Crystal structure of the M₅ muscarinic acetylcholine receptor

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The human M₅ muscarinic acetylcholine receptor (mAChR) has recently emerged as an exciting therapeutic target for treating a range of disorders, including drug addiction. However, a lack of structural information for this receptor subtype has limited further drug development and validation. Here we report a high-resolution crystal structure of the human M₅ mAChR bound to the clinically used inverse agonist, tiotropium. This structure allowed for a comparison across all 5 mAChR family members that revealed important differences in both orthosteric and allosteric sites that could inform the rational design of selective ligands. These structural studies, together with chimeric swaps between the extracellular regions of the M₅ and M₆ mAChRs, provided structural insight into kinetic selectivity, where ligands show differential residency times between related family members. Collectively, our studies provide important insights into the nature of orthosteric and allosteric ligand interaction across the mAChR family that could be exploited for the design of selective drugs.

Significance

The 5 subtypes of the muscarinic acetylcholine receptors (mAChRs) are expressed throughout the central and peripheral nervous system where they play a vital role in physiology and pathologies. Recently, the M₅ mAChR subtype has emerged as an exciting drug target for the treatment of drug addiction. We have determined the atomic structure of the M₅ mAChR bound to the clinically used inverse agonist tiotropium. The M₅ mAChR structure now allows for a full comparison of all 5 mAChR subtypes and reveals that small differences in the extracellular loop regions can mediate orthosteric and allosteric ligand selectivity. Together, these findings open the door for future structure-based design of selective drugs that target this therapeutically important class of receptors.

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homology of the mAChR orthosteric site residues (1) and in part due to a lack of detailed structural information for all 5 receptor subtypes. While structures of the M1 to M4 mAChRs have been previously determined, there are no available structures for the M5 mAChR. Therefore, to provide a complete structural comparison of all 5 family members, we determined a high-resolution crystal structure of the M5 mAChR, which revealed differences in the extracellular loop (ECL) regions that could mediate orthosteric and allosteric ligand selectivity. Based on these differences and the fact that some medicines are now known to have different binding rates between mAChR subtypes that can result in clinically relevant “kinetic” selectivity, we also made chimeric swaps of the ECL regions between the M2 and M5 mAChRs to investigate the role of these regions in mediating this important mode of drug selectivity.

Results

Crystalization and Determination of the M5 mAChR Structure. To determine the M5 mAChR structure, we designed a construct in which residues 225 to 430 of intracellular loop 3 were removed and replaced with a T4 lysozyme (T4L) fusion protein. Additionally, to promote crystallization, the first 20 N-terminal amino acids were cleaved by a tobacco etch virus protease site engineered into the receptor (SI Appendix, Fig. S1). The inverse agonist, tiotropium, was used to stabilize the inactive state as it has a slow dissociation rate at the M5 mAChR (20) and was also used in the determination of the M1, M3, and M4 mAChR structures (21, 22). The M5-T4L-tiotropium complex was crystallized in lipidic cubic phase (LCP), and crystals were obtained within 1 to 2 d; however, despite many rounds of optimization, diffraction was limited to 7 Å. To improve the resolution, we built upon a study from Kajiwara et al. (23) that predicted that mutation of the amino acid at position 3.39 [numbered according to Ballesteros-Weinstein (24)] to Arg would create a thermo-stabilized receptor by promoting an ion-bond between this residue and the highly conserved D2.50 residue. Recently, the same S3.39R mutation was applied to the M2 mAChR, resulting in a series of higher resolution structures (25). Although introduction of the S1173.39R mutation resulted in a construct that binds the antagonists N-methyl scopolamine (NMS) or tiotropium with a slightly reduced affinity relative to the wild-type (WT) M5 mAChR, the effect of the mutation on reducing ACh affinity was substantially more pronounced (SI Appendix, Fig. S1), consistent with the ability of the construct to favor an inactive over an active state. Similar differential effects on antagonist versus agonist affinity were previously observed for S3.39R at the M2 mAChR (25). Notably, introduction of the S1173.39R mutation increased our M5 mAChR yields during purification and resulted in crystals that diffracted to a resolution of 3.4 Å. Data were collected from ~130 crystals, and the structure was determined by molecular replacement using the M2 structure [Protein Data Bank (PDB) ID 4U15] and an ensemble of T4L structures as templates (Fig. 1A and SI Appendix, Table S1).

