G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody

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G-protein-coupled receptors are the largest class of cell-surface receptors, and these membrane proteins exist in equilibrium between inactive and active states1–13. Conformational changes induced by extracellular ligands binding to G-protein-coupled receptors result in a cellular response through the activation of G proteins. The A2A adenosine receptor (A2AAR) is responsible for regulating blood flow to the cardiac muscle and is important in the regulation of glutamate and dopamine release in the brain14. Here we report the raising of a mouse monoclonal antibody against human A2AAR that prevents agonist but not antagonism binding to the extracellular ligand-binding pocket, and describe the structure of A2AAR in complex with the antibody Fab fragment (Fab2838). This structure reveals that Fab2838 recognizes the intracellular surface of A2AAR and that its complementarity-determining region, CDR-H3, penetrates into the receptor. CDR-H3 is located in a similar position to the G-protein carboxy-terminal fragment in the active opsin structure and to CDR-3 of the nanobody in the active β2-adrenergic receptor structure, but locks A2AAR in an inactive conformation. These results suggest a new strategy to modulate the activity of G-protein-coupled receptors.

The structures of G-protein-coupled receptors (GPCRs) in an inactive conformation solved recently10–12 greatly advance our understanding of the molecular signalling mechanisms of the receptors. The first details of GPCR activation were provided by the structure of bovine opsin in an active conformation complexed with a G-protein C-terminal peptide (GzCT). Most recently, determination has been made of the crystal structures of β2 adenosine receptor (β2AR) in an active state with a camelid antibody fragment (nanobody Nb80) and with a heterotrimeric Gs protein15. In these structures, the complementarity-determining region (CDR-3) of Nb80 and the C-terminal α-helix of a subunit (Gzα) of Gs protein were located in the same pocket as was GzCT in the opsin structure. Nb80 and Gs protein change the conformational equilibrium of β2AR toward the active state in a similar manner, thereby substantially increase their agonist affinities15,16.

A2AAR is responsible for regulating blood flow to the cardiac muscle and is important in the regulation of glutamate and dopamine release in the brain14. Caffeine is a well-known antagonist of this receptor and is important in the regulation of glutamate and dopamine release in the brain14. Here we report the structure of A2AAR with complete ICL3 in complex with a mouse monoclonal-antibody Fab fragment, Fab2838. A2AAR was expressed in Pichia pastoris and the antibody was raised against the purified receptor with antagonist (ZM241385) bound using the conventional mouse hybridoma system combined with improved immunization and screening methods (Methods). Fab2838, a Fab fragment generated from one (IgG2838) of the obtained antibodies, completely inhibited binding of the agonist ([3H]-5′-N-ethylcarboxamido adenosine ([3H]-NECA) but did not affect binding of the antagonist ([3H]-ZM241385 (Fig. 1a, d and Supplementary Fig. 2). The results were confirmed by competition binding assays (Supplementary Discussion and Fig. 1). These findings suggest that Fab2838 induces an inactive conformation (that is, one to which agonist cannot bind) of the A2AAR ligand-binding pocket without blocking the ligand-binding site.

We crystallized A2AAR with Fab2838 in the presence of ZM241385 and solved the structure at a resolution of 2.7 Å (Supplementary Table 2). Because the occupancy of ZM241385 in the structure was low for unknown reasons, we repeated the experiments and obtained a higher occupancy structure at 3.1 Å (Supplementary Table 2 and Supplementary Figs 3 and 4). Except for the occupancy of the ligand, the two structures are almost identical (root mean squared deviation of Cα, 0.57 Å) (Supplementary Table 2). ZM241385 occupies the ligand-binding pocket on the extracellular side by making hydrophobic interactions with Phe 16829 and Ile 27439 and hydrogen bonds with Asn 25335 as observed in the A2AAR-T4L structure (Supplementary Fig. 4) (superscripts indicate residue numbers as per the Ballesteros–Weinstein scheme9). Although the overall structure of A2AAR in the A2AAR–Fab2838 complex is similar to that of A2AAR-T4L (Protein Data Bank code, 3EMI; root mean squared deviation of Cα, 0.85 Å), there is a major difference around the intracellular portions of helices V and VI; these are connected by ICL3, which in A2AAR-T4L is replaced with T4L (Supplementary Fig. 5). In our structure, ICL3 forms two regular helices—effectively continuations of helices V and VI, respectively—connected by a short turn (Supplementary Fig. 6a).

