Activation and allosteric modulation of a muscarinic acetylcholine receptor

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Despite recent advances in crystallography and the availability of G-protein-coupled receptor (GPCR) structures, little is known about the mechanism of their activation process, as only the β2-adrenergic receptor (β2AR) and rhodopsin have been crystallized in fully active conformations. Here we report the structure of an agonist-bound, active state of the human M2 muscarinic acetylcholine receptor stabilized by a G-protein mimetic camelid antibody fragment isolated by conformational selection using yeast surface display. In addition to the expected changes in the intracellular surface, the structure reveals larger conformational changes in the extracellular region and orthosteric binding site than observed in the active states of the β2AR and rhodopsin. We also report the structure of the M2 receptor simultaneously bound to the orthosteric agonist iperoxo and the positive allosteric modulator LY219620. This structure reveals that LY219620 recognizes a largely pre-formed binding site in the extracellular vestibule of the iperoxo-bound receptor, inducing a slight contraction of this outer binding pocket. These structures offer important insights into the activation mechanism and allosteric modulation of muscarinic receptors.

Muscarinic acetylcholine receptors (M1–M5) are GPCRs that regulate the activity of a diverse array of central and peripheral functions in the human body, including the parasympathetic actions of acetylcholine1. The M2 muscarinic receptor subtype has a key role in modulating cardiac function and many important central processes, such as cognition and pain perception1. As it was among the first GPCRs to be purified2 and cloned3, the M2 receptor has long served as a model system in GPCR biology and pharmacology. Muscarinic receptors have attracted particular interest owing to their ability to bind small-molecule allosteric modulators4. Because allosteric sites are often less conserved than the orthosteric binding site, some ligands binding to allosteric sites show substantial subtype selectivity5–8. Several agents hold promise for the development of drugs for the treatment of conditions such as those of the central nervous system and for metabolic disorders. Although crystal structures were recently obtained for inactive states of the M2 and M3 muscarinic receptors7,8, there are no structures of a GPCR bound to a drug-like allosteric modulator.

The binding of an agonist to the extracellular side of a GPCR results in conformational changes that enable the receptor to activate heterotrimeric G proteins. Despite the importance of this process, only the β2AR and rhodopsin have been crystallized in fully active conformations9–11. Crystallization of active-state GPCRs has been challenging due to their inherent conformational flexibility and biochemical instability12. To understand the mechanistic details underlying GPCR activation and allosteric modulation better, we solved X-ray crystal structures of the M2 receptor bound to the high-affinity agonist iperoxo13 alone and in combination with LY2191620, a positive allosteric modulator.

Conformational selection of nanobodies

Initial crystallization attempts with M2 receptor bound to agonists were unsuccessful, probably due to the flexibility of the intracellular receptor surface in the absence of a stabilizing protein. We thus sought to obtain a G-protein mimetic nanobody for the M2 receptor, analogous to that used to facilitate crystallization of the β2AR in an active conformation11. Llamas were immunized with M2 receptor bound to the agonist iperoxo, and a post-immune single variable domain (VH1) nanobody complementary DNA library was constructed and displayed on the surface of yeast (Fig. 1a).

An essential component for the selection of active-state stabilizing nanobodies was simultaneous staining of yeast with both agonist and inverse-agonist occupied M2 receptor populations, which were distinguishably labelled with separate fluorophores. This allowed the use of fluorescence-activated cell sorting (FACS) to select those clones binding only agonist-occupied receptor (Fig. 1b; see Methods). To ensure that the different fluorophore-conjugated receptors represent distinct receptor populations requires that at least one receptor population must be bound to an exceptionally high-affinity or irreversible ligand. We therefore developed a covalent muscarinic receptor agonist for use in selection experiments. This has preceded in an acetylcholine mustard16, which is thought to react with the binding-site residue Asp1033,2 (superscript numerals refer to the Ballesteros–Weinstein numbering system) to form a covalent adduct17. Accordingly, we synthesized an analogous ‘iperoxo mustard’, which we call FAUC123 (Supplementary Methods). We found that FAUC123 bound covalently and was able to induce activation of the M2 receptor (Extended Data Fig. 1), thereby allowing simultaneous staining of yeast with agonist- and antagonist-bound M2 receptor labelled with distinct fluorophores for each population.

After nine rounds of conformational selection, almost all remaining yeast cell clones preferentially bound FAUC123-occupied receptor (Fig. 1d). Three clones in particular, Nb9-1, Nb9-8 and Nb9-20
A key feature of GPCR activation is an outward movement of the intracellular portion of transmembrane (TM) helices 5 and 6, creating a cavity large enough to accommodate the carboxy terminus of the G protein α-subunit\(^{15,13}\). Although several GPCRs have been crystallized in complex with agonists, only the β₂AR and rhodopsin show a fully active state with adequate space to allow G-protein binding (Extended Data Fig. 2). As anticipated on the basis of functional studies (Fig. 2), Nb9-8 binds to the intracellular surface of the receptor (Fig. 3a). There is a significant outward displacement at the intracellular side of TM6, together with a smaller outward movement of TM5 and a rearrangement of TM7 around the NPXXY motif (Fig. 3b, d).

Like the active states of rhodopsin and the β₂AR, the active M2 receptor shows rearrangements of the highly conserved DRY motif at the intracellular side of TM3 and the NPXXY motif in TM7 (Fig. 3c, d).

