

Chapter 2

Structural Insights into Activation and Allosteric Modulation of G Protein-Coupled Receptors

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Abstract G protein-coupled receptors (GPCRs) are cell-surface receptors that regulate neurotransmission, cardiovascular function, metabolic homeostasis, and many other physiological processes. Due to their central role in human physiology, these receptors are among the most important targets of therapeutic drugs, and are they among the most extensively studied integral membrane proteins. To better understand GPCR signaling at a molecular level, we have undertaken structural studies of a prototypical GPCR family, the muscarinic acetylcholine receptors. These studies have led to crystal structures of muscarinic receptors in both inactive and active conformations, as well as the first structure of a GPCR in complex with a drug-like allosteric modulator. In addition, we have recently developed new approaches in combinatorial biology to create protein modulators of GPCR signaling. These studies shed light on the function of muscarinic receptors, and offer insights into the molecular basis for the regulation of GPCR signaling and activation in general.

2.1 Introduction

As critical regulators of human physiology, GPCRs have long been the subject of extensive pharmacological study. More recently, advances in protein expression and purification have made GPCRs amenable to investigation by biochemical methods, spectroscopic studies, and structural biology. GPCR signaling is typically initiated by binding of a ligand, such as a drug, to the extracellular surface of the receptor. This binding event leads to conformational changes in the receptor that allow the GPCR to interact with intracellular signaling proteins including G proteins, arrestins, and GPCR kinases [1]. While many aspects of these complex signaling pathways are understood in great detail, other important questions still remain unanswered, particularly surrounding molecular aspects of ligand binding, selectivity, and receptor activation.

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To better understand GPCR signaling at a molecular level, we have undertaken structural studies of a prototypical GPCR family, the muscarinic acetylcholine receptors. These receptors were chosen as the subject of this work due to their propensity to bind allosteric modulators, and for their longstanding role as a model system for understanding GPCR signaling in general. Muscarinic receptors are also important targets in the treatment of diseases, including respiratory conditions and neurodegenerative diseases [2]. Muscarinic receptors are also important drug antitargets, since they are responsible for off-target side effects of various drugs, including first-generation antihistamines, which often cause dry mouth due to inhibition of the M_3 muscarinic receptor.

2.2 Results and Discussion

Initially, we sought to understand how small-molecule drugs bind to muscarinic receptors by using X-ray crystallography to determine structures of these receptors bound to different ligands. Unfortunately, muscarinic receptors and other GPCRs are poor candidates for such studies, because they are biochemically unstable and because they possess large, unstructured loops. To address this problem, we followed an approach developed by Rosenbaum et al. [3], and replaced the flexible third intracellular loop of the receptor with T4 lysozyme (T4L), a soluble, crystallizable protein (Fig. 2.1). This approach has been successfully applied to a large number of GPCRs, showing it to be a highly general method. Indeed, a majority of GPCR structures reported to date have been solved using the protein fusion approach [4].

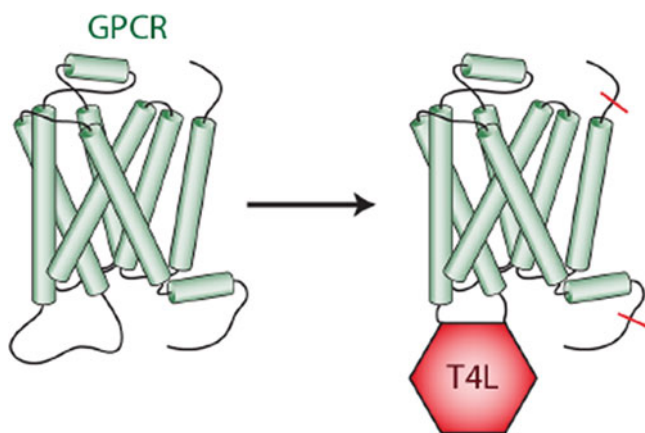


Fig. 2.1 Modification of a GPCR to facilitate crystallization. GPCRs typically contain flexible termini and loops. To address these liabilities, muscarinic receptors had the third intracellular loop replaced with T4 lysozyme (T4L), and had the amino- and carboxy-termini truncated in the case of the M_3 receptor

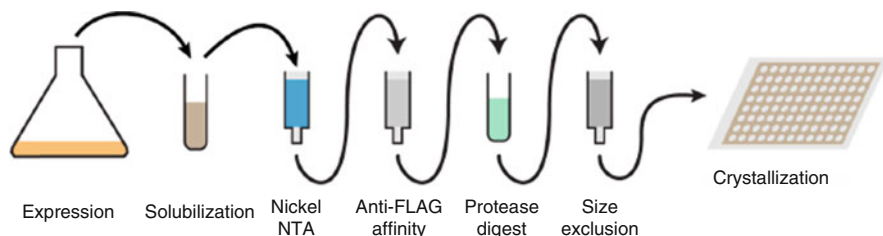


Fig. 2.2 Purification procedure. A typical GPCR purification procedure is diagrammed, showing major steps in the purification. Muscarinic receptors were purified in this manner

