronium and *N*-methylscopolamine to muscarinic acetylcholine receptors. *Mol Pharmacol* **38:**674–680.
Waelbroeck M (1994) Identification of drugs competing with *d*-tubocurarine for an allosteric site on cardiac muscarinic receptors. *Mol Pharmacol* **46:**685–692.

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