The parasympathetic branch of the autonomic nervous system regulates the activity of multiple organ systems. Muscarinic receptors are G-protein-coupled receptors that mediate the response to acetylcholine released from parasympathetic nerves1,2. Their role in the unconscious regulation of organ and central nervous system function makes them potential therapeutic targets for a broad spectrum of diseases. The M2 muscarinic acetylcholine receptor (M2 receptor) is essential for the physiological control of cardiovascular function through activation of G-protein-coupled inwardly rectifying potassium channels, and is of particular interest because of its extensive pharmacological characterization with both orthostERIC and allosteric ligands. Here we report the structure of the antagonist-bound human M2 receptor, the first human acetylcholine receptor to be characterized structurally, to our knowledge. The antagonist 3-quinuclidinyl-benzilate binds in the middle of a long aqueous channel extending approximately two-thirds through the membrane. The orthosteric binding pocket is formed by amino acids that are identical in all five muscarinic receptor subtypes, and shares structural homology with other functionally unrelated acetylcholine binding proteins from different species. A layer of tyrosine residues forms an aromatic cap restricting dissociation of the bound ligand. A binding site for allosteric ligands has been mapped to residues at the entrance to the binding pocket near this aromatic cap. The structure of the M2 receptor provides insights into the challenges of developing subtype-selective ligands for muscarinic receptors and their propensity for allosteric regulation.

Muscarinic receptors constitute a family with five subtypes, M1–M5 (ref. 1). M1, M3 and M5 subtypes couple with the G\textsubscript{i} family of G proteins, and M2 and M4 subtypes with the G\textsubscript{s}/G\textsubscript{q} family of G proteins. Previous work showing that the muscarinic action by a series of choline esters and other substances in various tissues could be differentiated from their nicotinic action2 led to muscarinic acetylcholine receptors being defined as a functional concept. Muscarinic receptors are now known to be G-protein-coupled receptors (GPCRs)3 and the nicotinic receptor a ligand-gated ion channel. Muscarinic receptors were initially defined biochemically as proteins that specifically bound the high-affinity inverse agonist R(-)-3-QNB, and IL3 was shown to have a flexible structure4. Using this modified M2 receptor bound to the high-affinity inverse agonist R(−)-3-QNB, we performed crystallization by hanging-drop vapour diffusion and obtained crystals that diffracted to around 9 Å, but were not able to improve the quality of these crystals. We subsequently replaced IL3 of the M2 receptor with T4 lysozyme (T4L) as initially described for the β2 adrenergic receptor5 (Supplementary Fig. 1a). This method has been used to obtain crystal structures of four other GPCRs: the adenosine A\textsubscript{2A} receptor6, the CXC4 receptor7, the dopamine receptor D3 (ref. 15) and most recently the histamine H\textsubscript{1} receptor8. The binding properties of M2-T4L with muscarinic ligands were essentially the same as for the wild-type M2 receptor (Supplementary Fig. 1b, c), indicating that the overall TM architecture of M2-T4L was minimally affected by introduction of T4L. The M2-T4L receptor was subsequently crystallized in lipidic cubic phase. A 3.0 Å structure was solved by molecular replacement from a data set obtained by merging diffraction data from 23 crystals.

As is typical for proteins crystallized by the lipidic cubic phase method, the lattice for the M2 receptor shows alternating aqueous and lipidic layers with M2 receptor molecules embedded in the latter while T4L is confined to aqueous regions (Supplementary Fig. 2). Within the membrane plane, receptor molecules are packed closely against one another, alternating orientations within the bilayer. There are abundant hydrophobic contacts between receptor molecules within the membrane, whereas polar interactions primarily involve contacts between T4L molecules as well as receptor–T4L interactions.