We crystallized the T4 lysozyme-fused M_2 and M_3 receptors using the lipidic cubic phase technique [5]. This approach involves reconstitution of the target protein in a lipid bilayer, followed by immersion of the protein/lipid mix in a precipitant solution that diffuses through the sample, promoting crystallization. In the case of the M_2 and M_3 receptors, extensive optimization of the purification procedures was required, leading to the purification procedure outlined in Fig. 2.2. Once homogenous biochemical preparations were obtained, crystallization and structure determination were relatively straightforward, leading to structures of both receptors [6, 7]. As is typical for many lipidic cubic phase crystals, muscarinic receptors formed crystals less than 100 μm in length and required micro-focus data collection methods [8]. The use of micro-focus X-ray sources at the Advanced Photon Source of Argonne National Lab was essential to allowing structure determination from such small crystals, since lipidic cubic phase crystallization drops often contain dozens to hundreds of crystals that cannot be separated from one another. The use of a micro-focus beamline this allows individual crystals to be irradiated sequentially, generating a series of “wedges” of data, each comprising 5° – 10° total rotation of the crystal.

Structures of the M_2 and M_3 receptors were solved with the receptors bound to two different antagonists: the clinical drug tiotropium (Spiriva) bound to the M_3 receptor, and the research compound quinuclidinyl benzilate (QNB) bound to the M_2 receptor. The two structures show a high degree of overall similarity, and possess the typical seven transmembrane fold seen in other GPCRs (Fig. 2.3). Closer inspection reveals that M_2 and M_3 receptors possess unique, deeply buried ligand-binding pockets, surrounded by transmembrane helices. The two receptors are virtually identical in terms of ligand binding site conformations (Fig. 2.4), offering an explanation for the difficulties faced by medicinal chemists in designing subtype-selective muscarinic receptor ligands. Key ligand contacts include a charge-charge interaction between Asp^{3.32} (superscripts denote Ballesteros-Weinstein numbering [9]) and the ligand amine, representing a feature conserved in all aminergic GPCRs as well as opioid receptors [10, 11]. The only other polar contact involves Asn^{6.52}, which engages in a pair of hydrogen bond interactions with the bound ligand in both muscarinic receptor subtypes. A striking feature of the muscarinic receptor ligand binding pocket is an abundance of aromatic amino acid side chains surrounding the

Fig. 2.3 Overall structure of muscarinic receptors. The overall structures of the muscarinic receptors are shown, with the M_2 and M_3 subtypes superimposed on one another. The overall folds are highly similar both to each other and to other Family A GPCRs

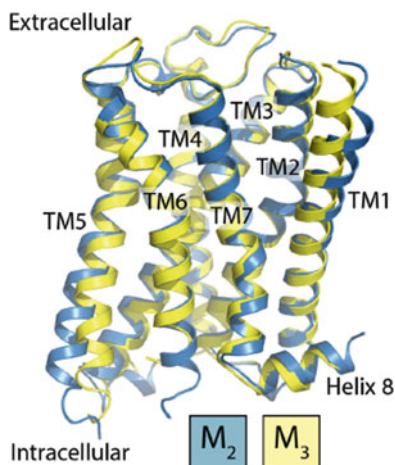
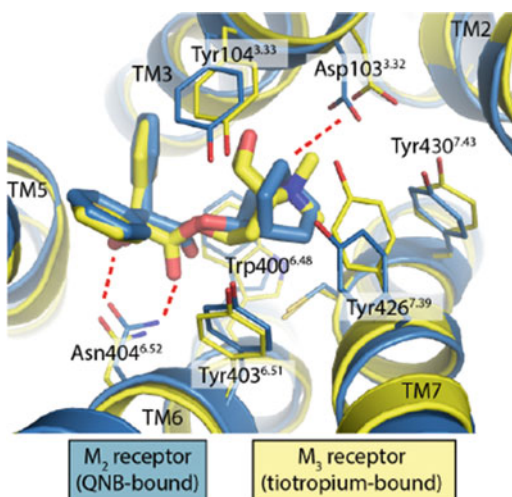


Fig. 2.4 Ligand recognition. The ligand binding pockets of the M_2 and M_3 muscarinic receptors are shown superimposed. Receptor amino acid side chains are shown in *thin sticks*, and the bound ligands (QNB and tiotropium) are shown in *thick sticks*. Residues are numbered according to the M_2 receptor sequence, with Ballesteros-Weinstein numbering in *superscripts*. Polar contacts are indicated with *dotted lines*



positively charge amine. Similar features are seen in the structures of other proteins that bind acetylcholine, suggesting convergent evolution toward a particular ligand recognition mode [6].

