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Regulation of Signal Transduction at M_2 Muscarinic Receptor

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Summary

Muscarinic acetylcholine receptors mediate transmission of an extracellular signal represented by released acetylcholine to neuronal or effector cells. There are five subtypes of closely homologous muscarinic receptors which are coupled by means of heterotrimeric G-proteins to a variety of signaling pathways resulting in a multitude of target cell effects. Endogenous agonist acetylcholine does not discriminate among individual subtypes and due to the close homology of the orthosteric binding site the same holds true for most of exogenous agonists. In addition to the classical binding site muscarinic receptors have one or more allosteric binding sites at extracellular domains. Binding of allosteric modulators induces conformational changes in the receptor that result in subtype-specific changes in orthosteric binding site affinity for both muscarinic agonists and antagonists. This overview summarizes our recent experimental effort in investigating certain aspects of M_2 muscarinic receptor functioning concerning i) the molecular determinants that contribute to the binding of allosteric modulators, ii) G-protein coupling specificity and subsequent cellular responses and iii) possible functional assays that exploit the unique properties of allosteric modulators for characterization of muscarinic receptor subtypes in intact tissue. A detailed knowledge of allosteric properties of muscarinic receptors is required to permit drug design that will modulate signal transmission strength of specific muscarinic receptor subtypes. Furthermore, allosteric modulation of signal transmission strength is determined by cooperativity rather than concentration of allosteric modulator and thus reduces the danger of overdose.

Key words

Cholinergic transmission • Allosteric modulation • Muscarinic receptor subtypes • G-proteins

Introduction

Muscarinic receptors belong to a family of GTP binding protein (G-protein) coupled receptors. A common feature of all G-protein coupled receptors (that together represent the largest family of plasma membrane receptors) is their general structure comprising seven transmembrane domains. Original classification of muscarinic receptors was based on binding and functional properties of different muscarinic ligands and discriminated three subtypes denoted M1, M2 and M3 receptors. Using advanced molecular biology techniques the existence of a maximum of five subtypes (M_1-M_5 encoded by five different genes) has been reported (Bonner *et al.* 1987, 1988, Peralta *et al.* 1987, Bonner 1989 a,b). Muscarinic receptors are widely expressed in both the central nervous system and in the periphery. They mediate various physiological functions ranging from higher nervous functions such as arousal, memory and alertness to vegetative processes such as regulation of

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heart rate and cardiac output, blood pressure, temperature regulation, perspiration, secretion of exocrine and endocrine glands, and motility of the gastrointestinal tract.

The natural extracellular signal for muscarinic receptors is acetylcholine released from cholinergic nerve terminals or from non-neuronal cells (Kawashima *et al.* 1989, 1990, Wessler *et al.* 1998, 2001, Kawashima and Fuji 2000, 2003) that then causes the activation of heterotrimeric GTP-binding proteins (G-proteins). Conformational changes in muscarinic receptors are induced by ligand binding and interaction with an alpha subunit of a preferred G-protein. This interaction results in an exchange of alpha subunit bound GDP for GTP and dissociation of the alpha and beta/gamma subunits of the G-protein. Both subunits can then exert regulatory influence on different effector molecules. G-protein activation is terminated by intrinsic GTPase activity within the alpha subunit causing cleavage of bound GTP and reassociation of subunits.

Individual subtypes of muscarinic receptors can be classified into two major groups according to preferential interactions with specific G-proteins (Jones *et al.* 1991, Jones 1993). Odd numbered muscarinic receptors interact with alpha subunit of $G_{q/11}$ class of G-proteins. This activates phosphatidylinositol-specific phospholipase C resulting in the production of the second messengers diacylglycerol and inositol phosphates. Even numbered muscarinic receptors are preferentially coupled, by means of alpha subunits of $G_{i/o}$ class of G-proteins, to adenylyl cyclase and their activation results in a decrease of cAMP production. The liberated complex of beta/gamma subunits can directly (without involvement of diffusible second messengers) regulate the function of potassium and calcium channels by so-called "membrane delimited action" (Beech *et al.* 1992, Hille 1992, 1994, Herlitze *et al.* 1996).