In the active state of M2, Arg 121^3.50 of the DRY motif adopts an extended conformation virtually identical to that seen in metarhodopsin II and the β₂AR–Gs complex (Fig. 3c, e), and Asp 120^3.49 is stabilized by a hydrogen bond with Asn 58^2.39 (Fig. 3c). To assess the importance of Asn 58^2.39 for stabilization of the active conformation, we mutated it to alanine. The resulting mutant displayed normal ligand-binding properties, but impaired ability to activate G protein (Extended Data Fig. 3a and Extended Data Table 2). Hence, it is likely that Asn 58^2.39 either directly stabilizes the active conformation, or engages in direct interactions with G protein.

Similar to the DRY motif, the NPXXY region in TM7 shows significant rearrangements on activation (Fig. 3d). Most striking is a partial ‘unwinding’ of TM7 around Tyr 440^7.53. This positions Tyr 440^7.53 of the NPXXY motif in close proximity to the highly conserved residue Tyr 206^5.58 of the β₂AR–Gs complex (Fig. 3c, e), and Asp 120^3.49 is stabilized by a hydrogen bond with Asn 58^2.39, as seen in the active-state structures of the β₂AR and rhodopsin. Indeed, the position of these two tyrosine residues is highly similar in the active structures of rhodopsin, β₂AR and the M2 receptor (Fig. 3f), indicating that this feature represents a hallmark of GPCR activation. In addition, a molecular dynamics study recently predicted that Tyr 206^5.58 and Tyr 440^7.53 interact in the active conformation of the M2 receptor\(^{19}\), although this model was in other ways dissimilar from the structures presented here.

To assess the importance of this interaction for M2 receptor activation, we mutated Tyr 206^5.58 to phenylalanine, eliminating its ability to interact with Tyr 440^7.53 via a bridging water molecule. The Y206F mutant receptor could no longer be activated by acetylcholine (Extended Data Fig. 3a) and gave only a very weak functional response on treatment with iperoxo. In addition, agonist affinity was reduced by greater than tenfold (Extended Data Table 2), whereas antagonist binding was largely unaffected. These results indicate that the Tyr 206^5.58–Tyr 440^7.53 interaction stabilizes the active conformation of the receptor in a manner reminiscent of the ‘ionic lock’ interaction\(^{20}\), which stabilizes the inactive conformation of family A GPCRs.
**Activation mechanism**

Whereas activation of β2AR and rhodopsin is associated with modest conformational changes in the orthosteric ligand-binding site, marked structural changes are observed in the M2 receptor. The activated M2 receptor shows a small orthosteric binding site, which completely occludes the agonist iperoxo from solvent (Fig. 4a, b). Indeed, the muscarinic inverse agonist quinuclidiny1 benzilate (QNB) is too large to be accommodated in this binding cavity, perhaps accounting for its ability to suppress basal activity of the M2 receptor.

Within the active orthosteric binding pocket, the agonist iperoxo adopts a bent conformation (Fig. 4c and Extended Data Fig. 4). Transmembrane helices 5, 6 and 7 move inward, towards the agonist, in the active M2 conformation. TM3, in contrast, undergoes a slight rotation about its axis, but has almost no inward motion towards the ligand. The largest differences between inactive and active states of the M2 receptor involve TM6, where an inward movement of 2 Å at the α-carbon of Asn 404.62 allows for formation of a hydrogen bond between its side chain and iperoxo.

Despite these activation-related structural changes, polar contacts between the agonist iperoxo and the receptor resemble those with QNB bound to the inactive M2 receptor. In particular, the conserved Asp 103.32 serves as a counter-ion to the ligand amine in both cases, and Asn 404.62 engages in hydrogen bonding with both ligands. The smaller size of iperoxo relative to QNB results in more limited hydrophobic contacts, however. This is particularly true along TM5, which engages the phenyl rings of QNB, but makes more limited hydrophobic contact with iperoxo in the active receptor conformation.

The hydrogen bond between Asn 404.62 and the iperoxo isoaxoline oxygen is analogous to the hydrogen bond between this residue and the QNB carbonyl in the inactive receptor state; however, the smaller size of iperoxo necessitates an inward motion of TM6 (Fig. 4d, e). To investigate the role of this hydrogen bond in receptor activation, we mutated Asn 404.62 to glutamine, which, due to the longer side chain, would allow TM6 to form a hydrogen bond with iperoxo in the inactive receptor. Consistent with a previous mutagenesis study24, the N404Q mutant receptor failed to bind detectable amounts of [3H]-NMS, but retained the ability to bind [3H]-QNB specifically, although with 163-fold reduced affinity (Extended Data Table 2). Similarly, the binding affinities for acetylcholine and iperoxo were reduced, and although the N404Q mutant was able to activate G protein in response to both iperoxo and acetylcholine, the concentration–response curves were shifted to the right by about 100-fold (Extended Data Fig. 3a), probably due to the reduced agonist-binding affinities. Nevertheless, it remains possible that a structural reorientation of Asn 404.62 also contributes to M2 receptor activation.

Like Asn 404.65, Asp 103.32 has a central role in receptor binding to iperoxo, engaging the trimethyl ammonium ion. Cation–π interactions with Tyr 104.33, Tyr 403.51 and Tyr 426.39 form an aromatic lid over the ligand amine (Fig. 4f). To assess the contribution of Asp 103.32 to receptor activation, we generated and analysed the D103E mutant M2 receptor, which abolished agonist-induced M2 receptor activation (Extended Data Fig. 3a). The D103E mutant receptor bound [3H]-NMS with wild-type-like affinity but showed greatly reduced affinities for acetylcholine (~120-fold) and iperoxo (~380-fold) (Extended Data Table 2), indicating that Asp 103.32 recognition of the ligand cation has a critical role in both agonist binding and receptor activation.