The binding site of tiotropium and QNB is referred to as the orthosteric ligand binding site, and this is also the binding pocket for the endogenous agonist acetylcholine. In addition to this site, structures of muscarinic receptors also revealed the existence of a large cavity situated directly above (extracellular to) the orthosteric binding site. This cavity, termed the “extracellular vestibule” is lined by residues that have been previously implicated in binding to allosteric modulators [12]. The location of this cavity is also consistent with the observation that many muscarinic allosteric modulators can slow dissociation of orthosteric ligands, resulting in slowed binding kinetics [12].

To probe the role of the extracellular vestibule in ligand binding, we performed long timescale molecular dynamics simulations of antagonists binding to and dissociating from the muscarinic receptors [7]. These studies suggested that ligands entering or leaving the orthosteric site may pause at the extracellular vestibule, indicating that this second site is also a potential target for small molecule drugs. In fact, experimental data have provided evidence that orthosteric ligands can also act as allosteric modulators at high concentrations [13], presumably binding to the extracellular vestibule and modifying the effects of orthosteric ligands.

More recently, Dror and colleagues have built upon this work and performed a detailed series of simulations and mutagenesis experiments to probe the action of negative allosteric modulators, which bind to the extracellular vestibule [14]. The simulations indicated that negative allosteric modulator binding involves cation- π interactions between the positively charged modulators and aromatic amino acid sidechains surrounding the extracellular vestibule. These simulations offer some of the most detailed views of negative allosteric modulator binding, but to date, no experimentally determined structure of a muscarinic receptor bound to a negative allosteric modulator has been reported.

While the first structures of muscarinic receptors offered important insights into the molecular details of ligand recognition, both structures represent inactive states. We next sought to understand activation of the M_2 muscarinic receptor, as well as regulation of receptor activation by positive allosteric modulators. Initial attempts to crystallize M_2 receptor bound to agonists were unsuccessful, likely due to conformational flexibility in the receptor. Such conformational heterogeneity has been extensively studied in the β_2 adrenergic receptor, a homolog of muscarinic receptors [15]. Based on this hypothesis, we sought to develop stabilizing single domain camelid antibody fragments (called nanobodies) to bind the intracellular side of the receptor and stabilize an active conformation, similar to a previously successful approach developed by Rasmussen et al. [16].

Initial attempts involving a standard immunization and phage display selection and screening approach were unsuccessful, resulting in nanobodies that could bind M_2 receptor but which lacked conformational selectivity. We next turned to a yeast surface display approach, expressing a library of nanobodies on the surface of yeast and staining the cells with M_2 receptor solubilized in detergent. Previously, we had developed this approach to create a high-affinity nanobody specific to the β_2 adrenergic receptor [17]. In the case of the muscarinic receptors, two receptor samples were prepared with distinct fluorophores covalently attached. Each population was bound to either a covalent agonist or a high affinity antagonist. By using a mix of active receptors and inactive receptors labeled with distinct fluorophores (Fig. 2.5), we were able to sort cells by fluorescence activated cell sorting (FACS) to select clones with the desired conformational selectivity [18].

After screening selected clones for the binding and conformational specificity, a high affinity, active-state stabilizing nanobody called Nb9-8 was identified. This nanobody was purified in complex with M_2 receptor and a bound agonist, and crystallized by the lipidic cubic phase method. The resulting structure revealed an active conformation M_2 receptor, showing a rotation of transmembrane helix 6,

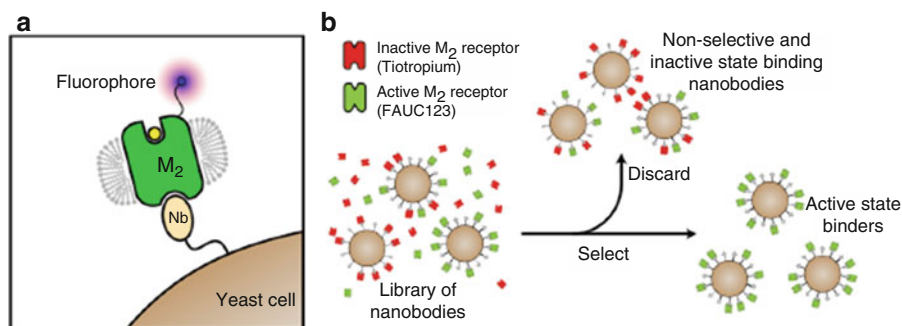
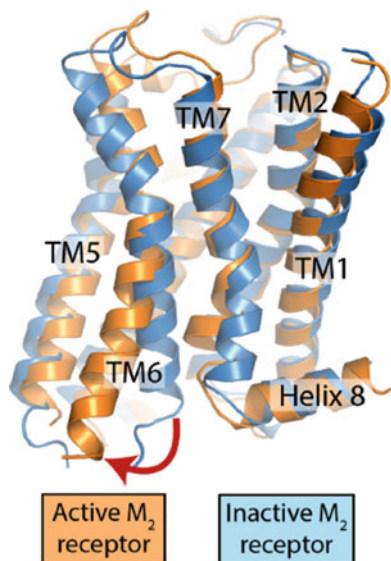


