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Muscarinic acetylcholine receptor X-ray structures: potential implications for drug development

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Abstract

Muscarinic acetylcholine receptor antagonists are widely used as bronchodilating drugs in pulmonary medicine. The therapeutic efficacy of these agents depends on the blockade of M₃ muscarinic receptors expressed on airway smooth muscle cells. All muscarinic antagonists currently used as bronchodilating agents show high affinity for all five muscarinic receptor subtypes, thus increasing the likelihood of unwanted side effects. Recent X-ray crystallographic studies have provided detailed structural information about the nature of the orthosteric muscarinic binding site (the conventional acetylcholine binding site) and an 'outer' receptor cavity that can bind allosteric (non-orthosteric) drugs. These new findings should guide the development of selective M₃ receptor blockers that have little or no effect on other muscarinic receptor subtypes.

Introduction

Many studies have shown that chronic obstructive pulmonary disease (COPD) and asthma are associated with increased pulmonary vagal tone [1–3]. As a result, muscarinic acetylcholine (ACh) receptor (mAChR) antagonists, including ipratropium and tiotropium, are of great clinical importance for the treatment of COPD and certain forms of asthma [1–3]. Various lines of evidence indicate that multiple mAChR subtypes are expressed in the airways of experimental animals and humans [4, 5].

In vitro and *in vivo* studies with mAChR knockout (KO) mice have provided convincing evidence that the bronchoconstricting effects of ACh are mediated predominantly by the M₃ mAChR subtype (M3R) (reviewed in [5]). Interestingly, in M2R-deficient mice, vagal stimulation resulted in enhanced bronchoconstrictor responses [6]. This finding is in good

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agreement with the concept that M2Rs present on pulmonary parasympathetic nerve endings function as inhibitory autoreceptors to limit ACh release [2]. It has also been reported that airways express additional mAChRs including the M1R. For example, a study with M1R KO mice strongly suggests that activation of a subpopulation of pulmonary M1Rs inhibits M3R-mediated bronchoconstriction, perhaps by stimulating the secretion of a bronchorelaxing agent from airway epithelia or pulmonary nerves [7].

At present, muscarinic antagonists that can block M3Rs with a high degree of selectivity are not available. Since blockade of pulmonary M2Rs (and perhaps M1Rs) is predicted to lower the therapeutic efficacy of muscarinic antagonists, the development of M3R antagonists with greatly reduced affinity for other mAChR subtypes appears an attractive therapeutic goal. The clinical use of selective M3R antagonists should also reduce the incidence of unwanted side effects mediated by non-M3R mAChRs which are widely distributed both in the central nervous system and in peripheral tissues [5].

Recently, X-ray crystallographic studies have led to important novel insights into mAChR structure [8–10]. These new studies provide detailed information about the structural features of mAChRs in their inactive (M2R [8] and M3R [9]) and their active (M2R) conformations [10]. Importantly, Kruse *et al.* [10] also reported the structure of the M2R in complex with an allosteric muscarinic modulator, providing the first direct structural information about how allosteric muscarinic agents interact with their target receptors. As discussed below, these recent structural studies offer new opportunities for the development of novel muscarinic drugs with increased affinity, efficacy, and/or mAChR subtype selectivity.

Structure of the M3R-tiotropium complex and implications for drug development

In 2012, X-ray crystallographic techniques yielded the first high-resolution mAChR structures, the structures of the inactive states of the human M2R [8] and the rat M3R [9]. The overall structures of the two receptors are similar to each other and to those of other biogenic amine G protein-coupled receptors (GPCRs) that have been crystallized during the past few years [8, 9] (Fig. 1a). The M2R and M3R were crystallized in complex with a muscarinic antagonist/inverse agonist (M2R, 3-quinuclidinyl benzilate [QNB]; M3R, tiotropium). A comparison of the two structures indicates that the configuration of the QNB/tiotropium binding pockets is virtually identical in the two receptors (Fig. 1b).