In the active state of the M2 receptor, the inward motion of the upper portion of TM6 allows Tyr 403.51 to form a hydrogen bond with Tyr 104.33, which in turn forms a hydrogen bond to Tyr 426.39 (Fig. 4f), resulting in closure of the aforementioned tyrosine lid over the agonist. Hydrogen bonding of this lid seems to be an important feature of agonist binding and activation in muscarinic receptors: mutation of any of the three tyrosines to Phe leads to impaired agonist binding in the homologous M3 muscarinic receptor22, and mutation of Tyr 104.33 and Tyr 403.51 in the M2 receptor has a similar effect21,24. It should be noted that the structure of active M2 receptor bound to other agonists, including acetylcholine, might show differences as compared to the iperoxo-bound structure presented here.

**Allosteric modulation**

Muscarinic receptors have long served as important model systems for understanding allosteric modulation of GPCR signalling6,23. The structures of the inactive M2 and M3 receptors confirmed that these
Figure 3 | Intracellular changes on activation of the M2 receptor. a, The overall structure of the active-state M2 receptor (orange) in complex with the orthosteric agonist iperoxo and the active-state stabilizing nanobody Nb9-8 is shown. b, Compared to the inactive structure of the M2 receptor (blue), transmembrane helix 6 (TM6) is substantially displaced outward, and TM7 has moved inward. Together, these motions lead to the formation of the extracellular vestibule. c, d, Conserved motifs likewise show substantial changes on activation, and adopt conformations similar to those seen in the two other active GPCR structures (e, f). In particular, an interaction between two conserved tyrosines (Tyr 5.58 and Tyr 7.53) is probably mediated by a water molecule (blue circle), as seen in the high-resolution structure of the active β2AR (ref. 18).

The extracellular vestibule shows a slight additional contraction around the allosteric ligand (Extended Data Fig. 6). This subtle change stands in contrast to the substantial closure of the extracellular vestibule in the two active structures relative to the inactive conformation (Fig. 5d). A notable exception is Trp 422 7.35, which adopts a vertical conformation in the presence of agonist. The extracellular vestibule shows a slight additional contraction around the allosteric ligand (Extended Data Fig. 6). This is largely due to the closure of the extracellular vestibule, which is directly superimposed on the orthosteric site, separated only by the tyrosine lid, with Tyr 426 7.39 interacting with both ligands.

The structure of the M2–iperoxo–LY2119620 complex is largely the same as that of receptor and agonist without LY2119620, indicating that the allosteric binding site is largely pre-formed in the presence of agonist. The extracellular vestibule shows a slight additional contraction around the allosteric ligand (Extended Data Fig. 6). This is largely due to the closure of the extracellular vestibule, which is directly superimposed on the orthosteric site, separated only by the tyrosine lid, with Tyr 426 7.39 interacting with both ligands.

The closed, active conformation of the extracellular vestibule is largely the consequence of the inward motion of TM6, which directly contacts the allosteric modulator, the orthosteric agonist, and probably the G protein as well. Stabilization of the closed extracellular vestibule by LY2119620 and other allosteric modulators may directly stabilize the open, active conformation of the intracellular side of TM6, accounting for the phenomenon of allosteric agonism in addition to positive
cooperativity with orthosteric agonists. However, although the differences in TM6 between inactive and active structures can be described as a rigid-body motion, we cannot exclude the possibility that TM6 is flexible, allowing independent conformational changes in the G-protein binding site, the orthosteric site and the extracellular vestibule.

Conclusions
The structures presented here offer insights into the structural basis for muscarinic receptor activation and allosteric modulation by a drug-like molecule. In contrast to rhodopsin and the β2AR, extensive changes are seen in the orthosteric binding site and in the extracellular vestibule.
vestibule upon M2 receptor activation. The structure of active M2 receptor bound to the allosteric modulator LY2119620 definitively establishes the extracellular vestibule as an allosteric binding site, and shows that the allosteric modulator induces several additional structural changes to those seen with orthosteric agonist alone. The structures presented here offer only a single view of an active muscarinic receptor; more work will be required to identify additional active states that may exist. Nonetheless, the information presented here provides a structural framework for future studies of GPCR activation and allostery, and may facilitate the development of novel therapeutics.

METHODS SUMMARY

The human M2 muscarinic receptor was expressed in S9 insect cells and purified to homogeneity by nickel affinity chromatography, followed by Flag affinity and size exclusion chromatography. The nanobody Nb9-8 was identified by yeast display using a library derived from peripheral blood lymphocytes of a llama immunized with purified, iperoxo-occupied M2 receptor. Recombinant Nb9-8 was expressed in E. coli as a fusion protein with a Flag tag, purified by nickel affinity chromatography followed by size exclusion chromatography. Nb9-8 was performed using lipidic mesoionic gradient methods, and data were collected by X-ray microdiffraction at Advanced Photon Source GM/CA beamlines 23ID-B and 23ID-D.

On-line Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 18 July; accepted 3 October 2013.

