Structure and dynamics of the M3 muscarinic acetylcholine receptor

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Acetylcholine, the first neurotransmitter to be identified¹, exerts many of its physiological actions via activation of a family of G-protein-coupled receptors (GPCRs) known as muscarinic acetylcholine receptors (mAChRs). Although the five mAChR subtypes (M1–M5) share a high degree of sequence homology, they show pronounced differences in G-protein coupling preference and the physiological responses they mediate²–⁴. Unfortunately, despite decades of effort, no therapeutic agents endowed with clear mAChR subtype selectivity have been developed to exploit these differences⁵–⁷. We describe here the structure of the Gq/11-coupled M3 mAChR (‘M3 receptor’, from rat) bound to the bronchodilator drug tiotropium and identify the binding mode for this clinically important drug. This structure, together with that of the Gi/o-coupled M2 receptor², offers possibilities for the design of mAChR subtype-selective ligands. Importantly, the M3 receptor structure allows a structural comparison between two members of a mammalian GPCR subfamily displaying different G-protein coupling selectivities. Furthermore, molecular dynamics simulations suggest that tiotropium binds transiently to an allosteric site en route to the binding pocket of both receptors. These simulations offer a structural view of an allosteric binding mode for an orthosteric GPCR ligand and provide additional opportunities for the design of ligands with different affinities or binding kinetics for different mAChR subtypes. Our findings not only offer insights into the structure and function of one of the most important GPCR families, but may also facilitate the design of improved therapeutics targeting these critical receptors.

The mAChR family consists of five subtypes, M1–M5, which can be subdivided into two major classes (Fig. 1a). The M1, M3 and M5 receptors show selectivity for G proteins of the Gq/11 family (that is, Gq and G11), whereas the M2 and M4 receptors preferentially couple to Gi/o-type G proteins (Gq and Go)²–⁴. The development of small molecule ligands that can selectively act on specific mAChR subtypes has proven more challenging than development of orthosteric GPCR ligands, due to the existence of differences in the three-dimensional architecture of the orthosteric sites of these receptors²–⁴. Nevertheless, the M3 receptor offers unique opportunities for the design of subtype-selective ligands, due to its unique structural features and the existence of differences in the three-dimensional architecture of the orthosteric site between the different mAChR subtypes. In fact, comparison of the structures of the M3 and M2 receptor ligand binding sites reveals structural divergences that might be exploited in the development of subtype-selective ligands.

Reflecting the difficulty in developing subtype-selective orthosteric ligands, the residues forming the orthosteric binding pocket are absolutely conserved among the five mAChR subtypes (Fig. 1f). However, this conservation at the amino acid level does not preclude the existence of differences in the three-dimensional architecture of the orthosteric site between the different mAChR subtypes. In fact, comparison of the structures of the M3 and M2 receptor ligand binding sites reveals structural divergences that might be exploited in the development of subtype-selective ligands.

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Figure 1 | Major structural features of the M3 receptor. a, Analysis of muscarinic receptor sequences divides them into two classes. b, The overall structure of the M3 receptor (green) is similar to that of the M2 receptor (orange). The M3-bound ligand, tiotropium, is shown as spheres coloured according to element, with carbon in yellow and oxygen in red. c, Comparison of the intracellular surfaces shows divergence in the cytoplasmic end of TM5. d, Comparison of the extracellular surfaces shows less deviation, with near perfect conservation of backbone fold of extracellular loops (ECLs). e, A solvent accessible surface for the M3 receptor bound to tiotropium (spheres) shows the receptor covering the ligand with a tyrosine lid (outlined in red). The surface of the receptor is shown in green and its interior in black. f, M3 receptor structure coloured by sequence conservation among the five mAChR subtypes. Poorly conserved regions are shown with larger backbone diameter. The orthosteric and allosteric sites are indicated in blue and red elliptical shaded areas, respectively, and the ligand tiotropium is shown as spheres.