To investigate the nature of NAM binding to the M5 mAChR, we attempted to obtain co-crystal structures. Given that the bis-ammonium alkane-type ligands tend to have higher affinities for the M2 mAChR than the prototypical modulator, galamine (26), we tried to obtain a ternary complex structure of the M5 mAChR with tiotropium and several bis-ammonium alkane ligands (Fig. 1B). We initially used the modulator 4B-C7/3-phth, which resulted in crystals that grew to a much larger size and diffracted to a resolution of 2.55 Å (Fig. 1 and SI Appendix, Table S2 and Fig. S2). Based on previous data (27), we predicted that 4B-C7/3-phth would bind in the extracellular vestibule (ECV). While there were regions of strong electron density present in the ECV, we could not unambiguously model 4B-C7/3-phth into the density as a molecule of the precipitant, polyethylene glycol 400 (PEG400), also likely binds in this site (22, 28) and may explain why researchers have had difficulty in obtaining co-NAM-bound structures for the mAChRs.

Subsequently, we designed 2 bis-ammonium alkane analogs using the higher affinity 4P-C7/3-phth scaffold (27) to try to improve modulator affinity (Fig. 1B) and detectability by X-rays. The first modification added 2 bromine (Br) atoms (4P-C7/3-bromo-phth) to increase the size of the pthalamides groups (29), and the second modification rigidified the flexible 7-carbon linker with an aromatic hydrocarbon (4P-aryl-C7/3-bromo-phth). When tested in functional inositol phosphate (IP3) assays and [3H]NMS competition radioligand binding, both ligands had a similar affinity in relation to the parent compound (4P-C7/3-phth) (SI Appendix, Figs. S3A and S4 and Table S3). Like 4B-C7/3-phth, the addition of either 4P-C7/3-bromo-phth or 4P-aryl-C7/3-bromo-phth to purified M5 mAChR and reconstitution into LCP yielded crystals that diffracted to a higher resolution (SI Appendix, Table S1). A full dataset for the 4P-aryl-C7/3-bromo-phth was collected at a wavelength of 0.92 Å to maximize the anomalous Br signal in a single wavelength anomalous diffraction experiment; however, no such signal was detected, suggesting that 4P-aryl-C7/3-bromo-phth was not present in the structure. Since the structure was solved by merging a large number of datasets, there is a possibility that the Br signal for the NAM would be averaged out if NAM occupancy is low. However, inspection of different datasets did not indicate that this was the case.

As an alternate strategy, we attempted to determine a co-crystal structure with the structurally diverse M5 mAChR-selective NAM, ML375 (19). In comparison to the bis-ammonium ligands, the addition of ML375 resulted in a slightly lower resolution structure (2.7 Å, SI Appendix, Table S1), and, as was the case with the bis-ammonium NAMs, we were not able to assign ML375 any electron density. Comparison of all M5 mAChR structures showed that they were nearly identical, with root mean square deviation values of 0.09 to 0.22 Å. The higher-resolution 2.55 Å M5-tiotropium (4B-C7/3-phth) structure was used for further comparison, as this was the best-resolved and modeled structure (SI Appendix, Fig. S5).

Family-Wide Comparison of All mAChR Subtypes. The solution of the M5 mAChR structure allows for a complete subtype-wide comparison of this important GPCR family. The structure of the M5 mAChR is similar to the previously determined structures of the M1 to M4 mAChR subtypes (21, 22, 30) with a root mean squared deviation of 0.5 to 0.8 Å (Fig. 2A) for the 7-transmembrane domain across all subtypes. The 5 mAChR subtypes are most similar

![Fig. 1. Structures of M5-T4L bound to tiotropium.](https://www.pnas.org/doi/abs/10.1073/pnas.1914466116)
in the orthosteric binding site, which is the most conserved region of the receptor. The fact that our M5 mAChR structure was obtained in complex with the same ligand (tiotropium) as the M1, M3, and M4 mAChR structures allowed for a specific, detailed comparison of residues lining this orthosteric binding site (Fig. 2 B and C). This comparison demonstrated that the residues within the orthosteric pocket are absolutely conserved between the receptors. Although there is no tiotropium-bound M2 mAChR structure, there are now 6 different inactive state M2 mAChR structures, which include structures bound with the nonselective ligands 3-quiniclidinyl benzilate (QNB) and NMS and the M2 mAChR selective ligand AF-DX384 (25). The 2.3-Å M2-NMS structure is most similar to the tiotropium-bound mAChR structures, although residues Y111 and Y182 of the “tyrosine lid” (Y111, Y182, and Y183) are positioned in a distinct conformation in comparison to the tiotropium-bound structures. These differences in the tyrosine lid positions are more pronounced in the M2 mAChR when compared to the M1 structure. Similarly, the roseine lid positions are more pronounced in the M2 mAChR compared to other subtypes like the M2 mAChR. For example, despite having similar equilibrium-binding affinities, [3H]NMS dissociates 18-fold more slowly at the M5 than at the M2 mAChR with half-lives of dissociation of 100 ± 11.6 and 5.7 ± 1 min, respectively (Fig. 2 F and G).

