

Structure–Function Studies of Muscarinic Acetylcholine Receptors

Katie Leach, John Simms, Patrick M. Sexton, and Arthur Christopoulos

Abstract There has been great interest in the structure–function relationships of the muscarinic acetylcholine receptors (mAChRs) because these prototypical Family A/class 1 G protein-coupled receptors (GPCRs) are attractive therapeutic targets for both peripheral and central nervous system disorders. A multitude of drugs that act at the mAChRs have been identified over the years, but many of these show minimal selectivity for any one of the five mAChR subtypes over the others, which has hampered their development into therapeutics due to adverse side effects. The lack of drug specificity is primarily due to high sequence similarity in this family of receptor, especially in the orthosteric binding pocket. Thus, there remains an ongoing need for a molecular understanding of how mAChRs bind their ligands, and how selectivity in binding and activation can be achieved. Unfortunately, there remains a paucity of solved high-resolution structures of GPCRs, including the mAChRs, and thus most of our knowledge of structure–function mechanisms related to this receptor family to date has been obtained indirectly through approaches such as mutagenesis. Nonetheless, such studies have revealed a wealth of information that has led to novel insights and may be used to guide future rational drug design campaigns.

Keywords Allosteric • Molecular modeling • Muscarinic receptor • Mutagenesis • Orthosteric • Structure-function

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1 Introduction

GPCRs comprise the largest family of membrane proteins in mammalian organisms (Fredriksson et al. 2003) and respond to a wide range of endogenous and exogenous ligands. Mammalian GPCRs are divided into three main classes based on similarities in their amino acid sequence (Foord et al. 2005). Family A (or class 1) GPCRs, which include the mAChRs, share sequence similarity to rhodopsin; Family B (class 2) GPCRs to secretin receptors; Family C (class 3) GPCRs to metabotropic glutamate receptors. Less than 10% of these three GPCR families already constitute the targets of approximately 30% of all drugs on the market (Harmar et al. 2009; Hopkins and Groom 2002) and, thus, there is ongoing incentive in understanding how the amino acid sequence of these proteins relates to their function and three-dimensional structure in order to facilitate drug discovery. The characteristic structural feature of all these receptors is the presence of an extracellular N-terminal region, intracellular C-terminal region and seven transmembrane (TM)-spanning α -helical domains connected by three extracellular and three intracellular loops. However, until very recently, detailed three-dimensional structural information on GPCRs has been hampered by difficulties in obtaining high-resolution crystal structures of these receptors. This is because they are highly unstable upon removal from their membranous environment and also because they dynamically isomerize between multiple conformations, both of which hinder the crystallization process (Congreve and Marshall 2010). To date, crystallization efforts have been successful for only a few GPCRs; rhodopsin (Palczewski et al. 2000), the β_1 (Warne et al. 2008) and β_2 (Cherezov et al. 2007; Rasmussen et al. 2007; Rosenbaum et al. 2007) adrenergic receptors (β -ARs), and the A_{2A} adenosine receptor (Jaakola et al. 2008).¹ As a consequence, computational approaches, such as homology modeling and associated methods, are the mainstay of rationalizing structural information derived at other GPCRs, such as the mAChR family. However, molecular models, in and of themselves, have only limited utility if not used in conjunction with molecular and biophysical techniques that can help to refine our structural and functional understanding of a protein. In this regard, the mAChRs remain a prototypical Family A GPCR model system that has been extensively explored by site-directed mutagenesis and related approaches, which is the focus of this chapter.

2 Amino Acids That Are Essential for Stabilization of the Receptor Structure

Substitution of amino acids that are essential for the structural stability and folding of a protein can lead to impairment in its assembly, maturation, and/or trafficking. In the mAChRs, substitutions of certain amino acid residues that are conserved

¹At the time of writing, the crystal structures of antagonist-bound chemokine CXCR4 and dopamine D₃ receptors have been solved but not published.

across all five mAChR subtypes cause a reduction in receptor expression, in particular mutation of Asp^{2.50}, Leu^{3.43}, Asp^{3.49}, Tyr^{3.51}, Trp^{4.50}, and Pro^{7.50} (Hulme et al. 2001, 2003a; Lu et al. 1997, 2001; Lu and Hulme 1999) [numbering in superscript corresponds to the Ballesteros–Weinstein system (Ballesteros et al. 1995)]. In fact, these residues are highly conserved throughout the TM domains of Family A GPCRs and thus likely serve an important role in maintaining the overall helical structure of these receptors. For instance, in the rhodopsin X-ray crystal structure, Asp^{2.50} interacts with Asn^{1.50} and Asn^{7.49} in a hydrogen-bonded network of residues mediated by water molecules that may assist in the initial folding of the receptor, whilst at later stages of the receptor lifetime these residues are implicated in signaling cascades. Asp^{3.49} and Tyr^{3.51} are two additional residues that are essential for the function of the majority of Family A GPCRs. In the mAChRs, only His, Asn, or Glu substitutions are tolerated at the position of Asp^{3.49}, although even in these instances receptor expression levels are reduced. Substitution with any other amino acid at this position generally results in undetectable levels of radioligand binding (Lu et al. 1997), suggesting that this residue is critical for maintaining a receptor conformation able to bind ligand.