Figure 2 | Orthosteric binding sites of the M2 and M3 receptors. In all panels, the M3 receptor is shown green with its ligand tiotropium in yellow, while the M2 receptor and its ligand QNB are shown in orange and cyan, respectively. a, Tiotropium binding site in the M3 receptor. A 2Fo – Fc map contoured at 2σ is shown as mesh. b, Chemical structures of ligands. A red arrow indicates the tropane C3 atom used as a tracking landmark in Fig. 3. Superimposing the receptor structures reveals that the two ligands adopt highly similar poses (bottom). c, There is a Phe (M2)/Leu (M3) sequence difference between the M2 and M3 receptors near the binding site. d, This produces an enlarged binding pocket in the M3 receptor, outlined in red and indicated with an arrow. e, Displacements of M3 Y529<sup>7.39</sup> and D147<sup>3.32</sup> are seen (black dashed lines). f, The displacement of Y529<sup>7.39</sup> may arise from a sequence difference at position 2.61 (Tyr 80 in M2 and Phe 124 in M3).
One such difference derives from the replacement of Phe181 in ECL2 of M2 with Leu225 in M3 (this residue is leucine in all mACHRs except M2). This creates a pocket in M3 not found in M2 (Fig. 2c, d). A second difference is a 2.8 Å shift of Tyr5297.39 relative to the position of the corresponding M2 residue (Tyr 426; Fig. 2e). This feature may derive from a difference in the identity of the residue in position 2.61 (Phe 124 in M3 and Tyr 80 in M2; Fig. 2f). This residue interacts directly with TM7, influencing the position of this helix and the residues within it, including Tyr5297.39. Notably, the residue at position 2.61 is not a part of the orthosteric binding pocket, but is positioned near a probable allosteric binding site17. Because tiotropium and QNB are structurally similar but not identical, the observed binding site differences must be interpreted with some degree of caution. However, site-directed mutagenesis studies with M1 and M3 receptors support the concept that the residue at position 2.61 plays a role in receptor activation18,19 and ligand binding selectivity20. This site does not appear to play a role in determining antagonist dissociation rates, because mutation of M3 F2.61 to tyrosine or of M2 Y2.61 to phenylalanine had no effect on dissociation rates for [3H]N-methyl scopolamine ([3H]NMS) or [3H]QNB.

We used molecular dynamics simulations to characterize the pathway by which tiotropium binds to and dissociates from the M2 and M3 receptors. Similar techniques have previously been shown to correctly predict crystallographic ligand binding poses and kinetics in studies of β-adrenergic receptors21. In both the M2 and M3 receptors, our simulations indicate that as tiotropium binds to or dissociates from the receptor, it pauses at an alternative binding site in the extracellular vestibule (Fig. 3, Supplementary Fig. 3). Intriguingly, this site corresponds to an allosteric site that has been previously identified by mutagenesis7, a finding consistent with pharmacological studies showing that orthosteric ligands can act as allosteric modulators at the M2 receptor22. Tiotropium adopts different preferred allosteric binding poses in M2 and M3 (Fig. 3d, Supplementary Fig. 4). These metastable binding poses, which appear independently in both binding and dissociation simulations, may represent the first structural view of a clinically used 'orthosteric' GPCR ligand binding to an experimentally validated allosteric site. Conceivably, therapeutic molecules could be rationally engineered to act independently as both allosteric and orthosteric ligands (in contrast to previously described bitopic ligands that bind at both orthosteric and allosteric sites simultaneously23).

Tiotropium dissociates from M3 receptors more slowly than from M2 receptors, a phenomenon thought to provide clinically important 'kinetic selectivity' of this drug for M3 receptors despite similar equilibrium binding affinities for both subtypes14. In simulations with tiotropium bound, the portion of ECL2 nearest the binding pocket proved more mobile in M2 than in M3 (Supplementary Fig. 5), probably proceeding into the orthosteric binding pocket; the agonist acetylcholine (ACh), a much smaller molecule, bound spontaneously to the orthosteric site in similar simulations (Supplementary Methods).