Muscarinic receptors have a classical (orthosteric) binding site for natural or exogenous ligands that is located deep in a pocket created by the transmembrane segments of the protein, in addition to one or several allosteric binding sites (Lazareno *et al.* 2000, 2002, Birdsall *et al.* 2001) for exogenous allosteric ligands which are located on the extracellular loops of the receptor. The allosteric nature of the protein is already apparent during interaction with G-proteins. When interacting with G-proteins, the affinity of the classical (orthosteric) binding site for agonists is high, but it decreases when GTP replaces GDP at the alpha subunit of G-protein resulting in dissociation of the receptor/G-protein complex.

The allosteric properties of exogenous ligands were originally observed and deduced from the unusual functional (Clark and Mitchelson 1976) and binding (Dunlap and Brown 1983, Stockton *et al.* 1983, Nedoma *et al.* 1985, 1986, Tuček *et al.* 1990) muscarinic properties of neuromuscular blocking agents (e.g. gallamine or alcuronium). Since then an increasing number of allosteric ligands that can decrease or increase in a subtype-specific manner classical muscarinic agonist and antagonist binding affinity has been described (Tuček *et al*. 1990, Proška and Tuček 1994, Dong *et al*. 1995, Jakubík *et al.* 1995, 1997, Lazareno and Birdsall 1995, Tuček *et al*. 1998). It has been demonstrated that the binding of allosteric ligands not only influences the binding characteristics of classical binding sites, but in the absence of classical agonists can also directly induce activation of G-proteins and this activation is not prevented by classical antagonists (Jakubík *et al.* 1996, 1998).

Specificity of M_2 receptor coupling to G-proteins

M_2 muscarinic receptors are linked to a modulation of cell function preferentially *via* $G_{i/o}$ class of heterotrimeric G-proteins. Their stimulation leads to an inhibition of adenylyl cyclase by means of a liberated alpha subunit resulting in a decrease of cAMP accumulation and in this way attenuates cAMP-dependent signaling. Other two well-known consequences of M_2 receptor stimulation are mediated by release of a complex of beta/gamma subunits which directly, without involvement of diffusible intermediates (membrane delimited action) activate or inhibit different potassium currents with resulting influence on excitability of different cells. This signaling pathway is characteristic for heart muscle where M_2 receptors represent the prevailing subtype of muscarinic receptors (Krejčí and Tuček 2002) and can also be found in some neurons of central and peripheral nervous system. In neuronal cells, mainly on synaptic terminals, liberated beta/gamma subunits interact directly with high voltage-activated calcium channels and inhibit calcium influx into nerve cells and terminals. This mechanism is responsible for presynaptic muscarinic autoinhibition of ACh release (Molenaar and Polak 1980) in both central and peripheral cholinergic neurons as well as for the presynaptic

inhibition of transmitter release by other presynaptic autoreceptors and heteroreceptors in various neuronal cells. All the above described effects can be inhibited by treatment with pertussis toxin (Tuček *et al.* 1987, Doležal *et al.* 1989, Michal *et al.* 2001) by a mechanism that catalyzes ADP-ribosylation of $G_{i/o}$ proteins, thereby blocking the interaction with the receptor. Likewise the action of non-hydrolysable analogues of GTP that preclude G-protein subunit reassociation, pertussis toxin treatment converts M_2 muscarinic receptors to their low affinity state.