In addition to traditional approaches that have relied on rationally guided systematic mutagenesis of the mAChR, a more recent, higher-throughput, random mutagenesis study identified a number of additional mutations that profoundly affected the expression of the M₃ mAChR (Li et al. 2007a), suggesting that there remains much to be learned about the structural determinants of mAChR stability

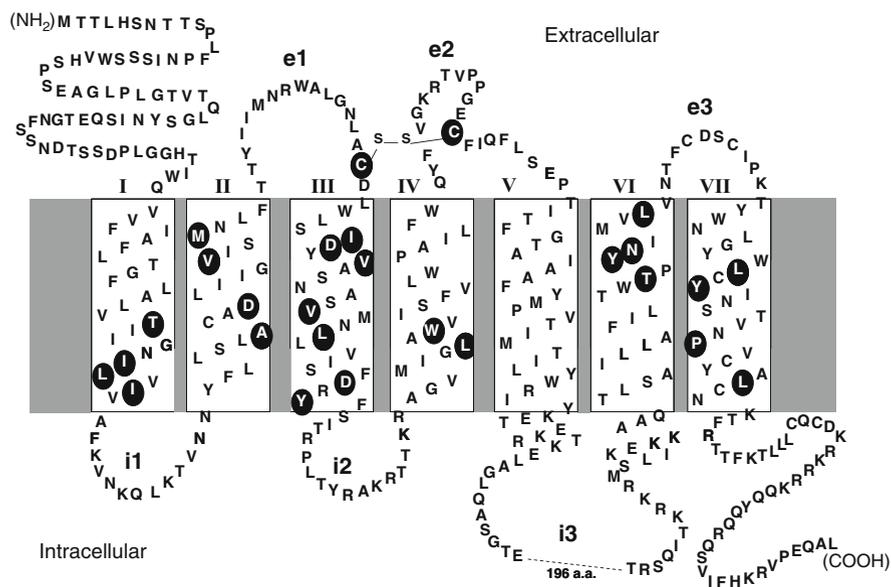


Fig. 1 Snake diagram of the M₃ mAChR. Residues labeled within the *black circles* indicate amino acids that have been implicated in the control of receptor expression

and expression. Many of these substitutions were for proline or charged amino acids, which are likely to disrupt important interactions that are essential for the folding of the receptor, including Thr^{1.46}Pro, Ile^{1.51}Asn, Leu^{1.52}Pro, Ile^{1.54}Asn, Ala^{2.47}Pro, Val^{2.55}Asp, Cys¹⁴⁰Gly, Ile^{3.31}Asp, Val^{3.34}Glu, Leu^{4.48}Pro, Cys²²⁰Ser, Thr^{6.49}Asn, Leu^{6.56}Pro, Leu^{6.56}Gln, Leu^{7.41}Pro, and Leu^{7.55}Pro (Fig. 1). Interestingly, mapping these positions onto models of the mAChR revealed that they are adjacent, in either 2D or 3D space, to conserved residues and may affect their local environment. In contrast to the above mutations, another series of positions (Met^{4.43}, Leu^{4.46}, Leu^{4.49}, Ala^{4.58}, Phe^{4.61}, and Thr^{7.47}) have also been shown to contribute to receptor conformation(s) that can result in an increase in the amount of cell surface expression.

3 Elucidation of the mAChR Orthosteric Binding Site

A wide range of structurally diverse ligands bind to the orthosteric site of GPCRs and, as such, a number of different domains may potentially form the ligand binding pocket, depending on the receptor. To date the high-resolution X-ray crystal structures for orthosteric inverse agonist-bound rhodopsin, the β_1 - and β_2 -ARs, and the A_{2A} adenosine receptor, have all been solved (Palczewski et al. 2000; Cherezov et al. 2007; Rasmussen et al. 2007; Jaakola et al. 2008). Rhodopsin is not a typical GPCR in that its ligand, 11-*cis*-retinal, is covalently bound to Lys^{7.43} via a Schiff base in the inactive form of the receptor. 11-*cis*-retinal also makes a number of additional contacts within the receptor that contribute to a binding pocket that shares similarities with the orthosteric binding site in the β -ARs (Rasmussen et al. 2007), being comprised of residues positioned predominantly in TMIII, TMV, and TMVI. Residues that form the binding crevice in rhodopsin and the β -ARs include 3.28, 3.29, 3.32, 3.33, 3.35, 3.36, 3.37, 5.41, 5.42, 5.43, 5.46, 5.47, 6.44, 6.48, 6.51, 6.52, 6.55, 7.35, 7.39, and 7.40. In contrast, the binding site for the A_{2A} receptor antagonist, ZM241385, is somewhat different and involves residues predominantly located in TMII, TMVI, and TMVII (Jaakola et al. 2008). Mutagenesis data support the hypothesis that the orthosteric binding site in mAChRs closely resembles that of rhodopsin and the β -ARs. Some of the first studies that investigated the location of the ACh binding site in mAChRs involved propylbenzilylcholine and acetylcholine mustards. These alkylating agents were used to highlight an important interaction that occurs between the common ammonium moiety that exists in all biogenic amines and Asp^{3.32} (Curtis et al. 1989; Spalding et al. 1994; Kurtenbach et al. 1990), conserved within TMIII of the biogenic amine receptors. A series of site-directed mutagenic studies have since identified additional amino acids that are equally critical for the binding of ACh to the mAChRs (Lu et al. 2001; Wess et al. 1991; Ward et al. 1999). Generation of a homology model of the M₁ mAChR based on the structure of bovine rhodopsin has predicted residues that most probably form direct contact points for ACh (Fig. 2a), including five residues in particular: Tyr^{3.33}, Thr^{5.39}, Thr^{5.42}, Tyr^{6.51}, and Tyr^{7.39}