**Figure 3** Molecular dynamics of ligand binding. Simulations suggest that the tiotropium binding/dissociation pathway for both receptors involves a metastable state in the extracellular vestibule. a. When tiotropium is pushed out of the binding pocket of M3, it pauses in the extracellular vestibule in the region outlined with a dashed circle. Spheres represent positions of the ligand’s C3 tropane atom at successive points in time. The direction of motion is indicated by an arrow. b. When tiotropium is placed in solvent, it binds to the same site in the extracellular vestibule. Our simulations are insufficiently long for it to proceed into the orthosteric binding pocket; the agonist acetylcholine (ACh), a much smaller molecule, bound spontaneously to the orthosteric site in similar simulations (Supplementary Methods). c. Schematic free-energy landscape for binding/dissociation. Differences between M2 and M3 are shown in orange and green, respectively, with the rest of the curve in black. d. Common binding poses for tiotropium in the extracellular vestibule of M2 (orange) and of M3 (green). Non-conserved residues that contact the ligand are shown as thin sticks. The location of the orthosteric site is indicated by tiotropium (as spheres).
owing to multiple sequence differences between the two receptor subtypes. This increased mobility disrupts a hydrophobic cluster involving a thiope ring of tiotropium, the ECL2 residue Phe 181(M2)/Leu 225(M3), and Tyr3.33, facilitating movement of Phe 181/Leu 225 away from the orthosteric site and rotation of Tyr3.33 towards TM4. In simulations of ligand dissociation, such motions clear a path for tiotropium’s egress from the orthosteric site to the extracellular vestibule. The increased mobility of ECL2 in M2 thus appears to facilitate tiotropium’s traversal of the largest energetic barrier on the binding/dissociation pathway (Fig. 3c). Experimental measurements with wild-type and mutant receptors (M2 L225F and M2 F181L) suggest that the Leu 225/Phe 181 sequence difference alone is insufficient to explain the difference in off-rates (for practical reasons these measurements were performed with QNB rather than tiotropium; see Methods).

One of the most interesting features of the M2 and M3 receptors is the fact that the two highly similar receptors display pronounced differences in G-protein coupling specificity. For this reason, the M2/M3 receptor pair has long served as an excellent model system to identify features contributing to the selectivity of coupling between GPCRs and G proteins. As no simple sequence elements have been identified as general determinants of coupling specificity across GPCR families, it is likely that recognition depends on features such as overall conformation in addition to specific inter-residue contacts.

The M2 and M3 receptor structures show a significant difference in the position of the cytoplasmic end of TM5 and of ICL2 (Fig. 4a, b). The highly conserved tyrosine residue at position 5.58 (M3 Tyr2505.58, M2 Tyr2065.58) shows a clear deviation between the two receptors, pointing towards the core of the protein in M2, and away from the receptor towards the surrounding lipid bilayer in M3. Interestingly, mutagenesis studies have identified a tetrad of residues (‘AALS’ in M3, ‘VTIL’ in M2) located on the cytoplasmic end of TM6 that are critical in determining G-protein coupling selectivity. In both structures, these residues interact directly with TM5 (Fig. 4a), and in the β2 adrenergic receptor–G complex two of the four corresponding residues make contact with the carboxy-terminal helix of Gαq, M3 Tyr 2545.62 at the bottom of TM5 also plays a role in activation of Gαq/11 (ref. 28). In the M2 receptor structure, the corresponding residue (Ser 2105.62) is displaced by approximately 4 Å relative to Tyr 2545.62 in M3 (Fig. 4a).

When we compared the position of TM5 in the M2 and M3 receptors to that in other GPCR structures, we found that it is M2-like in all Gαi/o-coupled receptors, whereas the two mammalian Gαq/11-coupled receptors solved to date exhibit another conformation (Fig. 4c, d). An important caveat here is that these structures have been solved using the T4L fusion strategy, and we cannot completely exclude the possibility that this approach perturbs the conformation of TM5 and TM6. However, in
molecular dynamics simulations of M2 and M3 receptors without T4L, each of the receptors adopts a set of conformations that includes its own crystallographically observed conformation (Supplementary Fig. 6, 7). These simulations suggest that the observed conformations are unlikely to be artefacts of the crystallization methodology, though the crystal structures probably represent only one conformation among many adopted by the receptors in a biological context.

The structure of the M3 receptor, together with that of the M2 receptor, offers a unique opportunity to directly compare the structural properties of two members of a mammalian GPCR subfamily endowed with different G-protein coupling selectivities. Examination of the M3 structure has provided structural evidence of differences between ligand binding sites of mAChR subtypes that could be exploited for the design of more selective therapeutics. Moreover, computational studies have identified a pathway by which the COPD drug tiotropium may bind to and dissociate from the M3 receptor, offering a structural view of an orthosteric GPCR ligand binding to an experimentally validated allosteric site. This information should facilitate the rational design of new muscarinic drugs exhibiting increased receptor subtype selectivity, potentially improving treatment for a wide variety of important clinical disorders.