Fig. 2.5 Conformational selection. (a) A library of nanobodies was expressed on the surface of *Saccharomyces cerevisiae* yeast, then stained with purified receptor in detergent. (b) This allowed selective isolation of only clones binding to activated receptors in the manner diagrammed here

Fig. 2.6 Activation mechanism. Comparison of inactive- and active-state structures of the M₂ receptor show the overall similar structure with a notable deviation at transmembrane helix 6. The outward rotation of this helix is seen in other activated GPCR structures, suggesting it is a common and conserved feature of GPCR activation



which is a hallmark of GPCR activation (Fig. 2.6). The most surprising feature of this structure is a large rearrangement of the extracellular region of the receptor, which had not previously been observed for other GPCRs. This region of the receptor is known to be the target of allosteric modulators, prompting a second series of crystallization trials with a positive allosteric modulator of agonists, called LY02119620 [19].

Allosteric modulators of GPCR function have become increasingly attractive as potential therapeutics, possessing properties often unachievable with conventional orthosteric ligands [20]. In particular, allosteric modulators can influence signaling while maintaining the native spatiotemporal regulation of agonist release. Allosteric

ligands may also show higher selectivity than orthosteric ligands, as they often bind to sites with lower sequence conservation than conventional ligands. This is particularly important in the case of muscarinic acetylcholine receptors, where drugs targeting specific subtypes have not been available. Although drugs targeting the M_1 muscarinic receptor have shown efficacy in the treatment of neurodegenerative diseases, their use in human patients is precluded by side effects due to activation of other muscarinic receptor subtypes. Ligands targeting less conserved allosteric sites are typically more selective for specific receptor subtypes, and may therefore offer a means of developing agonists and antagonists with selectivity toward particular muscarinic receptors.

Despite increased interest in allosteric modulators, no structural information regarding allosteric modulation of GPCRs has been available until recently. Using our engineered nanobody 9-8, we were able to obtain a second crystal structure of activated M_2 receptor, this time bound to the LY02119620 modulator. This structure revealed that the conformation of the receptor is highly similar irrespective of whether or not the modulator is bound, suggesting that the modulator is recognizing a binding site that is essentially “pre-formed” upon receptor activation [18, 21]. By stabilizing this site, the modulator may promote signaling and stabilize agonist binding, thereby accounting for its pharmacological profile (Fig. 2.7).

Taken together, these studies have shown structures of muscarinic receptors in both inactive and active conformations, as well as the first structure of a GPCR in complex with a drug-like allosteric modulator. The use of new approaches in combinatorial biology allowed the identification of nanobody modulators of GPCR signaling, facilitating structural studies of activated muscarinic receptors. Many questions remain, however, and ongoing studies will lead to a more complete understanding of muscarinic receptor function. In particular, the interactions between muscarinic receptors and their effectors (G proteins and arrestins) remain poorly

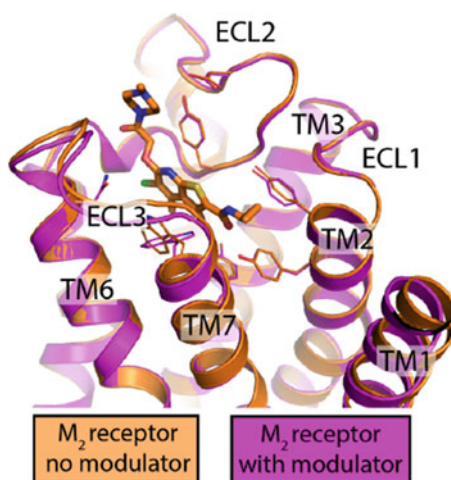


Fig. 2.7 Allosteric modulation. Comparison of active-state M_2 receptor structures with and without bound allosteric modulator LY02119620 (*thick sticks*). In each case, the overall structure of the receptor and the side chain conformations are highly similar (See Ref. [21] for more details)

understood, with little structural information regarding these interactions. In the long term, new insights into the mechanistic basis for muscarinic receptor signaling and GPCR function in general may facilitate the development of new and better therapeutics targeting these important receptors.

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