Published online 20 November 2013.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We acknowledge support from the National Science Foundation (graduate fellowship to A.C.K., and Award 1223785 to B.K.K.), the Stanford Medical Scientist Training Program (A.M. and A.M.R.), the American Heart Association (A.M.), the Ruth L. Kirshstein National Research Service Award (A.M.R.), National Institutes of Health grants NS02847123 and GM08311806 (B.K.K.), the Mihers Foundation (B.K.K., W.I.H. and K.G.C.), the Deutsche Forschungsgemeinschaft for the grant GM 13/10-1 (K.E., H.H., P.G.), the National Health and Medical Research Council (NHMRC) of Australia program grant 519461 (P.M.S. and A.C.), NHMRC Principal Research Fellowships (P.M.S. and A.C.) and the Howard Hughes Medical Institute (K.G.C.). This work was supported in part by the Intramural Research Program, NIDDK, NIH, US Department of Health and Human Services (J.H., K.H. and J.W.). We thank K. Leach for performing ERK assays, and B. Davie and P. Scammells for synthesis of iperoxo. We thank X. Zhao, K. Xiao, C. H. Croy and D. A. Schober for functional characterization of LY2119620. We thank T. S. Koblika for preparation of affinity chromatography reagents and F. S. Thian for help with cell culture.

Author Contributions A.C.K. expressed and purified M2 receptor for yeast display and crystallographic experiments, performed crystallization, data collection, and structure refinement, and performed radioligand binding assays to validate nanobody activity. A.M., A.M.R. and A.M. designed experiments to identify nanobodies by yeast display. A.M.R. performed all yeast selections, and expressed and purified Nb9-8 and other nanobodies. J.H. and K.H. performed site-directed mutagenesis and characterization of resulting mutants. K.E. synthesized FAUC123. H.H. performed cell assays and radioligand binding to characterize FAUC123. C.V. performed pharmacological characterization of LY2119620. P.M.S. and A.C. supervised pharmacological characterization of LY2119620. C.F. performed radioligand binding to characterize FAUC123. D.P. supervised synthesis and characterization of FAUC123. E.P. and J.S. performed llama immunization, cDNA production, and performed selections by phage display. W.I.W. supervised structure refinement. K.G.C. supervised yeast selection experiments. J.W. performed radioligand experiments and analyzed results. B.K.K. provided overall project supervision, and with A.C.K., A.M.R. and A.M. wrote the manuscript with assistance from A.C. and J.W.

Author Information Coordinates and structure factors for the active M2 receptor complex with Nb9-8 and iperoxo are deposited in the Protein Data Bank under accession code 4MQS, and the coordinates and structure factors of the same complex bound additionally to the allosteric modulator LY2119620 are deposited under accession code 4MTQ. Additional experimental information is available at www.nature.com/reprints. The authors declare competing financial interests; details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to B.K.K. (koblika@stanford.edu).
METHODS

Determination of M2 activation via inositol phosphate assays. Agonist-induced activation of the human M2 muscarinic receptor was studied in inositol phosphate (IP) accumulation assays as described. For M2 activation studies, COS-7 cells were transiently co-transfected with cDNAs encoding the human M2 receptor (Missouri S&T cDNA Resource Center) and the hybrid G protein Gβγδ5 (Gαq protein with the last five amino acids at the C terminus replaced by the corresponding sequence of Gαq; gift from The J. David Gladstone Institutes). Twenty-four hours after transfection, cells were transferred into 24-well plates at a density of 100,000 cells per well in a volume of 270 μL. After addition of 30 μM of myo-[3H]inositol (specific activity = 22.5 Ci mmol⁻¹, PerkinElmer), cells were incubated for 15 h. Then, medium was aspirated, the cells were washed with serum-free medium supplemented with 10 mM LiCl, and test compounds (diluted in serum-free medium supplemented with 10 mM LiCl) were added at 37 °C for 60 min. Cells were then lysed by adding 150 μL of ice-cold 0.1 M NaOH for 5 min. After neutralization with 50 μL of 0.2 M formic acid, the cell extract was diluted in buffer (5 mM sodium tetraborate, 0.5 mM Na-EDTA) and separated by anion-exchange chromatography using an AGI-X8 resin (Bio-Rad). After washing with water and elution buffer A (5 mM sodium tetraborate, 60 mM sodium formate) and again with water, total IP was eluted with 2.5 mL elution buffer B (1.0 M ammonium formate) and directly collected into scintillation counting vials. Radiolabeling was measured by scintillation counting after adding 2.5 mL of Emulsifier-Safe (PerkinElmer). Data were analyzed by normalizing disintegrations per minute (d.p.m.) values with 0% for the non-stimulated receptor and 100% for the full effect of the reference iperoxo. Concentration–response curves were fitted by nonlinear regression using the GraphPad Prism 5 software.

Irreversible activation of the M2 receptor was tested at 1 nM FAUC123 in comparison to the reversible ligand iperoxo (1 nM). After incubation for 30 min, the antagonist atropine (1 μM) was added to one-half of the sample (buffer was added to the other half) and incubations were continued for an additional 90 min. Total IP accumulation was determined as described above.

LY2119620 pharmacology. To characterize the allosteric interaction between LY2119620 and iperoxo, we performed radioligand binding and cellular functional assays at the wild-type human muscarinic receptor stably expressed in a CHO FlpIn cell line. Increasing concentrations of LY2119620 caused a modest reduction in the specific binding of the orthosteric antagonist, [3H]-NMS, indicating weak negative cooperativity, but robustly enhanced the potency of iperoxo to compete for [3H]-NMS binding, indicating positive cooperativity with the agonist (Extended Data Fig. 3b). Application of an allosteric ternary complex model to these data yielded the values shown in Extended Data Table 3 for ligand affinity and cooperativities with agonist and antagonist. We then investigated the functional effect of LY2119620 on M2 muscarinic receptor signalling via monitoring receptor-mediated [35S]-GTPγS binding to activated G proteins, or phosphorylation of ERK1/2 (pERK2). [35S]-GTPγS binding was chosen as a proximal measure of receptor activation, whereas the pERK1/2 assay was chosen because it measures a downstream response that is also a point of convergence of multiple cellular events.