METHODS SUMMARY

The M3 muscarinic receptor–T4 lysosome fusion protein was expressed in S9 insect cells and purified by nickel affinity chromatography followed by Flag antibody affinity chromatography and then size exclusion chromatography. It was crystallized using the lipidic cubic phase technique, and diffraction data were collected at the GM/CA-CAT beamline at the Advanced Photon Source at Argonne National Laboratory. The structure was solved by molecular replacement using merged data from 76 crystals. All-atom classical molecular dynamics (MD) simulations with explicitly represented lipids and water were performed using the CHARMM force field on Anton. Ligand-binding simulations included no artificial forces. Dissociation studies included a time-varying biasing term that gradually forces the ligand away from its crystallographic position, but not along any prespecified pathway or direction. Full details are provided in Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions A.C.K. cloned, expressed, and purified several M3 receptor crystalization constructs; developed the purification procedure; performed crystallization trials, collected diffraction data, solved and refined the structure of the M3 receptor, expressed and characterized several M3 receptor constructs in ligand binding and functional assays. A.C.P. and D.H.A. designed, performed and analysed MD simulations and assisted with manuscript preparation. D.M.R. assisted in design and characterization of initial M3–T4L fusion constructs. E.J. prepared, expressed and tested the pharmacology and stability of several M3 receptor constructs. E.R. performed binding assays and functional experiments together with J.H. P.S.C. developed and prepared neoprene glycolic detergents used for purifying the M3 receptor. R.O.D. oversaw, designed and analysed MD simulations. D.E.S. oversaw MD simulations and analysis. W.I.W. oversaw refinement of the M3 receptor structure and assisted in analysis of diffraction data. J.W. provided advice regarding construction design, protein expression and project strategy; and oversaw initial insect cell expression and pharmacological and functional characterization of M3 receptor constructs. B.K.K. was responsible for overall design of strategy, guided construction and crystallization of the crystals used with crystal harvesting and data collection. A.C.K, R.O.D, J.W. and B.K.K. wrote the manuscript.

Author Information Coordinates and structure factors for M3–T4L are deposited in the Protein Data Bank (accession code 4DAJ). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to B.K.K. (kobilka@stanford.edu) or J.W. (jwess@helix.nih.gov).
**METHODS**

Expression and purification of M3 muscarinic receptor. The wild-type M3 mAChR contains several long, poorly poorly ordered regions, including the extracellular amino-terminal domain and the third intracellular loop, making it a challenging candidate for crystallographic studies. To alleviate this problem, the M3 receptor from *R. norvegicus* was modified to include a TEV protease recognition site in the N terminus and a hexahistidine tag at the carboxy terminus. Moreover, the third intracellular loop (residues 260–481) was replaced with T4 lysozyme residues 1–161 in a manner described previously40, with two different fusions tested. These modifications are shown in Supplementary Fig. 1, which also shows the final crystallization conditions.

The pharmacological properties of the construct were tested and compared to those of the wild-type receptor (Supplementary Fig. 8, Supplementary Table 1; see below for methods details). Both constructs showed almost identical affinity for antagonists, while the crystallization construct (M3-crys) showed somewhat higher affinity for the agonist ACh than the wild-type construct. A similar observation has been noted previously in the β2 adrenergic receptor12. Studies with membranes prepared from transfected COS-7 cells showed that TEV cleavage of M3-crys (to remove most of the N-terminal tail) had no significant effect on ligand binding affinities (Supplementary Fig. 9). Moreover, the wild-type receptor and M3-crys, either cleaved with TEV or left uncleaved, showed very similar ¹H]QNB dissociation rate kinetics (Supplementary Fig. 10). As expected, the crystallization construct failed to stimulate agonist-dependent phosphoinositide hydrolysis in transfected COS cells (data not shown), probably because essential G-protein interacting regions in IC13 were omitted from the construct and also because the T4 lysozyme fusion protein sterically blocks G-protein association.