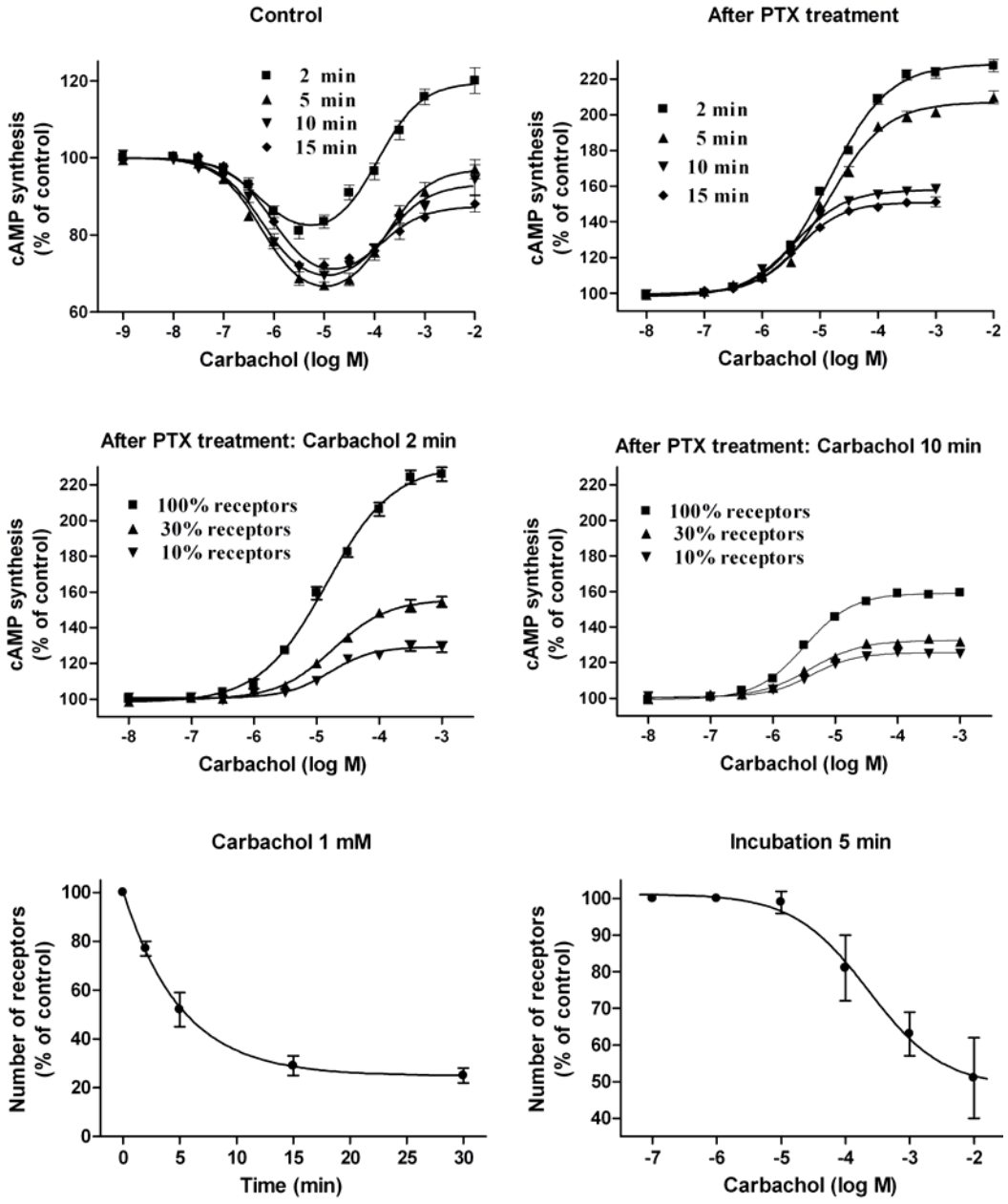

Fig. 1. Inhibitory and stimulatory effects of M2 receptor activation depend on time of incubation and on density of surface muscarinic receptors. **Upper** row: M_2-CHO cells were incubated for indicated time with increasing concentrations of carbachol. Synthesis of cAMP is expressed in per cent of control incubation in the absence of carbachol. Note that the degree of inhibition increases with time of incubation (left). When the cells were pretreated with pertussis toxin to inactivate Gi/o proteins only stimulatory phase persists. Note that in this case the degree of activation decreases with time of incubation (right). **Middle** row: The stimulatory effect of M_2 receptors measured in isolation in cells pretreated with pertussis toxin decreases after inactivation of a proportion (to 30 % and 10 % of controls) of surface receptors both during 2 min (right) and 10 min (left) incubations. **Lower** row: Incubation of M_2-CHO cells induces time- and concentration-dependent internalization of surface M_2 receptors. Left: cells were preincubated for indicated time in the presence of 1 mM carbachol and then the number of surface M_2 receptors was determined. Right: cells were preincubated for 5 min with indicated concentrations of carbachol before determination of surface M_2 receptors. Modified from Michal *et al.* (2001).