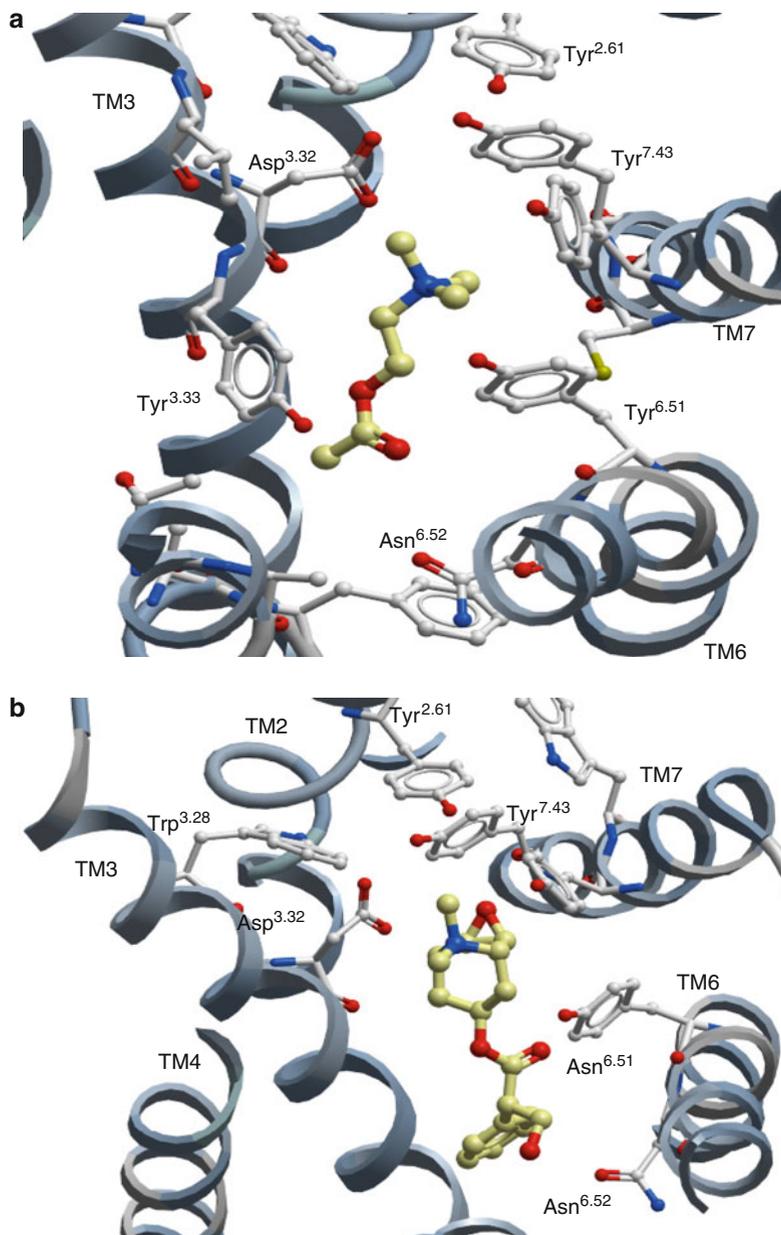


Fig. 2 The orthosteric binding pocket of mAChRs. A homology model of the M₂ mAChR was constructed using the crystal structure of the inactive-state β_2 adrenergic receptor as a template. Docking of (a) ACh or (b) NMS was performed and the key residues contributing to the respective pockets are also indicated

(Hulme et al. 2003a, b). Further residues that have been implicated in the binding of ACh have also been identified as Trp^{3.28}, Leu^{3.29}, Ser^{3.36}, Asn^{3.37}, Trp^{4.50}, Ser^{4.53}, Trp^{4.57}, Ala^{5.46}, Leu^{6.56}, Cys^{7.42}, and Tyr^{7.43} (Hulme et al. 2003a, b; Lu et al. 2001; Hulme and Lu 1998). However, visual inspection of homology models of the mAChR suggests that some of these amino acids lie outside of the orthosteric site and may thus affect the route of entry for the ligand into its main binding site crevice.

Many of the residues that are essential for ACh binding are equally as important for the binding of inverse agonists/antagonists such as *N*-methyl scopolamine (NMS), quinuclidinyl benzilate (QNB) and atropine to the mAChRs (Fig. 2b), although some subtle differences have been observed with regards to amino acids that contribute to the binding of these different ligands. For instance, although Asn^{6.52} is predicted to face into the ligand binding pocket and is important for the binding of atropine and NMS (Ward et al. 1999; Bluml et al. 1994a), it has a lesser role in ACh and QNB binding (Bluml et al. 1994a). Similarly, the binding of QNB is not significantly affected by substitution of Tyr^{6.51} (Ward et al. 1999), Tyr^{7.39}, Cys^{7.42}, or Tyr^{7.43} (Lu et al. 2001). Phe^{5.47}, on the other hand, which does not appear to interact with ACh and QNB, has been predicted to lie in close proximity to NMS and may be positioned at the very bottom of the NMS binding site, which extends deeper into the helical bundle than the ACh binding site (Goodwin et al. 2007). Thus, different ligands clearly form molecular interactions with different amino acid residues.