The crystallization construct was expressed in Sf9 cells using the baculovirus system in the presence of 1 μM atropine. M3 receptors expressed in Sf9 cells are known to exhibit functional and pharmacological properties similar to M3 receptors expressed in mammalian cells41. Infection was performed at 4 × 10⁶ cells per ml and flask were shaken at 27 °C for 60 h following infection.

Cells were harvested by centrifugation, then lysed by osmotic shock in the presence of 1 μM tiotropium bromide (PharmaChem), which was present in all subsequent buffers. Receptor was extracted from cells using a Dounce homogenizer with a buffer of 0.75 M NaCl, 1% dodecyl maltoside (DDM), 0.03% cholesterol hemisuccinate (CHS), 30 mM HEPES pH 7.5, and 30% glycerol. Iodoacetamide (2 mg ml⁻¹) was added to block reactive cysteines at this stage. Nickel-NTA agarose was added to the solubilized receptor without prior centrifugation, stirred for 2 h, and then washed in batch with 100 μl precipitant solution for 5 min each. Washed resin was poured into a glass column, and receptor was eluted in 0.1% DDM, 0.003% CHS, 20 mM HEPES pH 7.5, 0.75 M NaCl and 250 mM imidazole.

Nickel-NTA agarose resin-purified receptor was then loaded by gravity flow over anti-FLAG M1 affinity resin. Following extensive washing, detergent was gradually exchanged over 1.5 h into a buffer in which DDM was replaced with 0.01% lauryl maltose neopentyl glycol (MNG), and the NaCl concentration was lowered to 100 mM. MNG has been shown to be more effective at stabilizing muscarinic receptors than DDM42. Receptor was eluted with 0.2 mg ml⁻¹ Flag peptide and 5 mM EDTA. TEV protease (1:10 w/w) was added and incubated with receptor for 1.5 h at room temperature to remove the soluble N-terminal tail. Receptor was then separated from TEV by size exclusion chromatography (SEC) on a Sephadex S200 column (GE Healthcare) in a buffer of 0.01% MNG, 0.001% CHS, 100 mM NaCl and 20 mM HEPES pH 7.5. Tiotropium was added to a final concentration of 10 μM following SEC. The resulting receptor preparation was pure and monomeric (Supplementary Fig. 11). Purification of unliganded M3 receptor was also possible by this procedure, but the resulting preparation was polydisperse and unsuitable for crystallographic study.

Crystallization and data collection. Purified M3 receptor was concentrated to 60 mg ml⁻¹, then mixed with 1.5 parts by weight of a 10:1 mix of monoolein with cholesterol (Sigma) using the two syringe reconstitution method43. The resulting lipidic cubic phase mix was dispensed in 15 μl drops onto glass plates and overlaid with 600 nl precipitant solution using a Gryphon LCP robot (Art Robbins Instruments). Crystals grew after 2–3 days in precipitant solution consisting of 27–38% PEG 300, 100 mM HEPES pH 7.5, 1% (w/v) 1,2,3-heptanetriol, and 100 mM ammonium phosphate. Typical crystals are shown in Supplementary Fig. 12.

Data collection was performed at Advanced Photon Source GM/CA-CAT beamlines 23ID-B and 23ID-D using a beam size of 5 or 10 μm for most crystals. Diffraction quality rapidly decayed following exposure, and wedges of typically 5–10° were collected for most crystals. Some contamination of diffraction measurements due to the twin-related reflections was unavoidable, leading to slightly poorer merging statistics than is typical for data sets collected from many small crystals (Supplementary Table 2). Despite this, maps were generally of high quality and electron density was easily interpretable (Supplementary Figs 13, 14), in part due to the availability of non-crystallographic symmetry.

Analysis of <Fo>/〈F^2〉 along each of the three reciprocal space axes indicated that the diffraction was strong in two directions, and weak in the third direction, along the reciprocal space axis: 1/EV was confirmed by STS, PAGE, sF=E^2/<E^2> > 3 as a guide suggested a resolution cut-off of better than 3.2 Å along a* and b*, and of 4.0 Å along c*. We therefore applied an ellipsoidal truncation along these limits, and then applied an overall spherical truncation at 3.4 Å due to low completeness in higher resolution shells. Fortunately, fourfold non-crystallographic symmetry (NCS) allowed for improved map quality with map sharpening followed by NCS averaging, largely alleviating the effects of anisotropic diffraction and epitaxial twinning to give highly interpretable maps (Supplementary Figs 13, 14) and allowing details of ligand recognition to be clearly resolved (Supplementary Table 3).