Studies in native tissues on the coupling of muscarinic receptors with appropriate G-proteins and effectors are difficult because they always express more than one subtype of muscarinic receptor and there is a lack of known selectively acting muscarinic agonists. Molecular cloning of muscarinic receptors has enabled the creation of genetically modified Chinese Hamster Ovary Cells (CHO) which stably express individual muscarinic receptor subtypes (Buckley *et al*. 1989). Such cells represent a reliable and convenient model system for studying the signaling pathways activated by the muscarinic receptor subtypes starting with the ligand binding and ending with the functional response.

Using these CHO cells it has been found that a degree of inhibition of cAMP synthesis evoked by activation of M_2 or M_4 muscarinic receptors decreases at high concentrations of the agonist. After treatment with pertussis toxin (that selectively eliminates inhibitory transduction via $G_{i/o}$ proteins) the activation of M_2 or M_4 receptors leads to the stimulation of cAMP synthesis (Jones *et al*. 1991, Migeon and Nathanson 1994, Vogel *et al*. 1995, Jakubík *et al.* 1996). Analogous observations report that receptor coupling to more than one specific G-protein exists in both native tissues and genetically modified cells (Fraser *et al.* 1989, Eason *et al*. 1992, Pepperl and Regan 1993, Eason and Liggett 1995, Nasman *et al.* 1997, Sautel and Milligan 1998, Carruthers *et al.* 1999). A model permitting concentration-response curve prediction for a receptor interacting with two different G-proteins has been proposed by Tuček *et al.* (2001, 2002).

A detailed study by Michal *et al.* (2001) into the dual effect of M_2 receptor stimulation on cAMP synthesis pointed to a capability of the M_2 receptor to activate more than a unique G-protein and has revealed certain aspects of these interactions. A stimulatory effect of M_2 muscarinic receptor expressed in CHO cells on cAMP synthesis depends on the agonist used, duration of the stimulation, and also on the density of M_2 receptors present in plasma membrane. Results indicate that M_2 receptors expressed in CHO cells interact with high affinity with $G_{i/o}$ proteins, but also activate with lower affinity G_s proteins and cAMP synthesis. A decrease in EC_{50} for the stimulating effect of carbachol in M_2 cells pretreated with pertussis toxin (Fig. 1) supports this notion because more liganded receptors become available for interaction with G_s proteins. The inhibitory effect of carbachol, a non-hydrolysable analogue of acetylcholine, on forskolin-stimulated synthesis of cAMP increases with incubation time up to 15 min, whereas the activatory effect is most prominent after two minutes and then subsides in both control and pertussis toxin-treated cells. This difference in stimulatory and inhibitory effects is most likely due to a decrease in receptor density following the receptor internalization that, in the case of 1 mM carbachol, represents a decrease of plasma membrane receptor density of almost 50 % in five minutes. Experiments aimed at determining the effect on the inhibition and activation observed, caused by M_2 receptor density at the plasma membrane are also consistent with the notion that when liganded receptors are in excess, they do couple to G_s proteins. A coupling of M_2 receptors with G_s proteins and the resulting increase of cAMP production could explain the atypical positive inotropic effect of muscarinic receptor stimulation observed in native cardiac tissue (Imai and Ohta 1982, Eglen *et al*. 1988, Kenakin and Boselli 1990, Webb and Pappano 1995).

M_4 receptor mediates inhibition of acetylcholine release in striatum

The autoinhibition of transmitter release is a mechanism common to many different neurotransmitter systems (Illes 1986, Starke *et al.* 1989). The inhibition of transmitter release by a stimulation of presynaptic G-protein coupled receptors is elicited by a restriction of calcium influx through the specific high voltage-activated calcium channels that triggers transmitter release (Perney *et al.* 1986, Miller 1987, Dolphin 1990, Turner *et al.* 1993, Doležal and Tuček 1999). The inhibition of calcium channels is achieved by a shift in voltage dependence that can be removed by strong depolarizing prepulses (Bean 1989) and that is mediated by beta/gamma subunit of G_i/G_o class of G-proteins (Herlitze *et al.* 1996). Experiments with universal irreversible activator of G-proteins GTP-gamma-S has also indicated that other beta/gamma subunits can interact, in a transmitter receptor-independent manner, with calcium channels to modulate calcium currents (Mirotznik *et al.* 2000).