As a consequence of their roles in both the central and parasympathetic nervous systems, muscarinic receptors are targets for the treatment of a spectrum of disorders including Alzheimer’s disease, schizophrenia and Parkinson’s disease, and chronic obstructive pulmonary disease4,9. However, developing highly subtype-selective orthosteric drugs for muscarinic receptors has been challenging and thus far largely unsuccessful. Recent drug discovery efforts have therefore shifted to the development of small molecule allosteric modulators. Muscarinic receptors have long been a model system for studying allosteric regulation of GPCR signalling because of their exceptional propensity to bind allosteric ligands10,11. To understand better the structural basis for challenges in developing orthosteric drugs and the susceptibility for allosteric regulation, we obtained a crystal structure of the M2 receptor.

In our initial efforts to obtain the structure of the M2 receptor we expressed and purified M2 receptor lacking most of the third intracellular loop (IL3) and the native glycosylation sites. The central part of IL3 of the M2 receptor can be removed without impairing its ability to bind to agonists or activate G proteins12, and IL3 was shown to have a flexible structure13. Using this modified M2 receptor bound to the high-affinity inverse agonist R(−)-3-QNB, we performed crystallization by hanging-drop vapour diffusion and obtained crystals that diffracted to around 9 Å, but were not able to improve the quality of these crystals. We subsequently replaced IL3 of the M2 receptor with T4 lysozyme (T4L) as initially described for the β2 adrenergic receptor14 (Supplementary Fig. 1a). This method has been used to obtain crystal structures of four other GPCRs: the adenosine A\textsubscript{2A} receptor15, the CXC4 receptor16, the dopamine receptor D3 (ref. 15) and most recently the histamine H\textsubscript{1} receptor17. The binding properties of M2-T4L with muscarinic ligands were essentially the same as for the wild-type M2 receptor (Supplementary Fig. 1b, c), indicating that the overall TM architecture of M2-T4L was minimally affected by introduction of T4L. The M2-T4L receptor was subsequently crystallized in lipidic cubic phase. A 3.0 Å structure was solved by molecular replacement from a data set obtained by merging diffraction data from 23 crystals.

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The overall structure of the M2 receptor (Fig. 1a) is similar to that of rhodopsin and other recently crystallized inactive GPCR structures (compared in Supplementary Fig. 3). The cytoplasmic surface of the M2 receptor is in an inactive conformation, but as with most other GPCR structures, there is no interaction involving Arg 121\textsuperscript{3.50} (super-scripts indicate Ballesteros–Weinstein numbers) in the conserved...
E/DRY sequence in TM3 and Glu 382 in TM6 (Fig. 1b). Instead, the Arg 121 side chain forms a salt bridge only with Asp 120. In rhodopsin, the homologous residues form part of a charge–charge interaction that stabilizes the cytoplasmic ends of TM3 and TM6 in an inactive state. The second intracellular loop shows a helical conformation similar to that first seen for the turkey β1 adrenergic receptor.

GPCR crystal structures show the greatest differences in the extracellular surface (Supplementary Fig. 3). The M2 receptor has a relatively simple and open extracellular surface (Fig. 1c, d) with the longer extracellular loop (ECL2) stabilized by a conserved disulphide with Cys 96 and Cys 176 in the middle of ECL2. In addition, the second disulphide bond was detected between Cys 141 and Cys 146 in the ECL3. The extracellular surface of the M2 receptor most resembles that of the dopamine D3 receptor (Supplementary Fig. 3).

Crystal structures of GPCRs reveal a network of hydrogen bonding interactions that extend from the binding pocket to the cytoplasmic surface. However, a distinctive feature of the M2 receptor is that this network is part of a long, continuous aqueous channel extending from the extracellular surface to a depth of approximately 33 Å when measured from ECL2 (Fig. 1e). This channel contains the ligand binding pocket, but extends beyond the ligand and is separated from the cytoplasmic surface by a hydrophobic layer formed by three amino acids: Leu 65 in TM2, Leu 114 in TM4 and Ile 392 in TM6. Aqueous channel (green) extending from the extracellular surface into the transmembrane core is interrupted by a layer of three hydrophobic residues (blue spheres). Well-ordered water molecules are shown as red dots.