The structure of the M3 receptor was solved using the structure of the M2 muscarinic receptor* as the search model in Phaser39. The model was improved through iterative refinement in Phenix40 and manual rebuilding in Coot guided by both NCS averaged and unaveraged maps. NCS restraints were applied in initial refinement stages, and omitted in final refinement cycles to account for differences between NCS-related copies. The quality of the resulting structure was assessed using MolProbity37, and figures were prepared using PyMOL41.

Epitaxial twinning. Crystals of the M3 receptor showed hallmarks of epitaxial twinning, such as mixed sharp and split spots, poor indexing, and many unpredicted reflections in some frames. In some cases twinning from two distinct lattices was clearly visible, with a small fraction of reflections exactly superimposed from both lattices (Supplementary Fig. 16). In most cases one lattice dominated the diffraction pattern to such an extent that it could be easily processed as a single crystal. Intriguingly, the two indexing solutions were not equivalent but rather were two enantiomorph P1 cells (Supplementary Table 2).

As one of these two cells gave significantly better diffraction data than the other, data processing and refinement were only pursued in this case. Within the asymmetric unit, two layers of receptors and two layers of T4 lysozyme are present, but each of these four layers exhibits a different lattice packing (Supplementary Figs 17, 18). The order in which these layers are stacked in the crystal defines a unique direction along c, the axis normal to the membrane plane. As P1 is a polar space group, the positive direction along c is uniquely defined, and the two possible orientations of the stacked layers of membrane relative to the positive direction along c distinguish the two twin crystal forms.

Expression of M3 receptors in COS-7 cells, membrane preparation, and TEV treatment. COS-7 cells were cultured as described previously49. About 24 h before transfections, −1 × 10⁶ cells were seeded into 100-mm dishes. Cells were transfected with 4 μg per dish of receptor plasmid DNA using the Lipofectamine Plus kit (Invitrogen), according to the manufacturer’s instructions. The mammalian expression plasmid coding for the wild-type rat M3 receptor has been described previously41. Expression of the modified M3 receptor construct used for crystallization studies (M3-crys; see Supplementary Fig. 8, Supplementary Table 1) was inserted into the pcDNA3.1+ vector. Transfected cells were incubated with 1 μM atropine for the last 24 h of culture to increase receptor expression levels46. COS-7 cells were harvested −48 h after transfections, and membranes were prepared as described.

Membranes prepared from COS-7 cells transiently expressing M3-crys were resuspended in TEV protease digestion buffer (50 mM NaCl, 10 mM HEPES pH 7.5 and 1 mM EDTA) and incubated overnight with TEV protease (made in our laboratory, final concentration 1 μM) at 4 °C with rotation. Efficient removal of the two TEV-N-terminus fusions was confirmed by SDS–PAGE and immunoblotting using a monoclonal anti-Flag antibody directed against the N terminus of M3-crys. TEV-treated membranes were resuspended in either buffer A (25 mM sodium phosphate and 5 mM MgCl₂, pH 7.4) for radioligand binding studies or in sodium potassium phosphate buffer (4 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.4) for [¹H]QNB dissociation assays (see below).

Radioligand binding studies. [¹H]N-methylscopolamine ([¹H]NMS) saturation and competition binding studies were carried out essentially as described previously46. In brief, membrane homogenates prepared from transfected COS-7 cells (~10–20 μg of membrane protein per tube) were incubated with the muscarinic agonist NMS at a concentration of 300 nM, for 3 h at 22 °C in 0.5 ml of binding buffer containing 25 mM sodium phosphate and 5 mM MgCl₂ (pH 7.4). In saturation binding assays, we employed six different [¹H]NMS concentrations ranging from 0.1 to 6 nM. In competition binding assays, we studied the ability of tiotropium, atropine or acetylcholine to interfere with [¹H]NMS (0.5 nM) binding.
Incubations were carried out for 20 h in the case of tiotropium in order to achieve equilibrium binding (3 h for all other ligands). Non-specific binding was assessed as binding remaining in the presence of 1 μM atropine. Binding reactions were terminated by rapid filtration over GF/C Brandel filters, followed by three washes (~4 ml per wash) with ice-cold distilled water. The amount of radioactivity that remained bound to the filters was determined by liquid scintillation spectrometry.