In rat striatal cholinergic neurons, influx of calcium triggering acetylcholine release is mediated by N- and P/Q-type calcium channels and the stimulation of presynaptic muscarinic receptors inhibits acetylcholine release by inhibiting both channel types (Doležal and Tuček 1999). The subtype nature of the muscarinic receptor which mediates this inhibition was determined in

functional experiments (Fig. 2) that took advantage of the selective action of allosteric ligands. In an elaborate study Jakubík *et al*. (1997) have characterized the influence of several allosteric muscarinic modulators on the affinity of individual muscarinic receptor subtypes expressed in CHO cells for a number of orthosteric muscarinic agonists. It has been established that the allosteric modulator alcuronium decreases the affinity of furmethide binding on both M_2 and M_4 receptors. In contrast to alcuronium, another allosteric modulator brucine enhances the affinity of furmethide for the M_4 receptor, but largely decreases its affinity at M_2 receptors. Brucine also increases affinity for oxotremorine-M at M_4 receptor and decreases its affinity at M_2 receptor. Concentration-response curves for the inhibition of acetylcholine release (from rat striatal slices evoked by electrical stimulation) for oxotremorine-M and methylfurmethide in control medium or in medium containing allosteric modulators, display changes that implicate the involvement of M_4 receptor in the muscarinic receptor-mediated inhibition of acetylcholine release. In the presence of brucine both furmethide and oxotremorine-M concentration-response curves display leftward shift which corresponds to the increase of affinity at M_4 receptor and contrasts with a large decrease of affinity at M_2 receptor. In the presence of the allosteric modulator alcuronium (that decreases affinity for furmethide at M_4 as well as at M_2 receptor and thus does not discriminate between these subtypes) the predicted rightward shift of concentration-response relationship confirms apparently the functionality of the regulation in the opposite direction.

Involvement of the third extracellular loop in allosteric properties of M_2 receptor

In common with other G-protein coupled receptors, muscarinic receptors are formed by a protein that crosses the plasma membrane seven times. The membrane spanning domains are connected by three extracellular and three intracellular loops with a long C-terminus in the cytoplasma and shorter N-terminus orientated to the extracellular space. The C-termini contain conserved cysteine residues to which palmitate is covalently attached thus anchoring the receptors in the plasma membrane and creating the fourth intracellular loop. The C-termini together with the third intracellular loop determine the specificity of G-proteins interaction. The extracellular glycosylated N-terminus has a role in

Agonist	Allosteric modulator	Change of Kd at M_2 (fold increase)	Change of Kd at M_4 (fold increase)
Furmethide	alcuronium	8.4	9.3
Furmethide	brucine	71	0.2
Oxotremorine-M	brucine	3.3	0.2