The A2AAR–Fab2838 structure has a modified ‘ionic lock’ where Glu 22830 (helix VI) and Arg 10230 of the D/ERY motif (helix III) interact through a water molecule (W1; Fig. 2c, d). In the inactive bovine rhodopsin structure, the equivalent residues form a direct salt bridge (Supplementary Fig. 7). Arg 10230 of A2AAR–Fab2838 forms salt bridges or hydrogen bonds with Asp 10134 and Tyr 112 in ICL2

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Figure 1 | Effect of Fab2838 on A2AAR–ligand binding. a, Saturation binding curves for an antagonist [3H]-ZM241385 binding to A2AAR with (open circles) or without (filled circles) Fab2838. b, c, Inhibition of [3H]-ZM241385 binding by the antagonists theophylline (b) and SCH442416 (c) with (open circles) and without (filled circles) Fab2838. The binding of [3H]-ZM241385 in the absence of a competitor was set at 100%. d, As in a, but for the agonist [3H]-NECA. e, f, As in c and d, but for the agonists adenosine (e) and NECA (f). All data are the mean ± s.e.m. of three independent experiments performed in duplicate.

and with Thr 41 as observed in the A2AAR-T4L structure (Supplementary Fig. 5b). Because of the insertion of the water molecule, Glu 228 shifts towards the cytoplasmic space, as compared with the equivalent residue in rhodopsin (Glu 247), resulting in the formation of a salt bridge with Arg 220 in the short helical turn of ICL3. This interaction may be important in the formation of the helical structure in ICL3. The ionic lock has not been observed in the crystal structures of other inactive GPCRs, including A2AAR-T4L except for the D3 dopamine receptor. This may be because the ICL3 loops in the other structures were modified to stabilize the protein. While this paper was under review, the crystal structures of thermostabilized A2AAR mutants with native ICL3 were published. The antagonist-bound inactive structures have the ionic lock. Thus, the ionic lock of A2AAR seems to stabilize the inactive conformation of the protein, which is why the receptor has a low basal activity.

Fab2838 binds on the intracellular side of the receptor (Fig. 2a), CDR-H3 of Fab2838 is unusually long and penetrates a pocket formed by helices II, III, VI and VII (Fig. 2b). CDR-H3 interacts with the surrounding helices by forming six hydrogen bonds and eight van der Waals contacts (Fig. 2c, d). The most extensive interactions are with helix II (mainly through hydrogen bonds) and helix VI (mainly through van der Waals contacts). In addition, a hydrogen bond network including two water molecules is observed between CDR-H3 and helices III and VI (Fig. 2c, d). This hydrogen bond network together with the van der Waals interactions seem to stabilize the modified ionic lock interaction between Glu 228 (helix VI) and Arg 102 (helix III) discussed above. Other complementarity-determining regions further stabilize the A2AAR–Fab2838 complex by forming 14 hydrogen bonds with helices VI and VIII and ICL1, ICL2 and ICL3 (Fig. 2b).
The extensive interactions explain the high affinity of Fab2838 (dissociation constant, $K_d = 4.4 \text{nM}$) (Supplementary Fig. 8).

The binding site of Fab2838 CDR-H3 in A$_{2A}$AR is similar to those of Nb80 CDR-3 in $\beta_2$AR and GzCT in opsin$^1$. A critical difference is that Fab2838 stabilizes an inactive conformation whereas the others recognize active conformations of the receptors. These structures are compared in Fig. 3. In the opsin structure, GzCT, which forms a short $\alpha$-helix, fits into a large pocket formed by helices II, III, VI and VII interacting with the Arg residue of the D/ERY motif in helix III (Fig. 3, left panels). CDR-3 of Nb80 in the $\beta_2$AR structure binds in a similar position to GzCT although CDR-3 forms a $\beta$-hairpin$^1$ (Fig. 3, middle panels). CDR-H3 of Fab2838 also forms a $\beta$-hairpin but induces a differently shaped binding pocket (Fig. 3c). In the $\beta_2$AR structure, CDR-3 of Nb80 is positioned between helices III and VI, whereas in the A$_{2A}$AR structure CDR-H3 of Fab2838 is $\sim 6 \text{ Å}$ closer to helices II and VII (Fig. 3b and Supplementary Fig. 9). This allows the close association of helices III and VI and the formation of the modified ionic lock between Arg 102 in helix III and Glu 228 in helix VI, consequently stabilizing the inactive conformation. In the $\beta_2$AR/Gs$\alpha$ complex structure, the C-terminal $\alpha$-helix ($\alpha\beta$) of GzCT also binds in a similar position to CDR-H3$^{1,3}$ (Supplementary Fig. 10). The conformational changes of $\alpha\beta$ together with the GzC amino-terminal region induced by the activated receptor has been proposed to result in a nucleotide exchange from GDP to GTP in Gs$\alpha$, and to subsequent dissociation of the subunit from the receptor$^{20}$. Thus, the binding pocket formed by helices II, III, VI and VII seems to be the key site for the signal transfer between GPCRs and G proteins.