Figure 1 | The M2 receptor with bound QNB. a–e, The M2 receptor is shown as a blue ribbon and QNB as orange spheres. a, M2 receptor in profile. b, Extracellular view into QNB binding pocket. d, Extracellular view with solvent-accessible-surface rendering shows a funnel-shaped vestibule and a nearly buried QNB binding pocket. e, Aqueous channel (green) extending from the extracellular surface into the transmembrane core is interrupted by a layer of three hydrophobic residues (blue spheres). Well-ordered water molecules are shown as red dots.
accompanied by a conformational change of acetylcholine from the gauche to the trans form of the O–C2–C1–N dihedral angle. It remains to be determined in which pose acetylcholine binds to the M2 receptor or to the M2-receptor–G-protein complex, and whether acetylcholine hydrogen bonds with Asn 404 or other residues.

In a striking example of convergent evolution, the orthosteric site of the M2 receptor exhibits many features noted previously as common structural elements in unrelated acetylcholine binding proteins. Like the M2 receptor, a nicotinic acetylcholine receptor homologue bound to acetylcholine (Fig. 3b) shows an aromatic cage comprised of three tyrosines and a tryptophan, although it notably lacks a counterion to the choline group, whereas in the M2 receptor this role is filled by Asp 103. A bacterial acetylcholine binding protein, ChoX, from Sinorhizobium meliloti (Fig. 3c) also possesses an aromatic cage, and amino acids in the red boxes have been shown to reduce both antagonist and agonist binding by more than tenfold. Mutations of the amino acid in the purple boxes reduce agonist binding affinity by more than tenfold. Mutations of amino acids in the blue boxes reduce agonist binding by more than tenfold. Blue dotted lines indicate potential hydrophobic interactions and red lines indicate potential polar interactions.

**Figure 2| Binding interactions between the M2 receptor and QNB.**

a, b, Two views of the QNB binding pocket. Amino acids within 4 Å of the ligand are shown as light blue sticks, with QNB in orange. Nitrogen and oxygen atoms are coloured dark blue and red, respectively. Polar interactions are indicated by dashed lines. A 2Fo − Fc map is shown in wire at 1.5σ contour. c, A schematic representation of QNB binding interactions is shown. Mutations of amino acids in the red boxes have been shown to reduce both antagonist and agonist binding by more than tenfold. Mutations of the amino acid in the purple boxes reduce antagonist binding affinity by more than tenfold. Mutations of amino acids in the blue boxes reduce agonist binding by more than tenfold. Blue dotted lines indicate potential hydrophobic interactions and red lines indicate potential polar interactions.

**Figure 3 | Convergent evolution of acetylcholine binding sites.**

a, Acetylcholine is modelled into the crystal structure of the M2 receptor. b, Acetylcholine binding pocket in the crystal structure of the acetylcholine binding protein from the snail Aplysia californica (PDB accession 2XZ5). c, Acetylcholine binding pocket in the acetylcholine binding protein ChoX from the Gram negative bacterium Sinorhizobium meliloti (PDB accession 2RIN). d, Binding site for thio-acetylcholine in the enzyme acetylcholine esterase from the electric ray Torpedo californica (PDB accession 2C4H).
like the M2 receptor has an aspartate in close proximity to the amine engaging in a charge–charge interaction. Also like the M2 receptor, ChoX has an asparagine hydrogen bonding to the ligand carboxyl. Like these proteins, the enzyme acetylcholine esterase (Fig. 3d) uses an aromatic cage and a carboxylate to bind the choline group, while the (thio)acetyl group interacts with a phenylalanine, probably through π–π interactions. Taken together, these structures suggest that an aromatic cage and buried carboxylate are likely to be critical elements for acetylcholine recognition and binding in general.