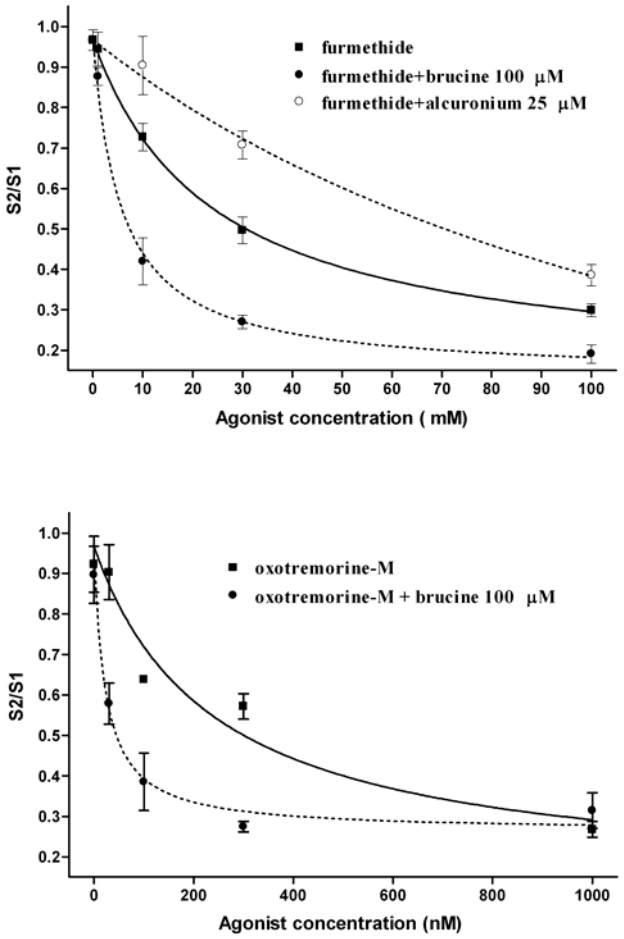

Fig. 2. Influence of allosteric modulators alcuronium and brucine on the inhibitory effect of orthosteric agonists furmethide and oxotremorine-M on acetylcholine release from rat striatal slices. **Upper** panel: changes of affinity of orthosteric agonists furmethide and oxotremorine-M elicited at M_2 and M_4 muscarinic receptors by allosteric modulators alcuronium and brucine as determined in CHO cells (modified from Jakubík *et al.* 1997). Middle panel: brucine enhances whereas alcuronium attenuates inhibitory action of the classical agonist furmethide on evoked release of acetylcholine. **Lower** panel: brucine enhances inhibitory action of oxotremorine-M on evoked acetylcholine release. Observed effects of allosteric modulators alcuronium and brucine on the concentration-response relationship of orthosteric agonists furmethide and oxotremorine-M are in concert with involvement of M_4 receptor (see upper part) in the presynaptic autoinhibition of acetylcholine release from rat striatal cholinergic neurons. Modified from Doležal and Tuček (1998).

protein targeting to plasma membrane and does not influence binding properties. Two cysteine residues in the first and the second extracellular loops create a disulfide bridge which is important for the affinity of classical ligands.

The affinity for allosteric modulators gallamine and alcuronium is considerably higher for the M_2 than for the M_3 receptor. Gallamine exerts a weaker degree of negative cooperativity with the orthosteric antagonists N-methyl scopolamine (NMS) at M_3 than M_2 receptors. Moreover, cooperativity of binding between alcuronium and NMS is positive at M_2 but negative at M_3 receptors (Table 1). Mutation studies in which the extracellular loops of M_3 receptor were modified to resemble the extracellular loops of M_2 receptor have revealed that certain amino acids play a role in the observed cooperativity and the higher affinity of gallamine and alcuronium binding to allosteric sites of the M_2 receptor.

Table 1. Amino acid sequences of individual extracellular loops of M_3 muscarinic receptor are shown. Mutated amino acids are shown in bold italics.

1st extracellular loop (o1)
(126)T–T–Y–I–I–M–N–***R***–W–A–L–G–N–L–A–C–D(142)
2nd extracellular loop (o2)
(208)Q–Y–F–V–G–K–R–T–V–***P***–***P***–G–E–C–F–I–Q–F–L–S–E–P(229)
3rd extracellular loop (o3)
(514)N–T–F–C–***D***–***S***–C–I–P–***K***–T–***F***–W–***N***(527)

	Gallamine			Alcuronium		
	pK_{diss} (M)	pK_A (M)	α	pK_{diss} (M)	pK_A (M)	α
Native M_3 receptor	3.91 ± 0.02	4.7 ± 0.08	4.3 ± 0.6	3.70 ± 0.04	3.9 ± 0.09	2.6 ± 0.3
Native M_2 receptor	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	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						
P217D+P218E	4.23 ± 0.06*	4.6 ± 0.06	4.0 ± 0.8	3.95 ± 0.02*	4.2 ± 0.03	2.1 ± 0.4
Entire o2 M_2	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	4.54 ± 0.003*	6.1 ± 0.06*	16.0 ± 1.9*	4.69 ± 0.04*	4.7 ± 0.20*	1.8 ± 0.3
DSKFN	4.21 ± 0.04*	6.3 ± 0.11*	25.1 ± 3.8*	4.67 ± 0.07*	4.4 ± 0.12*	0.3 ± 0.03*