Since airway M3Rs are a major target for drug therapy, the following paragraphs will focus on the key structural features of the M3R-tiotropium complex. Tiotropium, like other muscarinic antagonists, is highly effective in the treatment of COPD [1–3, 11]. Tiotropium blocks M3Rs and all other mAChR subtypes with subnanomolar affinity [12]. Like atropine and other conventional muscarinic antagonists, tiotropium can be considered an inverse agonist, since it is able to inhibit basal, ligand-independent mAChR signaling [13]. Tiotropium is characterized by a very slow dissociation rate from the M3R, as compared to the M2R (dissociation half-lives at room temperature: ~27 hr for the M3R and ~2–3 hr for the M2R, respectively) [12]. These kinetic properties explain the drug's long duration of

action (it is administered as a bronchial spray only once a day) and probably account for the observation that tiotropium causes few non-M3R-mediated side effects [1–3].

Structurally, tiotropium is an ester between a benzoic acid derivative and a modified tropane moiety containing a positively charged quaternary ammonium group. This structure is similar to that of many other high-affinity muscarinic antagonists such as atropine or QNB (Fig. 1c).

In the M3R/tiotropium complex, the tiotropium binding pocket is buried within the transmembrane (TM) receptor core, similar to the binding mode of other biogenic amine ligands [9]. The M3R amino acid side chains that contact the tiotropium ligand are located on TM3, 4, 5, 6, and 7 (Fig. 1b). Strikingly, these amino acids are fully conserved among all five mAChR subtypes [9]. This observation provides a molecular explanation for the fact that efforts to develop orthosteric, muscarinic antagonists (or agonists) endowed with a high degree of mAChR selectivity have not proven successful in the past.

The key M3R-tiotropium contact sites are summarized in Fig. 1b, with polar contacts indicated by dotted lines. The side chain of N507^{6.52} (superscript numerals refer to the Ballesteros-Weinstein numbering system) is engaged in paired hydrogen bonds with the tiotropium ketone and hydroxyl groups. The negatively charged side chain of D147^{3.32} forms a salt bridge with the positively charged amine of tiotropium (note that this type of interaction is conserved among all biogenic amine GPCRs). This ion pair is surrounded by a cage of three conserved tyrosine residues (Y148^{3.33}, Y506^{6.51} and Y529^{7.39}). Fig. 1d shows a cross-sectional side view of the M3R-tiotropium complex, illustrating that Y148^{3.33}, Y506^{6.51} and Y529^{7.39} form a lid above the tiotropium ligand present in the orthosteric binding site. This structural feature is predicted to interfere with the ability of tiotropium to dissociate from the M3R, providing a molecular basis for the high stability of the M3R-tiotropium complex. A rearrangement of the tyrosine lid structure is required to enable tiotropium to exit from the orthosteric binding site. This concept is consistent with recent mutagenesis data and molecular dynamics studies of the M3R-tiotropium complex [14]. Since the extracellular loops appear to be less flexible in the M3R as compared to the M2R [9], the energy barrier that needs to be overcome to achieve this conformational change (rearrangement of the tyrosine lid) is predicted to be higher for the M3R than for the M2R.

Notably, like the M2R-QNB structure, the M3R-tiotropium complex displays several features that appear to be unique for the mAChR family [8, 9]. Most importantly, the mAChR structures feature a rather large solvent-accessible vestibule which faces the extracellular space and is separated from the orthosteric binding pocket by the so-called tyrosine lid (Fig. 1d). Sequence alignments indicate that the residues lining this outer receptor cavity are less well conserved among the five mAChR subtypes, as compared to the amino acids surrounding the orthosteric binding site [9]. For this reason, this non-orthosteric (allosteric) mAChR surface represents an attractive target for the development of mAChR subtype-selective allosteric agents. In fact, during the past few years, several novel mAChR ligands endowed with a high degree mAChR subtype selectivity have been developed that are predicted to target this allosteric site [15–17]. These novel classes of drugs can act as

either allosteric (ectopic) agonists or as positive or negative allosteric modulators (PAMs or NAMs, respectively) of agonist-induced signaling via specific mAChR subtypes. Recently, the development of so-called 'bitopic' (or 'dualsteric') muscarinic ligands, which can interact with both allosteric and orthosteric mAChR sites simultaneously, has also been reported [18, 19]. Like allosteric muscarinic agents, bitopic muscarinic ligands, if properly designed, can preferentially recognize distinct mAChR subtypes [18, 19].