There is a growing interest in the development of allosteric ligands for GPCR targets. This is motivated by the ability to develop more subtype-selective drugs targeted at less conserved regions of the receptor. Moreover, allosteric ligands modulate the effects of natural hormones and neurotransmitters, and may therefore regulate receptor activity in a more physiological manner. As noted above, the orthosteric binding pocket is highly conserved among all muscarinic receptor subtypes. Allosteric regulation of GPCRs was first observed for the M2 receptor and this receptor has been one of the most extensively characterized allosteric model systems. Figure 4a shows the inner surface of the M2 receptor (blue), with QNB in orange spheres. The sequence conservation within the orthosteric site is apparent, while residues outside show more variability. b–d. Mutations that alter allosteric binding are shown with yellow carbons, and amino acids involved in QNB binding are shown with blue carbons as sticks or spheres. b, c. Different views of possible allosteric binding sites in the M2 receptor. The surface view in c shows the positions of possible allosteric binding sites (yellow) lining the path to the QNB binding pocket. d, Trp 422 (yellow spheres), implicated in binding of allosteric ligands, forms an edge-to-face aromatic interaction with Tyr 403, part of the aromatic cage (blue spheres) of the orthosteric site.

METHODS SUMMARY

Untagged human M2 muscarinic acetylcholine receptor was expressed in Sf9 cells with the IL3 replaced with T4 lysozyme, then extracted with digitonin and sodium cholate and purified by ligand affinity chromatography, then exchanged into decyl maltoside buffer. Purified receptor was crystallized by the lipidic cubic phase technique following addition of a stabilizing neopentyl glycol detergent. Data collection was performed at Advanced Photon Source beamlines 23ID-B and 23ID-D, and the structure solved by molecular replacement. Refinement statistics are given in Supplementary Table 2.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Contributions** K.H. purified M2 and M2-T4L receptors, characterized their ligand binding activity, and performed attempts to crystallize them with hanging drop and other methods for more than ten years. A.C.K. crystallized the M2-T4L receptors in lipidic cubic phase, collected and processed diffraction data, solved and refined the structure, and assisted with manuscript preparation. H.A. set up the expression system and expressed M2-T4L in large amounts using the insect cell baculovirus expression system. T.Y.-K. expressed M2 and M2-T4L receptors using a yeast expression system, and purified and crystallized M2 and M2-T4L receptors for five years. M.S. constructed several mutants of M2-T4L and evaluated their stabilities. C.Z. assisted with data collection and processing, W.I.W. oversaw data processing and refinement. T.O. gave advice to K.H. and T.H. on crystallization of the M2 receptor and interpretation of its structure. B.K.K. oversaw lipidic cubic phase crystallization, assisted with data collection, and wrote the manuscript together with T.H. and T.K.T., together with K.H., has engaged in biochemical studies of muscarinic receptors for more than thirty years. Prepared M2 and M2-T4L receptors, and wrote part of the manuscript. T.K. has been collaborating with T.H. for five years, designed the receptor production strategy with T.H., and wrote part of the manuscript.

**Author Information** Coordinates and structure factors for M2-T4L are deposited in the Protein Data Bank under accession code 3U0N. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to B.K.K. (kobilka@stanford.edu), T.H. (tatsuya.haga@gakushuin.ac.jp) or T.K. (t-ocboi@mfour.med.kyoto-u.ac.jp).
METHODS

Construction of M2-T4L expression vectors for S9 cells. The coding sequence of the human M2-T4L receptor fusion protein was designed to have N-linked glycosylation sites (Asn 2, Asn 3, Asn 6 and Asn 9) mutated to aspartic acid and cysteine-less T4L (C54T, C97A) residues 2–161 inserted into the IL3, replacing M2 residues 218–376. This construct was synthesized (TAKARA Bio), and cloned into the pFastbac1 S9 expression vector (Invitrogen) as illustrated in Supplementary Fig. 1a. A TAA stop codon was placed after the R466 codon, terminating translation. The synthesized M2-T4L receptor described above was confirmed by sequencing.