A possible inactivation mechanism of A$_{2A}$AR by Fab2838 is summarized as follows. Agonist binding induces large displacements of the intracellular ends of helices III, VI and VII$^{16,18}$, which are essential to form the G-protein binding pocket$^{13,20}$ (Supplementary Fig. 1). This indicates that the signal from the ligand-binding pocket is transferred through these helices and the conformations of the two pockets are strongly coupled. Our agonist- and antagonist-binding experiments indicate that this coupling also allows signal transfer in the reverse direction, from the G-protein-binding pocket to the ligand-binding pocket (Fig. 1). CDR-H3 of Fab2838 locks the positions of helices III, VI and VII from the cytoplasmic side, leading to an inactive conformation of the extracellular ligand-binding pocket to which agonists cannot bind, probably because of the rearrangement of the membrane. Bound ligands are shown as stick models in $\beta_2$AR and A$_{2A}$AR. The residues involved in the ionic lock formation are also shown. Nitrogen and oxygen atoms are coloured blue and red, respectively.

![Figure 3](image_url) **Figure 3 | Comparison of the structures of the opsin–GzCT, $\beta_2$AR–Nb80 and A$_{2A}$AR–Fab2838 complexes.** Left, middle and right panels show the structures of an active form of opsin (green) in complex with GzCT (yellow), an active form of $\beta_2$AR (brown) bound agonist BI-167107 in complex with Nb80 CDR-3 (blue) and an inactive form of A$_{2A}$AR (blue-grey) bound antagonist ZM241385 in complex with Fab2838 CDR-H3 (red). a, Views parallel to the membrane. Bound ligands are shown as stick models in $\beta_2$AR and A$_{2A}$AR. The residues involved in the ionic lock formation are also shown. Nitrogen and oxygen atoms are coloured blue and red, respectively. b, Cytoplasmic views of the complexes. c, Surface representations of cytoplasmic surfaces of the receptors. Surfaces within 4 Å of GzCT, CDR-3 or CDR-H3 are coloured red.
side chains at the bottom of the ligand-binding pocket including Trp 246–48, which is the toggle switch for activation (Supplementary Figs 1 and 11). A similar conceptual model of β2AR activation has been reported21. In the case of β-adrenergic receptors, the conformations of the ligand- and G-protein-binding pockets are less strongly coupled, as demonstrated in the structures of β2AR–agonist complexes22 and the β2AR/irreversible-agonist complex23. This may be because the A2AAR and β1AR or β2AR agonists interact with different helices in the binding pockets (Supplementary Discussion).

Antibody fragments (and nanobodies) such as Nb80 and Fab2838, which recognize conformational epitopes of GPCRs, have great potential for GPCR studies in vitro and in vivo. Although antibodies recognizing the intracellular surface are not suitable for direct therapeutic use, the CDR structures should provide useful information for the design of peptides or small-molecule compounds against their clearly defined pockets to control the activation states of GPCRs. The antibody fragments will also be useful tools to study ligand-binding kinetics of GPCRs because they can separate ligand binding from equilibrium shifts between different activation states of the receptors. Our approach based on the conventional mouse hybridoma system allows us to raise antibodies against various receptors in three to four months using standard laboratory equipment.

**METHODS SUMMARY**

Expression and purification. A2AAR^{N150S} (residues 1–316) was expressed in *P. pastoris* as described previously42 and purified as described in Methods.

Antibody generation. MRI/3b mice were immunized with the purified A2AAR with the antigen ZM241385. Antibodies were raised to recognize conformational epitopes of A2AAR using the conventional mouse hybridoma system25 in combination with new screening methods as described in Methods. The Fab fragments were obtained by papain cleavage and purified by anion exchange column chromatography.