In addition to the role of the TM domains in binding orthosteric ligands, there is some evidence that the extracellular domains of the mAChRs may contribute structural stability to the orthosteric binding site. Family A GPCRs possess two conserved cysteine residues that form a disulfide bond between the extracellular portion of TMIII and the second extracellular loop of the receptors. In rhodopsin, part of the second extracellular loop folds into the center of the helical bundle, with Glu¹⁸¹ (residues that lie outside the TM domains are indicated by their amino acid position) orientated toward 11-*cis*-retinal (Palczewski et al. 2000). Similarly, Thr^{5.34} at the junction of TMV and the second extracellular loop in the β_1 -AR is directed toward the ligand binding pocket, suggesting that this extracellular region may form a “cap” to that pocket. Thus, in most mAChR structural models that are based on homology with rhodopsin or the β -ARs, the second extracellular loop of these receptors defines a boundary of the orthosteric binding site that forms a lid-like structure over the top of the crevice. Although substitution of amino acid residues in the second extracellular loop does not significantly alter the binding affinity of prototypical orthosteric ligands, restriction of flexibility of this region in the M₂ mAChR (via engineering of an additional disulfide bond) was shown to substantially hinder the access of ligands such as NMS and ACh to the orthosteric binding site (Avlani et al. 2007). Residues that lie in close proximity to the cysteine residues responsible for the conserved disulfide bond have additionally been implicated in regulating the access of orthosteric ligands into the binding pocket. For instance, substitution of Asp^{3.26} reduces the binding of orthosteric ligands such as ACh, QNB, and NMS (Goodwin et al. 2007). It has been speculated that this

residue may be involved in the initial contact of positively charged orthosteric muscarinic ligands with their receptors before they enter into the main binding site (Lu et al. 2001; Goodwin et al. 2007; Jakubik et al. 2000). Structural predictions of the M₁ mAChR suggest that Ser^{4.53}, Trp^{4.57}, and Ile^{4.61} lie in close proximity to Asp^{3.26} and may also act to form this “peripheral” binding site (Lu et al. 2001).

The boundary between the top extracellular portion of TMII and the first extracellular loop in the mAChRs has also been implicated in the binding affinity of orthosteric ligands, with substitution of Trp⁹⁹ in the M₁ mAChR for Ala or Phe, or the equivalent Trp¹³³ for Gly in the M₃ mAChR, significantly reducing the binding affinity of ACh, NMS, and QNB (Li et al. 2007a; Matsui et al. 1995; Avlani et al. 2010). Similarly, mutation of Asn^{2.68} Ile at the junction of TMII and the first extracellular loop in the M₃ mAChR results in a reduction in the binding of [³H]NMS (Li et al. 2007a). Interestingly, recent *in silico* studies have suggested that the extracellular loops can have a strong influence on how TM helices pack together and, as such, perturbation of the extracellular loops may have an additional effect on the fine packing in the TM helices; it is thus possible that effects of extracellular loop mutations on orthosteric ligand binding may reflect such indirect perturbations of the orthosteric pocket.

4 Elucidation of mAChR Allosteric Binding Sites

In addition to the orthosteric binding site, it is now well established that GPCRs can possess topographically distinct allosteric sites (May et al. 2007a). Indeed, studies of the phenomenon at the mAChRs represent the earliest known examples in the field, dating back to the late 1960s and early 1970s when investigators described noncompetitive interactions between orthosteric mAChR agonists and the neuromuscular blocking agent, gallamine, or certain alkane-bis-ammonium compounds, exemplified by C₇/3-phth (Clark and Mitchelson 1976; Lullmann et al. 1969). Since that time, the actions of additional allosteric mAChR modulators have been characterized (Stockton et al. 1983; Lazareno and Birdsall 1995; Lazareno et al. 1998). Although beyond the scope of this chapter, it should be noted that there now exists a relatively rich, and expanding, allosteric pharmacology around the mAChRs, including prototypical negative allosteric modulators, such as gallamine and C₇/3-phth, as well as positive modulators of ACh, such as brucine and BQCA at the M₁ mAChR, LY2033298 at the M₄ mAChR, and VU0238429 at the M₅ mAChR (Lazareno et al. 1998; Birdsall et al. 1999; Chan et al. 2008; Gharagozloo et al. 1999; Leach et al. 2010; Ma et al. 2009; May et al. 2007b). In recent years, a number of putative allosteric agonists, which can activate the receptor in their own right, have also been identified (Chan et al. 2008; Leach et al. 2010; Ma et al. 2009; May et al. 2007b; Nawaratne et al. 2008; Jones et al. 2008; Langmead et al. 2006; Spalding et al. 2006; Sur et al. 2003; Thomas et al. 2008; Bridges et al. 2009).

There is compelling pharmacological evidence indicating that there are at least two allosteric binding sites on the mAChRs that can be targeted by small

molecule ligands. The best characterized site, referred to herein as the “prototypical modulator site,” recognizes compounds such as gallamine, C₇/3-phth, brucine and alcuronium, whereas the “second” allosteric site binds certain indolocarbazoles and the benzimidazole analogs, WIN 51,708 and WIN 62,577 (Lazareno et al. 2000; Lanzafame et al. 2006). To date, all mutagenesis studies of mAChR allosteric binding sites have focused on the prototypical modulator site, which is believed to comprise epitopes that are more extracellularly located than those within the TM-bound orthosteric pocket. The location of the “second” allosteric site is currently unknown, although a molecular modeling study has suggested an intracellular location (Espinoza-Fonseca and Trujillo-Ferrara 2005, 2006).