Expression and membrane preparation. Recombinant baculovirus was made from pFastbac1-M2-T4L using the Bac-to-Bac system (Invitrogen)31. The M2-T4L protein was expressed in baculovirus-infected S9 insect cells as described previously32. S9 insect cells were prepared at a density of 1.0 ± 10^6 cells ml^-1 and suspended in 5 litres of the IPL-41/SF900 II complex media or ES9211 insect media. Media containing S9 insect cells were transferred into the CELLBAG 22 L/O (GE Healthcare) and cultured for 4 days with the following culture conditions: 20 r.p.m., 8.5° rocking angle, 30% O2, 0.1% digitonin, 0.1% Na-cholate, 20 mM KPB, 150 mM NaCl in high resolution bins, inclusion of these reflections significantly improved map resolution. The final resolution cut-off was chosen on the basis of complete data collection and processing.

Measurement of ligand binding activity. Ligand binding activity of wild-type M2 and M2-T4L receptors was determined as described previously44. Briefly, the receptors solubilized from S9 membranes were incubated with 0.1–4 nM [3H]QNB with or without 1 μM atropine, or with 2 nM [3H]QNB with various concentrations of carbamylcholine or atropine in 0.1% digitonin, 20 mM KPB for 60 min at 30 °C (total volume 0.2 ml). The amount of [3H]QNB bound to receptors was assayed by using a small column of Sephadex G50 fine (2 ml). The density of [3H]QNB binding sites in the particulate fraction of M2-T4L was 17 pmol per mg of protein on average and ranged from 5.3–35 pmol per mg of total protein.

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Crystallization. QNB-bound M2-T4L was concentrated to 20 mg ml^-1 in decyl maltoside buffer in a volume of approximately 100 μl. A 10% stock solution of lauryl maltose neopentyl glycol detergent (MNG, Anatrace) with 100 mM NaCl and 20 mM HEPES pH 7.5 was then added to the protein to a final concentration of 1% (w/v) of MNG detergent. The sample was incubated for 1 h on ice, then diluted to 1 ml in 0.1% MNG buffer and reconstituted to 50 mg ml^-1 before reconstitution. The final volume of protein sample at this concentration was typically 20–30 μl. Protein was reconstituted in cubic phase by mixing with a 1.5-fold weight excess of a 1:10 monooolein:cholesterol mix by the twin-syringe method33. Briefly, the protein and lipid were mixed by passage through coupled syringes 100 times either by hand or using a Phyrnon LCP robot (Art Robbins Instruments). The reconstituted protein was dispensed using a modified ratchet device (Hilti) or using the Gryphon LCP robot in 40 nl drops to either 24-well or 96-well glass sandwich plates and overlaid with 0.8 μl precipitant solution. A single crystallization lead was initially identified using an in-house screen and then optimized. Crystals for data collection were grown in 25–35% PEG 300, 100 mM ammonium phosphate, 2% 2-methyl-2,4-pentanediol, 100 mM HEPES pH 7.0–7.8. Crystals reached full size and were harvested after 3–4 days at 20 °C. Typical crystals are shown in Supplementary Fig. 7.

Data collection and processing. Diffraction data were measured at the Advanced Photon Source beamlines 23 ID-B and 23 ID-D. Several hundred crystals were screened, and a final data set was compiled using diffraction wedges of typically 5 degrees from the 23 most strongly diffracting crystals. Data reduction was performed using HKL200034. The data collection and processing was performed with Coot and phenix.refine, molecular replacement model was further fitted by rigid body refinement followed by Coot and phenix.refine.

Structure solution and refinement. The structure was solved by molecular replacement using Phaser35,36 with the structure of the inactive δ2 adrenergic receptor and T4L used as search models (PDB accession 2RHI). The initial molecular replacement model was further fitted by rigid body refinement followed by simulated annealing and restrained refinement in Phenix37. Iterative manual rebuilding and refinement steps were performed with Coot and phenix.refine, respectively. Figures were prepared with PyMOL, and Ramachandran statistics were calculated with MolProbity.

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