Structural features of the agonist-activated M2R

Recently, Kruse *et al.* [10] also solved the structure of an agonist (iperoxo)-bound, active state of the human M2R stabilized by a G protein-mimetic antibody fragment (Fig. 2a). Iperoxo is an orthosteric agonist that displays extraordinarily high potency at the M2R [20].

The key features of the active conformation of the M2R are a significant outward displacement of the cytoplasmic end of TM6, together with a smaller outward movement of the C-terminal portion of TM5 and a rearrangement of the highly conserved NPXXY (TM7) and DRY (cytoplasmic end of TM3) motifs [10]. Similar conformational changes have been reported for the active-state conformations of rhodopsin [21, 22] and the β_2 -adrenergic receptor [23, 24].

Interestingly, iperoxo binding to the M2R leads to striking changes in the structure of the orthosteric binding site [10]. These structural changes are more pronounced than those observed in the active conformations of rhodopsin and the β_2 -adrenergic receptor. In particular, iperoxo binding to the M2R leads to a significant contraction of the orthosteric binding site, which completely occludes the agonist ligand from solvent (Fig. 2b). In the active M2R conformation, TM5, TM6, and TM7 move inward toward the iperoxo ligand and TM3 undergoes a slight rotation about its axis (Fig. 2c). Most importantly, the inward movement of TM6 allows the side chain of N404^{6.52} to form a hydrogen bond with the isoxazoline ring of iperoxo (Fig. 2c). Other important M2R-iperoxo contact sites are also shown in Fig. 2c. The inward motion of the exofacial portion of TM6 leads to the formation of a hydrogen network between Y403^{6.51}Y104^{3.33} and Y426^{7.39} (Fig. 2d), resulting in the closure of the tyrosine lid above the agonist ligand.

Mode of binding of an allosteric modulator to the M2R

During the past decades, the M2R has served as a model system for studying the regulation of GPCR function by small allosteric modulators [15–17]. As discussed above, the inactive M2R and M3R feature a large extracellular vestibule, which is predicted to be involved in the binding of allosteric muscarinic ligands. Strikingly, agonist activation of the M2R triggers a pronounced contraction of this outer cavity, primarily due to the inward movement of the extracellular portion of TM6 [10].

Besides elucidating the structural basis of M2R activation, this latter study [10] also provides the first structural view of how a drug-like allosteric ligand binds to a GPCR. Specifically, the structure of the iperoxo-occupied M2R was solved in complex with LY2119620, a positive allosteric modulator (the chemical structure of this compound is shown in Fig. 3a). LY2119620 displays similar pharmacological properties as its structural

congener, LY2033298, which has been characterized in previous studies (see, for example, ref. [25]). LY2119620 shows strong positive cooperativity with iperoxo and selectively enhances the affinity of the orthosteric agonist for the M2R [10]. At saturating concentrations, LY2119620 enhances the affinity for iperoxo by ~25-fold [10]. Moreover, LY2119620 is able to directly activate the M2R, although with ~500-fold lower potency and significantly reduced efficacy (by ~50%, as determined in a GTP γ S assay) as compared to iperoxo [10].