Given that many prototypical modulators interact with all five mAChR subtypes, it is likely that some conserved residues may be involved in their actions. An early study at the M₁ mAChR proposed that Trp^{3.28} and Trp^{7.35}, which lie at the extracellular end of TMIII and TMVII, respectively, may serve such a role, at least with respect to the binding of gallamine (Matsui et al. 1995). However, another key aspect of allosteric modulator action is that these compounds typically display greater degrees of selectivity across mAChR subtypes than do orthosteric ligands, and thus nonconserved amino acids must also contribute to modulator binding and/or actions. For example, gallamine binds with higher affinity to the M₂ mAChR than to the other mAChR subtypes and this preference has been attributed, in part, to the interaction of gallamine with residues located in the second extracellular loop of the M₂ mAChR, predominantly Tyr¹⁷⁷ and to a lesser degree ¹⁷²Glu-Asp-Gly-Glu¹⁷⁵, as well as residues at the junction of the third extracellular loop and the top of TMVII, namely Asn^{7.32}, Trp^{7.35}, and Thr^{7.36} (May et al. 2007b; Voigtlander et al. 2003; Huang et al. 2005; Prilla et al. 2006; Valant et al. 2008). Similarly, in the M₄ mAChR, Ser^{7.36} has been implicated in gallamine binding (Buller et al. 2002), whilst Glu^{7.32} in the M₁ mAChR has been implicated in the transmission of positive cooperativity between brucine and ACh (Stewart et al. 2010).

The binding site for the allosteric modulator/agonist, LY2033298, may also overlap with the prototypical allosteric site, because the interaction between LY2033298 and C₇/3-phth appears competitive (Leach et al. 2010). In support of this hypothesis, alanine substitution of Phe¹⁸⁶ in the second extracellular loop of the M₄ mAChR, which corresponds to Tyr¹⁷⁷ in the M₂ mAChR, markedly attenuates the binding of LY2033298 (Nawaratne et al. 2010). Interestingly, the equivalent position in the M₁ and M₃ mAChRs is also an aromatic residue, suggesting that aromaticity is an important characteristic in this region of the second extracellular loop of most mAChRs. Moreover, alanine substitution of the Tyr in this position of the M₁ mAChR extracellular loop greatly diminished the potency of BQCA as an allosteric modulator of ACh (Ma et al. 2009). Also in agreement with prior studies on the M₁ mAChR that focused on gallamine, substitution of the conserved Trp^{3.28} and Leu^{3.29} in the M₄ mAChR with alanine decreased the affinity of both C₇/3-phth and LY2033298 (Leach et al. 2011). However, alanine substitution of Asp^{7.32} in the M₄ mAChR to the corresponding Asn^{7.32} in the M₂ mAChR had no significant effect on the binding affinity of LY2033298 (Chan et al. 2008). Similarly, mutation

of Ser^{7.36} (M₄ mAChR) to the corresponding Thr^{7.36} in the M₂ mAChR did not alter the interaction between LY2033298 and ACh (Chan et al. 2008). Thus, as with orthosteric ligands, it appears that allosteric ligands can recognize a common site but, nonetheless, adopt different poses within that site such that they display differential sensitivity to specific mutations.

More recently, a novel class of ligand has been described that can bridge *both* orthosteric and allosteric sites concomitantly. Such ligands have been termed “bitopic,” and it is possible that a number of putative “allosteric agonists” may actually fall into this category (Voigtlander et al. 2003). A good example of this phenomenon has been noted with the functionally selective mAChR agonist, McN-A-343. Although exhibiting many properties commensurate with a competitive (orthosteric) mode of action, there have been provocative examples in the literature to suggest that McN-A-343 can also interact allosterically with the M₂ mAChR (May et al. 2007b; Birdsall et al. 1983; Waelbroeck 1994). A subsequent study revealed that the molecule is actually a hybrid composed of orthosteric (trimethylammonium) and allosteric (3-chlorophenylcarbamate) moieties (Lanzafame et al. 2006), thus providing a possible explanation of previous studies; depending on the experimental conditions, McN-A-343 can adopt a binding pose that bridges both orthosteric and allosteric sites (Fig. 3) or a second pose that only interacts allosterically with a prebound orthosteric ligand. Importantly,

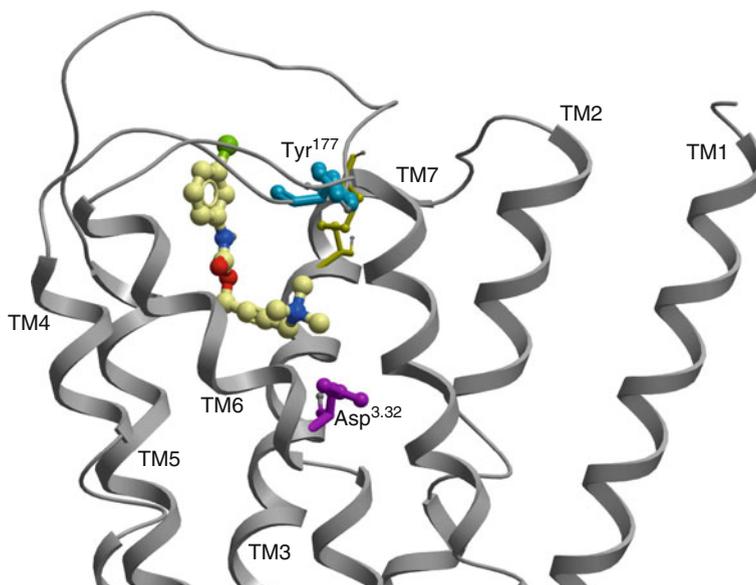


Fig. 3 A possible bitopic binding mode for McN-A-343 at the M₂ mAChR. Two key residues affecting the actions of the agonist in the orthosteric (Asp^{3.32}) and allosteric (Tyr¹⁷⁷) pockets are highlighted. Coordinates taken from Valant et al. (2008)

the binding of McN-A-343 is sensitive to mutation of both the key orthosteric site residue, Asp^{3.32}, and key allosteric site residue, Tyr¹⁷⁷, in the M₂ mAChR (May et al. 2007b; Valant et al. 2008).