Ligand binding data were analysed using the nonlinear curve-fitting program Prism 4.0 (GraphPad Software Inc.).

Tiotropium bromide was purchased from W/St PharmaChem, Inc. [3H]NMS (specific activity: 85.0 Ci mmol⁻¹) was obtained from PerkinElmer Life Sciences.

Atropine sulphate and acetylcholine chloride were from Sigma-Aldrich. Tiotropium bromide was from Sigma-Aldrich.

Simulations were carried out at 37 °C in a total volume of 620 μl using a buffer consisting of 4 mM Na₃HPO₄ and 1 mM KH₂PO₄ (pH 7.4). Membranes prepared from transfected COS-7 cells (final protein concentration, 10 μg protein per ml) were prelabelled with 1 nM [3H]QNB for 30 min. Dissociation of the labelled ligand was initiated by the addition of atropine (final concentration, 3 μM). Incubations were terminated by filtration through GF/C Brandel fibre filters that had been prewetted with 6.1% polyethyleneimine, followed by two rinses with ice-cold distilled water. The amount of radioactivity that remained bound to the filters was determined by liquid scintillation spectrometry.

Molecular dynamics. In all simulations, the receptor was embedded in a hydrated lipid bilayer with all atoms, including those in the lipids and water, represented explicitly. Simulations were performed on Anton,[46] a special-purpose computer designed to accelerate standard MD simulations by orders of magnitude.

System set-up and simulation protocol. Simulations of the M2 receptor were based on the crystal structure of the QNB-M2 complex, and simulations of M3 were based on the structure of the tiotropium-M3 complex (chain A). These crystal structures were determined using a T4 lysozyme (T4L) fusion strategy in which intracellular loop 3 (ICL3) of each receptor was replaced by T4L. The T4L sequence was omitted in our simulations. Residues 6.31–6.33 near the intracellular end of TM6 were unresolved in the M3 crystal structure, and residues 6.27–6.30 were resolved in an unstructured conformation packed against T4L. Residues 6.27–6.36 were modelled manually as a helical extension of TM6, with side chains then placed using Prime. Hydrogens were added to the crystal structures using Maestro (Schrödinger LLC), as described in previous work.[47] All titratable residues were left in the dominant protonation state at pH 7.0, except for Asp 692.50 in M2 and Asp 114 2.50 in M3, which were protonated. Asp 69 2.50 and Asp 114 2.50 correspond to rhodopsin Asp 83* 50, which is protonated during the entire photocycle.[48]

Prepared protein structures were inserted into an equilibrated POPC bilayer as described,[49] Sodium and chloride ions were added to neutralize the net charge of the system and to create a 150 mM solution.

Simulations of the M3 receptor initially measured 80 × 80 × 87 Å and contained 163 lipid molecules, 26 sodium ions, 41 chloride ions and approximately 9,897 water molecules, for a total of ~56,000 atoms. Simulations of the M2 receptor initially measured 79 × 79 × 85 Å and contained 156 lipid molecules, 24 sodium ions, 33 chloride ions and approximately 9,165 water molecules, for a total of ~53,000 atoms. To simulate M2 with tiotropium bound, we removed the co-crystallized ligand, QNB, and docked in tiotropium using Glide (Schrodinger LLC).

All simulations were equilibrated using Anton in the NPT ensemble at 310 K (37 °C) and 1 bar with 5 kcal mol⁻¹ Å⁻² harmonic position restraints applied to all non-hydrogen atoms of the protein and the ligand (except for the tiotropium-M2 complex, where the ligand was unrestrained); these restraints were tapered off linearly over 50 ns. All bond lengths to hydrogen atoms were constrained using CHARMM.[50] A RESP-embedded[51] was used with a time step of 2 fs, and long-range electrostatics computed every 6 fs. Production simulations were initiated from the final snapshot of the corresponding equilibration runs, with velocities sampled from the Boltzmann distribution at 310 K, using the same integration scheme, long-range electrostatics method, temperature and pressure. Van der Waals and short-range electrostatic interactions were cut off at 13.5 Å and long-range electrostatic interactions were computed using the k-space Gaussian Split Ewald method[52] with a 32 × 32 × 32 grid, σ = 3.33 Å, and e = 2.33 Å.