**Structural Differences between the ECVs of the M2 and M5 mAChRs**

An alternative strategy to generating selective ligands is to target nonconserved allosteric sites (31). This has been extensively explored for the mAChR family in which a palette of both positive and negative allosteric modulators has been identified (32, 33). Structural and mutagenesis studies have established that many of these ligands bind to a “common” allosteric site that is located above the orthosteric site and within an ECV (Fig. 3 and SI Appendix, Fig. S6) (34). In fact, the M3 mAChR has often served as a model system for early research into understanding the binding mode and mechanism of selectivity for prototypical modulators, such as the bis-ammonium alkane ligands (Fig. 1 B), that have higher sensitivity for modulating the M2 mAChR and lower sensitivity for the M5 mAChR (26, 35–38). These studies identified nonconserved residues in ECL2 (P179<sup>−1</sup>, E182<sup>−1</sup>, and Q184<sup>−1</sup>), superscript indicates the position of ECL2 residues relative to the conserved Cys in ECL2) and TM7 (V474<sup>3.32</sup> and H484<sup>7.43</sup>) as residues that can account for M2/M5 subtype selectivity. Comparison of the ECV between the M2<sub>−1</sub> and M5<sub>−1</sub> mAChRs confirms differences in the orientations and positions of these residues that could mediate the selectivity. Namely, P179<sup>−1</sup> in ECL2 restricts
the position of E182\(^{-1}\), forcing the residue into the ECV near Q184\(^{+1}\). Residue Q184\(^{+1}\), which is a F/Y residue for the M1 to M4 mAChRs subtypes, is a key residue for the activity of many allosteric modulators. Other major differences between the M2/M3 ECVs are in the positions of nonconserved residues lining the top of TM6 starting from S465\(^{6.58}\) across ECL3 and down to residue H478\(^{7.36}\) in TM7. At the M5 mAChR, these residues are bulkier and point more inwardly, constricting the overall size of the ECV (Fig. 3).

Role of the M5 and M2 mAChR ECL Regions in Orthosteric and Allosteric Ligand Binding. The effect of ECL regions on orthosteric ligand access and egress has significant biological and clinical relevance (39). Therefore, to investigate the role of the ECLs in modulating the slower dissociation kinetics of the M5 mAChR in comparison with the M2 mAChR, we designed full ECL1, ECL2, and/or ECL3 chimeric swaps between the 2 subtypes (Fig. 4 and SI Appendix, Fig. S7). The ECL chimeras had similar levels of expression and binding of \(^{[3]H}\)NMS to WT receptors (SI Appendix, Table S4). As previously noted, the M5 mAChR has a shorter half-life for \(^{[3]H}\)NMS dissociation in comparison with the M2 mAChR (Fig. 2F). Incorporation of the M2 ECL1 or ECL3 into the M5 mAChR increased \(^{[3]H}\)NMS dissociation, while the reciprocal chimeric swap decreased \(^{[3]H}\)NMS dissociation at the M5 mAChR. Unexpectedly, it was the ECL1 swaps that had the largest effect on \(^{[3]H}\)NMS dissociation between the 2 subtypes, particularly at the M5 mAChR (Fig. 4 and SI Appendix, Table S5). A possible structural explanation for this observation could be that R95\(^{ECL1}\), which is a conserved Tyr residue at the M5 to M2 subtypes, is capable of forming an ionic bond with either the M2 ECL2 residue D181\(^{-2}\) or, in the case of the M2 ECL1 chimera, residue D173\(^{3}\) (Fig. 3 and SI Appendix, Fig. S6). Such an interaction could tether ECL1 and ECL2, limiting their overall dynamics and thus reduce rates of orthosteric ligand dissociation. It is important to note that R95\(^{ECL1}\) is involved in an ionic interaction mediated through the crystal lattice with a neighboring T4L molecule (SI Appendix, Fig. S2 D–F), and as a result it does not directly interact with D181\(^{-2}\) in the M5 mAChR structure although it is well positioned to do so.