Crystallographic data collection and structure determination. Purified A2AAR was mixed with the Fab fragment and the A2AAR–Fab complex was purified twice by gel filtration chromatography. Crystals were grown by vapour diffusion under the conditions described in Methods. Diffraction data were collected from a single cryo-cooled crystal on beamline I24 at the Diamond Light Source, UK. The structures were solved by molecular replacement using the receptor from the A2AAR-T4L structure (PDB code, 3EML) and an antibody Fab fragment structure (PDB code, 1P7K) as search models. Data collection and refinement statistics are summarized in Supplementary Table 2.

**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** S.I. and T.M. designed the original research project. T.Y.-K. and T.S. purified and crystallized the receptor/Fab-fragment complex. S.W., T.A. and C.I.-S. purified and characterized antibodies. N.N. sequenced antibodies. T. Hino, T.A. and C.I.-S. expressed, purified and characterized the receptor. H.I., Y.N.-N., O.K.-A. and T. Harakubou purified and characterized antibodies. T. Hino, T.A. and C.I.-S. purified and characterized antibodies. N.N. sequenced antibodies. T. Hino, T.A. and T.S. purified and characterized the receptor/Fab-fragment complex. S.W., A.D.C. and S.I. performed data collection. T. Hino refined and refined the structure. T. Hino, S.I. and T.M. wrote the manuscript and all authors provide editorial input. The project was managed by T.K., T. Harakubou, S.I. and T.M.

**Author Information** Atomic coordinates and structure factors for the A2AAR–Fab structure have been deposited in the Protein Data Bank under the accession codes 3VGO (2.7 A˚) and 3VGA (3.1 A˚). 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 T.M. (t.murata@faculty.chiba-u.jp) or S.I. (so_iwata@mac.com).
METHODS

Construction of A2AAR expression vectors for Pichia pastoris. The coding sequence of A2AAR from residues 1 to 316 including the N-terminal signal peptide, FLAG-tag sequence and C-terminal 10×His-tag was synthesized by optimization of codon usage for P. pastoris (Takara Bio). In the construct, Asn 154 was also replaced by Gln to eliminate N-linked glycosylation. The DNA fragment was inserted into the multiple cloning site of the pPIC3K vector, and the linearized vector was transformed into the P. pastoris strain SMD1163 (Invitrogen) as described previously. The transformed cells were stored as glycerol stocks at −80 °C.

Expression and purification of A2AAR. A2AAR was expressed in P. pastoris as described previously. Cells were suspended in buffer A (50 mM sodium phosphate, 100 mM NaCl, 5% glycerol, 2 mM EDTA, protease inhibitor cocktail (Roche); pH 7.4) and disrupted with glass beads (0.5 mm; Biospec) by vigorous agitation with a conventional orbital shaker at 350 r.p.m. for 2 h at 4 °C. Following removal of unbroken cells and cell debris at 10,000g, membrane pellets were isolated by ultracentrifugation at 100,000g for 45 min. Membrane pellets were resuspended in buffer B (20 mM HEPES, 500 mM NaCl, 30% glycerol, EDTA-free protease inhibitor cocktail (Roche); pH 7.0) and solubilized using 1% n-dodecyl β-D-maltoside (DDM; Anatrace) containing 0.2% cholesterol hemisuccinate (CHS; Sigma) in the presence of 4 mM mephytoline (antagonist) for 1–2 h at 4 °C. After ultracentrifugation, the supernatant was supplemented with solid imidazole to a final concentration of 40 mM and incubated overnight with a TALON immobilized metal ion affinity chromatography resin (Clonetech) at 4 °C with gentle rotation (1 ml of TALON resin per 150 mg of total protein). The resin was washed with buffer C (20 mM HEPES, 250 mM NaCl, 10% glycerol, protease inhibitor cocktail, 0.05% DDM, 0.01% CHS; pH 7.0) containing 20 mM imidazole, and the bound A2AAR was eluted with buffer C containing 300 mM imidazole. The purified sample was incubated overnight with ConA resin at 4 °C to remove contaminating glycosylated proteins and was collected in the flow-through fraction. The final purified sample was dialysed against buffer D and concentrated to approximately 5 mg ml−1 by ultrafiltration (ULTRA-4 100 K, Millipore).

Construction, expression, and purification of A2AAR-T4L. A2AAR-T4L is a variant of A2AAR in which the ICL3 region is replaced with a bacteriophage T4 lysozyme (T4L). Asn 2 to Tyr 161 of T4L were inserted between Leu 208 and Arg 222 within the ICL3 region, replacing residues Lys 209 to Ala 221. A2AAR-T4L was expressed in P. pastoris and purified as described above.