Similarly to McN-A-343, there is some evidence that the putative allosteric agonists, AC-42 and its congener 77-LH-28-1, may also bridge both the orthosteric and allosteric binding sites at the M₁ mAChR, and thus are better classed as bitopic, rather than allosteric, agonists. Although the binding of AC-42 is relatively insensitive to orthosteric site mutations at Tyr^{3.33} and Tyr^{6.51} (Spalding et al. 2002, 2006), recent studies have suggested that AC-42 and 77-LH-28-1 could still interact with the key orthosteric site residue, Asp^{3.32}, but bind with a significantly different pose to prototypical orthosteric agonists (Lebon et al. 2009). In agreement with this model, the binding affinity of AC-42 is decreased by mutation of Leu^{3.29}, whilst that of AC-42 and 77-LH-28-1 is increased by Ala substitution of Trp^{3.28} (Avlani et al. 2010; Spalding et al. 2006; Gregory et al. 2010), indicating that they interact with a region that may border the orthosteric and allosteric binding sites. To accommodate this binding mode, it has been proposed that Trp^{3.28}, which would normally face toward the center of the helical bundle, may “flip” outwards and be stabilized by Phe^{2.56} in the M₁ mAChR, which is located on the external side of TMII (Avlani et al. 2010).

Collectively, these recent studies of bitopic ligands highlight a number of important considerations. First, it is possible that ligands previously classed as “functionally selective” may achieve such selectivity as a consequence of a bitopic mechanism of action. Second, caution must be exercised when classifying any novel agonist as “allosteric,” unless rigorous pharmacological data are available to suggest that such a compound’s agonism indeed arises directly from an interaction with an allosteric site (and not the orthosteric site, as would be expected for a bitopic ligand). Third, it should be possible to rationally design bitopic ligands by purposefully utilizing appropriate orthosteric and allosteric fragments joined together by an optimal linker. A number of recent elegant studies have indeed provided proof of concept for this approach (Disingrini et al. 2006; Steinfeld et al. 2007; Antony et al. 2009).

5 Effects of Mutations on Signaling

The molecular details underlying the activation mechanisms of GPCRs remain largely unknown, and thus represent a major ongoing field of research. Much of the problem lies with the fact that GPCRs are highly dynamic proteins that can adopt multiple active states, each associated with different intracellular interacting partners and functional outcomes. Thus, any interpretation of mutational studies on GPCR signaling must be tempered by the fact that it will be influenced by the choice of functional assay used as a measure of receptor activation.

In general, ACh binding is predicted to initially elicit conformational changes in the mAChRs that result in a reduced pocket volume between key residues,

specifically Tyr^{3.33}, Thr^{5.39}, Thr^{5.42}, Tyr^{6.51}, and Tyr^{7.39}, that surround the bound ligand (Lu et al. 2002). Not surprisingly therefore, substitutions of these amino acids result in reduced ACh efficacy (Lu et al. 2001; Nawaratne et al. 2010; Gregory et al. 2010; Spalding et al. 1998). However, the efficacy of other agonists need not be affected by mutation of some or all of these residues, as they are likely to adopt differing poses to ACh, depending on their structures. Recent disulfide cross-linking experiments performed on the rat M₃ mAChR also indicated that Ser^{3.36} and Cys^{7.42} lie in close proximity when the receptor is in an active conformation (Han et al. 2005a), consistent with movement of residues that point into the orthosteric binding pocket toward one another. Ser^{3.36} is predicted to face directly into the core of the orthosteric binding pocket thus it may serve as a secondary contact point for ACh when receptor activation is triggered. Indeed, substitution of Ser^{3.36} for Ala in the M₁ (Lu and Hulme 1999), M₂ (Gregory et al. 2010), and M₄ mAChRs (Leach et al. 2011) leads to a large attenuation in the signaling of ACh and other agonists.

The movement of key “inner shell” residues toward ACh causes a reorientation of amino acids located in TMVII, in particular those located in the highly conserved Asn^{7.49}-Pro^{7.50}-X-Cys^{7.52}-Tyr^{7.53} motif, which mediates a large conformational change at the intracellular end of TMVII. Agonist binding triggers movement of Tyr^{7.53} toward Val^{1.53}, whilst residues in TMVII that are predicted to face the lipid bilayer move opposite TMI, suggesting a rotational movement of the cytoplasmic end of TMVII (Han et al. 2005b) and concomitant movement of helix VIII away from TMI (Li et al. 2007b). An M₁ model based on homology with rhodopsin predicted that the Asn^{7.49}-Pro^{7.50}-X-Cys^{7.52}-Tyr^{7.53} motif constrains the inactive receptor conformation by forming a network of hydrogen bonds that connect TMVII to TMI, TMII, and TMIII (Lu et al. 2001). In support of this, Ala substitution of Asn^{7.49}, Pro^{7.50}, and Tyr^{7.53} increases the affinity of ACh for the M₁ mAChR (Lu et al. 2001). However, although Asn^{7.49} interacts with Thr^{6.43} and Asp^{6.44} in the inactive state of rhodopsin, an interaction between Asn^{7.49} and Asp^{2.50} is observed in opsin through crystal waters (Urizar et al. 2005), suggesting that this residue forms new contacts upon receptor activation that are important for the stability of the active receptor state. An identical interaction seems likely in the M₁ mAChR (Bee and Hulme 2007), and the significant reduction in agonist efficacy following mutation of Asn^{7.49} in the mAChRs confirms that this residue is indeed important for stabilizing an active receptor conformation.