For the first round of selection, counter-selection was performed against the β2 receptor to remove yeast clones that bind nonspecifically to membrane proteins or to secondary staining reagents. 1.0 × 10⁹ of induced yeast were washed with PBEM buffer and then stained in 5 mL of PBEM buffer containing 1 μM biotinylated β2 receptor liganded with carazolol for 1 h at 4 °C. Yeast were then washed with streptavidin-647 as a secondary reagent and magnetically labelled with anti-647 microbeads (Milenyi) as described previously. Positively labelled yeast were then removed by the use of an LS column (Milenyi); the cleared flow-through was then used for subsequent selection. Positive selection for clones recognizing the active state of the M2 receptor was performed by staining with 2 μM biotinylated M2 receptor bound to the agonist iperoxo in 5 mL PBEM buffer supplemented with 2 μM iperoxo for 1 h at 4 °C. Yeast were then washed, stained with streptavidin-647, and magnetically labelled with anti-647 microbeads, including binding in the PBEM buffer at all steps. Magnetic separation of M2 receptor–binding yeast clones was performed using an LS column (Milenyi) following the manufacturer’s instructions. Magnetically sorted yeast were re-suspended in SDCAA medium and cultured at 30 °C. Rounds 2–4 were selected in a similar manner, counter-selecting against 1 μM biotinylated β2 receptor bound to carazolol and positively selecting using 1 μM biotinylated M2 receptor bound to iperoxo. For these rounds, the scale was reduced tenfold to 1 × 10⁶ induced yeast and staining volumes of 0.5 μL.

Conformational selection was performed for rounds 5–9. For rounds 5–8, yeast were stained with 1 μM biotinylated M2 receptor pre-incubated with the high-affinity antagonist atropine (1 μM) and 10 μM tiotropium for 1 h at 4 °C. Yeast were then fluorescently labelled with either streptavidin-647 or streptavidin-PE, and magnetically labelled with the corresponding anti-647 or anti-PE microbeads (Milenyi). Depletion of inactive-state binders was carried out using an LS column. The cleared yeast were then positively selected by staining with 0.5 μM (rounds 5–7) or 0.1 μM (round 8) M2 ligand.
receptor pre-bound to iperoxo for 1 h at 4 °C. Yeast were then fluorescently labelled with either streptavidin-PE or streptavidin-647, using a fluorophore distinct from that used in the previous counter-selection step. Magnetic separation of agonist-occupied M2 receptor was performed using an LS column, as for steps 1–4. For round 9, two-colour FACS was performed. Induced yeast were simultaneously stained from that used in the previous counter-selection step. Magnetic separation of agonist-occupied M2 receptor was performed using an LS column, as for steps 1–4. For round 9, two-colour FACS was performed. Induced yeast were simultaneously stained with a 1 μM Alexa647-labelled M2 receptor reacted with iperoxo mustard and a 1 μM Alexa488-labelled M2 receptor pre-bound with tiotropium for 1 h at 4 °C. Alexa647 positive/Alexa488 negative yeast were purified using a FACS jell (BD Biosciences) sorter. Post-sort yeast were plated onto SDCAA agar plates and the nanobody-encoding sequences of several colonies were sequenced. Full sequences of clones confirmed to enhance agonist affinity are: Nb9-1, QVQLQESGGGLVQAGDSLRLSCAASGHTFSSARMYWVRQAPGEREFVAAISRSGFTYSADVSFKRTSRSRDANNVYLYQMSNLQPETDAYTICAYYLYLYFNDYTHYWLGQTIVVSS; Nb9-20, QVQLQESGGGLVQPEGSLSLTAOCTDGSFTMNYAIAYFWRQAPIEKREGLATISIDGTTYAYDSVFKRTSRSRDKNMVLYQMNLRPFTAYYCVSGDPSDYSYDGESEYWVQGQTIVVSS.

Expression of MBP-nanobody fusions in E. coli. Nanobody sequences were subcloned into a modified pMalp2x vector (New England Biolabs) containing an N-terminal, 3C protease-cleavable maltose binding protein (MBP) tag and a C-terminal 8×His tag. Plasmids were transfomed into BL21(DE3) cells and protein expression induced in Terrific Broth by addition of IPTG to 1 mM at an OD600 of 0.8. After 24 h of incubation at 22 °C, cells were collected and periplasmic protein was obtained by osmotic shock. MBP–nanobody fusions were purified by Ni-NTA chromatography and MBP was removed using 3C protease. Cleaved MBP was separated from the 8×His tagged nanobodies by an additional Ni-NTA purifi-
cation step. The 8×His tag was subsequently removed using carboxypeptidase A. Expression and purification of G protein. Heterotrimeric G was prepared by expression using a single baculovirus for the human Gαi1 subunit and a second, bicistronic vector for human Gβγ subunits. G protein was expressed in HighFive insect cells, and then purified as described previously for G, (ref. 10). In brief, G protein was extracted with cholate, purified by Ni-NTA chromatography, detergent exchanged into dodecyl maltoside buffer, and then purified by ion exchange and dialysed before use.