Data are derived from binding experiments with ^{3}H-NMS. pK_{diss} values indicate the negative logarithm of the molar concentration of the allosteric modulator which causes a 50 % decrease in the k_{off} of ^{3}H-NMS determined in the absence of the allosteric modulator and reflects affinity of occupied receptor. pK_A values indicate negative logarithm of equilibrium dissociation constant for the binding of the allosteric ligand to free receptors. α, factor of cooperativity between allosteric ligand and ^{3}H-NMS. Data are means ± S.E.M. of 3-9 experiments. NM, not measurable because the binding curves were flat; *, significantly different from wild-type M_3; [#], although clearly different from wild-type M_3, significance could not be evaluated numerically because of flat binding curves. Modified from Krejčí and Tuček (2001).

Substitution of a single amino acid in the first extracellular loop (R133G) of M_3 receptor has no influence on either the affinity of alcuronium or its cooperativity. However, it augments the affinity for gallamine derived from deceleration of NMS dissociation, but the cooperativity with NMS derived from equilibrium binding experiments becomes neutral. These findings point to an involvement of the first extracellular loop in the affinity for gallamine.

Replacement of the entire second extracellular loop of the M_3 receptor by that of the M_2 receptor substantially increases the affinity of both allosteric ligands, but has no effect on their cooperativity. Double mutation P217D and P218E has similar, although a slightly smaller, effect on gallamine and alcuronium affinity and again no effect on their cooperativity. These observations indicate a role for the second extracellular loop in the binding affinity of these allosteric ligands but not in their cooperativity.

The exchange of a single amino acid in the third extracellular loop of the M_3 receptor for its counterpart in the M_2 receptor (K523N) significantly increases the affinity for both allosteric modulators, but results in a change of cooperativity corresponding to M_2 receptor only in the case of gallamine. Substitution of five amino acids (DSKFN) for the appropriate counterparts in the third extracellular loop of M_3 receptor introduces the appearance of the positive cooperativity of alcuronium typical for M_2 receptor and the further decrease of cooperativity of gallamine which even exceeds that at native M_2 receptor, in addition to the increase in affinity. Taken together these results demonstrate the importance of the third extracellular loop in the binding of allosteric modulators gallamine and alcuronium and in particular in the positive cooperativity of alcuronium regarding NMS binding at M_2 receptor.

Conclusions

An investigation of the allosteric properties of muscarinic receptors (but not only of them) is of utmost importance for several reasons. From an experimental point of view the subtype specificity of allosteric modulators for classical ligands can be exploited for the identification of muscarinic receptor subtype in isolated tissues. This approach was successfully used to identify the role of the M_4 subtype in inhibiting acetylcholine release in the rat striatum (Doležal and Tuček 1998). These findings fully agree with data obtained using a knock-out approach (Zhang *et al.* 2002) and thus justify the applicability and usefulness of allosteric ligands in determining subtype physiological functions in tissues isolated from intact animals. Another future practical application of muscarinic allosteric ligands is their potential use in human medicine. Their strict subtype selectivity is likely to enable the development of drugs that will increase or decrease the affinity of specific subtypes of muscarinic receptors for both the endogenous mediator acetylcholine and exogenously applied orthosteric ligands. Additional advantages of allosteric ligands reside in that both stimulatory and inhibitory muscarinic effects depend primarily on cooperativity and not so much on concentration. Unlike an application of exogenous orthosteric ligands, provided that cholinergic innervation is at least partially preserved and operating, allosteric modulation of acetylcholine binding should maintain both temporal and spatial pattern of cholinergic signaling. Even when exogenous application of classical muscarinic drug is required, allosteric modulators should permit a lower dosage thus reducing muscarinic side effects elicited by the non-selective influence of exogenous drugs on individual muscarinic receptor subtypes.

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