In conjunction with the conformational changes associated with TMVII, relocation of the bottom of TMVI away from the helical bundle and toward TMV takes place upon receptor activation. The crystal structures of rhodopsin and opsin show significant differences in the position of TMVI relative to TMIII (Palczewski et al. 2000; Park et al. 2008), particularly within the regions that comprise the retinal binding pocket. The movement of TMVI is driven, in part, by alterations in a region that contains an aromatic cluster of amino acids (Cys^{6.47}-Trp^{6.48}-Leu^{6.49}-Pro^{6.50}-Tyr^{6.51}-Ala^{6.52} in rhodopsin) located toward the extracellular portion of TMVI (Ruprecht et al. 2004). This leads to the development of a kink at the highly conserved Pro^{6.50}, which causes the cytoplasmic end of TMVI to tilt away from

the center of the helical bundle (Park et al. 2008). In the mAChRs, mutations in the Thr^{6.47}-Trp^{6.48}-Thr/Ala^{6.49}-Pro^{6.50}-Tyr^{6.51}-Asn^{6.52} motif lead to reduced agonist efficacy or nonfunctional receptors (Spalding et al. 1998; Wess et al. 1992), as do substitutions at surrounding residues, including Lys^{6.29}, Ala^{6.34} and Ser^{6.38} in the M₃ mAChR and Val^{6.55} and Val^{6.57}, Val^{6.59} in the M₃ and M₅ mAChRs (Spalding et al. 1998; Schmidt et al. 2003). In contrast, mutations at other residues in TMVI, including Glu^{6.30} in the M₁ mAChR, Gln^{6.35} in the M₃ mAChR and Ile^{6.40}, Ala^{6.43}, Phe^{6.44}, and Ser^{6.58} in the M₅ mAChR, result in constitutive activity (Spalding et al. 1998; Schmidt et al. 2003; Hogger et al. 1995), suggesting that these residues help to stabilize the ground state of the receptor. Overall, these observations highlight the importance of the cytoplasmic end of TMVI in mAChR activation.

Substitution of conserved amino acids throughout TMIII, TMIV, and TMV, including Asp^{3.26}, Asp^{3.32}, Ile^{3.46}, Trp^{4.57}, Pro^{4.59}, Thr^{5.37}, Ile^{5.38}, Ala^{5.46}, and Ile^{5.61} can have particularly detrimental effects on agonist efficacy (Lu and Hulme 1999; Nawaratne et al. 2010; Spalding et al. 1998; Schmidt et al. 2003; Lu and Hulme 2000; Page et al. 1995). However, Pro^{4.59} is predicted to face into the lipid bilayer (Wess et al. 1991), thus the effect of mutations at this position may be indirect. In the M₁ mAChR, Ala substitution of Trp^{3.28} also greatly reduces the signaling efficacy of ACh (Lu and Hulme 1999).

More recently, a number of mutations that disrupt the function of the M₃ mAChR were identified in TMI and TMII, including mutations at Thr^{1.46}, Ile^{1.47}, Asn^{1.50}, Val^{1.53}, Asn^{1.60}, Asn^{2.38}, Asn^{2.39}, Ser^{2.45}, Ala^{2.49}, Asp^{2.50}, Leu^{2.51}, Ser^{2.57}, Met^{2.58}, Asn^{2.59}, Phe^{2.61}, Ile^{2.66}, and Asn^{2.68} (Li et al. 2007a). Furthermore, residues in the second extracellular loop of the M₃ mAChR are critical for the functional activity of the receptor, including Gln²⁰⁷, Gly²¹¹, Arg²¹³, Gly²¹⁸, Ile²²², Phe²²⁴, Leu²²⁵, and Pro²²⁸ (Scarselli et al. 2007). This is consistent with observations that conformational changes in the second extracellular loop occur upon activation of rhodopsin, whereby movement of TMV and disruption of the proposed ionic lock between TMIII and TMVI causes rearrangement of the hydrogen bond network that connects the extracellular ends of TMIV, TMV, and TMVI to the second extracellular loop (Ahuja et al. 2009).

In addition to inactivating mutations, amino acid substitutions in TMIII can result in increased constitutive activity of mAChRs, including Leu^{3.43} and Ser^{3.47} in the M₁ mAChR (Lu and Hulme 1999), suggesting a role for these residues in constraining the inactive receptor state. In particular, the highly conserved Glu/Asp-Arg-Tyr^{3.51} motif, which is found in approximately 70% of Family A GPCRs, has been implicated in stabilizing the inactive receptor state and enabling a switch to an active receptor conformation. In rhodopsin, the β -ARs and the A_{2A} adenosine receptor, Arg^{3.50} forms a hydrogen bond with the adjacent Glu/Asp^{3.49} (Cohen et al. 1993; Ballesteros et al. 2001; Scheer et al. 1996). In rhodopsin, Arg^{3.50} also forms a key salt bridge with Glu^{6.30} (although this interaction was not present in the β -ARs or A_{2A} adenosine receptor structures) that is broken upon receptor activation as TMVI moves apart from TMIII. In opsin, Arg^{3.50} interacts instead with Tyr^{5.58} and potentially with Tyr^{7.53}. Accordingly, mutation of Arg^{3.50} in the M₁ mAChR leads to significant reductions in agonist

efficacy (Jones et al. 1995), suggesting that this residue is essential for stabilizing the active receptor state in the mAChRs. However, and in contrast to observations at other GPCRs, significant effect on the signaling efficacy of ACh is not observed in the M₁ or M₅ mAChRs upon mutation of Asp^{3.49} (Lu et al. 1997; Burstein et al. 1998), suggesting that the postulated hydrogen bond between Asp^{3.49} and Arg^{3.50} does not necessarily exist in the mAChRs.