M2 receptor radioligand binding assays with G protein and nanobody. M2 receptor was expressed and purified as described above. Receptor was then reconstituted into LDL particles consisting of apolipoprotein A1 and a 3:2 (mol:mol) mixture of the lipids POPC:POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine:1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1’-rac-glycerol) respectively, Avanti Polar Lipids). Binding reactions contained 50 fmol functional receptor, 0.6 nM [3H]N-methylscopolamine (NMS), 100 mM NaCl, 20 mM HEPES pH 7.5, 0.1% BSA, and ligands and nanobodies as indicated. Concentration-dependent effects of nanobodies were measured in the presence of 10 nM iperoxo. All reactions were performed in a 500 μl volume. For samples containing G protein, purified Gi hetero-
dimers were added to the radioligand mixture at a 1.000-fold dilution from a 200 μM stock, resulting in a large stoichiometric excess over receptor and diluting G protein below the detergent CMC to allow incorporation into LDL particles, essentially as described previously. Reactions were mixed and then incubated for 2 h. Samples were then filtered on a 48-well harvester (Brandel) onto a filter which had been pre-treated with 0.1% polyethyleneimine. All measurements were taken by liquid scintillation counting, and experiments were performed at least in triplicate. Site-directed mutagenesis. A mammalian expression plasmid coding for the human M2 muscarinic receptor (M2R-pcDNA3.1+) was obtained from the Missouri S&T cDNA Resource Center. Mutant M2 receptors were generated by using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The identity of all mutant M2 receptor con-
structs was confirmed by DNA sequencing.

Transient expression of receptor constructs in COS-7 cells. Wild-type and mutant M2 receptors were transiently expressed in COS-7 cells grown in 100 mm dishes, as described previously. For functional studies, the various receptor con-
structs (3 μg each) were co-expressed with a chimaeric G protein α-subunit (Gαqi) (3 μg plasmid DNA) in which the last five amino acids of Gαq were replaced with the corresponding Gαs sequence.

Radioligand binding studies of mutant and wild-type M2 receptors. Acetyl-
choline bromide was purchased from Sigma. Iperoxo was a gift of Bristol Myers Squibb. [3H]NMS (85.5 Ci mmol−1) and 3-quinuclidinyl benzilate ([3H]-QNB; 47.4 Ci mmol−1) were from PerkinElmer Life Sciences (Downers Grove). Radioligand binding studies were carried out with membranes prepared from transiently transfected COS-7 cells as described. Forty-eight hours after transfection, cells were collected and re-
suspended in 25 mM sodium phosphate buffer (pH 7.4) containing 5 mM MgCl2.

Membrane homogenates were prepared and re-suspended in the same buffer. [3H]-NMS or [3H]-QNB binding reactions were carried out in the presence of 9 μg of membrane protein for 3 h at room temperature (total volume of the incubation mixture: 0.5 ml). In saturation binding studies, six different concentrations of the radioligand were used ([3H]-NMS, 0.3 nM to 10 nM; [3H]-QNB, 0.05 nM to 20 nM). In competition binding assays, membrane homogenates were incubated with ten different concentrations of acetylcholine (13 nM to 1 mM) or iperoxo (0.13 nM to 10 μM) in the presence of a fixed concentration of radioligand (2 nM [3H]-NMS or [3H]-QNB for N404Q; 15 nM [3H]-QNB for N404Q and 0.5 nM [3H]-QNB for wild-type M2 receptor). Nonspecific binding was deter-
mined in the presence of 10 μM atropine. Reactions were stopped by rapid filtra-
tion through GF/C filters. Data were analysed using Prism 4.0 software (GraphPad Software, Inc.).

Calcium mobilization assay. COS-7 cells co-expressing wild-type or mutant M2 receptor and the hybrid G protein, Gα(i1) (ref. 41), were incubated with increasing concentrations of agonists (acetylcholine, 5 nM to 50 μM; iperoxo, 50 pM to 0.5 μM), and increases in intracellular calcium levels were determined in 96-well plates using FLIPR technology (Molecular Devices), as described in detail previ-
ously. Agonist concentration–response curves were analysed using Prism 4.0 software.

Cryltralization. Purified M2 receptor was reconstituted into lipidic cubic phase by mixing with a 1.5-fold excess by mass of 10:1 (w/w) monoolein cholesterol lipid mix. Protein and lipid were loaded into glass syringes (Art Robbins Instruments), and then mixed 100 times by the coupled syringe method. Samples of 30–100 nl in volume were spotted onto 96-well glass plates and overlaid en bloc with 600 nl precipitant solution for each well. Precipitant solution consisted of 10–20% PEG300, 100 mM HEPES pH 7.2–7.9, 1.2% 1,2,3-heptanetriol, and 20–80 mM Ficoll 400. Identical conditions were used to crystallize LY2119620-receptor complexes, except that the overlay precipitant solution was supplemented with 500 μM LY2119620. Crystals grew in 24 h, and reached full size within 2 days. Crystals were then harvested in mesh grid loops (MiTeGen) with 10–50 crystals per loop and stored in liquid nitrogen before use.

Data collection. Grids of crystals were rastered at Advanced Photon Source beamlines 23ID-B and 23ID-D. Initial rasting was performed with an 80 μm by 30 μm beam with fivefold attenuation and 1-s exposure, and regions with strong diffraction were sub-rastered with a 10 μm collimated beam with equivalent X-ray dose. Data collection was similarly performed with a 10 μm beam, but with no attenuation and exposures of typically 1–5 s. An oscillation width of 1–2 degrees was used in each case, and wedges of 3–10 degrees were compiled to create the final data sets.