A hallmark feature of an allosteric ligand that modulates orthosteric ligand affinity is the ability to either increase or decrease the rate of dissociation of an orthosteric ligand. To examine the effect of allosteric modulators on NMS dissociation across the M5 and M2 ECL chimeras, we used the bis-ammonium alkane ligand 4P-C\(^{7/3}\)-phth, which had been previously studied at the M2 mAChR and had high affinity for the M5 mAChR (SI Appendix, Table S3) or the M2 selective modulator ML375 (19, 27). In the presence of ML375, \(^{[3]H}\)NMS dissociation was reduced at the M5 mAChR and had no effect at the M2 mAChR, whereas the addition of 4P-C\(^{7/3}\)-phth reduced radioligand dissociation at the M2 mAChR but not at the M5 mAChR (Fig. 4 and SI Appendix, Table S5). The ECL1 and ECL3 chimeric swaps had little effect on the activity of ML375 for either receptor subtype and slightly increased the activity of 4P-C\(^{7/3}\)-phth at the M2 mAChR. For the ECL2 chimeras, there was no effect on activity of ML375. However, there was a loss of 4P-C\(^{7/3}\)-phth activity at the M5 mAChR and a corresponding gain of activity at the M2 mAChR. These results are in line with previous studies and highlight the importance of residues in ECL2, particularly M2-Y177 and M5-E184, on modulating the activity of bis-ammonium alkane ligands. Interestingly, when all 3 ECLs were swapped, the resulting M2 and M5 chimeric constructs functioned more like their swapped receptor counterpart. That is, for the M2–M5–all-ECL construct, 4P-C\(^{7/3}\)-phth had little effect, and although ML375 did not retard \(^{[3]H}\)NMS dissociation, it slightly increased the rate of \(^{[3]H}\)NMS dissociation, suggesting an allosteric mode of action (Fig. 4 and SI Appendix, Table S5). Conversely, for the M2–M2–all-ECL construct, 4P-C\(^{7/3}\)-phth retarded radioligand dissociation, and, surprisingly, ML375 had no effect. While none of the chimeric constructs ever fully switched the basal dissociation rate of \(^{[3]H}\)NMS or ML375 activity to that observed for the corresponding WT constructs, the data nonetheless suggest that the ECL regions modulate the overall conformational changes of mAChRs and directly influence the dissociation of ligands from the orthosteric site.

Discussion

Individual mAChR subtypes have long been pursued as drug targets for a range of CNS disorders, and recent studies have begun to validate the M3 mAChR as a target for the treatment of drug addiction (4, 40). In this study, we have determined a high-resolution crystal structure of the M5 mAChR, thus allowing a subtype-wide comparison for any aminergic GPCR subfamily. Introduction of the inactive state stabilizing mutation S1173\(^{7.38}\)R, which was recently used to stabilize the M2 mAChR (25), was crucial to obtaining well-diffraction crystals and suggests that this mutation could be applied to aid the determination of inactive state structures for other related GPCRs. We further improved the resolution of the M5 mAChR structure by adding allosteric modulators to the purified protein prior to crystallization. Despite the consistent increase in resolution that each of the allosteric modulators provided, we were not able to model any of the modulators into electron density. From a pharmacological perspective, a lack of modulator binding is not surprising, as all of the modulators tested in this study showed strong negative cooperativity with tiotropium (SI Appendix, Fig. S3B). Nevertheless, it is still paradoxical that the addition of an allosteric modulator can clearly improve receptor crystallization and diffraction yet not be visible in any resulting structures. This phenomenon has been noted at other GPCRs, such as the M3 mAChR that was crystallized in the presence of the modulator alcuronium and the CC chemokine receptor 2A that was crystallized in the presence of the modulator AZD-6942, but where neither modulator could be observed in the resulting structures (25, 41).
Comparison of all 5 mAChR structures further confirms the well-conserved transmembrane core and orthosteric binding site that has made the discovery of highly selective drugs for these receptor subtypes incredibly challenging. The most apparent structural differences between the mAChR subtypes are in the ECL regions. Although these differences are generally quite subtle, they are important because they open up the possibility for designing selective molecules in a way that has not previously been possible (SI Appendix, Fig. S6). For example, a recent crystal structure of the M2 mAChR bound to the M2-selective antagonist AF-DX384 revealed that selectivity is mediated by differential interactions between the ligand and residues in ECL2, which lead to an outward displacement in ECL2 and the top of TM5 (Fig. 2D) (25). Likewise, by utilizing knowledge of a single amino acid difference in ECL2 between the M2 and M3 mAChRs, molecular docking and structure-based design led to the discovery of an M2-selective antagonist with 100-fold selectivity over the M3 mAChR (42). These results are similar to the structure-based design of biased ligands targeting the D2 dopamine receptor that were designed by utilizing specific amino acid-ligand contacts in ECL2 and TM5 (43). Taken together, these findings indicate that the differential targeting of ECL residues may be a path forward for creating selective mAChR ligands. This is well supported by the fact that many mAChR-selective allosteric modulators interact with the ECL regions (27, 34) and suggests that designing orthosteric ligands linked to allosteric pharmacophores, known as bitopic ligands, is a potential strategy for future structure-based drug design.