In the M2R-iperoxo-LY2119620 complex, the allosteric modulator is located directly above the orthosteric agonist (Fig 3a). LY2119620 engages in extensive interactions with the extracellular vestibule. Such contacts include aromatic stacking, hydrogen bond, and charge–charge interactions (for structural details, see Fig. 3b). Similar interactions have been found in a recent study examining the binding of several allosteric M2R modulators via atomic-level simulations and site-directed mutagenesis studies [26]. Interestingly, the structure of the M2R–iperoxo–LY2119620 complex is very similar to that of the M2–iperoxo complex, indicating that the binding site for LY2119620 is largely pre-formed after binding of the orthosteric agonist iperoxo. The iperoxo-stabilized contraction of the extracellular vestibule enables LY2119620 to engage in far more extensive interactions with this outer receptor cavity, as compared to the inactive state of the M2R. These findings support the concept that LY2119620 and, most likely, other muscarinic positive allosteric modulators enhance the receptor affinity of orthosteric agonists by stabilizing the active conformation of the receptor and slowing agonist dissociation from the orthosteric binding pocket.

Since LY2119620 and other allosteric modulators are predicted to stabilize the 'contracted configuration' of the extracellular receptor vestibule, it is possible that this conformational change can be propagated to the intracellular surface of the receptor protein. Such a mechanism could explain that certain allosteric muscarinic agents, including LY2119620, can act also act as direct mAChR activators [15–17].

Concluding remarks

Recent X-ray crystallographic studies with two mAChR subtypes (M2R, M3R) have offered detailed insights into the structural configuration of the orthosteric and allosteric muscarinic binding sites. This new structural information should guide the development of novel muscarinic agents endowed with a high degree of selectivity for distinct mAChR subtypes. Such agents could include PAMs, NAMs, direct allosteric agonists, or bitopic muscarinic ligands. All muscarinic antagonists in current clinical use in pulmonary medicine also retain high affinity for M2Rs whose blockade may lead to enhanced ACh release in the airways and trigger bronchoconstriction [2]. Thus, the development of novel muscarinic drugs that inhibit M3R activity with high efficacy but do not interfere with M2R function appears to be a very attractive goal.

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Highlights

- Blockade of airway M₃ muscarinic receptors (M3Rs) triggers bronchodilation.
- We solved the structure of the M3R-tiotropium (a clinically used drug) complex.
- We reported the structure of the active M₂ muscarinic receptor (M2R).
- We elucidated the structures of the orthosteric and allosteric binding sites (M2R).
- These new findings should facilitate the development of selective M3R antagonists.

Figure 1.

Comparison of the M2R and M3R structures in their inactive states. (A) The overall structure of the M3R bound to tiotropium (orange) is similar to that of the M2R subtype both in overall fold and in the specific binding site contacts, shown in (B). Extracellular view of the orthosteric ligand binding site. Polar contacts are indicated with dotted lines. Residues are numbered according to the rat M3R sequence. (C) The chemical structures of tiotropium and QNB, the two inverse agonists used to stabilize the M3R and M2R, respectively, for crystallographic studies. (D) A cross-section through the M3R shows the buried orthosteric ligand binding pocket and the large extracellular vestibule located superficial to it. This latter site is the target of a wide variety of allosteric modulators.

Figure 2.

Activation of the M2R. (A) The overall structure of the active M2R is shown in orange. The active state-stabilizing nanobody (Nb9-8) is highlighted in magenta. The bound orthosteric agonist iperoxo is represented by yellow spheres. (B) A cross-sectional view through the active M2R shows that the ligand binding pocket has closed completely over the orthosteric agonist. (C) The binding pocket conformation for the M2R is shown in both active (orange) and inactive (blue) conformations. Polar contacts are indicated with dotted lines, and major conformational changes associated with M2R activation are highlighted with red arrows. (D)

M2R activation involves closure of the tyrosine lid over the orthosteric agonist, forming a hydrogen bond network that occludes the agonist from solvent.

Figure 3.

Allosteric modulation of the M2R. (A) The structure of the active M2R bound to both the orthosteric agonist iperoxo and the allosteric modulator LY2119620 is shown with transmembrane domains 6 and 7 removed for clarity. The chemical structures of the two compounds are shown to the right. (B) Contacts between the allosteric modulator and the M2R are shown, with polar contacts indicated by dotted lines.