Data reduction and refinement. Diffraction data were processed in HKL2000, and statistics are summarized in Extended Data Table 1. The structure was solved using molecular replacement with the structure of the inactive M2 receptor (Protein Data Bank accession 3UON) and N880 (Protein Data Bank accession 3POG) as search models in Phaser. The resulting structure was iteratively refined in Phenix and manually rebuilt in Coot. Final refinement statistics are summarized in Extended Data Table 1. Figures were prepared in PyMol (Schrödinger).
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Extended Data Figure 1 | Characterization of FAUC123. a, Activation of M2 receptor by the prototypical muscarinic agonist carbachol, the high-affinity agonist iperoxo, and an irreversible iperoxo analogue (FAUC123) shows that iperoxo and FAUC123 are exceptionally potent full agonists at the M2 muscarinic receptor. Points indicate mean ± s.e.m. of three independent measurements, each performed in triplicate. 

b, S9 membranes expressing the human M2 receptor were incubated overnight at 4 °C with either no ligand, 100 µM iperoxo, or 100 µM FAUC123. Membranes were then washed three times in buffer without ligand, and incubated with a saturating concentration (20 nM) of [3H]-NMS. Incubation with iperoxo had no effect on radioligand binding, whereas FAUC123 blocked almost all [3H]-NMS binding sites. Bars indicate mean ± s.e.m. of three independent measurements. 

c, FAUC123 was tested for its ability to induce M2 receptor activation after covalent modification. Whereas iperoxo-induced inositol phosphate production was blocked by 1 µM atropine, FAUC123-induced activation was not susceptible to atropine blockade. Bars indicate mean ± s.e.m. of three independent measurements.
Extended Data Figure 2 | Comparison to other active GPCR structures.
Structures of all activated GPCRs show similarities in conformational changes at the intracellular surface. In each case, the intracellular tip of transmembrane helix 6 (TM6) moves outward on activation, as seen in the view from the intracellular side (right panels). This creates a cavity to which a G protein can bind the receptor.
Extended Data Figure 3 | Pharmacology. a, Functional properties are shown for M2 receptors in which key residues were mutated. Agonist-induced increases in intracellular calcium levels were monitored via FLIPR using transfected COS-7 cells. Because some mutant receptors (N58A, D103E) were expressed at lower levels than the wild-type (WT) receptor, reference curves were obtained using cells transfected with either 3 μg DNA or 1 μg wild-type receptor DNA. The latter cells showed receptor expression levels comparable to those found with the N58A and D103E mutants (see Extended Data Table 2 for details). Data are given as means ± s.e.m. of three independent experiments, each carried out in triplicate. AU, arbitrary units. b, The interaction between LY2119620 and iperoxo was measured by radioligand binding and functional assays. LY2119620 enhances the affinity of iperoxo (top graph) and its signalling potency (bottom graphs), and is also able to activate M2 receptor signalling directly as measured by [35S]GTPγS and ERK1/2 phosphorylation. Experiments were carried out with CHO cells stably expressing the human M2 receptor, and points are shown as mean ± s.e.m. of three independent experiments, each carried out in duplicate.
Extended Data Figure 4 | Binding-site diagram. M2 receptor residues interacting with the orthosteric agonist iperoxo and the positive allosteric modulator LY2119620 are shown. Polar contacts are highlighted as red dotted lines, and hydrophobic contacts are in green solid lines.
Extended Data Figure 5 | Electron density. a, b, $F_o - F_c$ omit maps are shown in grey, contoured at 2.5σ within a 2.5 Å radius of the indicated ligand.

c–f, $2F_o - F_c$ maps are shown in blue, contoured at 1.5σ within a 2.0 Å radius of the indicated region.
Extended Data Figure 6 | Comparison of M2 receptor structures with and without LY2119620 bound. Comparison of the structure of active M2 receptor with and without the allosteric modulator LY2119620 reveals that there are few differences outside the extracellular vestibule. The overall structures are compared in a. Within the extracellular vestibule, there is a slight contraction in the presence of the modulator, and Trp 422.7.35 undergoes a change of rotamer (panel b, red arrow). The orthosteric ligand-binding site, c, and intracellular surface, d, show few differences.
Extended Data Table 1 | Data collection and refinement statistics

|                          | M<sub>2</sub> receptor:Nb9-8 complex | M<sub>2</sub> receptor:Nb9-8 complex bound to LY2119620 |
|--------------------------|-------------------------------------|-----------------------------------------------------|
| **Data collection**      |                                     |                                                     |
| Number of crystals       | 17                                  | 18                                                  |
| Space group              | P2<sub>1</sub> 2<sub>1</sub>         | P2<sub>2</sub> 2<sub>2</sub>                        |
| Unit cell dimensions     |                                      |                                                     |
| a, b, c (Å)              | 62.9, 78.1, 163.5                   | 59.0, 77.4, 163.8                                   |
| α, β, γ (°)              | 90, 90, 90                          | 90, 90, 90                                          |
| Resolution (Å)           | 33 – 3.5 (3.6 – 3.5)                | 36 – 3.7 (3.8 – 3.7)                                |
| R<sub>work</sub> (%)     | 18.8 (74.4)                         | 19.5 (60.5)                                         |
| <i>I>/<i>o> (%)          | 5.8 (1.4)                           | 5.6 (2.1)                                           |
| CC<sub>1/2</sub> (%)     | 99.1 (35.0)                         | 99.0 (54.1)                                         |
| Completeness (%)         | 95.9 (83.1)                         | 93.0 (80.1)                                         |
| Redundancy               | 4.8                                 | 4.8                                                 |
| **Refinement**           |                                     |                                                     |
| Number of reflections    | 10237                               | 7867                                                |
| R<sub>work</sub>/R<sub>free</sub> (%) | 24.9 / 29.8                  | 25.0 / 30.1                                         |
| No. atoms                |                                      |                                                     |
| Protein                  | 3020                                | 3013                                                |
| Ligand(s)                | 14                                  | 57                                                  |
| Average B factors (Å<sup>2</sup>) |                         |                                                     |
| Receptor                 | 109.7                               | 102.3                                               |
| Nb689                    | 137.4                               | 129.3                                               |
| Iperoxo                  | 105.5                               | 107.6                                               |
| LY2119620                | -                                   | 119.0                                               |
| RMS deviation from ideality |                                    |                                                     |
| Bond length (Å)          | 0.004                               | 0.004                                               |
| Bond angles (°)          | 0.86                                | 0.81                                                |
| **Ramachandran statistics** |                                    |                                                     |
| Favored (%)              | 97.0                                | 95.9                                                |
| Allowed (%)              | 3.0                                 | 4.1                                                 |
| Outliers (%)             | 0                                   | 0                                                   |