Drug discovery has typically focused on optimizing ligand affinity and selectivity; however, it is now apparent that binding kinetics can play a critical role in these events (39, 44–46). This is illustrated in 2 ways with the drug tiotropium as a pertinent example. First, tiotropium has slow rate of dissociation from the M3 mAChR, which is a key feature of the drug that allows for a once daily dosing for the treatment of chronic obstructive pulmonary disease (47). Second, although tiotropium has the same equilibrium-binding affinity for the M3 and M2 mAChRs, it exhibits kinetic selectivity for the M3 over the M2 mAChR by having substantially different rates of dissociation. This kinetic selectivity over the M2 mAChR is postulated to be due to differences in the electrostatics and dynamics of the ECL region (47). The M3 mAChR is similar to the M2 mAChR with respect to having slow rates of orthosteric ligand dissociation (20), and data from our M2/M3 ECL chimeras support the idea of the ECL regions underpinning kinetic selectivity as [3H]NMS dissociation was switched between the M2 and M3 mAChRs (Fig. 4). Notably, none of the combined ECL chimeras could ever fully switch the dissociation kinetics between subtypes, suggesting that other mechanisms are operative such as the global conformation of the ECLs. Our results also highlight the importance of the ECL regions on conferring selectivity to allosteric modulators across different subtypes. By swapping out the entire ECL region between the M2 and M3 mAChRs we were able to completely alter the sensitivity of a modulator that is selective for the M2 versus the M3 mAChR and vice versa. These results are in line with previous studies using similar M2/M3 mAChR ECL chimeras (35–38, 48) and, collectively with our findings, highlight the importance of the ECL region for conferring subtype selectivity for different types of mAChR ligands.
Materials and Methods

Detailed information on cloning, receptor purification, synthesis of the bis-ammonium alkane ligands, and molecular pharmacology experiments is provided in SI Appendix, Materials and Methods.

M5 Receptor Expression and Purification. M5-T4L with the S1173.39R was purified similarly to previous methods (22).

Crystallization and Structure Determination. Purified M5-T4L S1173.39R bound to tigotropin was crystalized using LCP. For allosteric modulator cocystalization, the modulator was added to purified protein at a final concentration of 2.5 mM. The sample was incubated on ice for 3 h before it was mixed into 10:1 (w/vtwt) monoolein:cholester to 1:1.5 wtv salt:protein:lipid ratio. LCP crystallization was performed by spotting 25 to 30 nl of sample onto a siliconized 96-well glass plate overlaying the samples with 600 nL of precipitant solution using the Gryphon LCP (Art Robbins Instruments). Seeded crystals were incubated at 20 °C. Crystals appeared in the first 24 h and grew to full size in the following 1 to 2 d. The best diffracting crystals grew in 100 mM DL-Malic acid, pH 6.0; 220 to 280 mM ammonium tartrate dibasic; and 37 to 41% PEG 400. For the data collection, whole drops were harvested in a 2m j map; SI Appendix, Fig. S5) past the β-carbon were truncated. Structure figures were prepared using the program PyMol. Electrostatic and surface potential of M2 and M3 mAChR (+5kTe in blue and −5kTe in red) mapped on the surface of the receptors calculated at pH 7.0 were calculated using PDBePQR and APBS (56).

Data Availability. Atomic coordinates and structure factors have been deposited in the Protein Data Bank, http://www.rcsb.org (PDB ID code 6OL9 for M5-T4L). Detailed methods are provided in SI Appendix and data are available on request.

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In summary, our reported M3 MACHR crystal structure has allowed for the comparison of all 5 mAChR subtypes and has revealed that subtle differences in the ECL regions are a major determinant in ligand selectivity, regardless of the ligand being orthosteric or allosteric. As the M1, M2, and M3 mAChRs continue to emerge as exciting drug targets for the treatment of CNS disorders, it will be important to understand both the structural and the dynamic differences between all 5 mAChR subtypes in order to aid design of safer and more effective small-molecule therapeutics.
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