Spontaneous binding of tiotropium and acetylcholine. We performed simulations where tiotropium was placed arbitrarily in the bulk solvent (at least 40 Å from the entrance to the extracellular vestibule) and allowed to diffuse freely until it associated spontaneously with the M2 or M3 receptor, following methodology as described.[42] In these simulations (Supplementary Table 4, conditions D and E), the co-crystallized ligand was removed and four tiotropium molecules were placed in the bulk solvent. A tiotropium molecule bound to the extracellular vestibule at least once in each simulation. In the longer simulations, tiotropium bound to and dissociated from the extracellular vestibule multiple times. Tiotropium assumed several different poses when bound to the extracellular vestibule of either M2 or M3 (Supplementary Fig. 4). Tiotropium never entered the orthosteric binding pocket, presumably because the simulations were not of sufficient length.

The fact that tiotropium associated with and dissociated from the vestibule multiple times, but did not enter the binding pocket, suggests that tiotropium must traverse a larger energetic barrier to enter the binding pocket of M2 or M3 from the extracellular vestibule than to enter the vestibule from bulk solvent. This contrasts with earlier simulations on alprenolol binding to the β2-adrenergic receptor, in which the largest energetic barrier (by a small margin) was between the bulk solvent and the extracellular vestibule.[53] This difference probably reflects the fact that ligands must pass through a much tighter passageway to enter the binding pocket of the M2 and M3 receptors from the vestibule than is the case for the β2-adrenergic receptor. Tiotropium lost the majority of its hydration shell as it entered the vestibule (Supplementary Fig. 19), as observed previously for ligands binding to β2-adrenergic receptors.[52]

We followed a similar protocol in a simulation of the M3 receptor in the presence of the agonist ACh, a smaller molecule which might be expected to bind faster (Supplementary Table 4, condition F). Indeed, an ACh molecule bound in the orthosteric binding pocket after 9.5 μs and remained there for the remainder of the 25-μs simulation. Although ACh quickly passed through the extracellular vestibule on route to the binding pocket, it did not exhibit metastable binding in the vestibule. ACh exhibited significant mobility in the binding pocket, probably reflecting the low affinity of the crystallized inactive state for agonists.

Forced dissociation of tiotropium. To identify the entire binding/dissociation pathway, we ‘pushed’ tiotropium out of the binding pocket of both the M2 and M3 receptors.[53,53] Production simulations were initiated from configurations of the corresponding unbiased trajectory. These simulations employed a time-dependent harmonic biasing potential, U(t):

\[ U(t) = \frac{k}{2} (d(t) - d_0(t))^2 \]

where \( t \) is time, \( k \) is a force constant in units of kcal mol⁻¹ Å⁻², \( d \) is the distance between the centre-of-mass of the heavy atoms of tiotropium and the centre-of-mass of the protein Cx atoms, and \( d_0(t) \) varied linearly over 1.0 μs, from 9.6 Å to 33 Å for M2 and from 8.6 Å to 32 Å for M3. This biasing term does not impose any preferred direction of ligand exit. We performed seven such simulations for each of M2 and M3, with \( k = 5 \), starting from configurations extracted from the tiotropium bound simulations of M2 and M3. Each initial configuration was separated in time by 36 ns. Results were similar across all simulations.

Forced dissociation of tiotropium. We performed a clustering analysis on the 14.2-μs spontaneous binding simulation of M2 (Supplementary Table 4, condition D) and the 16.0-μs spontaneous binding simulation of M3 (Supplementary Table 4, condition E). In Supplementary Fig. 4, we performed a clustering analysis on the 14.2-μs spontaneous binding simulation of M2 (Supplementary Table 4, condition D) and the 16.0-μs spontaneous binding simulation of M3 (Supplementary Table 4, condition E). We performed k-means clustering on the set of trajectory snapshots in which a tiotropium molecule was in the extracellular vestibule, using the positions of atoms indicated in Supplementary Fig. 4. Clusters representing highly similar poses were merged.

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