* Highest shell statistics in parentheses.
† As calculated by Molprobity.
Radioligand binding studies were carried out with membranes prepared from COS-7 cells transiently expressing the indicated mutant M2 receptor constructs. The wild-type M2 receptor was expressed at two different densities to allow for a more straightforward interpretation of the functional data shown in Extended Data Fig. 3 (see Methods for details). Acetylcholine and iperoxo binding affinities ($K_i$) were determined in radioligand competition binding assays as indicated. Acetylcholine and iperoxo binding affinities ($K_i$) were determined in [3H]-QNB competition binding assays for the N404Q6.52 mutant, which did not bind [3H]-NMS with sufficient affinity. Data are given as means ± s.e.m. from two or three independent experiments, each performed in duplicate.

* No detectable [3H]-NMS binding activity.

### [3H]-NMS binding

| Receptor   | $K_D$ (nM) | $B_{\text{max}}$ (pmol/mg of protein) | $K_i$ (μM) | $K_i$ (μM) |
|------------|------------|-------------------------------------|------------|------------|
| WT M2      | 1.48 ± 0.31 | 1.79 ± 0.17                          | 2.74 ± 0.13 | 0.0073 ± 0.0006 |
| WT M2 (1 μg DNA) | 1.40 ± 0.02 | 0.60 ± 0.14                          |            |            |
| N58A5.59   | 1.15 ± 0.05 | 0.62 ± 0.14                          | 0.84 ± 0.21 | 0.0053 ± 0.0012 |
| D103E5.32  | 2.57 ± 0.58 | 0.51 ± 0.10                          | 327 ± 91    | 2.76 ± 0.82 |
| Y206F5.58  | 1.67 ± 0.26 | 1.87 ± 0.26                          | 31.8 ± 0.71 | 0.52 ± 0.20 |
| N404Q6.52  | N.D.*      |                                     |            |            |

### [3H]-QNB binding

| Receptor   | $K_D$ (nM) | $B_{\text{max}}$ (pmol/mg of protein) | $K_i$ (μM) | $K_i$ (μM) |
|------------|------------|-------------------------------------|------------|------------|
| WT M2      | 0.058 ± 0.015 | 1.63 ± 0.19                          | 1.25 ± 0.07 | 0.0130 ± 0.0070 |
| N404Q6.52  | 9.47 ± 2.22  | 1.24 ± 0.12                          | 34.3 ± 10.3 | 1.70 ± 0.29  |

Extended Data Table 2 | Ligand binding properties of mutant M2 receptors

- For the N404Q6.52 mutant, [3H]-NMS binding activity was not detectable.
- Acetylcholine and iperoxo binding affinities ($K_i$) were determined for the N404Q6.52 mutant in [3H]-QNB competition binding assays.
Extended Data Table 3 | Pharmacological characterization of LY2119620

**a**

| Parameter          | Value     |
|--------------------|-----------|
| \(pK_a^*\)         | 5.77 ± 0.10 |
| \(pK_r^*\)         | 8.51 ± 0.04 |
| \(\log\alpha^\ddagger\) | 1.40 ± 0.09 (\(\alpha = 25\)) |
| \(\log\alpha^\dagger\) | -0.26 ± 0.03 (\(\alpha' = 0.6\)) |

**b**

| Parameter | |\[^{[35]S}GTP\gamma S| ERK1/2 |
|-----------|-----------------|-----------------|
| \(pK_a^*\) | 5.73 ± 0.11 | 5.84 ± 0.18 |
| \(\log\alpha^\ddagger\) | 1.42 ± 0.09 | 1.30 ± 0.20 |
| \(\log\beta\) | (\(\alpha \beta = 26\)) | (\(\alpha \beta = 20\)) |
| \(\log\gamma\) | -0.28 ± 0.05 | 0.33 ± 0.09 |

a. Allosteric ternary complex model binding parameters for the interaction between LY2119620, iperoxo and \(^{[35]}\text{H}\)-NMS at the human M2 receptor. b. Operational model parameters for the functional allosteric interaction between iperoxo and LY2119620 at the human M2 receptor. Estimated parameter values represent the mean ± s.e.m. of three experiments performed in duplicate.

* Negative logarithm of the equilibrium dissociation constant of LY2119620.
† Negative logarithm of the equilibrium dissociation constant of iperoxo.
‡ Logarithm of the binding cooperativity factor between LY2119620 and iperoxo (antilogarithm shown in parentheses).
§ Logarithm of the binding cooperativity factor between LY2119620 and \(^{[35]}\text{H}\)-NMS (antilogarithm shown in parentheses).
|| Logarithm of the product of the binding cooperativity (\(\alpha\)) and activation modulation (\(\beta\)) factors between iperoxo and LY2119620. Antilogarithm shown in parentheses.
* Logarithm of the operational efficacy parameter of LY2119620 as an allosteric agonist.