The bulk of the studies described above have focused on activation mechanisms thought to be “universal” to agonists of the mAChRs. However, recent studies of allosteric and other novel functionally selective agonists of these receptors have begun to identify residues that contribute to receptor activation in a more ligand or pathway-specific manner. For instance, Phe^{2.56} in the M₁ mAChR is essential for the activity of AC-42 and 77-LH-28-1 but not ACh or pilocarpine (Avlani et al. 2010), whilst the efficacy of the allosteric agonist, LY2033298, but not that of ACh or McN-A-343, at the M₄ mAChR is selectively sensitive to mutations of extracellular loop 1 residues Ile⁹³ and Lys⁹⁵ (Nawaratne et al. 2010). Conversely, a recent study of the M₂ mAChR identified Tyr^{3.33} as a key residue selectively linking activation of the receptor to the phosphorylation of ERK1/2, irrespective of the nature of the activating ligand (Gregory et al. 2010). These findings indicate that there are likely to be a number of agonist and pathway-specific mechanisms that contribute to receptor signaling, consistent with the hypothesis that mAChRs can adopt multiple active states that are differentially stabilized by various classes of ligand and/or intracellular interacting proteins.

6 The G Protein Binding Interface

The only crystallographic evidence of the interaction between a GPCR and its G protein comes from the structure of opsin and metarhodopsin II in combination with a synthetic peptide composed of the first 11 amino acids of transducin, the cognate G α subunit for this receptor (Scheerer et al. 2008). This study indicated that the second and third intracellular loops, the cytoplasmic ends of TMIII, TMV, and TMVI and the amino-terminal segment of helix 8 are all involved in G protein binding events. Specifically, an interaction between Arg^{3.50} and a Cys residue in the G α peptide corresponding to Cys³⁴⁷ in transducin was observed, confirming the importance of Arg^{3.50} in receptor signaling and underlining its importance in stabilizing an active receptor state. Likewise, interactions were observed between the transducin peptide and Leu²²⁶, Val²³⁰, Ala²³³, Thr²⁴², Thr²⁴³, Ala²⁴⁶, and Val²⁵⁰ in the third intracellular loop of the receptor.

In agreement with the binding of the transducin peptide to opsin, mutagenic studies suggest an interaction between full-length G α_q and TMV, TMVI and helix 8 of the M₃ mAChR. Residues located at the junctions between the third intracellular loop and TMV and TMVI are particularly important for the recognition by G α_q proteins of the M₃ mAChR, including Tyr^{5.64}, Ala^{6.32}, Ala^{6.33}, Leu^{6.36},

and Ser^{6,34} (Bluml et al. 1994b, c; Blin et al. 1995; Kostenis et al. 1997). Similar observations have been made in the M₅ mAChR (Burstein et al. 1995, 1996; Hill-Eubanks et al. 1996). A recent cysteine cross-linking study additionally identified Leu¹⁷³ and Arg¹⁷⁶ in the second intracellular loop and Thr⁵⁴⁹, Thr⁵⁵², and Thr⁵⁵⁶ in the amino-terminal segment of helix 8 of the M₃ mAChR as residues that directly interact with the carboxy-terminal portion of G α q (Hu et al. 2010). These interactions were observed in the absence and presence of agonist and even in the presence of the inverse agonist, atropine, suggesting that inactive mAChRs can exist in complex with G α proteins. However, upon agonist stimulation, an interaction between Ala^{6,33} and the carboxy-terminal region of G α q was promoted, whilst interactions between Thr⁵⁴⁹ and Thr⁵⁵² and the G protein were enhanced. This is consistent with the concept that agonists trigger movement of the cytoplasmic end of TMVI away from the TM bundle, which enables the carboxy-terminus of G α to interact with TMV and TMVI. A weaker interaction was detected between Lys⁵⁴⁸ at the junction of TMVII and helix 8 of the M₃ mAChR and Asp³²¹ in the carboxy-terminus of G α q, which was also enhanced by agonist treatment. Interestingly, the same study identified an interaction between Leu¹⁷³ in the second intracellular loop of the M₃ mAChR and Arg³¹ in the amino-terminal region of the G α q subunit, again in the absence of agonist or inverse agonist, indicating that multiple regions of the G protein are involved in coupling to the receptor.

7 Conclusions

A wealth of mutagenesis-derived information continues to provide insight into the structural and functional role of diverse receptor regions in the mAChRs. Coupled with direct crystallographic information obtained for other Family A GPCRs, we are starting to gain an understanding of the intramolecular interactions that exist in the mAChRs, how mAChR ligands bind to their receptors, and how ligand binding triggers conformational changes in the mAChR structure that ultimately lead to intracellular signaling events. No doubt, given the recent advances in the field of GPCR structural biology, high-resolution structures of ligand-bound mAChRs are a likely outcome in the not-too-distant future. Irrespective of the nature of the experimental paradigm, the most likely gage of success in the area of receptor structure–function analyses is the use of information gained to successfully explain and predict biological events, and to rationally design drugs that can alleviate diseases associated with the mAChRs.

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