Antibody generation. All animal experiments described in this study conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals of Japan and were approved by the University of Tokyo Animal Care Committee (approval no. RAC07101).

To raise antibodies against conformational epitopes of A2AAR, we modified existing protocols for immunization and screening of mouse monoclonal antibodies. A detailed description of these modified protocols will be published elsewhere. Briefly, MRL/lpr mice were immunized with 0.1 mg purified A2AAR antigen ZM241385 complex three times at two-week intervals. The immunized mice were killed and single-cell suspensions were prepared from their spleens. These cells were fused with NS-1 myeloma cells using polyethylene glycol (PEG) according to conventional methods.

To screen antibodies that specifically recognize native receptors, we developed a novel ELISA method using protein liposomes. For ‘liposome-ELISA’, we purified A2AAR reconstituted into liposomes containing biotinyl phosphatidylethanolamine (Avanti) to maintain the protein in its native conformation and effectively immobilize liposomes onto Streptavidin-coated plates (Nunc). To eliminate antibodies recognizing flexible loops, N- and C-termini or unstructured regions of A2AAR, we performed ELISA using A2AAR denatured with 1% sodium dodecyl sulphate. Denatured ELISA-negative cells were collected and evaluated using a BIAcore T100 (GE Healthcare) as described below. The selected cells were isolated in 50 mM sodium phosphate buffer (pH 7.4) and purified as described above.

Ligand binding assays. Ligand-binding assays were performed using radioligands of the antagonist [3H]ZM241385 and the agonist [3H]NECA (GE Healthcare). For single-point binding assays, 5 nM [3H]ZM241385 and 5 µM [3H]NECA were incubated in 50 µl of buffer D containing 5 or, respectively, 50 nM purified A2AAR with or without 500 nM antibody for 1 h on ice. For saturation–binding assays, varying concentrations of [3H]ZM241385 and [3H]NECA were incubated in 50 µl of buffer D containing 5 or, respectively, 50 nM purified A2AAR with or without 500 nM antibody (Fab2388) for 1 h on ice. Receptor-bound ligands were separated by gel filtration and radioactivity was measured using a LS6500 scintillation counter (Beckman). Data were analysed by nonlinear-regression-fitting program using the GraphPad PRISM software. Competition assays with antagonists (ZM241385) and agonists (NECA, adenosine) were performed in the presence of 1.0 nM [3H]ZM241385 for A2AAR or 1.5 nM [3H]ZM241385 for A2AAR-Fab (corresponding to the respective Kd values).

Purification and crystallization of the A2AAR–Fab complex. Purified A2AAR and the Fab fragments were mixed in a 1:1.2 molar ratio and were incubated on ice for 1 h. The mixture was loaded onto a Superdex 10/300 column (GE Healthcare) equilibrated with buffer C and eluted using the same buffer. The gel filtration step was repeated twice to ensure successful crystallization of the A2AAR–Fab complex. Fractions containing the complex were concentrated to approximately 20 mg ml−1 by ultrafiltration (ULTRA-4 100 K, Millipore). Initial crystals were obtained using MemGold (Molecular Dimensions). After optimization, well-diffracting crystals were obtained in hanging drops by vapour diffusion at 20 °C with the protein solution containing 0.3–0.6% octylthioglucoside and the reservoir solution (1 µl) containing 30% PEG400, 0.1 M MES (pH 6.5) and 0.2 M MgCl2. Crystals appeared after one day and grew to maximum dimensions in one week before being flash-frozen and stored in liquid nitrogen.

Data collection and structure determination. Diffraction data were collected from single cryo-cooled crystals (100 K) on beamline I24 at Diamond Light Source, UK, using a 10-µm focused beam (wavelength, 0.9795 Å) and a PILATUS 6M detector (Dectris). Data were processed using MOSFLM and SCALA from the CCP4 program suite. The structure was initially solved using the data at 2.7 Å. Molecular replacement was carried out with Phaser using the receptor from the A2AAR-T4L fusion structure (PDB code, 3EML) and an antibody fragment (PDB code, 1P7K) as search models. Iterative cycles of model building and structure refinement were performed using COOT, REFMAC5 and phenix.refine in the PHENIX program package. The final model from this refinement was used as the initial model for refinement against the data at 3.1 Å. The refinement was carried out as above. Model validation was performed using PROCHECK and MOLPROBITY. The resulting crystallographic and refinement statistics are summarized in Supplementary Table 2. Disordered region of A2AAR was predicted by the RONN program. Figures were prepared using PYMOL.

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